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Crosstalk of TGF-β and Estrogen Receptor Signaling in Breast Cancer

Arja M. Band · Marikki Laiho

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Abstract Estrogen receptor-α (ERα) and transforming growth factor (TGF)-β signaling pathways are major regulators during mammary gland development, function and tumorigenesis. Predominantly, they have opposing roles in proliferation and apoptosis. While ERα signaling supports growth and differentiation and is antiapoptotic, mammary gland epithelia cells are very sensitive to TGF-β—induced cell cycle arrest and apoptosis. Their regulatory pathways intersect, and ERα blocks TGF-β pathway by multiple means, including direct interactions of its signaling components, Smads. However, relatively little is known of the dysfunction of their interactions in cancer. A better understanding would help to develop new strategies for breast cancer treatment.

Keywords Estrogen receptor · TGF-β signaling · Breast cancer · Smad · SnoN

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A. M. Band · M. Laiho
Molecular Cancer Biology Program, Biomedicum Helsinki and Haartman Institute, University of Helsinki,
Helsinki, Finland

M. Laiho
Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine,
Baltimore, MD, USA

M. Laiho (✉)
Department of Radiation Oncology and Molecular Radiation Sciences, The Johns Hopkins University School of Medicine,
1550 Orleans Street, Room 444,
Baltimore, MD 21231, USA
e-mail: mlaiho1@jhmi.edu

Abbreviations

AF	Activation function
EMT	Epithelial-to-mesenchymal transition
ERα	Estrogen receptor-α
ERβ	Estrogen receptor-β
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
MAPK	Mitogen activated protein kinase
PI3K	Phosphatidylinositol 3-OH kinase
R-Smad	Receptor regulated Smad
SnoN	Ski-related novel gene
TAM	Tamoxifen
TGF-β	Transforming growth factor-β

Introduction

Estrogen receptors (ERα and ERβ) are ligand-inducible transcription factors and members of the nuclear hormone receptors. ERα signaling is essential for the development of the adult mammary gland. ERα knock-out mouse lacks the development that occurs during pre- and postpubertal stages [1, 2]. Due to its ability to stimulate proliferation ERα is also a driving force during mammary gland tumorigenesis. Approximately 70% of breast cancers are ERα positive and estrogen-dependent. Therefore anti-estrogens like tamoxifen metabolites (TAM) acting as competitive inhibitors of the receptor, or suppression of the synthesis of the ligand by aromatase inhibitors, have been the mainstay of endocrine therapy for breast cancer. ERβ has been identified relatively recently [3]. It may act as a repressor of ERα transcriptional activity [4] and as a tumor suppressor in other organs, like the prostate [5]. It's expression pattern only partly overlaps with that of ERα

but is expressed in stromal cells in the mammary gland [6]. ERβ knock-out mouse models suggest that it has a role in the development of ovaries, bladder, and prostate but not the mammary gland [2, 7, 8]. Much less is known about the role of ERβ tumorigenesis than that of ERα.

TGF-β belongs to the TGF-β superfamily of cytokines. It consists of more than 30 factors including nodal, activins, inhibins, and bone morphogenic proteins. TGF-β is a major regulator in many essential cellular processes including proliferation, differentiation, migration, immune response, and apoptosis. The role of TGF-β in cancer is rather complex. It has a dual role in tumor progression. Due to its anti-proliferative effect on most cells it acts as a tumor suppressor, but due to its ability to promote epithelial-to-mesenchymal transition (EMT) and to suppress immune surveillance, it appears to convert to a tumor promoter in later stages of cancer [9].

ERα and TGF-β have opposing roles in cell proliferation and apoptosis of epithelial cells. Normal ERα—expressing luminal cells rarely proliferate [10]. However, prolonged exposure to estrogen promotes breast tumorigenesis and ERα has a strong mitogenic activity in breast cancer cells. ERα signaling enhances the transcriptional activation of cyclin D1 and c-Myc [11–13]. ERα prevents apoptosis by controlling both the extrinsic and intrinsic apopotic pathways and by promoting cell survival [14], whereas TGF-β causes cell cycle arrest by inhibiting cyclin dependent kinase activities and by reducing the expression levels of c-Myc in epithelial cells [15]. TGF-β can also promote apoptosis or cell survival in a cell-type and context-dependent manner [16].

Considering the intricate roles of these major signaling pathways, ERα and TGF-β, in mammary epithelial cells biology and tumorigenesis, and their extensive interactions, a deeper understanding how these pathways crosstalk and are dysregulated would be highly relevant for a better clinical translation. In this article we review the current knowledge and raise aspects for future consideration.

Regulation of TGF-β Signaling Pathway

TGF-β family members signal through cell membrane receptors through a cascade of positive and negative regulation steps that culminate in activation of transcriptional activator complexes. Active TGF-β binds to serine/threonine kinase type II receptor, which leads to the recruitment of the type I serine/threonine kinase receptor to the complex and its subsequent phosphorylation. The signaling cascade is mediated by the phosphorylation of receptor regulated Smads (R-Smads), which, for the TGF-β family, are represented by Smad2 and Smad3. Phosphorylated R-Smads form a complex with co-Smad, Smad4. Once formed, the complex is translocated to the nucleus where it recruits transcriptional co-activators like p300 and CBP to induce acetylation and activation of the expression of TGF-β target genes [17, 18].

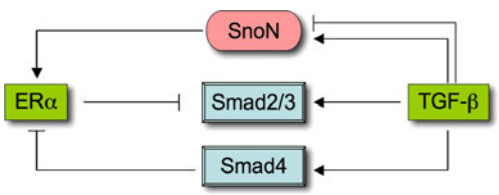

Figure 1 Intersections between TGF-β and ERα pathways

Well-controlled TGF-β signaling cascade is essential for cell and tissue homeostasis. Therefore the duration and timing are governed by negative regulators, Smad7, SnoN (Ski-related novel gene) and Ski [18, 19]. Upon TGF-β stimulation Smad7 is transported from the nucleus to the plasma membrane where it binds the TGF-β type I receptor [20]. At the plasma membrane it recruits phosphatases or ubiquitin ligases leading to degradation or dephosphorylation of the type I receptor [21–23]. Smad7 also prevents the formation of the functional transcription complex at the Smad binding element [24]. The negative feedback loop is reinstated by TGF-β-induced production of Smad7 to a steady state level.

Similarly to Smad7, SnoN and Ski provide negative feedback loops. TGF-β induces rapid proteasomal degradation of SnoN to allow induction of its target genes [25–30]. SnoN and Ski can block TGF-β induced transcription through various mechanisms. They recruit N-CoR-histone deacetylase (HDAC) complex to the promoter [31–33]. Alternatively, they may bind to the phosphorylated Smads and prevent the translocation of the Smad complex to the nucleus, or prevent the formation of the active Smad complex at the promoter [34]. Consequent to the initial decrease of SnoN, TGF-β induces the production of SnoN to the steady state level (Fig. 1). In some cell types, TGF-β induces degradation of also Ski [35, 36]. In addition, SnoN and Ski modify the TGF-β signaling cascade by regulating Smad7 production. Both block the Smad-dependent transcriptional induction of Smad7 [37, 38]. Conversely, degradation of SnoN or Ski increases production of Smad7 and causes downregulation of TGF-β receptors [37–39]. Thus, upregulation of SnoN and Ski shuts down the production of Smad7 and thus closes down the feedback loop of TGF-β signaling.

Although Smad signaling is the main mediator of TGF-β responses, TGF-β activates also other pathways, like mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt and Rho-like GTPase signaling pathways [40].

Regulation of ERα Signaling Pathway

ERα nuclear hormone receptor consists of several functionally relevant domains. Two acidic activation domains are located in the amino- and carboxy-termini (activation function 1 and 2, AF-1 and AF-2), which mediate the ligand-dependent transcriptional activation. AF-1 contributes to the constitutive transcriptional activity of the ERα while AF-2 is ligand-dependent. DNA-binding domain is located in a central zinc finger region whereas the ligand-binding domain overlaps with AF-2 [41].

Upon ligand binding the conformation of ERα changes and promotes receptor dimerization and high-affinity binding to estrogen response element (ERE) [42]. ERα regulates gene transcription by formation of a multiprotein complex containing general transcription factors, co-activators, co-repressors, co-integrators, histone acetyltransferases (HATs) and HDACs that provide tissue- and context-specific activation of ERα signaling [43]. Transcriptional activation requires relaxation of the chromatin to loosen the interaction between histones and DNA. This is facilitated by acetylation of the histones. Co-activators enhance transcription by recruiting HATs to the chromatin or contain themselves HAT activity. Co-repressors, on the other hand, may recruit HDACs to remove acetylation and turn off the transcription. Histone methyltransferase also modulates the promoter for transcription. Chromatin immunoprecipitation assays have revealed that up to 46 transcription factors cycle on and off the *pS2* gene promoter in a cyclic manner [44]. Thus, acetylation and methylation status regulate the permissiveness of the promoter and are dependent on the specific association of multiprotein complexes.

In addition to the chromatin remodeling proteins, ERα interacts with and is regulated by several co-activators that enhance its transcriptional activity. Many of these contain a LXXLL motif, a so called nuclear receptor binding domain that ERα binds to [45, 46]. The best characterized co-activators are the p160 family that consists of steroid receptor co-activators (SRC-1, SRC-2), GRIP1 and AIB1 (SRC-3).

In addition to the classical model where ERα bind to EREs in the promoter region, ERα can also regulate gene expression by binding to other transcription factors that are already bound to DNA. These transcription factors include Sp1, AP1, Runx1 and FOXA1. The whole genome contains tens of thousands of putative ERα binding sites but the number of sites at which ERα binds to the chromatin is much less, perhaps thousands [47, 48]. Genome-wide maps for ERα binding sites has indicated that most of the ERα binding to the promoters requires DNA binding capacity of ERα and are dependent on accessibility factors such as Runx1 or FOXA1 [49, 50].

Apart from co-activators and co-repressors, ERα activity can be also modulated by phosphorylation, acetylation and methylation. Several kinases have been implicated in the phosphorylation and transcriptional activation of ERα reporters. These kinases include protein kinase A, MAPK and cyclin A-CDK2 [51–53], opening up the possibility of different signaling pathways to modulate ERα signaling. Acetylation of ERα may either enhance or attenuate its transcriptional activity [54–56]. Methylation of ERα leads to its stabilization [57].

Rapid response to estrogen by several protein kinases has lead to the identification of a membrane bound pool of ERs which may consist of 5–10% of the total amount of ER in cell [58]. Membrane-localized ER dimerizes upon ligand-binding and transactivates several protein kinase cascades. These include MAPK, PKA, protein kinase C, PI3K and Src kinase [58]. Collectively, these are referred to as non-genomic mechanism of ERα because they do not require DNA binding ability or transcriptional activation by ERα. Both nuclear and membrane bound pools of ER contribute the overall cellular response to estrogen. In cancer, intervention of both pathways may be relevant.

Crosstalk Between TGF-β and ERα Pathways in the Breast

The finding that ERα—expressing luminal cells rarely express proliferation markers is intriguing and indicative that ERα mainly functions to conserve and support the differentiated state. However, the breast is an organ that is highly responsive to hormonal stimulation. In order to explore the relationship between proliferation, ERα and TGF-β signaling, Ewan and colleagues detected their expression in mouse mammary glands at estrus, and showed co-localization of phosphorylated Smads and nuclear ERα. This indicated co-regulation of the pathways and suggested that TGF-β could act to restrict ERα-mediated proliferation [59]. Furthermore, the proliferation rate of mammary gland cells was significantly increased in a mouse model of heterozygous TGF-β1 expression, and proliferation marker Ki67 expressing cells frequently co-expressed ERα.

Several other observations have been made over the years for the crosstalk between ER activity and TGF-β signaling. Estrogen receptor activation has been reported to inhibit transcriptional activity of TGF-β reporter assays by up to 60% [60–63]. It inhibits TGF-β—induced cell migration [61]. Furthermore, microarray analysis in MCF-7 breast cancer cells revealed that TGF-β treatment increased more than two fold the expression of 956 genes whereas estrogen treatment reduced the expression of 683 genes of these [63], indicating that ERα is a major modifier of TGF-β

signaling cascade. Similarly, activin and ER signaling reciprocally suppress each other [64].

Overexpression of Snail transcription factor, which drives EMT, in ERα-expressing MCF-7 cells led to downregulation of ERα and increased expression of TGF-β signaling components [65]. Additionally, it has been reported that inhibition of breast cancer cell growth by antiestrogens TAM and ICI 182.780, is mediated by TGF-β [66]. Treatment of MCF-7 cells with antiestrogen leads to increased secretion of active forms of TGF-β1 and TGF-β2 as well as increased expression of TGF-β type II receptor [67, 68]. These antiestrogen activities are mediated through p38 MAPK [68]. Furthermore, expression levels of the type II receptor in ER-negative tumors correlate with more aggressive tumors [68].

TGF-β is also a potent immunosurveillance factor. In mouse mammary tumor models generated by deletion of the type II receptor, accrual of immune cells in the tumor, and notably a subset of tumor-promoting immune suppressive myeloid cells, was observed [69]. Therefore an important feature of TGF-β is to regulate immune responses. Bierie and colleagues conducted molecular profiling of breast cancer expression signatures and compared to those of TGF-β response signatures [70]. Intriguingly, the TGF-β response signature correlated with ER-negative tumors and poor prognosis. However, complete absence of TGF-β signaling correlated with ER-positive and lymph node-positive tumors and poor prognosis. The latter profile appeared to result from increased expression of chemokines driving the recruitment of the myeloid suppressor cells [69]. On the other hand, also antiestrogens may induce immunosuppression in the tumor microenvironment in a manner that depends on TGF-β signaling [71]. While the explicit connections of these ER regulatory events to TGF-β—regulated pathways remain to be established, they indicate that both pathways are interlinked in their ability to maintain epithelial tissue homeostasis and govern also the metastatic ability of breast cancer cells.

Smad-ERα Interactions

Estrogen inhibits TGF-β signaling (Fig. 1). Estrogen treatment has been reported to reduce the phosphorylation of Smad2 and Smad3 [61, 63]. ERα physically interacts with Smad2 and Smad3 but only when ERα is in a ligand activated form [60, 63]. This interaction leads to recruitment of ubiquitin ligase, Smurf1, to the complex and ubiquitylation of Smad2/3 and subsequently Smad degradation through ubiquitin–proteasome pathway [63]. However, also contrasting reports on the ability of ERα to reduce Smad2/3 levels exist [62, Band and Laiho, unpublished]. The ability of ERα to enhance the degradation of Smads and to reduce TGF-β signaling does not require ERα DNA binding or transcriptional activity. Therefore ERα appears to regulate TGF-β signaling also through a non-genomic mechanism. Interestingly, also ERβ may inhibit TGF-β signaling by enhancing Smad2/3 degradation in an estrogen-dependent manner [63]. Consequently, activation of ERα signaling leads to the reduction of both TGF-β induced migration and invasion of breast cancer cells [61, 63].

Smad4 acts as an inhibitor of ERα. Smad4 interaction with ERα is activated by antiestrogens, occurs at the ERE binding sites, and negatively regulates ERα transcriptional activity [72, 73]. In contrast to Smad2/3, the interaction with ERα does not lead to its degradation [72]. It is believed that overexpression of Smad3 or inhibition of Smad4 leads to a change of role of TGF-β from a repressor to an activator of the ERα signaling cascade [73]. On the other hand, Smad4 can induce apoptosis in an ERα-dependent manner in ERα positive but not in ERα negative breast cancer cells. Smad4 promotes expression of the pro-apoptotic proteins, Bim and Bax, as well as release of cytochrome c [74].

SnoN in Breast Cancer

The negative regulator of TGF-β signaling, SnoN, is widely expressed in adult and embryonic cells. SnoN is structurally and functionally homologous to Ski [75, 76]. Four isoforms of Sno exist in human cells: SnoA, SnoN, SnoN2 and SnoI. The level of SnoN is directly linked to its ability to repress TGF-β signaling. Overexpression of SnoN or stabilization of SnoN, by mutation of its three lysine residues that are ubiquitylated, leads to resistance to TGF-β—induced growth arrest [25, 27]. Several ubiquitin ligases are recruited to SnoN by the Smad2/3 and responsible for the proteasomal degradation [30]. The inability of TGF-β to cause SnoN degradation results in resistance to TGF-β—induced growth arrest in esophageal cancer cells [77]. A high level of SnoN expression has been found in many cancers and cancer cell lines including breast cancer [75, 78, 79]. Like TGF-β, SnoN has a dual role in cancer. It has been reported to act as both a tumor promoter and a tumor suppressor. When the levels of SnoN are reduced by RNA interference, the ability of the cells to respond to TGF-β induced growth arrest is restored [79]. A reduction of SnoN expression also reduces anchorage-independent growth of MDA-MB-231 breast cancer cells in soft agar and tumor growth in nude mice [79]. Furthermore, elevated SnoN levels co-operate with polyoma middle T-antigen in the formation of aggressive multifocal adenocarcinomas in mouse mammary glands [80]. On the other hand, Sno+/−heterozygous mice are hypersensitive to chemical carcinogens and develop spontaneous lymphomas [81]. This suggests that SnoN may also act as a tumor suppressor. Furthermore, downregulation of SnoN leads to

increased EMT and metastasis during breast tumorigenesis [79].

SnoN is ubiquitously expressed in adult and embryonic tissues albeit at low levels. In mammary gland, its expression is detected in luminal epithelial cells of the ducts and in lobular cells [78]. In mouse, SnoN expression in the mammary gland peaks in late pregnancy and early lactation and is then rapidly downregulated [80]. These associations indicate that its expression, and likely function, is coupled to mammary epithelial cell differentiation. We have previously investigated SnoN levels in human breast cancer [78]. We analyzed SnoN expression by immunohistochemistry in a tissue microarray of 1122 breast carcinomas. Ninety percent of tumors contained a moderate to high level of SnoN. Low levels of SnoN expression correlated with longer distant disease free survival than in patients whose tumors contained high levels of SnoN. Interestingly, this phenomenon was only observed in ERα positive tumors. Consequently, we have recently investigated the possible mechanism of crosstalk between TGF-β and ERα signaling pathways. We find that SnoN contains two highly conserved nuclear receptor binding LxxLL-like motifs. SnoN interacts with ERα at *pS2* target gene promoter and is a potent transcriptional activator of ERα (Band and Laiho, unpublished). These findings indicate that SnoN acts as ERα-coactivator. Therefore TGF-β and ERα pathways intersect also through SnoN, which further underscores its importance in breast cancer.

Conclusions

Crosstalk between ERα, insulin-like growth factor and epidermal growth factor/HER2 pathways have been intensively studied. Relatively little is known of the interaction between ER and TGF-β signaling cascade although they are two major regulators during the mammary gland development and tumorigenesis.

Antiestrogen therapy such as TAM has been a successful endocrine treatment for breast cancer for decades. However, its long-term use is limited by TAM resistance which develops for most of the treated women within three years. The relevance of the plasma membrane bound ER, and its effects on calcium signaling, cAMP and activation of several kinases open up the possibility of ER signaling to influence several signaling pathways. New strategies have been developed using combination treatments blocking other signaling pathways with estrogen. Dysregulation of these crosstalk mechanisms may affect the outcome for the breast cancer treatment. Therefore, further understanding of crosstalk of ER and other pathways is needed to be able to develop these treatments.

While evidence is accruing that estrogen signaling inhibits TGF-β pathway by multiple means through the Smads (direct interaction, decreased phosphorylation and activity, increased degradation), these responses are challenging to convert to a translation of their implications in breast cancer. The more so considering the dual aspects of TGF-β acting both as a tumor suppressor and tumor promoter during breast carcinogenesis and the profound effect of the tumor microenvironment on these activities. Therefore, more molecular models, combined with analyses of the interaction at different stages of breast tumorigenesis, and addressing of their relevance in the clinical setting will take forward our understanding of these signaling cascades and their clinical translation.

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