Identification of a Gadd45β 3-prime enhancer that mediates SMAD3 and SMAD4 dependent transcriptional induction by TGFβ*

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Running Title: Gadd45β enhancer mediates TGFβ induction.

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SUMMARY

Gadd45β regulates cell growth, differentiation, and cell death following cellular exposure to diverse stimuli, including DNA damage and Transforming Growth Factor-β (TGFβ)[1]. We examined how cells transduce the TGFβ signal from the cell surface to the Gadd45β genomic locus and describe how Gadd45β contributes to TGFβ biology. Following an alignment of Gadd45β genomic sequences from multiple organisms, we discovered a novel TGFβ-responsive enhancer encompassing the third intron of the Gadd45β gene. Using three different experimental approaches, we found that SMAD3 and SMAD4, but not SMAD2, mediate transcription from this enhancer. Three lines of evidence support our conclusions. First, over-expression of SMAD3 and SMAD4 activated the transcriptional activity from this enhancer. Second, silencing of SMAD protein levels using short interfering RNAs revealed that TGFβ-induced activation of the endogenous Gadd45β gene required SMAD3 and SMAD4, but not SMAD2. In contrast, we found that the regulation of plasminogen activator inhibitor type I (PAI1) depended upon all three SMAD proteins. Lastly, SMAD3 and SMAD4 reconstitution in SMAD-deficient cancer cells restored TGFβ induction of Gadd45β. Finally, we assessed the function of Gadd45β within the TGFβ response and found that Gadd45β deficient cells arrested in G2 following TGFβ treatment. These data support a role for SMAD3 and SMAD4 in activating Gadd45β through its third intron to facilitate G2 progression following TGFβ treatment.

INTRODUCTION

Normal epithelial cells are in constant communication with their surrounding environment, largely through the detection, interpretation, and response to extracellular signaling molecules. The TGFβ superfamily of growth factors comprises 42 such signaling molecules in humans, many of which play fundamental roles in development and adult tissue homeostasis. The epithelial response to members of this family is highly varied and includes such diverse cellular processes as proliferation, movement, differentiation, and apoptosis. Indeed, cells harboring mutations within the signal transduction proteins or the TGFβ target genes either fail to respond or respond inappropriately to the TGFβ signal, often leading to developmental problems, oncogenesis, fibrotic disease, metastasis, and autoimmune disorders. Greater understanding of how cells interpret the TGFβ signal will facilitate the prevention, detection, and treatment of various human diseases.

The central elements of TGFβ signal transduction are now known (1,2). TGFβ activates the serine/threonine kinase activity of a multimeric receptor complex. Activation of this complex initiates a cascade of intracellular events that culminate in altered gene expression. The SMAD proteins form the foundation of this signaling network, as they are the only proteins directly phosphorylated by the receptor complex. However, these transcription factors are by no means sufficient to impart a TGFβ response. To specifically target a gene for transcriptional regulation, the SMADs require assistance by accessory factors. Consequently, the presence and activity of these accessory factors is as important to the TGFβ transcriptional program as are the SMAD proteins. By designing the system in such a way, cell specific responses to TGFβ can be

achieved. Further, the logic of the TGFβ signaling network explains how the cell integrates multiple signals to generate highly specific phenotypic responses.

In an attempt to better understand how TGFβ regulates gene transcription, and how those gene products contribute to TGFβ biology, we have partially defined the TGFβ transcriptional profile in normal human mammary epithelial cells (HMEC). cDNA microarray expression analysis of TGFβ treated HMEC revealed a set of genes involved in cellular proliferation, differentiation and apoptosis. One of these genes, Gadd45β/hMyD118 is regulated by TGFβ in multiple cell types, thus suggesting that this gene is of central importance to the TGFβ response.

Gadd45β, and two similar small acidic nuclear proteins, Gadd45α and Gadd45γ, make up the Gadd45 gene family (3). All three proteins regulate diverse cellular mechanisms including cell growth, DNA repair, differentiation, and apoptosis – four phenotypes that are also controlled by TGFβ signaling. Aside from sequence similarity, these genes share transcriptional regulation by DNA damage insult and growth factors. Gadd45β is, however, the only member of this family regulated by TGFβ (4,5). Gadd45β was first discovered as a transcript rapidly induced by either TGFβ treatment or the onset of terminal differentiation in M1 murine myeloid cells (6,7). Subsequent studies employing antisense mediated silencing established Gadd45β as an important regulator of the G2/M checkpoint following genotoxic stress (8) and apoptosis during M1 myeloid cell terminal differentiation (6,9). Human Gadd45β, which was first identified in a complex containing the p38 activating kinase MTK1 (MEKK4), is now a well-established regulator of p38 activity, and consequently p38 regulated biology (5,10,11). TGFβ activates p38 kinase activity and induces apoptosis in normal murine hepatocytes, but not in hepatocytes derived from Gadd45β knock out mice (11). An initial characterization of the molecular

mechanism by which TGFβ induces Gadd45β transcription has recently been reported. First, reconstitution of SMAD4 expression in SMAD4-null pancreatic cell lines restored Gadd45β induction by TGFβ (5). The nature of the TGFβ-SMAD-Gadd45β link appears to be direct; exogenously expressed SMAD2 and SMAD4, or SMAD3 and SMAD4 induce Gadd45β proximal-promoter activity three to four fold (11). However, the relative importance and function of each SMAD protein to the transcriptional activation of the endogenous Gadd45β gene is not known.

Utilizing RNA interference and reconstitution of SMAD3 and SMAD4 protein expression in SMAD deficient cell lines, we exclude SMAD2 and include SMAD3 and SMAD4 as transcription factors involved in the TGFβ induction of Gadd45β. Additionally, through a genomics-based approach, we identified a SMAD-dependent TGFβ responsive enhancer encompassing the third intron of Gadd45β. The importance of this enhancer is indicated by a three-fold greater transcriptional induction following TGFβ treatment than transcriptional effects mediated by 5-prime promoter sequences. Finally, using a cell system that does not undergo TGFβ induced apoptosis but does respond to TGFβ by Gadd45β transcriptional induction, we establish an apoptosis-independent role for Gadd45β as an important mediator of G2/M progression following TGFβ treatment.

MATERIALS AND METHODS

Cell Culture and Drug Treatments. The following cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 2.0 μM L-glutamine, 1.0 μM sodium pyruvate, penicillin, streptomycin and split every third day or at 80% confluency: Mv1Lu (CCL64), HaCaT, HeLa, 293, and 10T1/2. HT29 adenocarcinoma colon cells were cultured in McCoys media supplemented with 10% fetal bovine serum. The HepG2 and JAR cell lines were cultured in MEM and RPMI supplemented with 10% fetal bovine serum, respectively. We obtained all the cells lines from ATCC except for the following: HaCaT immortalized keratinocyte cell line was a kind gift from D. Grossman (University of Utah, Salt Lake City, Utah), the Mv1Lu cells were a kind gift from D. Ayer (University of Utah, Salt Lake City, Utah), and the JAR cells were a kind gift from E. Adashi (University of Utah, Salt Lake City, Utah). Human mammary epithelial cells (HMEC) were obtained from BioWhittaker (Maryland) and cultured in complete Mammary Epithelial Growth Media (MEGM). HMEC were seeded at passages 7 or 8 and harvested at no greater than 80% confluency for all experiments. For treatments with TGFβ (isoform type 1; Peprotech, New Jersey), we found little to no differences with respect to gene transcription if the cells had been previously serum starved. The vehicle control for TGFβ comprised 4mM HCl, 1mg/ml BSA. Cyclohexamide and actinomycin D (Calbiochem) were used at 10μg/ml and 5μg/ml, respectively, and treated as described in the text.

RNA Interference. siRNAs were designed to specifically target either Smad2, Smad3, or Smad4 in accordance with the guidelines developed by Tuschl *et. al.* (12). Because the sequence of mink Smad2 and Smad3 cDNAs is unknown, siRNAs designed against the human sequences.

The human-designed Smad2 and Smad3 siRNAs efficiently and specifically silenced mink Smad2 and Smad3 protein expression, thus indicating that these sequences are conserved in mink. We designed the Smad4 siRNA-A and siRNA-B against the mink sequence, and consequently they do not silence human Smad4 (data not shown). The control siRNA (siScr) specifically recognizes human Smad4, and thus does not affect mink SMAD2, SMAD3, or SMAD4 expression. The sequence of the chemically synthesized and HPLC purified RNA oligomers are as follows (sense strand shown): Smad2 5'-UCUUUGUGCAGAGCCCCAAtt; Smad3 5'-ACCUAUCCCCGAAUCCGAUtt; Smad4-A 5'-GGACGAAUAUGUUCAUGACtt; Smad4-B 5'-UUGGAUUCUUUAAUAACAGtt; siScr 5'- GGAUGAAUAUGUGCAUGACtt. To silence gadd45β expression, three siRNAs were designed (sense strand shown): siGadd45β-A 5'-GUU GAU GAA UGU GGA CCC Att; siGadd45β-B AUC CAC UUC ACG CUC AUC Ctt; siGadd45β-C CUU GGU UGG UCC UUG UCU Gtt. Of these three siRNAs, siGadd45β-A was the most efficacious and used to generate the data seen in Figure 8. All RNA oligomers were reconstituted and annealed following the protocol of Tuschl *et. al*. (12). Mv1Lu cells were plated 24 hours prior to transfection and transfected at 70% confluency. All siRNAs were transfected using 18μL of Lipofectamine 2000 per 10cm^2 plate according to manufacturer's guidelines (Invitrogen). For the SMAD silencing experiments, total RNA or protein was isolated 40 to 48 hours after transfection. In time course experiments, we found that maximal silencing occurred 36 hours after transfection for all three SMAD proteins (data not shown). For Gadd45β silencing, 3 hours after the start of Gadd45β siRNA transfection, cells were treated with vehicle or TGFβ for an additional 2 hours prior to RNA isolation, or 12 hours prior to flow cytometry.

Plasmids and Genomic Alignments. We electronically cloned the human, murine, and rat gadd45β genomic loci from publicly available sequence databases. Approximately 8 kilobases of the genomic loci, starting at 5000 kilobases upstream of the transcriptional start site, were aligned using the MAVID alignment algorithm (13,14). The portion of this piece of genomic DNA showing conservation among all three species is shown in Figure 7A. The G45β-1 (-1470bp, +362bp), G45β-2 (-972bp, +362bp), G45β-3 (-476bp,+362bp), G45β-A (-1535bp, -1042bp), G45β-B (-572bp, -79bp), and G45β-C (+941bp, +1428bp) reporter constructs were created as follows, the indicated region of the human gadd45β genomic locus was PCR amplified from HMEC genomic DNA and cloned into the pCR2.1-TOPO vector (Invitrogen). These DNAs were then subcloned into pGL3basic, sequence verified, and utilized in subsequent dual luciferase assays. J. Massague generously provided the 3TPLux reporter construct (Memorial Sloan-Kettering Cancer Center, New York). The murine Smad7 cDNA (generously provided by R. Derynk, University of California, San Francisco, California) was subcloned into pcDNA3.1. Similarly, the flag-tagged Smad expression vectors used in the reporter experiments were created by subcloning the cDNAs from constructs provided by D. Satterwhite into pCMV2-flag (University of Utah, Salt Lake City, Utah). For luciferase assays, all reporters were co-transfected with a Simian virus 40 (SV40)-Renilla luciferase reporter plasmid that was used to normalize transfection efficiencies. For retroviral infections, we PCR amplified the Smad3 or Smad4 open reading frames from HMEC cDNA and then cloned them into the pBabe retroviral vector. D. Ayer generously provided the GFP-pBabe vector (University of Utah, Salt Lake City, Utah).

Luciferase Assays. Fugene 6 (Roche Biochemicals) was used to transfect HaCaT cells as instructed by the manufacturer. We seeded cells at a density of 80,000 cells per well in twenty-four well plates and transfected the next day. Transfections were performed using 0.6μg DNA (including either 0.1μg normalization vector and 0.5μg reporter vector, or 0.1μg normalization vector, 0.2μg reporter vector 0.3μg expression vector), and harvested twenty hours after the start of transfection. For TGFβ treatment, media containing either TGFβ (200pM) or an equal volume of vehicle was added to cells 3 hours after the start of transfection. Luciferase values were analyzed using a Dual Luciferase Assay System (Promega). Dividing the Firefly luciferase activity from each well by the Renilla luciferase activity from the same well normalized transfection efficiencies. Data in each experiment are presented as the mean +/- S.D. of triplicates from a representative experiment. All experiments were performed at least three times producing qualitatively similar results.

Retroviral Transduction. Expression of the GFP, Smad3, or Smad4 retroviral constructs was verified by Western blot in a transient assay prior to virus production. To produce the retrovirus, Phoenix helper cells were seeded in 60mm^2 plates 24 hours prior to transfection with Lipofectamine 2000. 24 hours after transfection began, we split the cells 1:3 to 10cm^2 plates. 48 hours after the cells had been split, virus-containing media was removed from the Phoenix cells, filtered (0.22μM, low protein binding filter) and added to a 6-well plate containing the HT29 or JAR target cells (at 60% confluence). We added Polybrene (4μg/ml) to the virus immediately before transduction of the target cells to facilitate infection. 24 hours after infection, the target cells were split to 10cm^2 plates and placed under selection with 750ng/ml puromycin for 10 days.

Quantitative RT-PCR. Trizol (Invitrogen) was used to isolate the total RNA from the HT29 and JAR retroviral polyclonal stables according to the manufacturer's guidelines. cDNA was synthesized from 2 μg of total RNA using Superscript III (Invitrogen). Real-time PCR was performed using the Roche Light Cycler instrument and software, version 3.5 (Roche Diagnostics). Intron-spanning primers (Gadd45β: forward, 5'-CGGTGGAGGAGCTTTTGGTG-3'; reverse, 5'-CACCCGCACGATGTTGATGT-3', 18S rRNA: forward, 5'-GGTGAAATTCTTGGACCGGC-3', reverse, 5'-GACTTTGGTTTCCCGGAAGC-3') were designed to amplify 200-bp products in order to minimize contamination from genomic DNA.

PCR was performed in duplicate (or triplicate for 18S rRNA) with a master mix consisting of cDNA template, buffer (500 mM Tris pH 8.3, 2.5 mg/mL BSA, 30 mM $MgCl_2$), dNTPs (2 mM), TaqStart antibody (Clontech), Biolase DNA polymerase (Bioline), gene-specific forward and reverse primers (10 μM) and SYBR Green I (Molecular Probes). The PCR conditions are as follows: 35 cycles of amplification with 1 second denaturation at 95 °C, and 5 second annealing at 57 °C for Gadd45β, and 53°C for 18S rRNA. A template-free negative control was included in each experiment. We determined the copy number by comparing gene amplification with the amplification of standard samples that contained 10^3 to 10^7 copies of the gene or 10^5 to 10^9 for 18S rRNA. The relative expression level of each gene was calculated by averaging the replicates then dividing the average copy number of Gadd45β by the average copy number of 18S rRNA. Standard error of the ratios was calculated using a confidence interval.

Northern and Western blotting. Total RNA was isolated using Trizol following the manufacturer's protocol (Invitrogen). Where indicated, total RNA isolation was followed by

poly-A RNA selection using a PolyAT Tract mRNA Isolation kit (Promega). Total RNA or poly-A RNA was fractionated through formaldehyde-containing agarose gels and transferred onto N+Hybond nylon membranes (Amersham Pharmacia). Labeled probes were generated using the Rediprime II random prime labeling system (Amersham Pharmacia) supplemented with ^{32}P-dCTP (ICN). To generate Northern blot probes, we PCR amplified gene specific sequences from human, mink, or murine cDNA. Mink Gadd45β was PCR amplified using the following degenerate primers: 5'-CTGCARATYCACTTCACSCT and 3'-GGRAYCCAYTGGTTDTTGC. Hybridizations with ^{32}P-labeled probes were carried out using ULTRAhyb buffer (Ambion) as recommended by the manufacturer. For Western blotting, protein lysates were harvested in a buffer containing 25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM CaCl2. 1% Triton X-100, 0.1mM phenylmethylsulfonyl fluoride, 0.1mM benzamidine, 1mg/ml Pepstatin A, and 1mg/ml phenantholine. The resulting whole cell lysates were centrifuged at 20,800Xg for 10 minutes at 4°C. Following protein quantitation using the DC Protein Assay (BioRad), equal amounts of protein were fractionated through Tris-Glycine 4%-12% gradient NuPAGE gels using the MES buffer system (Invitrogen). The following antibodies were used to detect the Smad proteins in both Mv1Lu and human cell lines: Smad3 (Zymed; cat# 51-1500), Smad2 (Transduction Laboratories; cat# S66220), Smad4 (Santa Cruz; cat# sc-7966), and β-Catenin (Transduction Laboratories; cat#610153). Immune complexes were visualized using a secondary antibody conjugated to horseradish peroxidase (Amersham) and Western Lighting Chemiluminescence Reagent (PerkinElmer).

RESULTS

Gadd45β is a primary TGFβ responsive gene in normal human mammary epithelial cells. TGFβ induces a G1 cell cycle arrest and epithelial to mesenchymal transition, but not apoptosis, in primary normal human mammary epithelial cells grown in culture (15 and data not shown). To understand the mechanisms behind these TGFβ induced phenotypes, we partially defined the TGFβ transcriptome in normal human mammary epithelial cells (HMEC). Specifically, we used cDNA microarray expression analysis to determine the relative expression of 7,000 genes at two and twelve hours after TGFβ treatment in HMEC. Data analysis revealed 54 up-regulated and 10 down-regulated TGFβ regulated genes. Genes included in this list had a fold change of greater than 1.3 or less 0.7 at both time points and a p-value of less than 0.05 at both time points (Supplemental Table 1 and methods therein). Next, we identified genes within this data set that were in common to TGFβ regulated genes identified through transcriptional profiling in other TGFβ-responsive cell systems. We surmised that because genes in this subgroup were regulated by TGFβ irrespective of cell origin or transformation status, they would be of central importance to the TGFβ cytostatic program. Plasminogen activator inhibitor-1 (PAI1) is a well-established TGFβ induced gene and was induced eight-fold two hours after TGFβ treatment in HMEC (Supplemental Table 1). Consequently, PAI-1 served as an important positive control in the microarray, Northern blots, and in subsequent experiments (Fig. 1A). A second common TGFβ target gene identified in our expression analysis was Gadd45β/hMyD118. In addition to our studies in primary normal HMEC, previous findings indicate that Gadd45β is a TGFβ induced gene in transformed cell lines derived from myeloid, breast, skin, breast, pancreas, and bone (4,5,11,16). Because of its frequent presence in the TGFβ transcriptional response, and because

of its previously described role in growth arrest, differentiation, and apoptosis, we chose to characterize the upstream signal transduction pathway necessary for Gadd45β transcriptional induction and second, to examine the role of Gadd45β in the TGFβ response.

We first determined the scope of Gadd45β transcriptional activation. Specifically, we monitored its induction by TGFβ in several cell lines, and by other members of the TGFβ superfamily in HMEC. To determine if other TGFβ responsive cell lines responded similarly to HMEC with respect to Gadd45β transcription, several cell lines were treated with TGFβ or vehicle for 1 hour. The Gadd45β and PAI1 transcripts were induced by TGFβ in the following cell lines: HMEC, HaCaT, Mv1Lu, PANC-1, primary breast organoid outgrowths and to a lesser extent in HepG2 and HeLa cells (data not shown and Fig. 1A). The MDCK and 293 cell lines did not respond to TGFβ stimulation by inducing either Gadd45β or PAI1. TGFβ treatment of 10T1/2 murine fibroblasts caused a moderate increase in PAI1 transcription but did not affect Gadd45β mRNA levels. We also asked if other members of the TGFβ superfamily of growth factors could regulate Gadd45β transcription. Figure 1C illustrates that both TGFβ and BMP2 induced Gadd45β transcription. However, the kinetics of Gadd45β induction, as well as the strength of induction differed between the two ligands. Finally, of the three genes that comprise the Gadd45 family, only Gadd45β was found to be TGFβ inducible in HMEC; Gadd45α was not affected by TGFβ treatment and Gadd545γ was not detected (Fig. 1B). Gadd153/Chop10, a GADD family member by virtue of its induction by cellular stress, was transiently repressed by TGFβ.

To distinguish whether TGFβ treatment resulted in increased Gadd45β transcription or increased Gadd45β mRNA stability, we measured the Gadd45β mRNA half-life before and after

TGFβ treatment. HMEC were treated with TGFβ for one hour before the addition of the transcription inhibitor actinomycin D for various periods of time. Quantitative analysis of the Northern blot revealed that TGFβ failed to stabilize the Gadd45β mRNA (Fig. 2A and Fig. 2B). The accumulation of Gadd45β mRNA within two hours of TGFβ treatment suggested that it is an immediate-early TGFβ-induced target gene. To test this idea, we pre-treated HMEC with the protein translation inhibitor cyclohexamide 15 minutes before a three-hour combined TGFβ/cyclohexamide treatment. We found that the levels of Gadd45β increased in a TGFβ-dependent manner irrespective of cyclohexamide pre-treatment, indicating that new protein synthesis is not required for TGFβ induction of Gadd45β (Fig. 2C). These data indicate that Gadd45β is a direct TGFβ transcriptional target.

Gadd45β is partly dependent upon SMAD3 and independent of SMAD2 in its regulation by TGFβ. We first sought to determine whether specific inhibition of SMAD2, SMAD3 and SMAD4 abrogated Gadd45β responsiveness to TGFβ. To approach this, we employed siRNA-mediated silencing of the SMAD2, SMAD3, and SMAD4 proteins. Because we were unable to achieve silencing greater than 60% of wild-type levels in HMEC, we chose to use Mv1Lu cells for our siRNA studies. Transfection of Mv1Lu cells with siRNAs specific to SMAD2 or SMAD3 reduced the respective protein expression to nearly undetectable levels (Fig. 3A). Loss of SMAD2 caused a 70% decrease in the induction of PAI1 by TGFβ. In contrast, siRNA silencing of SMAD2 had no significant effect on Gadd45β induction following TGFβ treatment (Fig. 3A). SMAD3 deficient cells however, responded to TGFβ stimulation with reduced levels of induction for both Gadd45β and PAI-1. Although the decrease in PAI-1 induction by TGFβ observed in the SMAD2 and the SMAD3 single-knockout cells was enhanced in the double-

knockout cells, the SMAD2/SMAD3 double-knockout cells behaved similarly to SMAD3 deficient cells with respect to Gadd45β induction (Fig. 3A). Dose-response curves with the Smad3 siRNA (IC_{50} ~ 1nM) further demonstrated that TGFβ activates PAI-1 and Gadd45β through a mechanism that is partly dependent upon SMAD3 (Fig. 3B).

SMAD4 silencing prevents Gadd45β and PAI1 induction by TGFβ. Of the many proteins involved in mediating the different facets of TGFβ signal transduction, SMAD4 is considered central to many of the responses. Two different siRNAs were designed against mink SMAD4 and the efficacy of their silencing was tested in Mv1Lu cells by Western blot (Fig. 4A). Consistent with the central role of SMAD4 in TGFβ signaling, siRNA silencing of SMAD4 resulted in a dramatic loss of Gadd45β transcriptional induction following TGFβ treatment (Fig. 4B). As a confirmation of specificity, a human-specific SMAD4 siRNA, which contains mismatches at 2 positions relative to the mink sequence, did not affect SMAD4 protein expression or TGFβ regulated transcription of Gadd45β or PAI1. siSmad4-A and siSmad4-B both robustly silenced SMAD4 protein expression, and did not interfere with SMAD3 protein expression (Fig. 4A). Examination of the Gadd45β and PAI1 transcript levels in these SMAD4 deficient cells revealed a clear necessity for SMAD4 in targeting these genes for transcription following TGFβ stimulation (Fig. 4B). The small difference between siSmad4-A and siSmad4-B in silencing SMAD4 protein expression directly reflected the levels of Gadd45β and PAI1 induction by TGFβ. The siSmad4-A silences SMAD4 protein expression with an IC_{50} of less than 1nM, which is consistent with the IC_{50} of silencing imparted by siSmad3 (Compare Fig. 4C to Fig. 3B). The induction of Gadd45β and PAI1 transcripts by TGFβ in these cells showed close correlation with each other and with the levels of SMAD4 protein (Fig. 4C).

Finally, we asked if Mv1Lu cells lacking SMAD2, SMAD3, and SMAD4 responded differently to TGFβ with respect to Gadd45β transcriptional induction than cells deficient in only one or two of the SMADs. Mv1Lu cells were transfected with siRNAs directed against each of the SMADs alone, and in all combinations thereof (Fig. 5A). Northern blot analysis of Gadd45β again demonstrated a SMAD3 and SMAD4 dependency for TGFβ induced transcription. Loss of SMAD2 in these SMAD3/SMAD4 deficient cells had no further effect on Gadd45β induction. Interestingly, even though PAI1 depends partly upon SMAD2 for TGFβ induced transcription (Fig. 3A), loss of SMAD2 did not affect PAI1 induction in cells lacking SMAD3 and SMAD4 (Fig. 5B).

Our data generated with siRNA mediated silencing has revealed no differences between Gadd45β and PAI1 with respect to their regulation by SMAD3 and SMAD4 (Fig. 3 and Fig. 4). To examine the role of SMAD3 and SMAD4 more closely, we asked if loss of SMAD3 in a SMAD4 reduced background would further inhibit Gadd45β and PAI1 induction by TGFβ. Mv1Lu cells were transfected with a constant amount of siSmad4-A (15nM) in the presence of an increasing concentration of siSmad3 (Fig. 5C). siRNA mediated silencing of SMAD3 in a SMAD4 reduced background had no effect on PAI1 induction (Fig. 5C and Fig. 5D). SMAD3 silencing in these SMAD4 deficient cells did however, further repress the transcriptional induction of Gadd45β following TGFβ treatment. These data support a transcriptional model that distinguishes Gadd45β from PAI1 in their regulation by SMAD3 and SMAD4.

SMAD3 and SMAD4 expression in SMAD3 and SMAD4-null cancer cells reconstitutes TGFβ mediated induction of Gadd45β. The second approach we utilized to study the transcriptional regulation of Gadd45β by TGFβ relied upon the preponderance of inactivating mutations within

the SMAD proteins in human cancer cell lines. HT29 colon adenocarcinoma cells do not express SMAD4 protein because of a nonsense mutation that renders the transcript unstable (17). JAR cells, on the other hand, do not express SMAD3 (18). TGFβ treatment of these cell lines results in the phosphorylation of SMAD2, indicating that both cell lines express functional TGFβ receptor complexes and that SMAD2 phosphorylation is not dependent upon SMAD3 or SMAD4 (Fig. 6A). Retroviral transduction followed by polyclonal selection of these cells with either a GFP-encoding retrovirus or a SMAD3 or SMAD4 encoding virus provided an experimental approach to further examine the role of the SMADs in Gadd45β transcription. Two weeks after the transduced cells were placed under selection, expression of the transduced genes was verified by fluorescence microscopy (for GFP expression, data not shown) and Western blot (Fig. 6B). RNA harvested in parallel to the protein samples analyzed in Figure 6B was reverse transcribed and used in real time quantitative PCR to measure the Gadd45β transcript levels. TGFβ treatment of JAR-SMAD3 cells revealed a small but statistically significant increase in Gadd45β message levels (Fig. 6C). The SMAD4-HT29 cells responded to TGFβ through a robust induction of Gadd45β (Fig. 6C). Northern blot analysis of these RNAs confirmed the quantitative PCR results (data not shown).

Gadd45β contains a TGFβ responsive enhancer that encompasses the third intron. Next, we analyzed the Gadd45β genomic locus for transcriptional responsiveness to TGFβ. First, 1500 basepairs of the proximal promoter of gadd45β was cloned upstream of firefly luciferase for use in reporter assays (G45β-1; Fig. 7A). TGFβ stimulation of HaCaT or Mv1Lu cells increased the transcriptional activity of G45β-1, G45β-2, and G45β-3 approximately two-fold (Fig. 7B and Mv1Lu data not shown). In contrast, the endogenous Gadd45β transcript levels increased 8 to

15 fold in responsive cell lines following TGFβ treatment (Fig. 1A). We were unable to see increased reporter activity when other portions of this 5-prime flanking region were analyzed, or in numerous other cell lines, or when the cells were treated for different lengths of time with TGFβ (Fig. 7A and data not shown). We reasoned that because the Gadd45β coding sequence is highly conserved between human, mouse, rat, and zebrafish, the region of the genomic locus mediating TGFβ responsiveness might also be conserved. To address this possibility, we aligned the human, mouse and rat Gadd45β genomic sequences and plotted the degree of conservation utilizing the MAVID algorithm (13,14). In addition to the coding regions, three domains of the Gadd45β genomic locus demonstrate high conservation between species. Each of these regions was cloned upstream of Firefly luciferase and used in reporter experiments (Fig. 7A). Remarkably, TGFβ robustly activated transcription off of G45β-C, which contains part of the third exon, the complete third intron and part of the fourth exon, but not off of G45β-A or G45β-B (Fig. 7B, Supplemental Figure 1 and Mv1Lu data not shown). We took two approaches to test if SMAD proteins were mediating TGFβ-dependent transcriptional induction off of G45β-C. First, the inhibitory SMAD7 protein was over-expressed to block SMAD activation. SMAD7 over-expression inhibited TGFβ-induced activation of the 3TPLux reporter, which contains the PAI-1 promoter, and the G45β-C reporter, but did not affect a SV40 driven luciferase construct (Fig. 7C). Second, over-expression of SMAD3 and SMAD4 greatly enhanced G45β-C reporter activity in HaCaT cells (Fig. 7C) and in HeLa cells (data not shown). In contrast, SMAD2 expression did not affect the transcriptional activity. Interestingly, the increase in reporter activity was dependent upon both SMAD3 and SMAD4 because neither one alone significantly affected the G45β-C transcriptional activity. These data support a role for SMAD3 and SMAD4 in regulating Gadd45β transcription through a 3-prime enhancer that contains the third intron.

Indeed, sequence analysis of G45β-C revealed four conserved putative SMAD binding elements (SBEs, Supplementary Figure 1).

Gadd45β regulates G2 progression following TGFβ stimulation. To examine the contribution of Gadd45β to the TGFβ phenotype, a siRNA was designed to silence GADD45β expression. Dose-response analysis revealed potent (IC_{50}~1nM) and specific knockout of TGFβ induced GADD45β expression (Fig. 8A). TGFβ rapidly induces a G1 cell cycle arrest, but not apoptosis, in Mv1Lu cells. We asked if Mv1Lu cells deficient in Gadd45β would undergo a G1 cell cycle arrest. Introduction of a scrambled siRNA had no detectable effect on TGFβ induced cell cycle arrest (Fig. 8B). However, cells containing reduced levels of Gadd45β demonstrated a slight reduction in G1 accumulation and failed to progress through G2 following TGFβ treatment (Fig. 8B). Loss of Gadd45β transcript did not affect cell cycle progression in the absence of TGFβ treatment. Dose response analysis further verified this finding; 0.01nM and 0.1nM siRNA did not significantly affect Gadd45β transcript levels or cell cycle progression following TGFβ stimulation. These findings indicate that Gadd45β is an important regulator of cell cycle progression following TGFβ treatment.

DISCUSSION

The intracellular domain of a ligand-bound TGFβ receptor complex ignites an intertwined cascade of signaling events that induces one of many possible phenotypic responses (1,2). Consequently, the mechanism by which a cell decides how to respond to TGFβ is fundamental to many aspects of eukaryotic life. One approach to decipher the cellular interpretation of the TGFβ signal, and how that interpretation might be altered in a diseased tissue is to define and utilize the TGFβ-target genes as a starting point in a retrograde molecular characterization of the upstream transcriptional program. Concurrent studies would assess the gene function as it contributes to the phenotypic response. We have employed this approach to the Gadd45β gene. We found that Gadd45β transcriptional induction by TGFβ was dependent upon SMAD4, and to a lesser extent on SMAD3, but independent of SMAD2. Further, SMAD3 and SMAD4 mediated the transcriptional induction of Gadd45β through an enhancer that encompasses the third intron of the Gadd45β gene. Finally, TGFβ stimulation of Gadd45β deficient cells, but not of Gadd45β expressing cells, resulted in the activation of a G2/M checkpoint.

We used RNA interference as a tool to probe the upstream signal transduction components necessary for Gadd45β and PAI1 transcriptional induction following TGFβ stimulation. We chose Mv1Lu cells as a cell system for these studies rather than a human cell line such as HMEC or HaCaTs because we found that in these cells our siRNAs were more efficacious as compared to a panel of TGFβ responsive human cells. In fact, silencing SMAD protein expression in HaCaT cells to 30% of wild type levels resulted in no detectable effect on PAI1 or Gadd45β transcription following TGFβ treatment[2]. These results and our findings presented in Figure 4 argue that with respect to the transcriptional activation of Gadd45β and

PAI1, the SMAD proteins are expressed in excess. Coupled to the immediate-early transcriptional induction of Gadd45β and PAI1 by TGFβ, these data suggest that the Gadd45β and PAI-1 promoters share a relatively high affinity for the SMAD proteins. Further, this provides a possible molecular mechanism explaining how Gadd45β and PAI1 are regulated by TGFβ irrespective of tissue type. Analogous findings have recently been discovered in *C. elegans* where the FoxA protein, PHA-4, achieves transcriptional discrimination among target genes through a differential affinity to gene promoter sequences (19). Consequently, high affinity PHA4 promoters are responsive to relatively low levels of PHA4 protein expression. Further studies are in progress to classify TGFβ transcriptional targets by their sensitivity to changes in SMAD protein expression.

With the exception of a few genes, such as p15 (20) and MMP2 (21), most well characterized immediate-early TGFβ regulated genes appear to depend upon SMAD3 and SMAD4, but not SMAD2, for TGFβ transcriptional regulation. Our work places Gadd45β within this SMAD2-independent, SMAD3/SMAD4-dependent class of TGFβ responsive genes. Our conclusion that Gadd45β is a SMAD4 dependent TGFβ target gene agrees with Yoo *et. al.* and Takekewa *et. al.* who have also reported SMAD4 dependency in Gadd45β regulation, although through different experimental approaches (5,11). Conversely, our findings that TGFβ regulated Gadd45β independently of SMAD2 contradicts previous findings. Yoo *et. al.* recently reported that over-expression of SMAD2 and SMAD4 together, but not separately, induced a Gadd45β reporter construct in a TGFβ dependent fashion. Although additional work is necessary to reconcile these outcomes, they could result from cell type specific responses to TGFβ (hepatocytes *versus* keratinocytes and fibroblasts). It is important to note however, that SMAD2 deficient fibroblasts show Gadd45β transcriptional induction following TGFβ with

kinetics and efficacy similar to that of wild-type cells, an observation that is consistent with a SMAD2-independent model of Gadd45β regulation (22).

In contrast to Gadd45β, we found that SMAD2, SMAD3, and SMAD4 all contributed to TGFβ regulation of PAI1, although to varying degrees (Fig. 3 through Fig. 5). Extensive research on PAI1 has not implicated SMAD2 (23-25) in its regulation with the notable exception that fibroblasts derived from Smad2 knockout mice failed to induce PAI1 following TGFβ treatment (21). The ability of Smad2 siRNAs to phenocopy the Smad2 knock out fibroblasts in this respect strongly supports the use of siRNA-mediated gene silencing in future TGFβ transcriptional studies. Clearly, genome wide analysis of TGFβ responsiveness in SMAD-silenced or SMAD-knockout cells will be of great importance.

Yoo *et. al.* have recently shown that 220 base pairs of the Gadd45β proximal promoter is activated by TGFβ, and that this activation is enhanced by over-expression of SMAD2, SMAD3 and SMAD4, but not dominant negative forms of SMAD2 or SMAD3 (11). Our data supports their results in that we have also found the 5-prime promoter sequence to be TGFβ responsive (Fig. 7B). However, through a genomics-based alignment strategy, we identified a second TGFβ-responsive domain encompassing the highly conserved third intron of the Gadd45β gene. In contrast to the two-fold activation we observed with 5-prime promoter sequences, the 3-prime enhancer is activated five to seven-fold following TGFβ treatment. It will be important to determine which of the conserved transcription factor binding sites within this enhancer account for induction by TGFβ. Notably, we identified four conserved SMAD binding elements, three of which are located in exonic sequence (Supplementary Figure 1). The endogenous Gadd45β gene may likely respond to TGFβ through a concerted action of the 3-prime enhancer and 5-prime promoter sequences. A similar transcriptional model has been reported for the Gadd45α gene

where highly conserved sequences within the third intron or fourth exon facilitate transcriptional induction following genotoxic stress (26,27) and vitamin D3 (28), respectively. Thus, in addition to primary sequence and genomic organization, Gadd45α, Gadd45β, and Gadd45γ might also share an intronic/exonic enhancer as an important transcriptional regulatory element.

Lastly, utilizing the power of siRNA mediated gene silencing, we discovered that Mv1Lu cells made deficient for Gadd45β arrested at the G2/M checkpoint following TGFβ treatment. Previous research has established Gadd45β as a negative regulator of cell cycle progression, and several molecular mechanisms behind this inhibition have been put forth (3). Following genotoxic stress, Gadd45β acts to inhibit cdc2/CyclinB1 kinase to induce a G2/M cell cycle checkpoint in RKO lung carcinoma cells (8). In contrast, normal fibroblasts microinjected with a Gadd45β expression vector fail to undergo a G2/M arrest, although Gadd45β was found to associate with Cdc2 in these cells (29). Our findings support this previous data in that we also see a Gadd45β-dependent effect on the G2/M cell cycle checkpoint. However, we show that Gadd45β acts to promote G2/M progression following TGFβ treatment in Mv1Lu cells (Fig. 8). This data supports the notion that Gadd45β does not act to modulate cell cycle progression in isolation, but rather the presence of other proteins might ultimately determine how cells respond to increases in Gadd45β protein levels (3). Indeed, Gadd45β associates with many nuclear proteins involved in cell cycle progression, including PCNA, p21, Gadd45α, and cdc2/CyclinB2 (8,30,31).

Perhaps the most well understood function of the Gadd45 family of proteins is their ability to regulate apoptosis through the activation of MTK1 (MEKK4) (32) and subsequently p38 kinase (5,10). Although Gadd45β is rapidly induced by TGFβ in Mv1Lu and HMEC, we have not detected an apoptotic response following TGFβ treatment in these cells[2]. An apoptosis-

independent cellular response to Gadd45β induction was recently shown where TNFα signaling through NFκB induced Gadd45β transcription to prevent JNK activation and cell death (33,34). Further, several research labs have been successful in generating Gadd45β over-expression systems and have not observed cell death (35). Future studies utilizing siRNA silencing of Gadd45β following transcriptional agonists other than TGFβ will be invaluable in determining the functional consequences of Gadd45β expression.

REFERENCES

1. Massague, J. (2000) *Nat Rev Mol Cell Biol* **1**, 169-178
2. Shi, Y., and Massague, J. (2003) *Cell* **113**, 685-700
3. Liebermann, D. A., and Hoffman, B. (2002) *Leukemia* **16**, 527-541
4. Zhang, W., Bae, I., Krishnaraju, K., Azam, N., Fan, W., Smith, K., Hoffman, B., and Liebermann, D. A. (1999) *Oncogene* **18**, 4899-4907
5. Takekawa, M., Tatebayashi, K., Itoh, F., Adachi, M., Imai, K., and Saito, H. (2002) *Embo J* **21**, 6473-6482
6. Selvakumaran, M., Lin, H. K., Sjin, R. T., Reed, J. C., Liebermann, D. A., and Hoffman, B. (1994) *Mol Cell Biol* **14**, 2352-2360
7. Abdollahi, A., Lord, K. A., Hoffman-Liebermann, B., and Liebermann, D. A. (1991) *Oncogene* **6**, 165-167
8. Vairapandi, M., Balliet, A. G., Hoffman, B., and Liebermann, D. A. (2002) *J Cell Physiol* **192**, 327-338
9. Zhan, Q., Lord, K. A., Alamo, I., Jr., Hollander, M. C., Carrier, F., Ron, D., Kohn, K. W., Hoffman, B., Liebermann, D. A., and Fornace, A. J., Jr. (1994) *Mol Cell Biol* **14**, 2361-2371
10. Takekawa, M., and Saito, H. (1998) *Cell* **95**, 521-530
11. Yoo, J., Ghiassi, M., Jirmanova, L., Balliet, A. G., Hoffman, B., Fornace, A. J., Jr., Liebermann, D. A., Bottinger, E. P., and Roberts, A. B. (2003) *J Biol Chem*
12. Elbashir, S. M., Harborth, J., Weber, K., and Tuschl, T. (2002) *Methods* **26**, 199-213
13. Bray, N., and Pachter, L. (2003) *Nucleic Acids Res* **31**, 3525-3526
14. Bray, N., Dubchak, I., and Pachter, L. (2003) *Genome Res* **13**, 97-102

15. Hosobuchi, M., and Stampfer, M. R. (1989) *In Vitro Cell Dev Biol* **25**, 705-713

16. Zawel, L., Yu, J., Torrance, C. J., Markowitz, S., Kinzler, K. W., Vogelstein, B., and Zhou, S. (2002) *Proc Natl Acad Sci U S A* **99**, 2848-2853

17. Woodford-Richens, K. L., Rowan, A. J., Gorman, P., Halford, S., Bicknell, D. C., Wasan, H. S., Roylance, R. R., Bodmer, W. F., and Tomlinson, I. P. (2001) *Proc Natl Acad Sci U S A* **98**, 9719-9723

18. Xu, G., Chakraborty, C., and Lala, P. K. (2002) *Biochem Biophys Res Commun* **294**, 1079-1086

19. Gaudet, J., and Mango, S. E. (2002) *Science* **295**, 821-825

20. Feng, X. H., Lin, X., and Derynck, R. (2000) *Embo J* **19**, 5178-5193

21. Piek, E., Ju, W. J., Heyer, J., Escalante-Alcalde, D., Stewart, C. L., Weinstein, M., Deng, C., Kucherlapati, R., Bottinger, E. P., and Roberts, A. B. (2001) *J Biol Chem* **276**, 19945-19953

22. Yang, Y. C., Piek, E., Zavadil, J., Liang, D., Xie, D., Heyer, J., Pavlidis, P., Kucherlapati, R., Roberts, A. B., and Bottinger, E. P. (2003) *Proc Natl Acad Sci U S A* **100**, 10269-10274

23. Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) *Embo J* **17**, 3091-3100

24. Song, C. Z., Siok, T. E., and Gelehrter, T. D. (1998) *J Biol Chem* **273**, 29287-29290

25. Stroschein, S. L., Wang, W., and Luo, K. (1999) *J Biol Chem* **274**, 9431-9441

26. Hollander, M. C., Alamo, I., Jackman, J., Wang, M. G., McBride, O. W., and Fornace, A. J., Jr. (1993) *J Biol Chem* **268**, 24385-24393

27. Chin, P. L., Momand, J., and Pfeifer, G. P. (1997) *Oncogene* **15**, 87-99

28. Jiang, F., Li, P., Fornace, A. J., Jr., Nicosia, S. V., and Bai, W. (2003) *J Biol Chem*

29. Yang, Q., Manicone, A., Coursen, J. D., Linke, S. P., Nagashima, M., Forgues, M., and Wang, X. W. (2000) *J Biol Chem* **275**, 36892-36898

30. Kovalsky, O., Lung, F. D., Roller, P. P., and Fornace, A. J., Jr. (2001) *J Biol Chem* **276**, 39330-39339

31. Vairapandi, M., Azam, N., Balliet, A. G., Hoffman, B., and Liebermann, D. A. (2000) *J Biol Chem* **275**, 16810-16819

32. Mita, H., Tsutsui, J., Takekawa, M., Witten, E. A., and Saito, H. (2002) *Mol Cell Biol* **22**, 4544-4555

33. Jin, R., De Smaele, E., Zazzeroni, F., Nguyen, D. U., Papa, S., Jones, J., Cox, C., Gelinas, C., and Franzoso, G. (2002) *DNA Cell Biol* **21**, 491-503

34. De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D. U., Jin, R., Jones, J., Cong, R., and Franzoso, G. (2001) *Nature* **414**, 308-313

35. Yang, J., Zhu, H., Murphy, T. L., Ouyang, W., and Murphy, K. M. (2001) *Nat Immunol* **2**, 157-164

36. Karpf, A. R., Peterson, P. W., Rawlins, J. T., Dalley, B. K., Yang, Q., Albertsen, H., and Jones, D. A. (1999) *Proc Natl Acad Sci U S A* **96**, 14007-14012

37. Miller, G. S., and Fuchs, R. (1997) *Comput Appl Biosci* **13**, 81-87

FOOTNOTES

[1] The abbreviations used are: TGF, Transforming Growth Factor; HMEC, human mammary epithelial cells; CX, cyclohexamide; ActD, actinomycin D; siRNA, short interfering RNA; Scr, scrambled; JNK, c-Jun N-terminal kinase; TNF, tumor necrosis factor.

[2] M. B. Major and D. A. Jones, unpublished findings.

FIGURE LEGENDS

Figure 1. Gadd45β is a TGFβ inducible gene. (A) Various established cell lines that have been previously reported to be TGFβ sensitive by some measure were treated with TGFβ for 1 hour prior to total RNA isolation and Northern blotting for Gadd45β and PAI-1. The PAI-1 3.2kb transcript is shown; we could not detect the 2.2kb PAI-1 transcript in Mv1Lu or 10T1/2 using human, mink or murine PAI-1 probe. Extended exposure of the Northern blots and additional experiments not shown verified that the human PAI1 and Gadd45β Northern probes are capable of recognizing their respective orthologues. The 18S ribosomal band was visualized in the ethidium bromide stained gel prior to Northern blotting and serves as the loading control. (B) Randomly cycling HMEC were treated with either TGFβ (200pM) or an equal volume of vehicle. At the indicated time, total RNA was isolated from the cells. Following mRNA purification, Northern blot analysis was performed to visualize the relative transcript abundance of the indicated genes. Both the 3.2 kb and the 2.2 kb alternatively spliced forms of the mature PAI1 mRNA are shown. (C) HMEC were treated with TGFβ (200pM for 2 hours) or BMP-2 (4nM) for the indicated times before RNA isolation and Northern blot analysis for Gadd45β transcript. GAPDH serves as a loading control. The Gadd45β and GAPDH signals were quantitated using a Phosphoimager and the resulting Gadd45β/GAPDH ratio was plotted below the Northern blots.

Figure 2. Transcriptional activation of Gadd45β by TGFβ. (A) Following a two hour treatment with TGFβ (200pM) or an equal volume of vehicle, HMEC were treated with 5 μg/ml Actinomycin D for the indicated times. RNA was subsequently harvested and analyzed for the

Gadd45β and GAPDH transcripts. (B) Plotting the GAPDH-normalized Gadd45β mRNA levels from Figure 2A demonstrates that TGFβ does not stabilize the Gadd45β mRNA. (C) HMEC were treated with 10 μg/ml cyclohexamide (CX) for 15 minutes prior to a three hour combined TGFβ (200pM)-CX treatment. RNA was then isolated and analyzed for the Gadd45β and GAPDH transcripts.

Figure 3. Gadd45β induction by TGFβ is independent of SMAD2 and dependent upon SMAD3 expression. (A) Total cellular protein and total RNA was isolated from Mv1Lu cells transfected with the indicated siRNAs (15nM). 1 hour prior to RNA and protein harvest the designated plates received 200 pM of TGFβ. The protein lysates and RNA was then examined by Western blot and Northern blot for the SMAD2 or SMAD3 proteins, and the Gadd45β and PAI-1 transcripts, respectively. A graphical representation of the quantitated Gadd45β and PAI1 Northern blots is shown below. For both bar graphs, all values were normalized to the transcript expression level in the siScr and vehicle treated sample. The nonspecific band detected by the SMAD3 polyclonal antibody (indicated by *) and the ribosomal 28S RNA serve as loading controls. (B) Mv1Lu cells were transfected with the Smad3 siRNA at the indicate concentrations for 40 hours prior to a 1 hour vehicle (V) or TGFβ (T). Total cellular protein and total RNA was then analyzed for the expression of the SMAD proteins and the Gadd45β and PAI1 transcripts. β-catenin and the 18S ribosomal band serve as loading controls for the Western and Northern blots, respectively.

Figure 4. siRNA mediated silencing of Smad4 prevents Gadd45β and PAI1 induction by

TGFβ. (A) Two different siRNAs, siSmad4-A and siSmad4-B were transfected into Mv1Lu cells at 15nM for 40 hours. Western blot analysis of total cellular protein for the SMAD4 and SMAD3 proteins demonstrates the efficacious and specific silencing of SMAD4. (B) Total RNA isolated from vehicle or TGFβ treated SMAD4-silenced Mv1Lu cells was analyzed for the presence of the Gadd45β and PAI1 transcripts by Northern blot. These RNA samples were prepared in parallel to the protein extracts shown in Figure 4A. (C) Dose-response relationship between Gadd45β induction by TGFβ and SMAD4 protein levels in siRNA transfected Mv1Lu cells. Mv1Lu cells were transfected with siSmad4-A at the indicated concentration for 40 hours prior to a one-hour TGFβ (200 pM) treatment. Protein and total RNA were harvested in parallel. β-catenin and the 18S ribosomal band serve as loading controls for the Western and Northern blots, respectively.

Figure 5. SMAD3 silencing in SMAD4-deficient cells represses Gadd45β transcriptional induction by TGFβ. (A) Mv1Lu cells were transfected with the indicated siRNA at 15nM for 40 hours. The siSmad4-A siRNA was used to silence Smad4 expression. Total cellular protein was harvested and analyzed by Western blot for the indicated proteins. (B) RNA was harvested from either vehicle or TGFβ treated Mv1Lu cells transfected with the indicated siRNAs (15nM) in parallel to the protein seen in Figure 5A. The Gadd45β and PAI1 transcripts were detected by Northern blot. A graphical representation of the quantitated Gadd45β and PAI1 Northern blots is shown below. For both bar graphs, all values were normalized to the transcript expression level in the siScr and vehicle treated sample. The 18S ribosomal band serves as a loading control. (C) Mv1Lu cells were transfected with either the scrambled siRNA(15nM) alone or the Smad4-A siRNA(15nM) in the presence of increasing concentrations of Smad3 siRNA (at

0.01nM, 0.1nM, 1.0nM, 10.0nM, and 15.0nM) for 40 hours prior to TGFβ treatment for 1 hour. As before, the RNA and protein were harvested in parallel for Northern and Western blot analysis respectively. (D) The PAI1 and Gadd45β Northern blots seen in Figure 5C were quantitated by Phosphoimager analysis and normalized to the fold induction observed in Mv1Lu cells transfected with a scrambled siRNA (fold induction in lane 1 vs. lane 2).

Figure 6. Reconstitution of Smad3 and Smad4 expression in SMAD-null cancer cells restores TGFβ signaling to Gadd45β. (A) The Mv1Lu, HT29 and JAR cell lines were treated with TGFβ (200pM) for 1 hour prior to protein isolation and Western blot analysis for the indicated proteins. A ponceau S stained membrane (data not shown) and β-catenin verified that equal protein was loaded into each lane. (B) HT29 cells and JAR cells were infected with the indicated pBabe retrovirus and selected for 2 weeks in puromycin. Total cellular protein harvested from the polyclonal stables was analyzed by SDS-PAGE for the SMAD4 and SMAD3 proteins. GFP expression was visualized by fluorescence microscopy (data not shown). (C) The indicated polyclonal stable cell line was treated with either vehicle or TGFβ (200pM) for 1 hour before the total cellular RNA was harvested. Following reverse transcription, real time quantitative PCR for the Gadd45β transcript was performed. The Gadd45β transcript values were normalized to copies per 100,000 copies of 18S ribosomal RNA. The values plotted are the average of two independent experiments and the error bars represent the confidence interval of the ratios between the experiments. * p-value < 0.02, ** p-value < 0.001.

Figure 7. Gadd45β is activated by TGFβ through a 3-prime enhancer. (A) Schematic representation of the gadd45β genomic locus. The relative position of the exons and introns are

indicated above the graph. The MAVID algorithm was used to determine the relative degree of sequence conservation between human gadd45β (x-axis) and rat gadd45β (bottom half of plot, y-axis) and murine gadd45β (top half of plot, y-axis). The degree of genomic conservation is indicated by the height of the black curve. Below the graph is a cartoon representation of 6 pieces of the gadd45β genomic locus that were cloned upstream of firefly luciferase for use in subsequent reporter assays. Of note, G45β-C contains 93bp of exon 3, the complete third intron (237bp) and 98bp of exon 4 of Gadd45β (Supplementary Figure 1). (B) HaCaT cells were transfected with the indicated reporter construct for 24 hours in the presence or absence of TGFβ (200pM) before Firefly luciferase values were quantitated, normalized, and plotted as fold induction by TGFβ. Qualitatively similar results were obtained with Mv1Lu cells (data not shown). (C) Reporter assay in HaCaT cells that were transiently co-transfected with either an empty vector (Vector) or a SMAD7 expression vector and the indicated luciferase reporter construct. TGFβ or vehicle control was added four hours after the transfection began. Luciferase values were read 24 hours after the TGFβ was administered. (D) HaCaT cells co-transfected with G45β-C and the indicated SMAD expression construct in the presence (black bars) or absence (open bars) of TGFβ. All firefly luciferase values were normalized to Renilla luciferase before data analysis. The error bars represent the standard deviation from three independent experiments. All luciferase reporter experiments were repeated a minimum of 5 times (each time in triplicate) and produced qualitatively identical results.

Figure 8. Gadd45β regulates cell cycle progression following TGFβ treatment. (A) Mv1Lu cells were transfected with a scrambled siRNA (siScr) or a Gadd45β-specific siRNA (siGadd45β) at the indicated concentration. Three hours after the start of transfection, the cells

received an equal volume of vehicle or TGFβ (200pM). TGFβ treatment lasted 2 hours at which point RNA was extracted and analyzed for the Gadd45β or GAPDH transcript by Northern blot. Quantitative representation of the GAPDH normalized Gadd45β Northern blot is plotted below. (B) Mv1Lu cells were transfected with Gadd45β siRNA at the indicated concentration for three hours prior to vehicle or TGFβ (200pM) treatment for an additional 12 hours. Samples were then harvested for flow cytometry analysis. The error bars represent the standard deviation across three independent experiments.

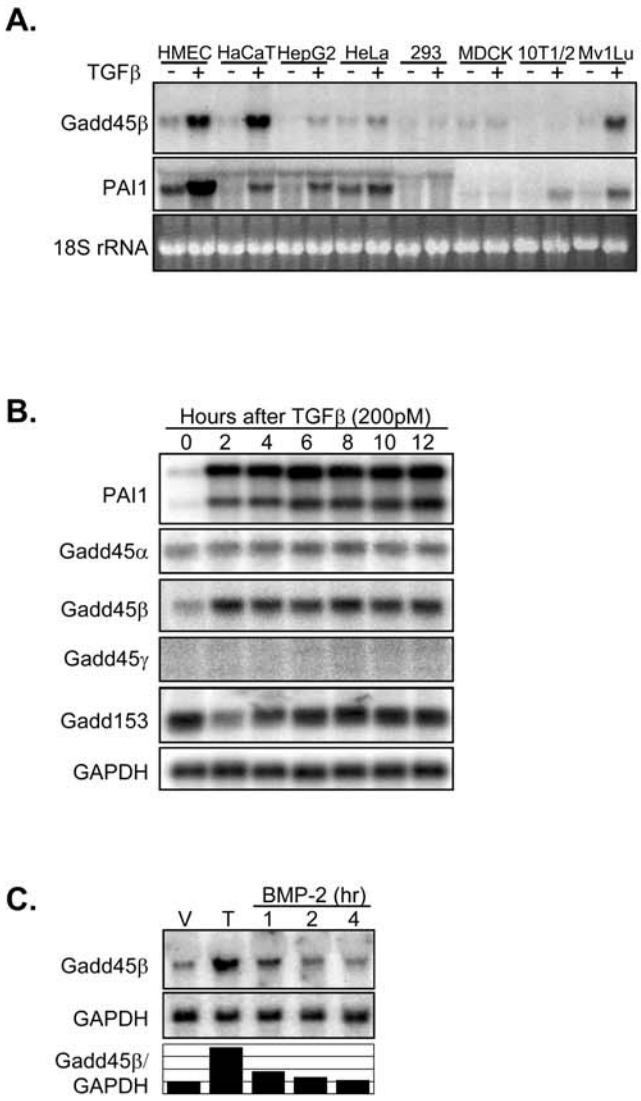
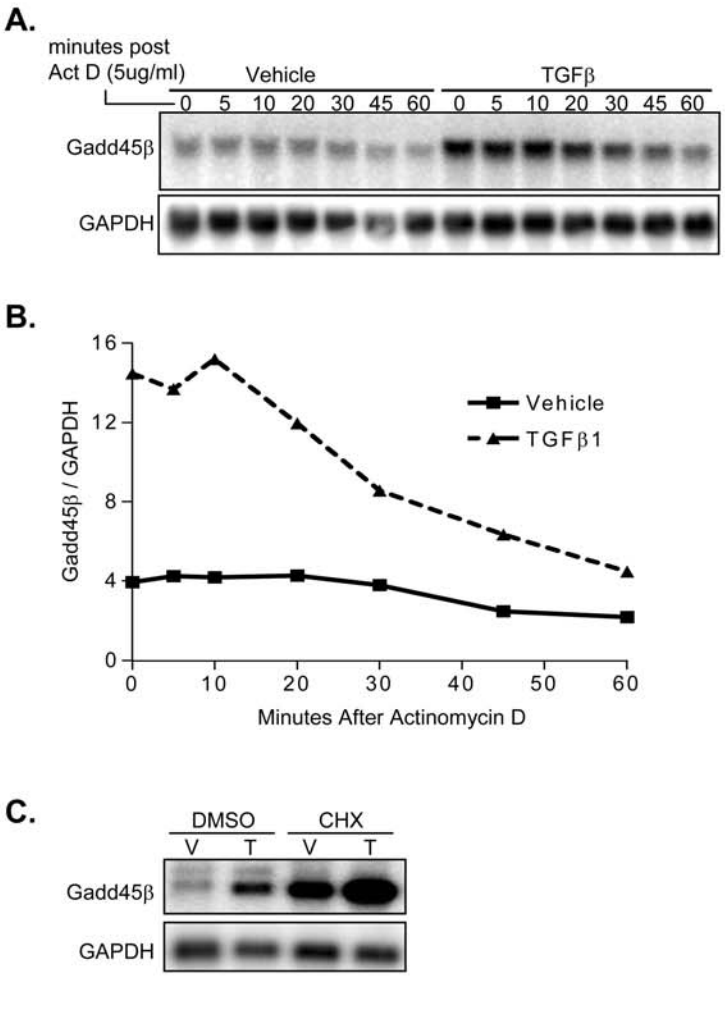


Figure 1: Major *et. al.*, 2003



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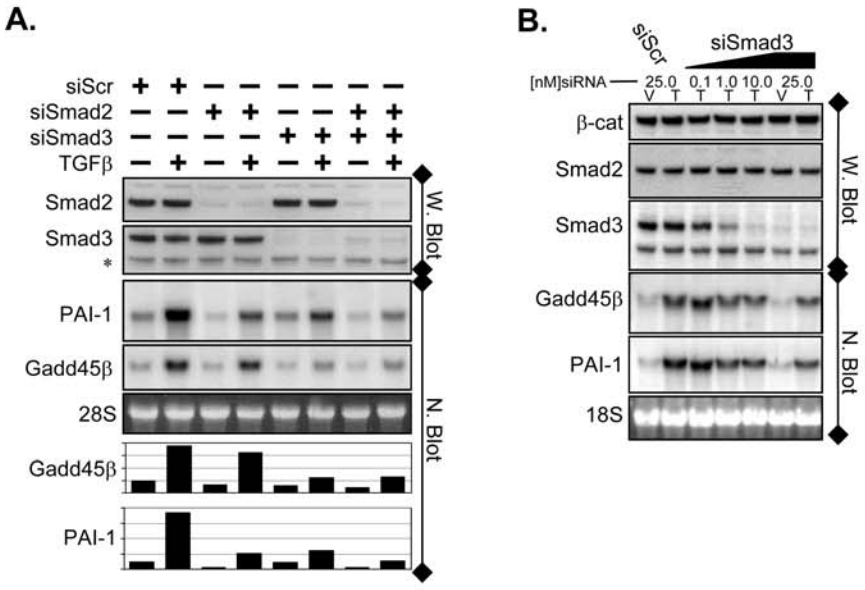

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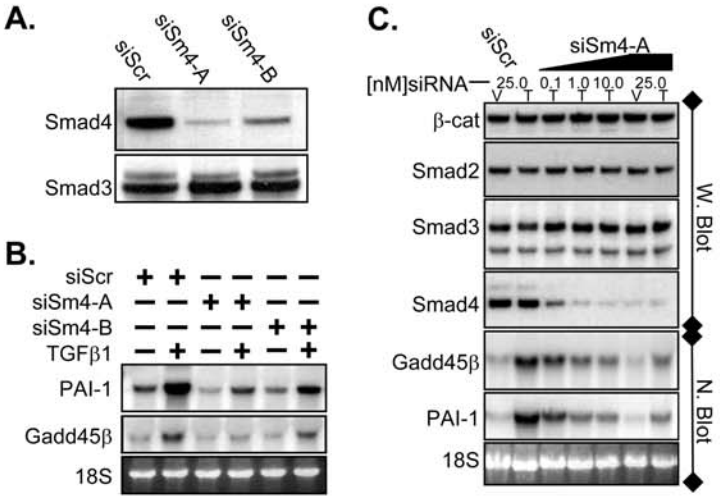

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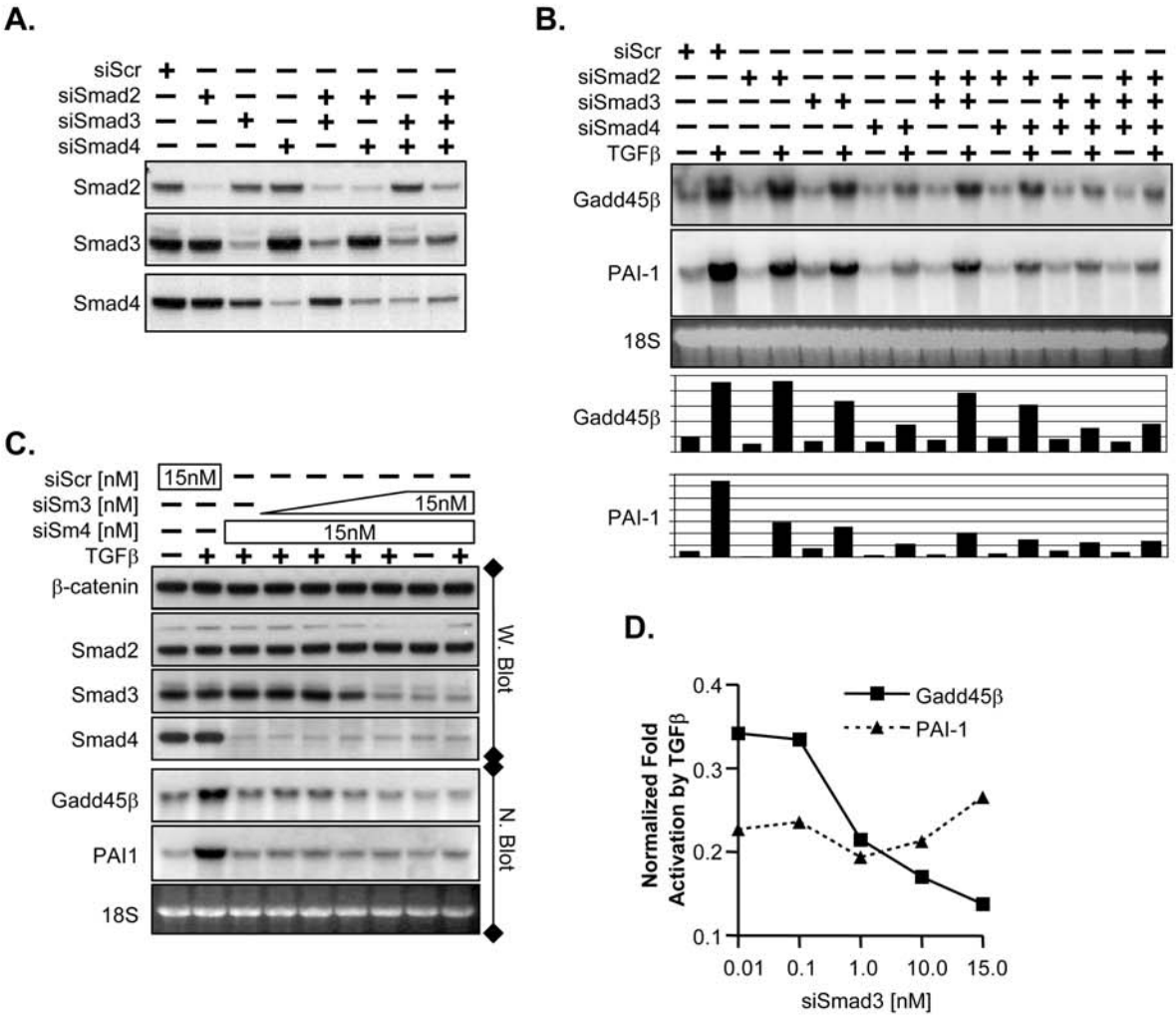

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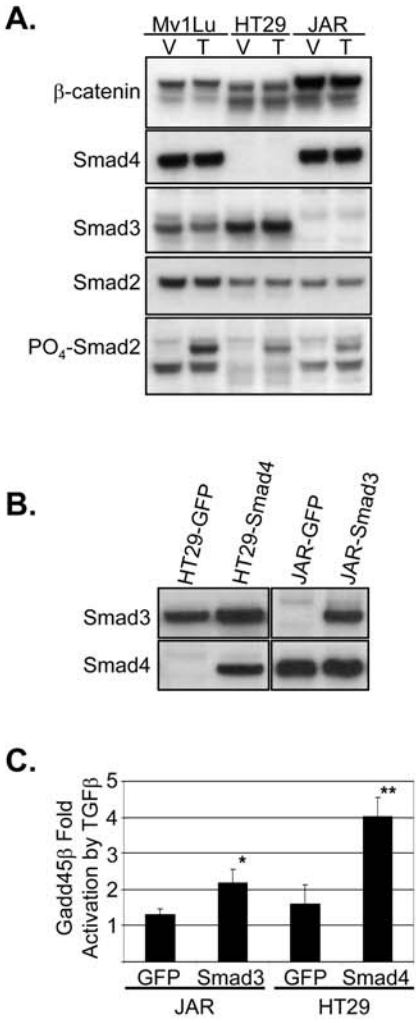

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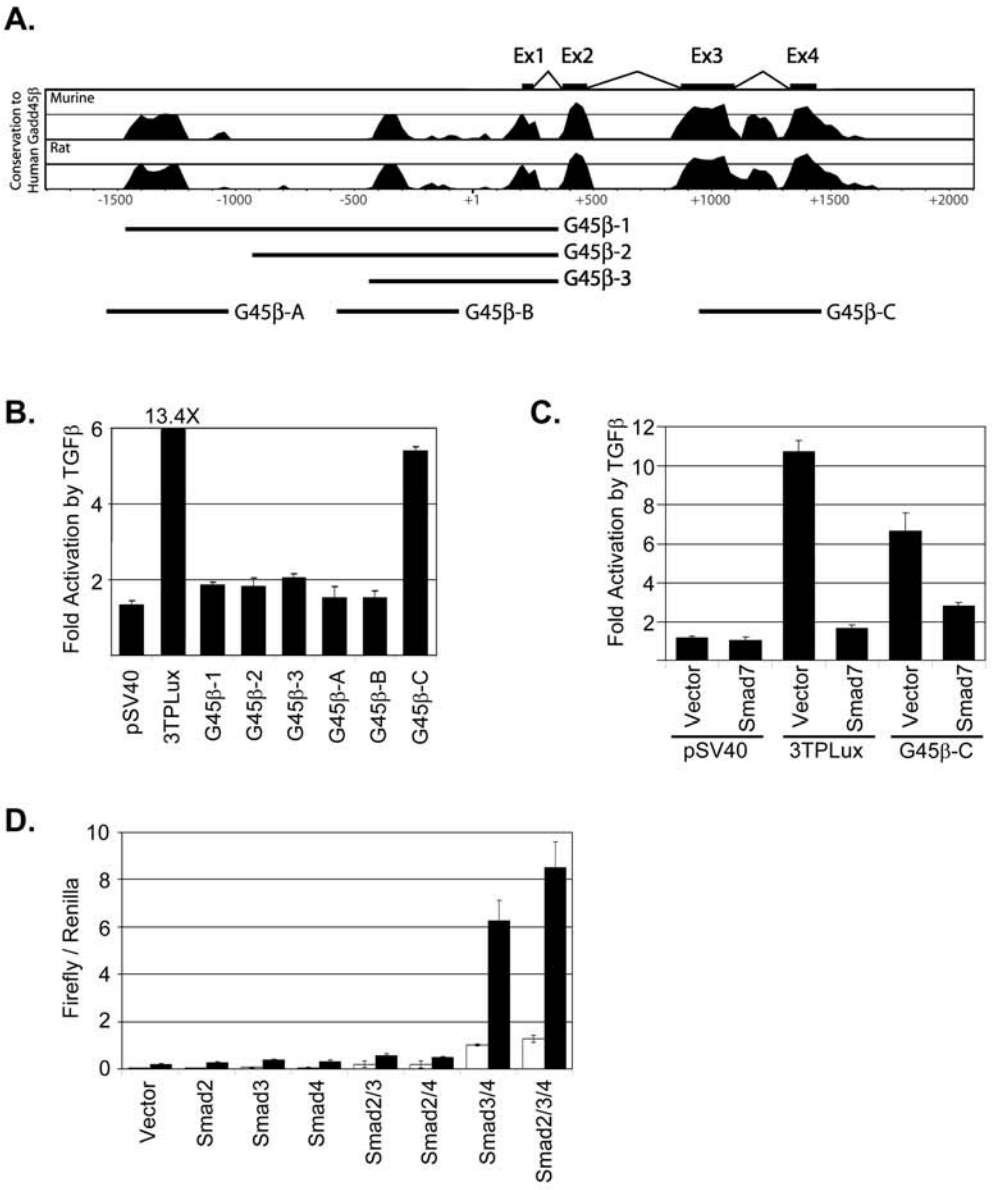


Figure 7: Major *et al*, 2003

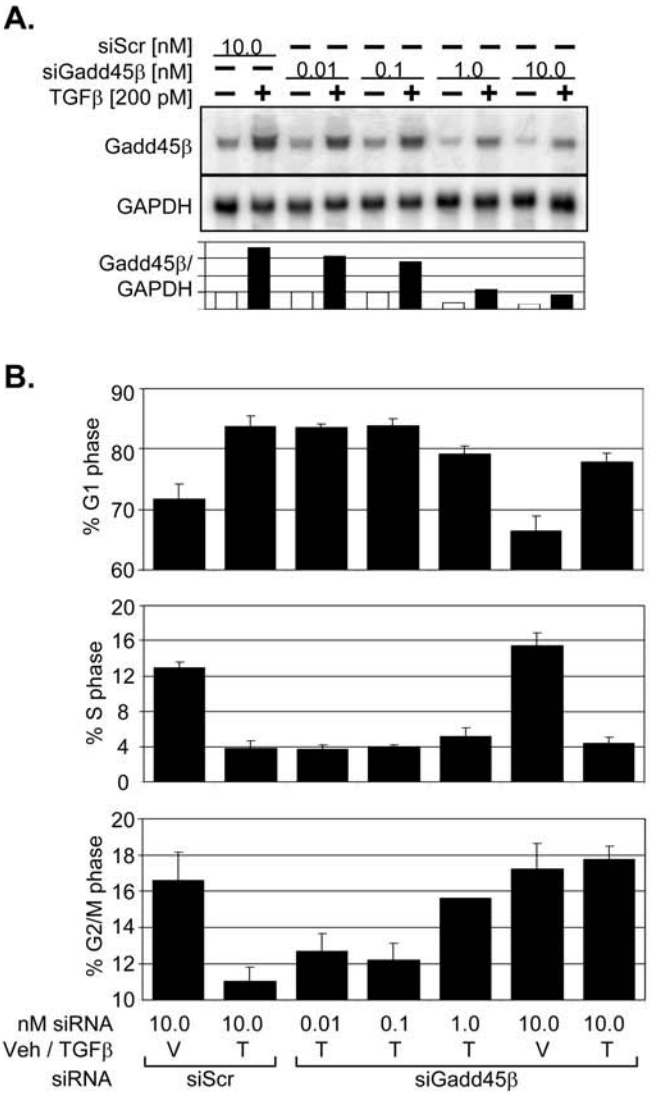


Figure 8: Major *et al*, 2003

SUPPLEMENTARY METHODS

cDNA Microarray Data Analysis. The construction of the microarrays, generation of the microarray probes, microarray hybridization, and scanning were performed as described previously (36). First stand cDNAs were generated from reverse-transcription of the mRNA samples in the presence of Cy3-dCTP or Cy5-dCTP. The resulting cDNAs were combined and simultaneously hybridized to one of two different microarray slides, each containing 6912 randomly selected and minimally redundant cDNAs from the UniGene set (37). Each of the 6912 genes was present in duplicate on the microarray and the comparisons were completed 4 separate times at each time point resulting in a total of 8 measurements for each gene (4 per time point). In each case, mRNA from TGFβ-treated HMEC (2 hours or 12 hours) was directly compared to vehicle-treated HMEC. The GeneSpring software program (version 5.1; SiliconGenetics) was utilized for all steps in the data analysis. To normalize the data, a Lowess curve was fit to the Log-intensity versus Log-ratio plot. 20% of the data was used to calculate a Lowess fit at each point. This curve was used to adjust the control value for each measurement. We selected TGFβ-responsive genes based on a statistical analysis using GeneSpring. We applied the inclusion criteria of 1.3 fold induced or 0.7 fold repressed at 2 hours and 12 hours after TGFβ treatment and a p-value of equal to or less than 0.05 (Students T-test) in defining TGFβ regulated genes (Supplementary Table 1).

Supplementary Table 1: Genes regulated by TGFβ

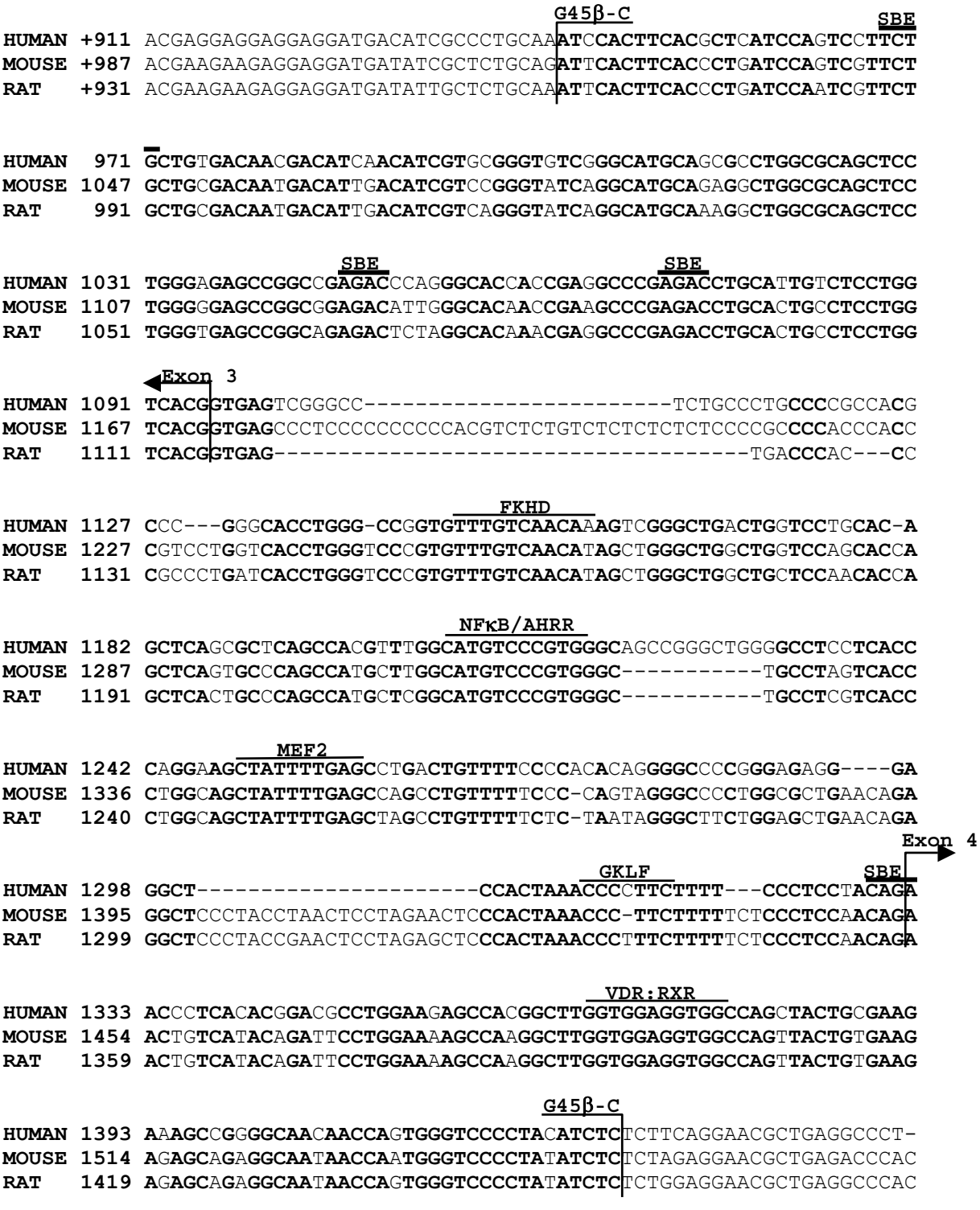
Human mammary epithelial cells were treated with TGFβ (200pM) for 2 hours and 12 hours. mRNA harvested from treated and untreated HMEC (2 independent replicates per time point) was reverse transcribed into cDNA in the presence of either a green or red fluorescent tag. Competitive hybridization of the labeled cDNAs on glass slides containing 13,816 cDNAs spotted in duplicate revealed the relative increase or decrease of specific mRNAs. Stringent data analysis (greater than 1.3 fold or less than 0.7 fold at both 2 hours and 12 hours post TGFβ treatment; p-value less than 0.05 across four replicates) facilitated the identification of 53 upregulated genes and 10 down regulated genes.

			Time after TGFβ treatment			
			2 Hour		12 Hour	
Gene Symbol	Gene Name	UniGene*	Fold Change	p-value[1]	Fold Change	p-value
PAI1	Plasminogen activator inhibitor, type I	Hs.414795	8.29	3.74E-07	14.02	8.96E-10
SLUG	Slug	Hs.360174	3.34	1.87E-03	1.84	4.59E-03
NET1	Guanine nucleotide regulatory protein (NET1)	Hs.25155	2.58	1.79E-06	1.84	2.42E-07
CD53	Connective Tissue Growth Factor	Hs.410037	2.42	1.86E-05	1.45	1.45E-05
MCM3AP	MCM3 minichromosome maintenance deficient 3 assoc protein	Hs.389037	2.38	1.83E-05	1.89	3.71E-07
ITGA2	Integrin, alpha 2	Hs.387725	2.10	2.23E-07	3.20	6.01E-06
JUNB	jun B proto-oncogene	Hs.400124	1.93	3.39E-03	1.51	2.37E-03
LDHA	lactate dehydrogenase A	Hs.2795	1.92	6.02E-07	1.32	1.72E-04
LOC255512	ESTs	Hs.28805	1.79	5.21E-05	1.74	8.92E-06
ACTG1	Actin, gamma 1	Hs.14376	1.78	2.54E-05	1.57	2.08E-05
LAMA3	laminin, alpha 3	Hs.83450	1.78	4.86E-03	4.20	1.57E-03
RHBDF1	rhomboid family 1	Hs.57988	1.67	2.41E-04	1.31	3.36E-04
	ESTs	AA127017	1.67	3.85E-03	1.77	5.78E-03
TUBA1	tubulin, alpha 1 (testis specific)	Hs.75318	1.66	1.30E-02	1.36	4.02E-02
	ESTs	R86898	1.62	4.62E-05	1.37	5.70E-05
ELF1	Ets related factor-1	Hs.124030	1.61	9.18E-06	1.60	8.36E-07
LAMC2	laminin, gamma 2	Hs.54451	1.58	5.46E-04	4.30	6.39E-04
FKBP1A	FK506-binding protein 1A (12kD)	Hs.374638	1.58	8.49E-04	1.36	8.90E-04
LLGL2	lethal giant larvae (Drosophila) homolog 2	Hs.3123	1.57	1.15E-02	3.13	5.40E-05
AQP1	Aquaporin-CHIP	Hs.76152	1.57	9.05E-05	1.40	3.17E-03
CNN1	calponin 1, basic, smooth muscle	Hs.21223	1.57	4.05E-06	1.60	4.42E-05
KRT6B	Keratin 6B	Hs.432677	1.53	8.17E-03	1.79	1.50E-03
	ESTs	Hs.113314	1.52	7.42E-04	1.46	2.95E-05
GFPT2	Glutamine-fructose-6-phosphate transaminase 2	Hs.30332	1.51	5.59E-05	3.27	7.75E-06
	ESTs	R98532	1.50	3.00E-05	1.46	1.55E-05
TGM1	Transglutaminase	Hs.22	1.50	3.01E-05	3.07	1.64E-06
GADD45B	growth arrest and DNA-damage-inducible, beta	Hs.110571	1.49	6.59E-05	1.32	5.74E-05
ITGB4	integrin, beta 4	Hs.85266	1.47	8.93E-05	1.44	2.51E-05
ANXA8	annexin A8	Hs.87268	1.47	1.59E-04	1.66	6.61E-06
MSN	moesin	Hs.170328	1.47	1.37E-04	1.49	2.82E-05
DUSP6	dual specificity phosphatase 6	Hs.447904	1.46	9.86E-03	4.15	3.10E-04
PLP2	proteolipid protein 2 (colonic epithelium-enriched)	Hs.77422	1.45	1.02E-03	1.32	8.01E-04
SPUVE	serine protease, umbilical endothelium	Hs.25338	1.45	3.34E-04	1.52	3.11E-04
EBI3	Human cytokine receptor - EBI3	Hs.185705	1.44	1.82E-05	1.58	1.21E-05
ITGA6	Integrin, alpha 6	Hs.212296	1.43	6.56E-05	1.32	4.42E-05
CD59	CD59 antigen p18-20	Hs.278573	1.43	5.05E-04	1.39	5.81E-05
RAC2	rho family, small GTP binding protein Rac2	Hs.301175	1.42	3.43E-05	1.48	1.21E-04
IVD	isovaleryl Coenzyme A dehydrogenase	Hs.410396	1.42	1.72E-04	1.55	2.95E-06
TFG	TRK-fused gene	Hs.446568	1.41	4.75E-05	1.53	6.13E-06
ANXA5	annexin A5	Hs.145741	1.41	3.13E-02	1.94	1.09E-03
MYH9	myosin, heavy polypeptide 9, non-muscle	Hs.146550	1.41	5.15E-05	2.01	1.48E-05
S100A11	S100 calcium-binding protein A11 (calgizzarin)	Hs.417004	1.41	6.69E-04	1.60	2.72E-06
FBXO26	F-box only protein 26	Hs.425352	1.40	1.49E-04	1.60	9.19E-07
MCAM	melanoma adhesion molecule	Hs.211579	1.38	9.67E-04	2.76	4.62E-06
IRF1	Interferon regulatory factor 1	Hs.80645	1.37	5.27E-02	1.39	8.50E-06
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	Hs.11899	1.37	1.64E-03	1.33	2.30E-04
	ESTs	H82876	1.34	6.08E-03	1.32	1.29E-03
FLNA	filamin A, alpha (actin-binding protein-280)	Hs.195464	1.34	6.67E-04	1.47	3.65E-05
COL4A1	Collagen, type IV, alpha 1	Hs.437173	1.34	3.95E-04	3.00	9.77E-06
ITGAV	Integrin, alpha V	Hs.436873	1.33	2.30E-04	1.65	6.49E-07
CALM1	Human calmodulin mRNA, complete cds	Hs.282410	1.33	1.33E-03	1.32	3.33E-04
ITGB1	Integrin, beta 1	Hs.287797	1.32	1.74E-04	1.55	6.86E-06
FN1	Fibronectin 1	Hs.418138	1.30	8.00E-03	2.08	9.66E-06
H1F0	H1 histone 1 family, member 0	Hs.226117	0.70	1.83E-03	0.69	8.65E-03
ITGB3	Integrin beta 3	Hs.87149	0.66	1.76E-03	0.67	2.45E-02
KRT19	keratin 19	Hs.309517	0.55	1.07E-03	0.56	8.93E-04
IL1B	Interleukin 1, beta	Hs.126256	0.55	1.07E-03	0.66	3.16E-04
KRT13	keratin 13	Hs.433871	0.55	1.51E-03	0.49	1.87E-04
RPL8	Ribosomal protein L8	Hs.178551	0.53	2.33E-03	0.59	2.02E-04
MYC	Myc	Hs.202453	0.53	7.17E-04	0.56	1.68E-04
RPL6	Ribosomal protein L6	Hs.416566	0.51	8.34E-04	0.68	1.27E-03
OPA1	Optic Atrophy 1	Hs.131273	0.46	4.57E-04	0.42	1.08E-04
SOD2	Superoxide dismutase 2, mitochondrial	Hs.384944	0.44	5.28E-04	0.41	1.05E-04

1 *p*-values were calculated using the Students T-test from 4 independent microarray expression ratios.

* For all genes except ESTs, UniGene Cluster identifiers (current as of 10/15/03) and the gene symbols are provided for cross referencing. For ESTs, the accession number is provided.

Supplemental Figure 1. Identification of conserved putative transcription factor binding sites in G45β-C. Using the MAVID algorithm, we aligned human, mouse and rat Gadd45β genomic sequences. The region corresponding to G45β-C as well as all intron/exon boundaries are indicated. Perfectly conserved nucleotides within G45β-C are shown in bold. All transcription factor binding sites were identified using the MatInspector algorithm (http://www.genomatix.de/index.html) and are shown above the sequence alignment. The transcription factor abbreviations used are: SBE (SMAD binding element), FKHD (Xenopus fork head domain factor 3), AHRR (Aryl hydrocarbon/Arnt heterodimers), NFκB (Nuclear Factor κ B), MEF2 (Myogenic MADS factor MEF-2) and GKLF(Gut-enriched Krueppel-like factor).



Supplementary Figure 1. Major *et. al. 2003*