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Transcriptional Regulation of Smad2 is Required for Enhancement of TGFβ/Smad Signaling by TGFβ Inducible Early Gene

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Abstract TGFβ inducible early gene (TIEG) is a novel Krüppel-like transcriptional repressor that was recently shown to increase the activity of the TGFβ/Smad signal transduction pathway by relieving negative feedback through repression of the inhibitory Smad7. Interestingly, while Smad7 is required for maximal enhancement of TGFβ/Smad signaling, we observe that TIEG is still capable of increasing Smad pathway activity in the absence of Smad7. Furthermore, while Smad7 is known to block both TGFβ and bone morphogenetic protein (BMP) signaling, we observe that TIEG specifically enhances only the TGFβ pathway. Similarly, while both TIEG and the related Krüppel-like factor, FKLF2, repress Smad7 transcription, only TIEG is capable of enhancing Smad signaling. In order to identify additional regulatory targets of TIEG important for this enhancement of the Smad pathway activity, we performed microarray analysis and identified Smad2 as a TIEG target gene. We now show evidence that TIEG increases transcription of the Smad2 gene but not the Smad3 or Smad4 genes. Furthermore, while the TGFβ/Smad pathway remains intact in Smad2 null cells, TIEG enhancement of Smad signaling is dramatically reduced. Thus we propose a new model whereby TIEG enhances Smad signaling by a dual mechanism involving both the repression of the inhibitory Smad7 as well as the activation of Smad2. J. Cell. Biochem. 87: 233–241, 2002. © 2002 Wiley-Liss, Inc.

Key words: transforming growth factor-β (TGFβ); Smad; TGFβ inducible early gene (TIEG); bone morphogenetic protein (BMP); Krüppel-like factor (KLF)

Transforming growth factor-beta (TGFβ) is a pleiotropic cytokine known to play an important role in development, immune function, differentiation, and tumorigenesis [Massague et al., 2000]. Consistent with the importance of TGFβ in regulating a diverse array of cellular processes, mutations, or the misexpression of downstream signaling mediators can result in embryonic lethality and/or tumorigenesis [Goumans and Mummery, 2000]. Regulation of gene expression by TGFβ is carried out primarily by the initiation of intracellular signal transduction and the activation of the Smad signaling pathway [Miyazono, 2000; ten Dijke et al., 2000]. Ligand binding to the type II receptor results in the formation of a heteromeric complex between the type II and type I TGFβ receptors [Derynck and Feng, 1997]. The type II receptor contains constitutive intrinsic kinase activity and activates the type I receptor by phosphorylation. The activated type I receptor in turn phosphorylates the receptor regulated proteins, Smad2 and 3 (R-Smads), which subsequently complex with the common mediator Smad4, translocate to the nucleus and regulate gene expression. Negative feedback to the TGFβ/Smad signaling pathway occurs through the inhibitory Smad7

Abbreviations used: TGFβ, transforming growth factor-β; TIEG, TGFβ inducible early gene; BMP, bone morphogenetic protein; FKLF-2, embryonic/fetal β-like globin gene activating Krüppel-like Factor-2; KLF, Krüppel-like factor; siRNA, small inhibitory RNA; SBE, Smad binding element.

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protein, which binds to the type I receptor thereby preventing activation of the R-Smads or inducing receptor degradation [Hayashi et al., 1997; Nakao et al., 1997; Kavsak et al., 2000; Ebisawa et al., 2001].

TGFβ inducible early gene (TIEG) is a novel zinc finger Krüppel-like transcription factor (KLF) and is a primary response gene for TGFβ in many cell types [Subramaniam et al., 1995; Subramaniam et al., 1998]. Overexpression of TIEG mimics the effects of TGFβ in many cell types by modulating markers of differentiation in osteoblast-like cells and inducing apoptosis in epithelial, pancreatic, and hepatic cells [Tachibana et al., 1997; Chalaux et al., 1999; Ribeiro et al., 1999; Hefferan et al., 2000]. One mechanism by which TIEG modulates the TGFβ/Smad signaling pathway is through inhibition of negative feedback to the Smad pathway by repression of the inhibitory Smad7 [Johnsen et al., 2002]. Indeed, TIEG binds to the Smad7 promoter and thereby represses Smad7 transcription. Interestingly, TIEG is still capable of partially enhancing R-Smad dependent transcription even when the levels of Smad7 are depleted, suggesting that TIEG modulates TGFβ/Smad signaling by an additional mechanism.

We now present evidence that TIEG specifically modulates TGFβ signaling by increasing the levels of the R-Smad, Smad2. Thus we have expanded the role of TIEG in TGFβ signaling by demonstrating a dual mechanism whereby TIEG enhances TGFβ/Smad signaling both by decreasing negative feedback through Smad7 and by increasing the pool of available R-Smads.

MATERIALS AND METHODS

Expression Vectors and Reporter Constructs

The pcDNA4/TO-Flag-TIEG and pSG5-FKLF2 expression vectors as well as the $CAGA_{12}$-MLP-Luc reporter construct have been described previously [Nakao et al., 1997; Dennler et al., 1998; Asano et al., 2000; Johnsen et al., 2002]. The untagged TIEG expression vector was created by PCR amplification of the TIEG coding region and subsequent cloning into the pSG5 expression vector (Stratagene, La Jolla, CA). The IdWT4F-luc reporter plasmid was constructed by cloning oligonucleotides containing the bone morphogenetic protein (BMP) inducible element between nucleotides −985 and −957 of the human Id1 promoter in quadruplicate into the XhoI site of the pGL3 Promoter construct (Promega, Madison, WI). The renilla luciferase internal control plasmids pRL-CMV and phRG-TK were purchased from Promega.

Cell Culture and Transfection

Smad2 null and wild-type mouse embryo fibroblasts were graciously provided by Dr. E. Piek and Dr. A. Roberts [Piek et al., 2001]. Development of the stable doxycycline inducible TIEG overexpressing Hs578T cells was described previously [Johnsen et al., 2002]. All cells were grown in DMEM/F12 (1:1) medium (Sigma, St. Louis, MO) containing 10% (v/v) FBS (Bio Whittaker, Walkersville, MD) and 1 × antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA). Cells were seeded in 12-well plates and transfected at 50% confluence with various combinations of plasmid DNA using Lipofectamine Plus (Invitrogen) according to the manufacturer's directions. All luciferase transfections included a plasmid containing renilla luciferase reporter as an internal transfection control.

RNA Interference

RNA interference studies were performed as previously described [Elbashir et al., 2001]. Duplex small inhibitory RNAs (siRNAs) directed against murine Smad7 (GenBank accession #AF015260), as well as the negative control siRNA (GL2), were synthesized by Dharmacon Research (Lafayette, CO). The ribonucleotide sequence of the Smad7 duplex siRNA is: Upper, GAAACUCAAGGAGCGGCAGdTdT; and Lower, CUGCCGCUCCUUGAGUUUCdTdT.

Northern Analysis and RT-PCR

Total RNA was isolated using the Tri-Reagent phenol–guanidine isothiocyanate solution (Molecular Research Center, Cincinnati, OH) as described previously [Subramaniam et al., 1995]. Ten micrograms of total RNA was run on a 1% (w/v) tris-acetate agarose gel and blotted to nylon membranes overnight in 20 × SSC. Prior to blotting, the gel was stained with ethidium bromide and photographed to ensure equal loading. Membranes were probed with a fragment containing the coding region of the human Smad2 cDNA. The probes were labeled with [α^{32}P]-dCTP to a specific activity of $> 10^8$ cpm/μg.

RT-PCRs for TIEG, Smad3, Smad4, and GAPDH were performed as previously described [Rickard et al., 1996]. The primers used were:

TIEG Forward, TTTGCCCGTTCTGATGAACT; TIEG Reverse, GGGGTTGGAGGTAGAGCAAT; Smad3 Forward, CTCTCCAATGTCAACAGGAATG; Smad3 Reverse, AACTGGTAGACAGCCTCAAAGC; Smad4 Forward, GTCTTTGATTTGCGTCAGTGTC; Smad4 Reverse, AGTCTAAAGGTTGTGGGTCTGC; GAPDH Forward, CAACTACATGGTTTACATGTTC; and GAPDH Reverse, GCCAGTGGACTCCACGAC. Amplification reactions were terminated during the linear phase of amplification after 25 cycles (TIEG, Smad3, and Smad4) and 20 cycles (GAPDH). PCR products were visualized on 1.5% (w/v) agarose gels stained with ethidium bromide.

Microarray Analysis

RNA was harvested from control (duplicate), 12 h (duplicate) and 24 h (single) doxycycline treated Hs578T Tet-TIEG cells. The abundance of mRNA transcripts was measured by the use of high-density oligonucleotide arrays containing probes representing ~6,800 genes (HuGeneFL array; Affymetrix, Inc., Santa Clara, CA) at the Mayo Microarrary Core Facility.

RESULTS

Partial Requirement of Smad7 for TIEG Enhancement of TGFβ/Smad Signaling

Overexpression of TIEG was recently demonstrated to increase the activation of the TGFβ/Smad signaling pathway as measured by the enhanced induction of a synthetic Smad binding element (SBE) reporter construct ($CAGA_{12}$-MLP-Luc) upon TIEG overexpression [Johnsen et al., 2002]. Mechanistically, TIEG appears to enhance TGFβ/Smad signaling by suppressing Smad7 transcription, thus relieving the negative feedback imposed by Smad7. In order to examine whether the effect of TIEG on TGFβ/Smad signaling is due solely to repression of Smad7 promoter activity, we tested the ability of TIEG to enhance TGFβ/Smad signaling in the absence of Smad7 expression. This was performed by co-transfecting the SBE reporter construct into AKR2B cells along with various combinations of an activated type I TGFβ receptor (ALK5TD) which has previously been shown to mimic the effects of TGFβ treatment [Wieser et al., 1995], TIEG, Smad7 siRNA, or the appropriate controls. As shown in Figure 1, depletion of endogenous Smad7 by co-transfecting the cells with Smad7 siRNA results in a decreased

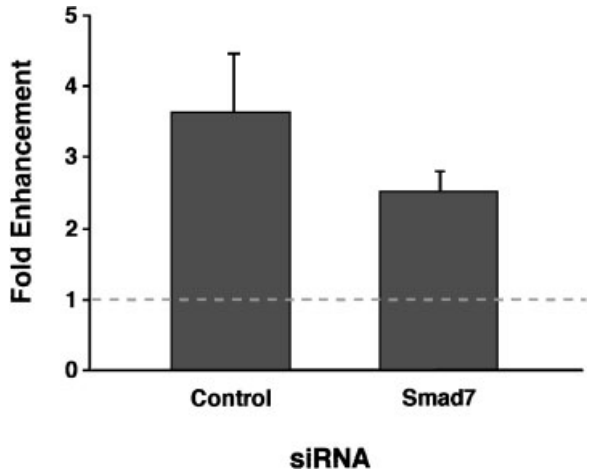

Fig. 1. TIEG mediated enhancement of Smad signaling is partially Smad7 independent. AKR2B cells were transfected with the $CAGA_{12}$-MLP-Luc (0.5 μg) reporter construct, the internal control plasmid phRG-TK (50 ng), ALK5TD (0.5 μg), and TIEG or empty vector (0.5 μg) along with 120 pmol Smad7 or control siRNAs as indicated. Normalized luciferase values were then compared between TIEG and vector transfected cells for each siRNA and graphed as Fold Enhancement. Data for a representative experiment are shown (n = 3). Error bars indicate SD.

ability of TIEG to enhance SBE induction in the presence of ALK5TD. Yet, while the effects of TIEG are diminished, TIEG expression is still capable of enhancing SBE reporter gene induction by more than twofold in the absence of Smad7. This suggests that TIEG functions by another mechanism to enhance Smad-mediated transcription other than Smad7 repression.

TIEG Does Not Enhance BMP Signaling

In addition to inhibiting TGFβ signaling, Smad7 also inhibits BMP signaling by binding to the type I BMP receptors [Casellas and Brivanlou, 1998; Souchelnytskyi et al., 1998; Fujii et al., 1999]. An element that is induced specifically by BMPs, but not TGFβ, was recently identified in the Id1 promoter [Katagiri et al., 2002; Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002]. In order to test whether TIEG overexpression also enhances BMP signaling, we transfected cells with a reporter construct containing multiple copies of the BMP inducible element (IdWT4F-luc), along with combinations of an activated type IA BMP receptor (ALK3QD) and TIEG or the respective control vectors. Overexpression of ALK3QD has previously been shown to mimic both the signal transduction and physiological effects of BMP treatment [Fujii et al., 1999]. As shown in Figure 2, overexpression of ALK3QD alone

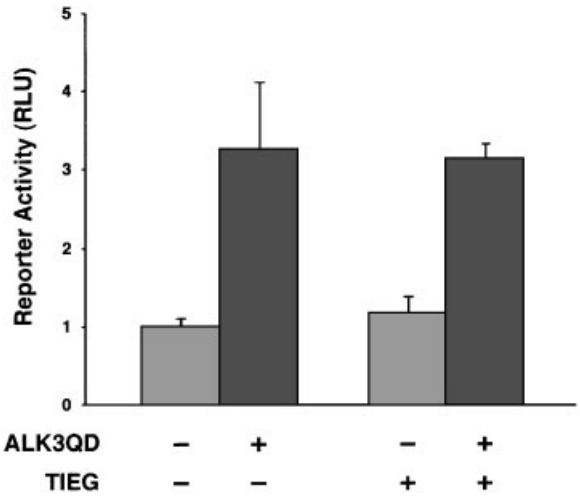

Fig. 2. TIEG does not enhance BMP signaling. AKR2B cells were transfected with the IdWT4F-luc (0.5 μg) reporter construct, the internal control plasmid pRL-CMV (25 ng), ALK3QD, or empty vector (0.5 μg), and TIEG or empty vector (0.5 μg) as indicated. Normalized relative luciferase values are shown for a representative experiment (n = 3). Error bars indicate SD.

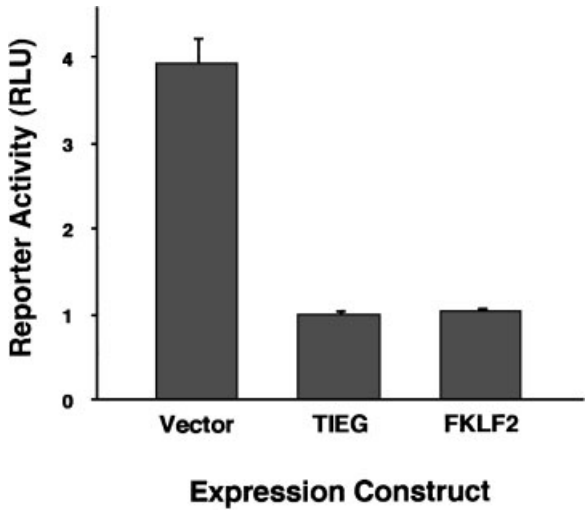

Fig. 3. TIEG and FKLF2 similarly repress Smad7 transcription. AKR2B cells were transfected with the murine Smad7 promoter reporter construct (0.5 μg), the internal control plasmid phRG-TK (50 ng), and TIEG, FKLF2, or empty vector (0.5 μg) as indicated. Normalized relative luciferase values are shown for a representative experiment (n = 3). Error bars indicate SD.

induces reporter gene activity more than threefold, and overexpression of TIEG has no effect on reporter gene induction. We conclude that TIEG specifically enhances TGFβ but not BMP signaling.

Smad7 Repression is not Sufficient for Enhanced TGFβ/Smad Signaling

TIEG was recently shown to belong to a subfamily of KLFs, which in addition to their highly conserved zinc finger DNA binding domain, share a common α-helical repression domain involved in the recruitment of histone deacetylase activity through mSin3A [Zhang et al., 2001]. Based on their structural and functional similarities they have been classified into the TIEG/BTEB subfamily of KLFs. Therefore, we sought to test whether one of these factors, FKLF2 (also called KLF13, RFLAT-1, and BTEB3) also functions in TGFβ/Smad signaling [Song et al., 1999; Asano et al., 2000; Kaczynski et al., 2001]. Initially we compared the abilities of the FKLF2 and TIEG proteins to repress Smad7 promoter activity. As shown in Figure 3, when a TIEG expression vector was co-transfected along with a reporter construct containing the mouse Smad7 promoter, a 75% decrease in Smad7 promoter activity relative to the vector control cells was observed. Transfection of an FKLF2 expression vector similarly decreases Smad7 promoter activity.

In order to test whether transcriptional repression of Smad7 is sufficient for enhancement of TGFβ/Smad signaling, and whether this effect is specific for TIEG, the TGFβ regulated SBE reporter construct ($CAGA_{12}$-MLP-Luc) was transfected into AKR2B cells along with various combinations of ALK5TD, TIEG, FKLF2, or empty expression vectors. As shown in Figure 4, overexpression of TIEG increases

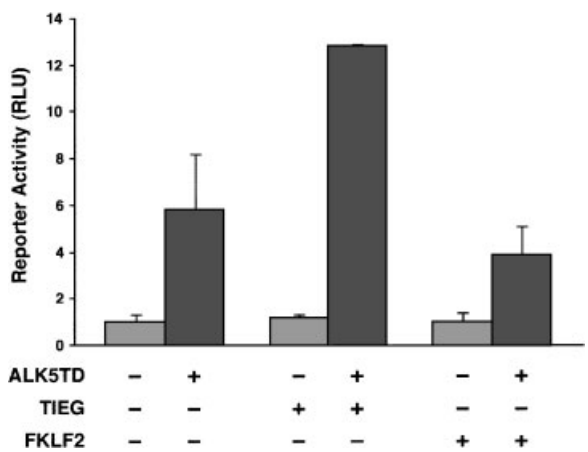

Fig. 4. TIEG, but not FKLF2, enhances SBE dependent transcription. AKR2B cells were transfected with the $CAGA_{12}$-MLP-Luc (0.5 μg) reporter construct, the internal control plasmid pRL-CMV (25 ng), ALK5TD, or empty vector (0.5 μg), and TIEG, FKLF2, or empty vector (0.5 μg) as indicated. Normalized relative luciferase values are shown for a representative experiment (n = 3). Error bars indicate SD.

the induction of SBE reporter activity by ALK5TD greater than twofold relative to vector transfected cells. In contrast, FKLF2 overexpression has no significant effect on SBE reporter induction. These data suggest that the related proteins, TIEG and FKLF2, are functionally different and that the ability to repress Smad7 expression does not fully account for TIEG's ability to stimulate SBE transcription.

Smad2 is a TIEG Regulated Gene

In order to identify additional regulatory targets of TIEG involved in the regulation of the TGFβ/Smad signal transduction pathway, we performed high-density oligonucleotide microarray analysis using RNA samples harvested from a doxycycline inducible TIEG overexpressing cell line treated with doxycycline for 0, 12, or 24 h. Interestingly, one of the genes observed to be increased in both the 12 h (3–4-fold) and 24 h (4–6-fold) samples was Smad2. In order to verify the regulation of Smad2 by TIEG, Northern blot analysis was performed. As shown in Figure 5A, the expression of both the 4.0 and 2.8 kb Smad2 transcripts is dramatically increased following induction of TIEG expression.

Since an increase in the transcription of Smad3 or Smad4 by TIEG would also be expected to enhance TGFβ/Smad signaling, the transcriptional regulation of these genes by TIEG was examined by RT-PCR. As shown in Figure 5B, the induction of TIEG expression with doxycycline in Hs578T-TIEG cells did not affect the expression of either Smad3 or Smad4. Thus, TIEG specifically upregulates Smad2 gene expression.

Smad2 is Required for Maximal TIEG Enhancement of TGFβ/Smad Signaling

Previous data have indicated that SBE dependent induction of gene expression remains intact in Smad2 null cells, presumably due to the presence of Smad3 [Piek et al., 2001]. In order to determine whether enhancement of TGFβ/Smad signaling by TIEG requires the induction of Smad2 mRNA, the SBE reporter construct along with combinations of ALK5TD and TIEG (or the appropriate control vectors) was transfected into wild-type and Smad2 null mouse embryo fibroblasts. As shown in Figure 6, TIEG co-transfection into wild-type cells increased ALK5TD triggered SBE reporter induction 2.5-fold compared to vector control. In contrast,

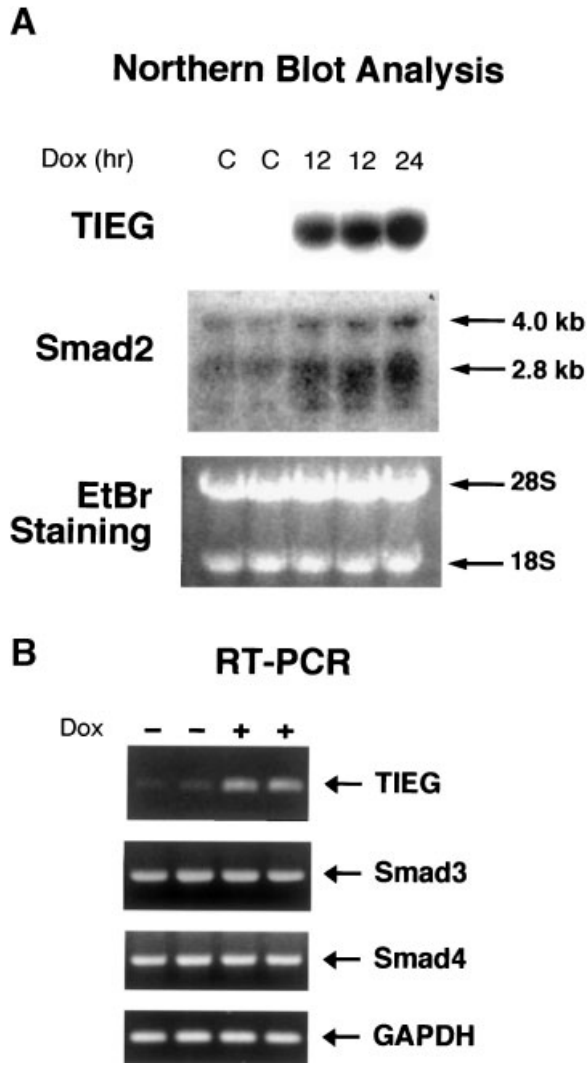

Fig. 5. TIEG specifically upregulates Smad2 transcription. **Panel A**: Hs578T-TIEG cells were treated with doxycycline (50 ng/ml doxycycline) for 0 (untreated), 12, or 24 h as indicated. Total RNA was extracted and analyzed by Northern blotting. Blots were probed for TIEG and Smad2 RNA transcripts. Prior to Northern blotting, the gel was stained with ethidium bromide to verify equal loading. **Panel B**: Total RNA was isolated from control and 24 h doxycycline (50 ng/ml) treated Hs578T-TIEG cells and reverse transcribed, and PCRs were performed in duplicate for TIEG, Smad3, Smad4, and GAPDH mRNA. The PCR products were separated on 1.5% (w/v) agarose gels and visualized with ethidium bromide.

in Smad2 null cells TIEG enhanced SBE reporter induction only marginally. These results indicate that TIEG's ability to regulate SBE transcription relies heavily on Smad2 upregulation.

DISCUSSION

TGFβ plays an important role in regulating a variety of cellular and developmental processes.

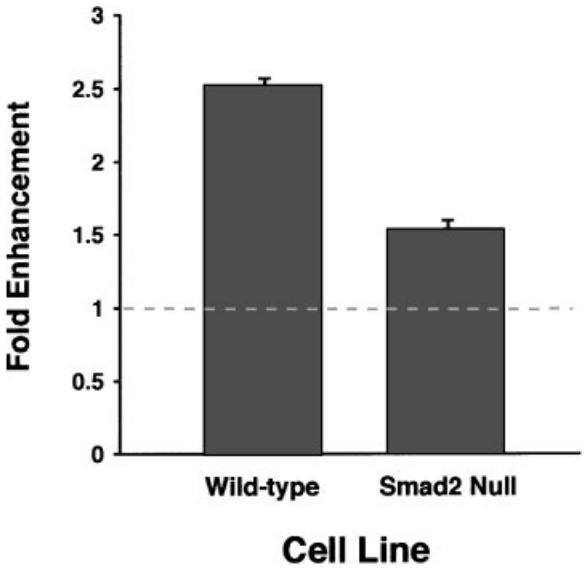

Fig. 6. Regulation of Smad2 is required for enhancement of SBE dependent transcription by TIEG. Wild-type and Smad2 null mouse embryo fibroblasts were transfected with the $CAGA_{12}$-MLP-Luc (0.5 μg) reporter construct, the internal control plasmid pRL-CMV (25 ng), ALK5TD (0.5 μg), and TIEG or empty vectors (0.5 μg) as indicated. Normalized luciferase values were then compared between TIEG and vector transfected cells for each cell line and graphed as Fold Enhancement. Data for a representative experiment are shown (n = 12). Error bars indicate SEM.

Most notably, misregulation of the TGFβ pathway can lead to tumor formation or increased tumorigenicity. For example, mutations or deletions in the TGFβ receptors, Smad2 or Smad4 are commonly observed in colorectal, pancreatic, and certain other types of cancer [Akhurst and Derynck, 2001]. Additional data suggests that Smad3 may also act as a tumor suppressor [Zhu et al., 1998]. Furthermore, overexpression of TGFβ signaling inhibitors, such as the inhibitory Smad6 and 7 proteins, c-Ski or SnoN, also enhances tumorigenicity or induces cellular transformation [Boyer et al., 1993; Kleeff et al., 1999b,c; Luo et al., 1999].

While insensitivity to TGFβ is common in cancer cells, some retain certain aspects of TGFβ responsiveness. For example, while normal mammary epithelial and many non-metastatic breast cancer cell lines are growth inhibited by TGFβ, many other, more invasive, breast cancer cell lines are not [Koli and Arteaga, 1996]. In fact, TGFβ often actually increases the aggressiveness or metastatic potential of the more advanced breast cancer cells [Koli and Arteaga, 1996]. This suggests that there is a switch in the responses elicited by TGFβ during the progression of certain types of cancer. The mechanism by which the cells change with respect to their TGFβ responsiveness is not known. One of the potential regulators of this process is TIEG. In support of this hypothesis, a decrease in the levels of TIEG protein correlates with an increase in the histological stage of breast cancer [Subramaniam et al., 1998]. Further support that TIEG serves a tumor suppressor role relates to the observation that TIEG is a negative regulator of cell growth in both pancreatic and osteosarcoma cells [Tachibana et al., 1997; Hefferan et al., 2000].

Previously, TIEG overexpression has been shown to mimic TGFβ action in osteosarcoma cells and to enhance TGFβ/Smad signaling, at least in part, by down-regulating negative feedback through the inhibitory Smad7 [Hefferan et al., 2000; Johnsen et al., 2002]. In the present study, we identified an additional role for TIEG in TGFβ signaling. We now show that the role of TIEG in TGFβ signaling contains both Smad7 dependent and Smad7 independent mechanisms since TIEG still enhances TGFβ/Smad signaling in the absence of Smad7 expression. Interestingly, we observed that while TIEG enhances TGFβ signaling, it does not enhance BMP signaling. This observation was initially surprising since Smad7 inhibits both TGFβ and BMP signaling [Nakao et al., 1997; Casellas and Brivanlou, 1998; Katagiri et al., 2000]. However, the induction of Smad7 by BMPs in many cell types is weaker than the induction by TGFβ [Takase et al., 1998]. This suggests that negative feedback to BMP signaling may primarily occur through Smad6 rather than Smad7. In this case, the effects of Smad7 repression may not appreciably affect BMP signaling. Further support that the function of TIEG in TGFβ/Smad signal transduction is not limited to repression of Smad7 expression is provided by the observation that, while both TIEG and FKLF2 repress Smad7 promoter activity, only TIEG enhances TGFβ signaling. Taken together these data suggest that there are additional mechanisms by which TIEG functions to differentially regulate the TGFβ and BMP signaling pathways.

The present study has identified one of these mechanisms as the upregulation of Smad2 expression. Consistently, the levels of Smad2 mRNA have been observed to increase following TGFβ treatment in certain cell lines [Kleeff et al., 1999a; Osaki et al., 1999]. Furthermore, the expression of Smad2 mRNA varies consider-

ably among different breast cancer cell lines and between different tissues [Takenoshita et al., 1998; Pouliot and Labrie, 1999]. Therefore, the subtle regulation of Smad2 mRNA levels may potentially play an important role in the types of responses elicited by TGFβ apart from the mutation or deletion of Smad pathway components.

It is also interesting that Smad2 mRNA levels are upregulated by TIEG, although TIEG has been described as a transcriptional repressor [Yajima et al., 1997; Cook et al., 1999; Zhang et al., 2001; Johnsen et al., 2002]. However, other KLF proteins, such as BTEB1 and FKLF2, possess both repressor and activator functions [Imataka et al., 1992; Asano et al., 2000; Zhang et al., 2001]. For example, FKLF2 is capable of binding both transcriptional co-activators and co-repressors [Zhang et al., 2001; Song et al., 2002]. The mechanisms by which FKLF2 functions as an activator in one context and a repressor in another context are not known, but may be related to a difference in the sequence of the two regulated elements [Asano et al., 2000; Kaczynski et al., 2001]. Similarly, while TIEG represses promoters containing a consensus Sp1 sequence, it also activates a basic transcription element, suggesting that TIEG may also function as both an activator and a repressor depending upon the regulated sequence [Blok et al., 1995; Yajima et al., 1997; Johnsen et al., 2002].

In conclusion, we have identified a new mechanism by which TIEG functions to enhance TGFβ/Smad signaling through the induction of Smad2 expression. These data broaden the role of TIEG in TGFβ signaling, and suggest that TIEG functions in a dual role in the Smad pathway by increasing Smad2 and decreasing Smad7 transcription. Thus, TIEG enhances Smad signaling by increasing both the pool of available Smad2 as well as the phosphorylation and activation of Smad2. These data may open a new area of research in cancer biology and may explain the differences in TGFβ responsiveness observed in different stages of cancer progression. Future studies will be necessary to determine whether a decrease, or loss, of TIEG expression is related to the switch in TGFβ responsiveness observed in the progression of metastatic breast cancer.

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