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Tieg3/Klf11 Induces Apoptosis in OLI-Neu Cells and Enhances the TGF-β Signaling Pathway by Transcriptional Repression of Smad7

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Abstract TGF-β signaling is indispensible for development of the nervous system since it regulates ontogenetic cell death. The recently identified TGF-β-inducible zinc finger protein Tieg3/Klf11 belongs to the family of Sp1/Klf-like transcription factors and shares all structural and functional features with other Tieg proteins. Using the established TGF-β-responsive oligodendroglial cell line OLI-neu, we analyzed the role of Tieg3/Klf11 in TGF-β signaling. In this report, we show that Tieg3/Klf11 mimics TGF-β effects by inducing apoptotic cell death accompanied by activation of caspase-3. Moreover, we demonstrate that Tieg3/Klf11 enhances TGF-β signaling by transcriptional repression of the inhibitory Smad7 and, thereby, disrupts the negative feedback loop of the TGF-β signaling pathway. Loss of the N-terminal repression domains of Tieg3/Klf11 abrogates the pro-apoptotic nature of this transcription factor and abolishes the enhancement of Smad-mediated TGF-β responses. In conclusion, we provide evidence that the recently identified transcription factor Tieg3/Klf11 is a downstream mediator of TGF-β-induced apoptosis in the oligodendroglial cell line OLI-neu. Since other signaling molecules are able to initiate transcription of members of the Tieg family, the ability of Tieg3/Klf11 to modulate TGF-β signaling by transcriptional inhibition of Smad7 might be an important clue for the understanding of the crosstalk between different signaling pathways. J. Cell. Biochem. 104: 850–861, 2008.

Key words: Tieg3; Klf11; TGF-β signaling; Smad7; apoptosis

Transforming growth factor β (TGF-β) signaling plays important roles during central nervous system development by regulating ontogenetic neuronal cell death [Krieglstein et al., 2000]. Different TGF-β isoforms exert their function by stimulating the formation of heteromeric complexes of type I (TβRI) and type II (TβRII) serine/threonine kinase receptors [Yamashita et al., 1994]. The constitutively active TβRII phosphorylates TβRI, which in turn phosphorylates the receptor-associated Smads (R-Smads), Smad2 and Smad3 [Wrana et al., 1994; Abdollah et al., 1997]. Activated R-Smads form a heterotrimeric complex with Smad4 and translocate to the nucleus, where they regulate the transcription of TGF-β target genes [Massague and Wotton, 2000]. TGF-β signaling is blocked by the inhibitory Smad7 [Nakao et al., 1997], which stably interacts with the TβRI and, thereby, prevents the association and phosphorylation of R-Smads [Hayashi et al., 1997]. In the recent years, Tieg proteins were described as TGF-β-inducible transcription factors that participate in TGF-β signaling. Human TIEG1/KLF10 [Subramaniam et al., 1995] and TIEG2/KLF11 [Cook et al., 1998] as well as murine Tieg1/Klf10 [Yajima et al., 1997; Fautsch et al., 1998] and Tieg3/Klf11 [Wang et al., 2004] are members of the family of Sp1/Krüppel-like zinc finger transcription factors. As a defining feature of the Sp1/Klf family, Tieg proteins share a highly conserved C-terminal DNA binding domain, consisting of three zinc finger motifs which facilitate interaction with GC-rich promoter elements. Another common feature of all Tieg proteins is

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the presence of three N-terminal repression domains [Cook et al., 1999], designated as R1, R2 and R3. Whereas the mechanisms of transcriptional repression mediated by R2 and R3 remain elusive, the inhibition of transcription by R1 is well understood. The R1 domain interacts via an alpha-helical motif with the corepressor mSin3A, which interferes with transcriptional initiation by modifying the chromatin structure of target genes via histone deacetylation [Zhang et al., 2001]. Therefore, R1 is often referred to as SID (mSin3A interacting domain).

It has been shown that Tieg proteins can act as growth inhibitory and/or pro-apoptotic proteins in different cell types [Tachibana et al., 1997; Chalaux et al., 1999; Fernandez-Zapico et al., 2003; Bender et al., 2004, Wang et al., 2007]. We have previously demonstrated that Tieg1/Klf10 is able to induce apoptosis in OLI-neu cells [Bender et al., 2004] and thereby mimics TGF-β effects [Schuster et al., 2002]. Moreover, Tieg proteins are able to modulate TGF-β responses by transcriptional silencing of Smad7 and disruption of the negative feedback loop within the TGF-β signaling pathway [Johnsen et al., 2002; Ellenrieder et al., 2004].

Tieg3 was recently identified as a new TGF-β-inducible member of the Sp1/Klf family of transcription factors in the oligodendroglial cell line OLI-neu [Wang et al., 2004]. It shares all the structural and functional properties of Tieg proteins [Spittau et al., 2007].

In the present study, we show that Tieg3 is a pro-apoptotic downstream mediator of TGF-β in OLI-neu cells. We further demonstrate that Tieg3/Klf11 increases the R-Smad activity by transcriptional repression of Smad7 and not by additional release of TGF-β. Moreover, these Tieg/Klf11-mediated effects are dependent on the presence of its N-terminal repression domains. Together, these results consolidate the role of Tieg3/Klf11 in TGF-β-mediated apoptosis in OLI-neu cells and imply that Tieg3/Klf11 is a potent modulator of the cellular TGF-β response by silencing Smad7 expression and disrupting the negative feedback loop of the TGF-β signaling pathway.

MATERIALS AND METHODS

Expression Vectors and Reporter Constructs

The pFLAG-CMV2 expression plasmid was purchased from Sigma (Deisenhofen, Germany), pEGFP-C2 vector was obtained from Clontech (Heidelberg, Germany). Generation of the plasmids pFLAG-Tieg3, pEGFP-Tieg3 and pEGFP-Tieg3ΔRD has been previously described [Spittau et al., 2007]. pGL3-SBE-Luc and pFLAG-Smad7 were kindly provided by Peter ten Dijke (Ludwig Institute for Cancer Research, Uppsala University, Sweden). Generation of Smad7 promoter constructs pGL3-Smad7-promoter and pGL3-Smad7-promoterΔSp1 was described by Brodin et al. [2000]. The NF-κB responsive reporter construct pGL3-HIV-LTR was a kind gift from Jakob Troppmair (Institute for Radiation and Cell Research, University of Würzburg, Germany) and contains the HIV long terminal repeat (LTR) sequences from −120 to +83, including two NF-κB motifs. The pCMV-βGal reporter driving expression of β-galactosidase under the control of the human cytomegalovirus promoter (CMV) was purchased from Clontech.

Cell Culture and Transfections

The oligodendroglial cell line, OLI-neu [Jung et al., 1995], was cultured in DMEM containing 1% horse serum, N2 supplement (GIBCO) and PSN (Sigma). Cells were grown in poly-L-lysine-coated flasks and incubated at 37°C and 5% CO_2. For individual experiments, cells were seeded on poly-L-lysine-coated glass coverslips or tissue culture plates. After cell transfection, the medium was replaced by DMEM containing 0.2% horse serum.

Luciferase Reporter Assay

The luciferase reporter assay system was used to determine the activity of several promoter constructs after transfection of OLI-neu cells with Tieg3 and Tieg3ΔRD expression plasmids. Sixty thousand cells per well were seeded on a 24-well tissue culture plate. After 24 h, cells were transfected with 800 ng of the expression vector, 200 ng of the luciferase reporter construct and 100 ng of pCMV-βGal internal control vector using 4 μl Lipofectamine™ 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. DNA complexes were removed after 5 h. After 19 additional hours, cells were harvested in lysis buffer (Tropix, Applied Biosystems) and cell lysates were analyzed for luciferase activity in a luminometer (LumatB5076, Berthold, Bad Wildbad, Germany). For every individual experiment,

luciferase activity was assessed in duplicates and normalized results were averaged. Mean firefly luciferase activity was normalized to the mean β-galactosidase activity of each sample to account for differences in transfection efficiency.

Detection of Activated Caspase-3

Active caspase-3 was detected using the Caspase-Glo 3/7 Assay (Promega). Briefly, 60,000 cells per well were seeded on a 24-well tissue culture plate and transfected with EGFP expression plasmids. After 24 h of incubation, the cells were washed with PBS and lysed with Lysis buffer (Tropix, Applied Biosystems). Protein lysates were treated according to the manufacturer's instructions. The activation of caspase-3 and caspase-7 was proportional to the luciferase activity of the cell lysates yielded. Results, expressed as relative light units, were normalized to native culture medium and are given as percentages of control. All measurements were performed in duplicates.

RNA Isolation and RT-PCR Analysis

Total RNA was extracted from OLI-neu cells using TRIzol reagent (GIBCO-BRL) according to the manufacturer's instructions. cDNAs were prepared from 1 μg RNA using 1 μl of Reverse Aid™ reverse transcriptase according to the manufacturer's instructions (Fermentas). PCR amplification was performed using Tieg3 N-terminal and C-terminal-specific primers (N-terminal forward: 5′-CCTGATCTACCAAAGGACTT-3′, N-terminal reverse: 5′-GATTACTTGGGAAGGAACAG-3′, C-terminal forward: 5′-CGGGATCCTTGTGTGTCCTGTGTGTGAC-3′, C-terminal reverse: 5′-CGGGATCCATTCCTGAAGAGGCTTT-3′) and Gapdh-specific primers (Gapdh forward: 5′-ATGACTCTACCCACGGCAAG-3′, Gapdh reverse: 5′-GATCTCGCTCCTGGAAGATG-3′). PCR products were separated on a 2% agarose gel and DNA was visualized with ethidium bromide staining.

Electrophoresis and Immunoblotting

Cells were lysed in lysis buffer supplemented with Complete Protease Inhibitor cocktail (Roche). Cell lysates (25 μg total protein/well) were loaded on a 9% SDS–acrylamide gel for electrophoresis. For Western blot analysis, proteins were transferred onto a PVDF membrane (Immobilon, Millipore). Membranes were blocked overnight at +4°C on a shaker in TTBS with 10% dry milk (Roth, Germany).

For detection of Smad7, membranes were incubated with a rabbit anti-Smad7 antibody (Abcam) at a dilution of 1:500 at +4°C overnight. The primary antibody treatment was followed by treatment with a HRP-conjugated donkey anti-rabbit antibody (GE Healthcare) at a dilution of 1:10,000 for 1 h at +4°C. Labeled proteins were detected using Immobilon Western chemilumnescent HRP substrate (Millipore). Protein levels of Smad1, Smad2 and Smad3 were determined using the mouse-anti-Smad1/2/3 antibody (Santa Cruz, sc-7960, 1:500) at +4°C overnight and a HRP-conjugated goat anti-mouse antibody (1:10,000, Abcam). For detection of phosphorylated Smad2, a rabbit anti-phospho-Smad2 antibody (Cell Signaling, 1:1,000) was used. To confirm equal protein loadings, membranes were reprobed with a Gapdh antibody (1:10,000, Abcam) and HRP-conjugated goat anti-mouse antibody (1:10,000, GE Healthcare). All blots were captured with Amersham Hyperfilm™ ECL (GE Healthcare).

TGF-β Release Assay

Mink lung epithelial cells (MLECs), stably transfected with an expression construct containing a truncated PAI-1 promoter fused to the firefly luciferase gene, were used to determine the release of TGF-β. The assay was performed as described by Abe et al. [1994]. Briefly, MLECs were seeded in a 96-well tissue culture plate (16,000 cells/well) and allowed to attach for 6 h at 37°C and 5% CO_2. Medium of untreated OLI-neu cells and OLI-neu cells transfected with the plasmids pEGFP, pEGFP-Tieg3 and pEGFP-Tieg3ΔRD were collected after 24 h of incubation. The different media were either left untreated for the detection of active TGF-β or were incubated with 1 M HCl to activate latent TGF-β, followed by treatment with 1 M NaOH to adjust the pH. MLECs were incubated for 18 h with the different media and harvested in lysis buffer (Tropix, Applied Biosystems). Luciferase activity was analyzed in duplicates with a luminometer (LumatB5076, Berthold, Bad Wildbad, Germany).

Fluorescence Microscopy

OLI-neu cells were seeded on poly-L-lysine-coated glass cover slips laid in each well of a 24-well tissue culture plate (60,000 cells/well).

After transfection with 800 ng pEGFP-C2 derived Tieg3 expression plasmids and 24 h of incubation, the cells were washed with PBS. Glass coverslips were mounted with Vecta-SHIELD® Mounting Medium containing DAPI (1:1,000) to counterstain the nuclei. Epifluorescence was visualized immediately with a fluorescence microscope (Zeiss).

Quantification of Apoptosis

Twentyfour hours after transfection of OLI-neu cells with pEGFP-C2, pEGFP-Tieg3 or pEGFP-Tieg3ΔRD the nuclei were counterstained with DAPI (1:1,000) to distinguish apoptotic cells with condensed and fragmented nuclei from normal cells. Quantification of apoptosis was achieved by counting the numbers of transfected cells and the numbers of apoptotic cells in corresponding visual fields. Ten randomly chosen fields were analyzed with a minimum number of 400 transfected cells counted for each transfection condition. The extent of apoptotis is given as percentage of transfected cells.

Statistics

Relative light units are given as percentage of control ± SEM. Statistical significances were determined with paired *t*-tests using the program GraphPad Prism4 (GraphPad Software Inc.). *P*-values <0.05 were considered as significant.

RESULTS

The N-Terminal Repression Domains of Tieg3 Are Essential to Induce Apoptotis

To analyze the biological role of Tieg3 in the oligodendroglial cell line OLI-neu, cells were transfected with the constructs pEGFP-C2 (control), pEGFP-Tieg3 (wild type) and pEGFP-Tieg3ΔRD, respectively. An overview of the plasmid constructs used is given in Figure 1A. We have previously reported that loss of the three N-terminal repression domains does not alter the subcellular distribution of Tieg3 but leads to a loss of Tieg3-mediated transcriptional repression in OLI-neu cells [Spittau et al., 2007]. Thus, the construct pEGFP-Tieg3ΔRD was used to overexpress a dominant negative Tieg3 mutant in contrast with wild type Tieg3. Overexpression of Tieg3 and Tieg3ΔRD was verified at RNA (Fig. 1B) and protein levels (Fig. 1C). Expression of EGFP alone did not change the morphology of OLI-neu cells after 24 h, as shown in Figure 1C. In contrast, overexpression of wild type Tieg3 was accompanied by nuclear shrinkage and chromatin condensation, serving as hallmarks of apoptotic cell death (Fig. 1C). This effect was not detectable after transfection of OLI-neu cells with pEGFP-Tieg3ΔRD, a construct coding for a truncated Tieg3 lacking N-terminal repression domains. Cells expressing EGFP-Tieg3ΔRD showed a normal nuclear morphology, as shown in Figure 1C. Nuclear localization of the mutant protein was not altered, indicating that N-terminal truncation of Tieg3 did not cause changes in the physiological intracellular localization.

To quantify the extent of apoptosis, the ratio of cells with typical morphological criteria among transfected cells was determined. EGFP-Tieg3 significantly increased the number of apoptotic cells ($46.86 \pm 7.86\%$) 24 h after transfection. In contrast, overexpression of EGFP-Tieg3ΔRD did not result in an increase in apoptosis ($9.78 \pm 7.13\%$). As shown in Figure 1D, this extent of apoptotic cell death was comparable to that observed after overexpression of EGFP alone ($12.70 \pm 3.39\%$). Recent studies have shown that Tieg-induced apoptotic cell death is accompanied by activation of caspase-3 [Bender et al., 2004; Wang et al., 2007]. Here we show that overexpression of EGFP-Tieg3 also results in activation of caspase-3, whereas EGFP-Tieg3ΔRD is not able to cause this effect (Fig. 1E). Bender et al. [2004] have previously shown that overexpression of Tieg1/Klf10 results in a decrease in NF-κB activity. In contrast, we show here that overexpression of FLAG-tagged- and EGFP-tagged Tieg3/Klf11 significantly increases the activity of the survival factor NF-κB in OLI-neu cells. Furthermore, these effects were dependent on the presence of the N-terminal repression domains of Tieg3/Klf11 (Fig. 1F).

These results indicate that Tieg3 is a protein with pro-apoptotic functions in the oligodendroglial cell line OLI-neu. Moreover, the observed data suggest that the N-terminal repression domains of Tieg3, mediating transcriptional silencing of target genes, are essential for its pro-apoptotic feature.

Overexpression of Tieg3 Results in Increased Activity of R-Smads

Human TIEG1/KLF10 and TIEG2/KLF11 have been shown to repress the transcription

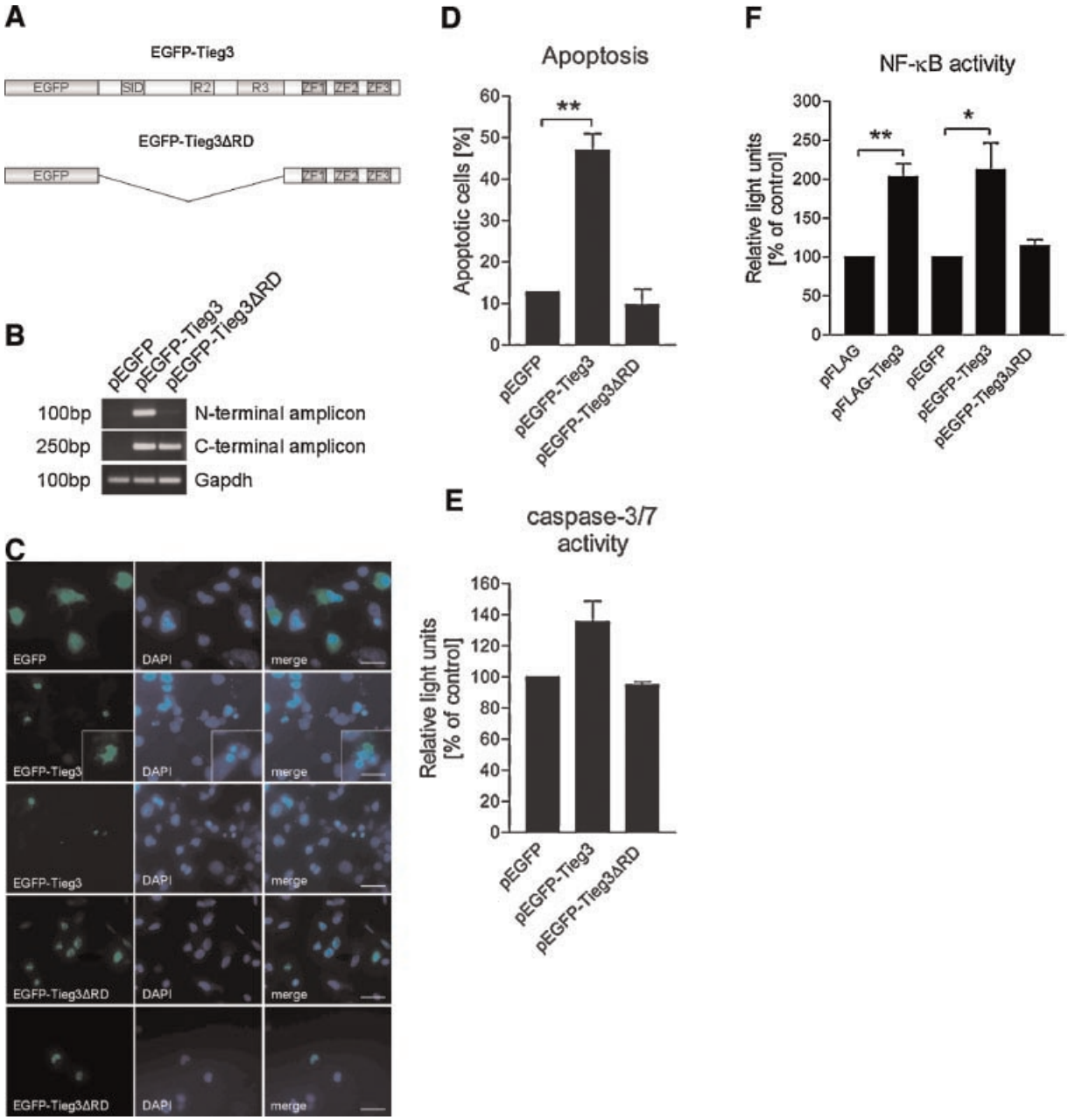

Fig. 1. The N-terminal repression domains of Tieg3 are essential for the induction of apoptosis. OLI-neu cells were transiently transfected with pEGFP, pEGFP-Tieg3 and pEGFP-Tieg3ΔRD, respectively. Schematic overview of the EGFP-Tieg3 and EGFP-Tieg3ΔRD mutant. SID: mSin3A interacting domain, R: repression domain, ZF: zinc finger (**A**). To confirm transcription from the different expression plasmids, RT-PCR analysis with N-terminal and C-terminal specific primers was performed 24 h after transfection with pEGFP-derived Tieg3-expression plasmids. Gapdh was used as control for equal cDNA loading per reaction (**B**). Wild type EGFP, EGFP-Tieg3 and EGFP-Tieg3ΔRD were detected by epifluorescence in OLI-neu cells 24 h after transfection. Counterstaining with DAPI facilitates analysis of the condensation state of chromatin after overexpression of EGFP fusion proteins (**C**). Scale bars: 20 μm for overall figure, 10 μm for insets. The pictures are representative of three independent experiments. To quantify the extent of apoptosis, the numbers of cells with condensed and fragmented nuclei were determined and are given as percentages of transfected cells from five independent experiments ± SEM (**D**). Tieg3-induced apoptosis is accompanied by activation of caspase-3 (**E**). To detect active caspase-3, the luciferase-dependent Caspase-Glo 3/7 assay (Promega) was used. Using the NF-κB responsive reporter construct pGL3-HIV-LTR, we observed that Tieg3 increases the activity of NF-κB in OLI-neu cells 24 h after transfection (**F**). Data are given as means of three independent experiments ± SEM. *P*-values derived from Student's *t*-test are $^{**}<0.01$, $^{*}<0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of the inhibitory SMAD, SMAD7, thereby, potentiating TGF-β signaling [Johnsen et al., 2002; Ellenrieder et al., 2004]. To analyze the potential of Tieg3 to influence the negative feedback loop within the TGF-β signaling pathway, we first determined R-Smad activity after overexpression of EGFP-Tieg3 and EGFP-Tieg3ΔRD using the pGL3-SBE-Luc reporter construct (Fig. 2A). Additionally, the construct pFLAG-Tieg3 was used to alleviate the concern that the relatively large EGFP could alter the physiological function of Tieg3 if expressed as a fusion protein. Figure 2B shows that the R-Smad activity was significantly increased in cells overexpressing FLAG-tagged Tieg3 (871.0 ± 68.94%) and EGFP-Tieg3 (549.1 ± 179.0%). Overexpression of EGFP-Tieg3ΔRD still resulted in a significantly increased R-Smad activity (206.9 ± 37.54%) compared to the effects after overexpression of wild type EGFP, but in a decreased activity compared with overexpression of EGFP-Tieg3 (Fig. 2B). To support these findings we determined the levels of phosphorylated Smad2 after overexpression of EGFP, EGFP-Tieg3 and EGFP-Tieg3ΔRD. Figure 2C shows that the levels of phospho-Smad2 were increased in the presence of EGFP-Tieg3. The dominant negative mutant EGFP-Tieg3ΔRD was not able to increase the level of phosphorylated Smad2. This implies that the N-terminal repression domains of Tieg3 are important for

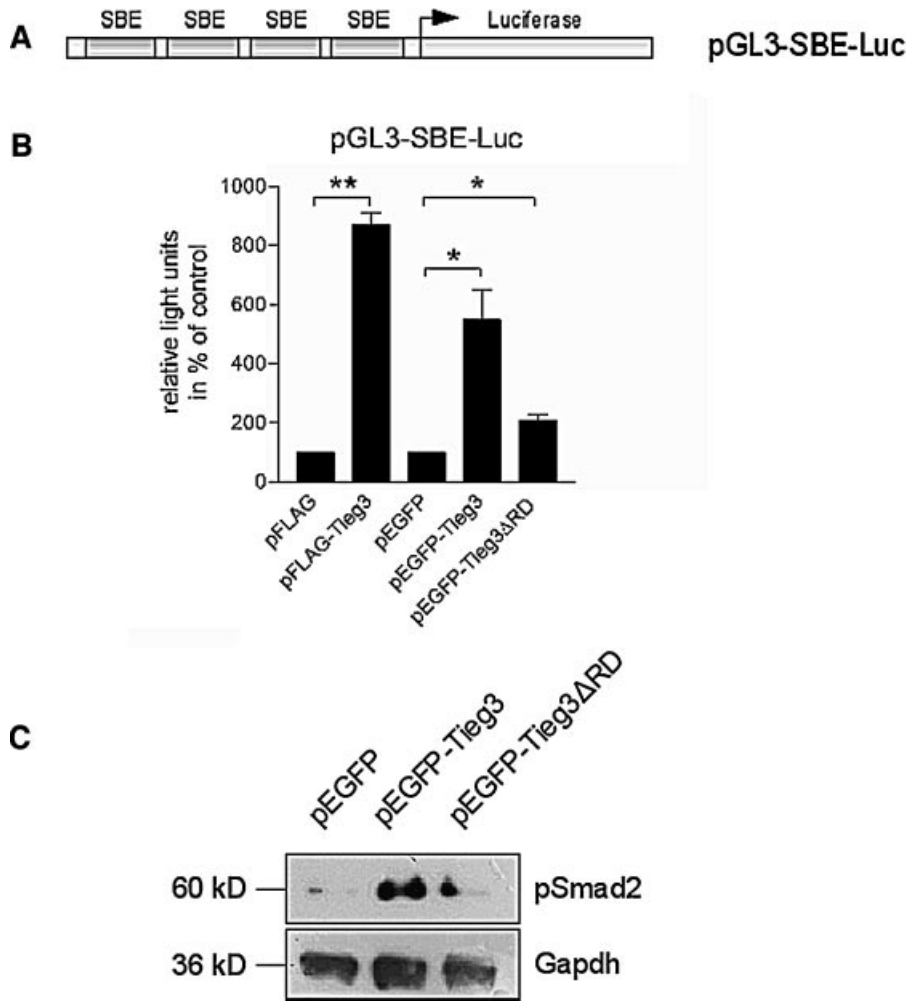

Fig. 2. Tieg3 enhances the activity of R-Smads. The reporter plasmid pGL3-SBE-Luc (**A**) was used to monitor R-Smad activity. This construct contains 4 Smad-binding elements (SBE) cloned upstream of the luciferase firefly gene. Overexpression of FLAG-tagged Tieg3 and EGFP-Tieg3 significantly increased the R-Smad activity in OLI-neu cells 24 h after transfection. In the presence of the dominant negative EGFP-Tieg3ΔRD the R-Smad activity was strongly reduced compared with the effect of EGFP-Tieg3 (**B**). Data are given as means of three different experiments ± SEM. *P*-values derived from Student's *t*-test are $^{**}<0.01$, $^{*}<0.05$ compared with control. (**C**) The protein levels of phosphorylated Smad2 are increased 24 h after overexpression of EGFP-Tieg3. The phospho-Smad2 antibody specifically detects Smad2 phosphorylated at Serine residues 465/467.

Tieg3-mediated enhancement of the TGF-β signaling pathway.

Increased R-Smad Activity Is Not Mediated by Tieg3-Induced Release of TGF-β

The promoters of the genes coding for TGF-β1 and TGF-β2 contain several Sp1-sites [Geiser et al., 1991; Noma et al., 1991]. Thus, we analyzed whether the increased R-Smad activity after Tieg3 overexpression could be the result of an increased secretion of TGF-β from OLI-neu cells. Using mink lung epithelial cells (MLEC), we determined the amount of TGF-β in the culture medium of transfected OLI-neu cells after 24 h. Neither FLAG-tagged Tieg3 nor EGFP-Tieg3 were able to increase the amount of TGF-β in the culture medium, as shown in Figure 3. However, this experiment demonstrated that OLI-neu cells secrete a basal level of TGF-β which explains the basal R-Smad activity observed in the luciferase experiments. Thus, the distinct increase of the R-Smad activity after Tieg3/Klf11 overexpression is not mediated by an increased release of endogenous TGF-β, indicating that secretion of TGF-β from OLI-neu cells is not regulated by Tieg3/Klf11.

Tieg3 Inhibits Expression of Smad7

Given the fact that TIEG1/KLF10 and TIEG2/KLF11 inhibit SMAD7 expression, we determined the expression of the luciferase gene driven by the murine Smad7 promoter in the presence of Tieg3 and the N-terminal deletion mutant Tieg3ΔRD. Figure 4A gives an overview of the Smad7-promoter constructs used. As shown in Figure 4B, FLAG-tagged Tieg3 as well as EGFP-Tieg3 significantly inhibited transcription driven by the Smad7 promoter ($57.92 \pm 10.97\%$ and $44.53 \pm 15.17\%$, respectively). In contrast, the dominant negative EGFP-Tieg3ΔRD had no significant influence on the activity of the Smad7 promoter. Since the Smad7 promoter contains several GC-rich Sp1-sites [Brodin et al., 2000], serving as possible binding sites for Tieg3, we, further, used the reporter construct pGL3-Smad7-promoterΔSp1 (Fig. 4A), where these Sp1-sites had been deleted. Using these truncated construct, neither FLAG-tagged Tieg3 nor EGFP-Tieg3 were able to reduce transcription of the firefly luciferase gene driven by this promoter (Fig. 4C). These observations indicate that the transcriptional repression of Smad7 is the result of a direct interaction of the N-terminal domains of Tieg3 with the Sp1-sites in the Smad7-promoter.

To confirm that Tieg3-mediated transcriptional repression of Smad7 has an impact on Smad7 protein levels, we performed Western blot analysis 24 h after transfection of OLI-neu

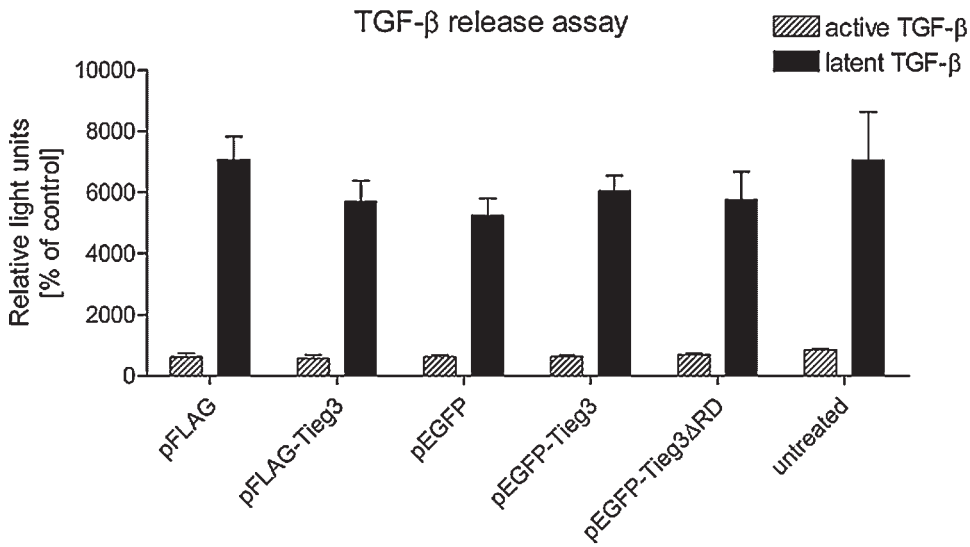

Fig. 3. Tieg3 has no influence on the TGF-β release from OLI-neu cells. To determine the TGF-β release from transfected OLI-neu cells, mink lung epithelial cells were incubated for 18 h with culture medium from OLI-neu cells 24 h after transfection with pFLAG- and pEGFP-derived Tieg3 expression plasmids. Active TGF-β was monitored in untreated media, whereas latent TGF-β was activated by treatment of media with 1 M hydrochloric acid. Data are given as means of three independent experiments ± SEM.

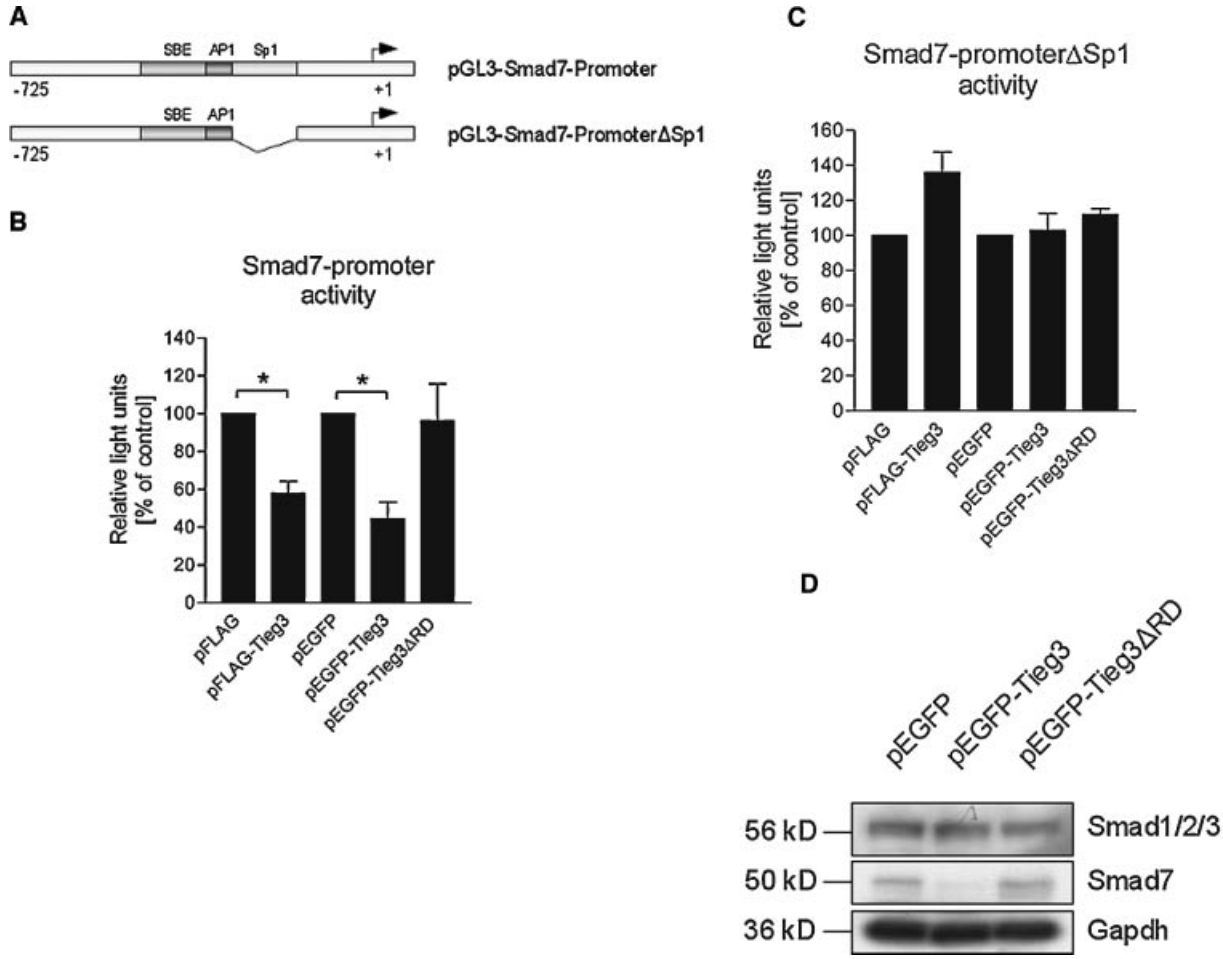

Fig. 4. Tieg3 represses the transcription of Smad7 in OLI-neu cells. To analyze the effect of Tieg3 on Smad7 promoter activity, the constructs pGL3-Smad7-promoter and pGL3-Smad7-promoterΔSp1 were used (**A**). Twentyfour hours after overexpression of FLAG-Tieg3 and EGFP-Tieg3, the activity of the Smad7 promoter was significantly reduced compared with effects after expression of FLAG and EGFP alone. The dominant negative EGFP-Tieg3ΔRD had no significant effect on the Smad7 promoter (**B**). Similar to the effect of EGFP-Tieg3ΔRD, deletion of the Sp1-sites within the Smad7-promoter abrogated the Tieg3-mediated transcriptional repression after 24 h (**C**). Data are given as means of three independent experiments ± SEM. *P*-values derived from Student's *t*-test are *<0.05 compared with control. Smad7 protein expression level is reduced 24 h after overexpression of EGFP-Tieg3 but not after overexpression of the dominant negative mutant EGFP-Tieg3ΔRD. The protein expression levels of Smad1/2/3 are not changed after overexpression of EGFP-Tieg3 or EGFP-Tieg3ΔRD (**D**). This blot is representative of three independent experiments with similar results. Gapdh was used as control for equal protein loading.

cells with pEGFP-derived Tieg3 expression plasmids. As shown in Figure 4D, overexpression of EGFP-Tieg3 resulted in a dramatic decrease in Smad7 protein expression levels. In contrast, the dominant negative EGFP-Tieg3ΔRD mutant did not change the protein levels of Smad7. The overexpression of FLAG-tagged Tieg3 also reduced the protein levels of Smad7 in OLI-neu cells after 24 h (data not shown). Further, Figure 4D shows that the levels of Smad1/2/3 are not altered after overexpression of EGFP-Tieg3 and EGFP-Tieg3ΔRD indicating that Tieg3/Klf11 has no regulatory effect on these genes in OLI-neu cells. Together, these data support that Tieg3/Klf11 reduces Smad7 protein levels by interacting with Sp1-sites in the Smad7 promoter and transcriptional inhibition via its N-terminal repression domains.

Overexpression of Smad7 Abrogates Tieg3-Dependent R-Smad Activation

To support the hypothesis that the increased R-Smad activity after overexpression of Tieg3 is mediated by the transcriptional repression of the inhibitory Smad7, we overexpressed Smad7

in the presence of Tieg3. For this purpose, OLI-neu cells were transiently transfected with pFLAG- and pEGFP-derived Tieg3-expression plasmids alone and in combination with pFLAG-Smad7. After 24 h, the cells were harvested and lysates were used to determine the SBE-dependent luciferase activity. As shown in Figure 5, overexpression of Smad7 strongly decreased the R-Smad activities induced by FLAG-tagged Tieg3 and EGFP-Tieg3, respectively. These results support the idea that transcriptional repression of the inhibitory Smad7 is responsible for increased R-Smad activity after overexpression of Tieg3.

DISCUSSION

Tieg3/Klf11 was originally identified as a TGF-β-inducible Sp1/Klf-like transcription factor in the oligodendroglial cell line OLI-neu [Wang et al., 2004] and shares all structural and functional features with other Tieg proteins [Spittau et al., 2007]. In the present study, we provide evidence for Tieg3/Klf11 being a pro-apoptotic transcription factor in the oligodendroglial cell line OLI-neu. We further show that the N-terminal repression domains of Tieg3/Klf11 are essential to activate caspase-3 and to induce apoptotic cell death. It has been shown that both TGF-β and Tieg1/Klf10 are able to induce caspase-3-dependent apoptosis in OLI-neu cells [Schuster et al., 2002; Bender et al., 2004]. Thus, Tieg3/Klf11 is able to mimic these effects and acts as a downstream mediator in TGF-β-induced apoptosis. Interestingly, overexpression of Tieg1/Klf10 and Tieg3/Klf11 result in the same effects in OLI-neu cells, indicating that these two proteins are functionally redundant at least in this cell line. This observation is in accordance with the silent phenotypes of the knock-out animals for Tieg1/Klf10 and Tieg3/Klf11, both of which show normal embryonic development into adulthood [Song et al., 2005; Subramaniam et al., 2005] suggesting that loss of either Tieg1/Klf10 or Tieg3/Klf11 is dispensable for normal development. However, the exact role of the two proteins in development and maintenance of adult tissues remains unclear and has to be further elucidated.

We demonstrate that the N-terminal repression domains, which are the defining feature of the Tieg family, are essential for the Tieg3/Klf11-mediated effects in OLI-neu cells. Loss of these domains might abrogate the interaction of Tieg3/Klf11 with the corepressor complex mSin3A which contributes to transcriptional silencing by modifying the chromatin structure via histone deacetylation. Although it has been shown that Tieg proteins can act as transcriptional repressors and activators depending on the cell type and the promoter analyzed [Noti et al., 2004; Neve et al., 2005], our results clearly demonstrate that transcriptional repression

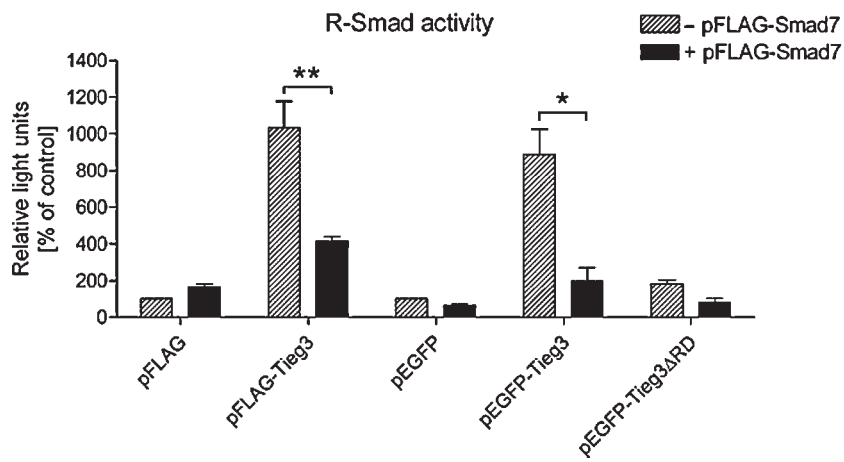

Fig. 5. Overexpression of Smad7 abrogates the Tieg3/Klf11-dependent effects on R-Smad activity. Twentyfour hours after transfection of pFLAG- and pEGFP-derived expression plasmid together with pFLAG-Smad7, the cells were harvested and lysates were used to determine the luciferase activity. In the presence of Smad7 the Tieg3-dependent R-Smad activity was significantly reduced. Data are given as means of three independent experiments ± SEM. *P*-values were derived from Student́s *t*-test analysis (**<0.01 and *<0.05).

mediated by Tieg3/Klf11 is necessary to activate caspase-3 and to induce apoptosis in OLI-neu cells.

Overexpression of Tieg3/Klf11 results in an increase in R-Smad activity in OLI-neu cells. The R-Smads, Smad2 and Smad3, are activated via phosphorylation upon binding of the TGF-β ligand to its receptors [Abdollah et al., 1997]. We demonstrated that no additional TGF-β was secreted after overexpression of Tieg3/Klf11, suggesting that TGF-β-dependent phosphorylation is not the only mechanism of the observed R-Smad activity. It has been shown that human TIEG1/KLF10 and TIEG2/KLF11 are able to inhibit the transcription of the inhibitory Smad, SMAD7 [Johnsen et al., 2002; Ellenrieder et al., 2004]. In light of these previous reports, we demonstrated that Tieg3/Klf11 represses the transcription of Smad7 in OLI-neu cells resulting in reduced protein levels of Smad7 and increased R-Smad activity. The Smad7 promoter contains several Sp1-sites [Brodin et al., 2000], which are essential for Tieg3/Klf11-mediated transcriptional repression as demonstrated using a Sp1-site-deficient Smad7 promoter construct. Truncation of the N-terminal repression domains of Tieg3/Klf11 also led to a loss of repression of transcription driven by the wild type Smad7 promoter. Thus, we propose that Smad7 is a target gene of the TGF-β-inducible transcription factor Tieg3/Klf11 in OLI-neu cells.

The functional role of Smad7 in TGF-β-induced apoptosis seems to be cell type and tissue specific. In B-lymphocytes, Smad7 is upregulated to inhibit TGF-β- and Activin-A-induced apoptosis [Ishisaki et al., 1998; Patil et al., 2000]. Lallemand et al. [2001] showed that Smad7 inhibits the survival factor NF-κB and potentiates TGF-β-mediated apoptosis in epithelial cells. In contrast, Smad7 mediates TGF-β-induced apoptosis in mesangial cells [Okado et al., 2002] and prostate carcinoma cells [Landström et al., 2000]. However, in OLI-neu cells Tieg3/Klf11-mediated apoptosis is accompanied by downregulation of Smad7 and activation of NF-κB. Furthermore, the increase in NF-κB activity is dependent on the presence of the N-terminal repression domains of Tieg3/Klf11. Interestingly, overexpression of Tieg1/Klf10 in OLI-neu cells results in a decrease in NF-κB activity [Bender et al., 2004]. An attractive explanation might be that both murine Tieg isoforms induce apoptosis in OLI-neu cells by activating different pro-apoptotic pathways. At the moment we are not able to give a satisfying explanation for the diverse effects of Tieg1/Klf10 and Tieg3/Klf11 on the regulation of NF-κB activity. Thus, a more detailed analysis of the molecular mechanism behind this phenomenon is necessary.

The fact that Tieg3/Klf11 and other members of the Tieg family inhibit the transcription of Smad7 and, thereby, enhance the intensity and duration of TGF-β signaling may provide important clues to understand the crosstalk between different signaling pathways. Interestingly, it has been shown that the induction of Tieg proteins is not restricted to TGF-β. Indeed, estrogen [Tau et al., 1998], BMP2, BMP4, Activin [Hefferan et al., 2000], CTGF [Wahab et al., 2005] and GDNF [Yajima et al., 1997] are also able to initiate transcription of Tieg encoding genes and may influence TGF-β signaling in a Tieg-dependent manner.

In conclusion, this study shows that the TGF-β-inducible transcription factor Tieg3/Klf11 induces apoptosis in the oligodendroglial cell line OLI-neu. Moreover, we observed an increase in R-Smad activity which was the result of Tieg3/Klf11-mediated transcriptional repression of the inhibitory Smad7. The presence of the N-terminal repression domains of Tieg3/Klf11 is essential for its pro-apoptotic nature and for the disruption of the negative feedback loop of the TGF-β signaling pathway by inhibition of Smad7 expression. These results clearly demonstrate that Tieg3/Klf11, like Tieg1/Klf10, mimics TGF-β effects in OLI-neu cells and, thereby, participates in TGF-β signaling. By enhancing the duration and intensity of the TGF-β response, Tieg3/Klf11 could be an important mediator between different signaling pathways and might contribute to the diverse biological effects of TGF-β in different cell types and tissues.

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REFERENCES

Abdollah S, Macias-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL. 1997. TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. J Biol Chem 272:27678–27685.

Abe M, Harpel JG, Metz CN, Loskutoff DJ, Rifkin DB. 1994. An assay for transforming growth factor-β using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. Anal Biochem 216:276–284.

Bender H, Wang Z, Schuster N, Krieglstein K. 2004. TIEG1 facilitates transforming growth factor-beta-mediated apoptosis in the oligodendroglial cell line OLI-neu. J Neurosci Res 75:344–352.

Brodin G, Ahgren A, ten Dijke P, Heldin CH, Heuchel R. 2000. Efficient TGF-beta induction of the Smad7 gene requires cooperation between AP-1, Sp1, and Smad proteins on the mouse Smad7 promoter. J Biol Chem 275:29023–29030.

Chalaux E, Lopez-Rovira T, Rosa JL, Pons G, Boxer LM, Bartrons R, Ventura F. 1999. A zinc-finger transcription factor induced by TGF-β promotes apoptotic cell death in epithelial Mv1Lu cells. FEBS Lett 457:478–482.

Cook T, Gebelein B, Mesa K, Mladek A, Urrutia R. 1998. Molecular cloning and characterization of TIEG2 reveals a new subfamily of transforming growth factor-β-inducible Sp1-like zinc finger-encoding genes involved in the regulation of cell growth. J Biol Chem 273:25929–25936.

Cook T, Gebelein B, Belal M, Mesa K, Urrutia R. 1999. Three conserved transcriptional repressor domains are a defining feature of the TIEG subfamily of Sp1-like zinc finger proteins. J Biol Chem 274:29500–29504.

Ellenrieder V, Buck A, Harth A, Jungert K, Buchholz M, Adler G, Urrutia R, Gress TM. 2004. KLF11 mediates a critical mechanism in TGF-beta signaling that is inactivated by Erk-MAPK in pancreatic cancer cells. Gastroenterology 127:607–620.

Fautsch MP, Vrabel A, Rickard D, Subramaniam M, Spelsberg TC, Wieben ED. 1998. Characterization of the mouse TGFβ-inducible early gene (TIEG): Conservation of exon and transcriptional regulatory sequences with evidence of additional transcripts. Mamm Genome 9:838–842.

Fernandez-Zapico ME, Mladek A, Ellenrieder V, Folch-Puy E, Miller L, Urrutia R. 2003. An mSin3A interaction domain links the transcriptional activity of KLF11 with its role in growth regulation. EMBO J 22:4748–4758.

Geiser AG, Kim SJ, Roberts AB, Sporn MB. 1991. Characterization of the mouse transforming growth factor-beta 1 promoter and activation by the Ha-ras oncogene. Mol Cell Biol 11:84–92.

Hayashi H, Abdollah S, Qui Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Jr., Wrana JL, Falb D. 1997. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. Cell 89:1165–1173.

Hefferan TE, Subramaniam M, Khosla S, Riggs BL, Spelsberg TC. 2000. Cytokine-specific induction of the TGF-beta inducible early gene (TIEG): Regulation by specific members of the TGF-beta family. J Cell Biochem 78:380–390.

Ishisaki A, Yamato K, Nakao A, Nonaka K, Ohguchi M, ten Dijke P, Nishihara T. 1998. Smad7 is an activin-inducible inhibitor of activin-induced growth arrest and apoptosis in mouse B cells. J Biol Chem 273:24293–24296.

Johnsen SA, Subramaniam M, Janknecht R, Spelsberg TC. 2002. TGFbeta inducible early gene enhances TGFbeta/Smad-dependent transcriptional responses. Oncogene 21:5783–5790.

Jung M, Krämer EM, Grzenkowski M, Tang K, Blakemore W, Aguzzi A, Khazaie K, Chlichlia K, von Blankenfeld G, Kettenmann H, Trotter J. 1995. Lines of murine oligodendroglial precursor cells immortalized by an activated neu tyrosine kinase show distinct degrees of interaction with axons in vitro and in vivo. Eur J Neurosci 7:1245–1265.

Krieglstein K, Richter S, Farkas L, Schuster N, Dünker N, Oppenheim RW, Unsicker K. 2000. Reduction of endogenous transforming growth factors beta prevents ontogenetic neuron death. Nat Neurosci 3:1085–1090.

Lallemand F, Mazars A, Prunier C, Bertrand F, Kornprost M, Gallea S, Roman-Roman S, Cherqui G, Atfi A. 2001. Smad7 inhibits the survival nuclear factor kappaB and potentiates apoptosis in epithelial cells. Oncogene 20: 879–884.

Landström M, Heldin NE, Bu S, Hermansson A, Itoh S, ten Dijke P, Heldin CH. 2000. Smad7 mediates apoptosis induced by transforming growth factor beta in prostatic carcinoma cells. Curr Biol 10:535–538.

Massague J, Wotton D. 2000. Transcriptional control by the TGF-β/Smad signaling system. EMBO J 19:1745–1754.

Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH, ten Dijke P. 1997. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. Nature 389:631–635.

Neve B, Fernandez-Zapico ME, Ashkenazi-Katalan V, Dina C, Hamid YH, Joly E, Vaillant E, Benmezroua Y, Durand E, Bakaher N, Delannoy V, Vaxillaire M, Cook T, Dallinga-Thie GM, Jansen H, Charles MA, Clement K, Galan P, Hercberg S, Helbecque N, Charpentier G, Prentki M, Hansen T, Pedersen O, Urrutia R, Melloul D, Froguel P. 2005. Role of transcription factor KLF11 and its diabetes-associated gene variants in pancreatic beta cell function. Proc Natl Acad Sci 102:4807–4812.

Noma T, Glick AB, Geiser AG, ÓReilly MA, Miller J, Roberts AB, Sporn MB. 1991. Molecular cloning and structure of the human transforming growth factor-beta 2 gene promoter. Growth Factors 4:247–255.

Noti JD, Johnson AK, Dillon JD. 2004. The zinc finger transcription factor transforming growth factor beta-inducible early gene-1 confers myeloid-specific activation of the leukocyte integrin CD11d promoter. J Biol Chem 279:26948–26958.

Okado T, Terada Y, Tanaka H, Inoshita S, Nakao A, Sasaki S. 2002. Smad7 mediates transforming growth factor-beta-induced apoptosis in mesangial cells. Kidney Int 62: 1178–1186.

Patil S, Wildey GM, Brown TL, Choy L, Derynck R, Howe PH. 2000. Smad7 is induced by CD40 and protects WEHI 231 B-lymphocytes from transforming growth factor-beta -induced growth inhibition and apoptosis. J Biol Chem 275:38363–38370.

Schuster N, Bender H, Philippi A, Subramaniam S, Strelau J, Wang Z, Krieglstein K. 2002. TGF-beta induces cell

death in the oligodendroglial cell line OLI-neu. Glia 40:95–108.

Song CZ, Gavriilides G, Asano H, Stamatoyannopoulos G. 2005. Functional study of transcription factor KLF11 by targeted gene inactivation. Blood Cells Mol Dis 34:53–59.

Spittau B, Wang Z, Boinska D, Krieglstein K. 2007. Functional domains of the TGF-beta-inducible transcription factor Tieg3 and detection of two putative nuclear localization signals within the zinc finger DNA-binding domain. J Cell Biochem 101:712–722.

Subramaniam M, Harris SA, Oursler MJ, Rasmussen K, Riggs BL, Spelsberg T. 1995. Identification of a novel TGF-β-regulated gene encoding a putative zinc finger protein in human osteoblasts. Nucl Acids Res 23:4907–4912.

Subramaniam M, Gorny G, Johnsen SA, Monroe DG, Evans GL, Fraser DG, Rickard DJ, Rasmussen K, van Deursen JM, Turner RT, Oursler MJ, Spelsberg TC. 2005. TIEG1 null mouse-derived osteoblasts are defective in mineralization and in support of osteoclast differentiation in vitro. Mol Cell Biol 25:1191–1199.

Tachibana I, Imoto M, Adjei PN, Gores GJ, Subramaniam M, Spelsberg TC, Urrutia R. 1997. Overexpression of the TGFβ-regulated zinc finger encoding gene, TIEG, induces apoptosis in pancreatic epithelial cells. J Clin Invest 99:2365–2374.

Tau KR, Hefferan TE, Waters KM, Robinson JA, Subramaniam M, Riggs BL, Spelsberg TC. 1998. Estrogen regulation of a transforming growth factor-beta inducible early gene that inhibits deoxyribonucleic acid synthesis in human osteoblasts. Endocrinology 139:1346–1353.

Wahab NA, Weston BS, Mason RM. 2005. Modulation of the TGFbeta/Smad signaling pathway in mesangial cells by CTGF/CCN2. Exp Cell Res 307:305–314.

Wang Z, Peters B, Klussmann S, Bender H, Herb A, Krieglstein K. 2004. Gene structure and evolution of Tieg3, a new member of the Tieg family of proteins. Gene 325:25–34.

Wang Z, Spittau B, Behrendt M, Peters B, Krieglstein K. 2007. Human TIEG2/KLF11 induces oligodendroglial cell death by downregulation of Bcl-X(L) expression. J Neural Transm 114:867–875.

Wrana JL, Attisano L, Wieser R, Ventura F, Massague J. 1994. Mechanism of activation of the TGF-beta receptor. Nature 370:341–347.

Yajima S, Lammers C, Lee S, Hara Y, Mizuno K, Mouradian MM. 1997. Cloning and characterization of murine glial cell-derived neurotrophic factor inducible transcription factor (MGIF). J Neurosci 17:8657–8666.

Yamashita H, ten Dijke P, Franzen P, Miyazono K, Heldin CH. 1994. Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. J Biol Chem 269:20172–20178.

Zhang J, Moncrieffe MC, Kaczynski J, Ellenrieder V, Pendergast FG, Urrutia R. 2001. A conserved α-helical motif mediates the interaction of Sp1-Like transcriptional repressors with the corepressor mSin3A. Mol Cell Biol 21:5041–5049.