Smad7 Inhibits Fibrotic Effect of TGF-β on Renal Tubular Epithelial Cells by Blocking Smad2 Activation

JIN H. LI,*† HONG-JIAN ZHU,‡ XIAO R. HUANG,*‡ KAR N. LAI,†
RICHARD J. JOHNSON,* and HUI Y. LAN,∗†

*Department of Medicine, Baylor College of Medicine, Houston, Texas; †Department of Medicine, The University of Hong Kong, Hong Kong; and ‡Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Victoria, Australia.

Abstract. It has been shown that transforming growth factor–β (TGF-β) is a potent mediator in renal fibrosis and that Smad proteins are critical intracellular mediators in TGF-β signaling. It is here reported that TGF-β mediates renal fibrogenesis in tubular epithelial cells (TEC) in association with the activation of Smad2 and that overexpression of Smad7 blocks this fibrotic process. Using a normal rat kidney tubular epithelial cell line (NRK52E), it was determined that TGF-β1 induces Smad2 phosphorylation and nuclear localization in both a dose- and time-dependent manner. The activation of Smad2 was evident at 5 min (20%), peaked at 15 to 30 min (85%), and declined to baseline levels by 2 h (5 to 10%). This was associated with de novo expression of collagens I, III, and IV and the transformation of TEC into a “myofibroblast” phenotype with de novo expression of α-smooth muscle actin (α-SMA) and with the loss of E-cadherin (≥50%). To investigate a negative regulatory role of Smad7 in renal fibrosis, the Smad 7 gene was stably transfected and its expression was tightly controlled by doxycycline into NRK52E cells. Overexpression of Smad7 induced by doxycycline results in marked inhibition of TGF-β–induced Smad2 activation (90%) with the prevention of collagen synthesis and myofibroblast transformation. Thus, Smad2 activation occurs in the fibrogenic response of TEC to TGF-β, and this process is blocked by overexpression of Smad7. This indicates that Smad signaling is a key pathway of TGF-β–mediated renal fibrosis and suggests that treatments targeting the inactivation of Smad2 by overexpression of Smad7 may provide a new therapeutic strategy for renal fibrosis.

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Correspondence to: Dr. Hui Y. Lan, Department of Medicine-Nephrology, Baylor College of Medicine, One Baylor Plaza, Alkek N520, Houston, TX 77030. Phone: 713-798-1303; Fax: 713-798-5010; E-mail: hlan@bcm.tmc.edu

Tubulointerstitial fibrosis is a final common pathway leading to end-stage renal failure regardless of the initial cause of the disease, and it is a major determinant of progressive renal injury (1). Tubulointerstitial fibrosis is histologically characterized by tubular loss and the accumulation of extracellular matrix (ECM) proteins, including fibronectin and collagens (types I, III, IV, V, and VII) (1). ECM is thought to be produced mainly by myofibroblasts, although tubular epithelial cells (TEC) can also synthesize a variety of ECM proteins in response to stimuli such as transforming growth factor–β (TGF-β) (2). It has been shown that TEC can express fibroblast specific protein–1 (FSP-1) (3). Furthermore, we have recently demonstrated that TEC are profibrotic cells capable of transforming into ECM-producing myofibroblasts (α-smooth muscle actin [α-SMA]*) and that they play an active role in the development of tubulointerstitial fibrosis (4).

TGF-β is a secreted signaling molecule with fibrotic properties, and it regulates a diverse range of cellular responses, including proliferation, differentiation, migration and apoptosis (5,6). There is increasing evidence that TGF-β is a key mediator of fibrosis in both experimental and human kidney diseases (5,6). This is clearly illustrated by the finding that renal fibrosis can be induced by the deliberate overexpression of TGF-β1 within the normal kidney (7). TGF-β stimulates ECM deposition by both increasing the synthesis of ECM proteins and by inducing the production of protease inhibitors, which block their degradation (6). In vitro, TGF-β has been shown to stimulate collagen synthesis and induce expression of FSP-1 in cultured TEC (8). Most recently, we demonstrated that TGF-β is able to induce normal TEC to transform into a myofibroblast phenotype (9). Tubular epithelial cells expressing a myofibroblast-like phenotype have also been identified in human glomerulonephritis with progressive tubulointerstitial fibrosis (10). These studies suggest that TGF-β may play an important role in the pathogenesis of tubulointerstitial fibrosis.

Recent studies have suggested a pivotal role of Smads as intracellular effector molecules of the TGF-β family members (11,12). TGF-β exerts its biologic effects by signaling through a heteromeric receptor complex of the type I and type II serine/threonine kinase receptors, TβRI and TβRII (11,12). In the absence of TGF-β, TβRI and TβRII form a latent receptor complex. Upon TGF-β binding, the receptors rotate relatively within the complex (11,12), resulting in phosphorylation of TβRI by the constitutively active and autoprophosphorylated TβRII and thereby activate TβRI. The activated TβRI then
directly signals to downstream intracellular substrates, Smad2 and Smad3 (R-Smads). Activated R-Smads heterologimerize with the common partner Smad4 (Co-Smad) and these complexes are translocated into the nucleus to target gene expression. Activation of the TGF-β signaling pathway can also result in the expression of inhibitory Smads (I-Smads), including Smad6 and Smad7. These inhibitory Smads appear to act by specifically inhibiting Smad2 and Smad3 phosphorylation by blocking their access to TβRI (11,12).

The involvement of Smads in TGF-β–mediated renal fibrosis has been demonstrated by the groups of Poncelet et al. (13,14) and Schiffer et al. (15), with the findings that Smads may play a role in TGF-β–induced collagen matrix synthesis in human mesangial cells and podocyte apoptosis during glomerulosclerosis. In this study, we have examined the role of Smad signaling in the fibrogenic response of TEC (NRK52E) to TGF-β. We report that TEC stimulated with TGF-β caused activation of Smad2 (phosphorylation and nuclear localization) with the de novo expression of collagens I, III, and IV and the transformation of TEC into a “myofibroblast” phenotype (α-SMA+) with the loss of E-cadherin. We also report that overexpression of Smad7 blocks Smad2 activation and prevents collagen synthesis and myofibroblast transformation. Thus, this study demonstrates the actual role for Smad signaling in regulating Smad7 and counter-regulating Smad7 tubular fibrogenesis in response to TGF-β.

Materials and Methods

Establishing Doxycycline-Regulated Smad7-Expressing TEC Cell Lines

The Doxycycline (Dox)–regulated Smad7-expressing cell line was established as described previously (16), but with a well-characterized normal rat kidney TEC line (NRK52E). Mouse Smad7 cDNA with a flag tag (m2) at its NH2 terminus in pcDNA3 was a generous gift from Dr. Peter ten Dijke (Ludwig Institute for Cancer Research, Uppsala, Sweden). To subclone the flag-tagged Smad7 cDNA into a tetracycline (Tet)–inducible vector, pTRE (Clontech, Palo Alto, CA), to obtain pTRE-Smad7, a BamHI-XbaI fragment encoding full-length Smad7 and flag tag at its NH2 terminus, and an Xhol-BamHI fragment from pTRE were ligated into pTRE at its Xhol and XbaI sites. An improved pTet-on vector (Clontech), pEF-purop-Tet-on, was generously provided by Dr. Gino Vario (Cerylid, Melbourne, Australia). Briefly, the gene encoding the “reverse” Tet repressor was subcloned into a pEF-BOS vector, pEFr-PGKpuropAv18, which confers puromycin resistance. Thus, the EF-1 promoter drives the expression of the reverse Tet repressor, and stable cell lines can be selected by puromycin. To obtain Dox (a Tet derivative)–induced Smad7-expressing NRK52E cell lines, pTRE-Smad7 and pEF-purop-Tet-on were cotransfected into NRK52E cells by electroporation, and then the stable transfected cells were selected in the presence of puromycin (2 μg/ml). Positive clones were confirmed by their ability to express Smad7 in the presence of Dox by Western blot analyses and immunohistochemistry using an anti-flag antibody m2 (IBI; Eastman Kodak, Rochester, NY). Three clones of stable Smad7–expressing NRK52E cell lines were obtained, and clone S7-7 was used in this study.

Cell Culture

The stable, Dox-regulated Smad7–expressing NRK52E cells were grown in a 5% CO2 atmosphere at 37°C in Dulbecco’s modified Eagle’s medium (Life Technologies BRL, Gaithersburg, MD) containing 0.5% fetal bovine serum, 60 μg/ml penicillin, and 100 μg/ml streptomycin in 6-well plastic plates or eight-chamber glass slides (Nunc, Naperville, CT). A recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) at concentrations of 0, 0.615, 1.25, 2.5, 5, and 10 ng/ml were added into the cell culture for periods of 0, 5, 15, 30, 60, and 120 min and 1, 3, and 5 d. To induce overexpression of Smad7, cells were treated with Dox at designated concentrations of 0, 0.125, 0.25, 0.5, 1, and 2 μg/ml for 24 h. TGF-β1 was then added at the designated concentrations for periods as described above. At least four experiments were performed throughout the study.

Reverse Transcriptase–PCR Analyses

Total RNA was isolated using the High Pure RNA Isolation Kit according to the manufacturer’s instructions (Roche Molecular Biochemicals, Indianapolis, IN). Contaminated DNA was removed by treating the samples with RNAase-free DNase I (Promega, Madison, WI). Reverse transcriptase–PCR (RT-PCR) was performed using a ThermoScrip RT-PCR Kit following the manufacturer’s instruction (Life Technologies BRL). The first-strand cDNA was synthesized by using oligonucleotide primers and M-MLV reverse transcriptase (Promega) before PCR amplification (35 cycles) using primers specific for mouse Smad2 (5'-GGTCAATCCAGCAAGGATC-3' and 5'-CTCATGCGTTGACATC-3'), Smad7 (5'-CATTACGAGGAGGAAGAGGA-3' and 5'-CTGGTTGAAGATGACTTCTAC-3'), rat collagen type I (5'-TGGCGGTACCTCAAGATG-3' and 5'-TGGCAGATGTGGT-3'), collagen type III (5'-CGTCGAAGATGACTTCTAC-3'), and α-SMA (5'-GAC CAT CGG TGG ATG AGT GAA-3' and 5'-CAG CTA ATG GGT GCA-3'), rat collagen type IV (5'-CTGGTTGAAGATGACTTCTAC-3'), and 5'-GCT CAT GAG ATG AGT GAA-3'), and GAPDH (5'-TGGCACCCTTCTGGATTG-3' and 5'-CAC GGA AGG CCC TGG TGG-3'). All samples were subjected to RT-PCR for housekeeping gene GAPDH as a positive control and as an internal standard. Afterward, RT-PCR products were resolved on 1.5% agarose gels in 1× Tris-borate-EDTA (TBE) buffer, visualized by ethidium bromide, photographed using a gel 1000 ultraviolet documentation system (Bio-Rad, Hercules, CA), and analyzed by densitometry.

Western Blot Analyses

NRK52E cells grown in six-well plates with or without TGF-β1 or Dox were analyzed by Western blotting as described previously (9). Cells were washed in phosphate-buffered saline (PBS) and then lysed in 1 ml of 1% Nonidet P-40, 25 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0, containing a 1:50 dilution of a protease inhibitor cocktail (P2714; Sigma, St. Louis, MO) for 30 min on ice. Samples were centrifuged at 14,000 × g for 5 min to pellet cell debris. Samples (20 μg) were mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for 5 min, electrophoresed on a 10% SDS polyacrylamide gel, and electroblotted onto Hybond-ECL nitrocellulose membrane (Amersham International, Buckinghamshire, UK). The membrane was blocked in PBS containing 5% skimmed milk powder and 0.02% Tween 20. To detect phosphorylated Smad2 (p-Smad2), total Smad2 and Smad7, collagen types I, III, and IV protein synthesis, and tubulin epithelial-
myofibroblast transformation (TEMT) with the de novo expression of α-SMA and the loss of E-cadherin, the membrane was incubated for 1 h with mouse monoclonal (mAb) to Smad2 (Transduction Laboratories, Lexington, KY) or polyclonal antibodies (Ab) to Smad7 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), p-Smad 2 (Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal antibodies to collagen types I, III, and IV (Santa Cruz Biotechnology), mAb to α-SMA (1A4, Sigma), and anti-E-cadherin antibody (Santa Cruz Biotechnology). After washing, the membrane was incubated with a 1:20,000 dilution of peroxidase-conjugated goat anti-mouse IgG or swine anti-rabbit IgG in PBS containing 1% normal goat serum and 1% fetal calf serum. The blot was then developed using the ECL detection kit (Amersham International) to produce a chemiluminescence signal, which was captured on x-ray film.

**Immunocytochemical Analyses**

NRK52E cells were cultured in eight-chamber glass slides in the presence or absence of TGF-β1 or Dox as described above. Cells were analyzed for activation of Smad2 using the anti–p-Smad2 antibody. Briefly, cells were fixed in 2% paraformaldehyde and preincubated with 10% fetal calf serum and 10% normal sheep serum to block nonspecific binding. Cells were then incubated with the anti–p-Smad2 Ab or an irrelevant isotype control rabbit IgG at 4°C overnight. After inactivation of endogenous peroxidase, cells were incubated with peroxidase-conjugated goat anti-mouse IgG or swine anti-rabbit IgG and then by the mouse or rabbit peroxidase anti-peroxidase complexes. After being washed, slides were developed with diaminobenzidine to produce a brown color, and the cell nucleus was counterstained with hematoxylin.

Microwave-based two-color immunostaining was used to examine TGF-β–induced TEC-myofibroblast transformation and the inhibition of this process by Dox-induced overexpression of m2-Smad7 (9,17). Briefly, after color developing with the anti–α-SMA mAb as described above, cells were treated with 10 min of microwave oven heating in 10 mM sodium citrate (pH 6.0) at 2450 MHz and 800 watts of power to block antibody cross-reactivity and to facilitate antigen retrieval. Cells were then incubated with the anti-m2 mAb (IBI, Eastman Kodak Co.) or control mAb (73.5, which recognized human CD45R antigen) for 60 min and then with alkaline phosphatase-conjugated goat anti-mouse IgG and mouse alkaline phosphatase anti-alkaline phosphatase complexes. Finally, sections were developed with Fast Blue BB Base (Sigma) and coverslipped in an aqueous mounting medium. All procedures were performed at room temperature.

**Quantitative Analyses**

For quantitative analyses of p-Smad2 transnuclear location, Smad7 expression, and α-SMA, the percentage of positive cells in eight-chamber slides stained for various Abs as described above was determined by counting at least 1000 cells under high power (×400) in each well. All scoring was performed blind on coded slides.

**Statistical Analyses**

Data obtained from this study are expressed as the mean ± SD. Statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Software, Inc. San Diego, CA). Differences in p-Smad2, Smad7, ECM, and E-cadherin expression were assessed by one-way ANOVA or by t test.

**Results**

**TGF-β1 Induces Activation of Smad2 in Renal Tubular Epithelial Cells**

To examine the kinetics of TGF-β–induced Smad2 activation in TEC, NRK52E cells were cultured with the TGF-β1 for periods of time and examined for Smad2 activation. As shown in Figure 1A, immunocytochemical analyses demonstrated that TGF-β1 (10 ng/ml) induced a rapid transnuclear location of p-Smad2 in a time-dependent manner, being evident at 5 min (20%), peaking over 15 to 30 min (85%), and then declining to normal levels by 2 h (5 to 10%). Consistent with these observations, Western blot analyses showed that TGF-β1–induced Smad2 phosphorylation in both a time-dependent and dose-dependent fashion (Figure 1B).
**Smad7 Inhibits TGF-β-Induced Smad2 Phosphorylation and Nuclear Localization**

Smad7 has been reported as an intracellular negative regulator of TGF-β signaling, particularly in R-Smad activation (11,12). To investigate the counter-regulating role of Smad7 on TGF-β-induced Smad2 activation in renal TEC, we established a cell line in which the expression of Smad7 in NRK52E cells was tightly regulated by Dox. We first examined the ability of Dox to induce Smad7 expression. As shown in Figure 2A, cells cultured in the presence of Dox for 24 h exhibited a strong Smad7 expression in a dose-dependent manner, with maximal expression at 2 μg/ml. We then examined the counter-regulating role of Smad7 in the inhibition of TGF-β-induced Smad2 activation. As demonstrated by Western blot analyses, TGF-β–induced Smad2 phosphorylation was inhibited by the addition of Dox in both a time-dependent and dose-dependent fashion, with marked inhibition of Smad2 phosphorylation at a dose of 2 μg/ml of Dox (Figure 2, B and C). This inhibitory effect is on the activation (phosphorylation), because Dox-induced Smad7 overexpression did not alter Smad2 expression at both protein (unphosphorylated) and mRNA levels (Figure 2, D and E). Immunostaining also showed that inducing overexpression of Smad7 by the addition of Dox caused a marked inhibition of Smad2 transnuclear location in a dose-dependent manner (Figure 2F). These data demonstrate that overexpression of Smad7 is able to negatively regulate TGF-β-induced Smad2 activation in NRK52E cells.

**Regulating Role of Smad Signaling on the Fibrogenic Effects of TGF-β on Renal Tubular Epithelial Cells**

We previously reported that TGF-β stimulates fibrotic transdifferentiation of renal tubular epithelial cells into myofibroblasts (9). We now examined whether this TGF-β–induced fibrotic process is regulated by Smad signaling. Using a Dox-regulated Smad7–expressing NRK52E cells, TGF-β1 (in the absence of Dox) induced NRK52E cells to transform into a myofibroblast phenotype as evidenced by de novo synthesis of α-SMA mRNA (Figure 3A) and protein (Figure 3B), a myofibroblast marker, and by the loss of the epithelial marker, E-cadherin mRNA and protein, a molecule that plays a key role in maintaining the integrity of epithelial cells (Figure 3, A and B). Furthermore, TGF-β1 also induced the synthesis of ECM mRNA (Figure 3A) and protein (Figure 3B) by TEC, including collagen types I, III, and IV in a dose-dependent manner. RT-PCR showed that a significant increase in α-SMA and collagen (types I, III, and IV) mRNA expression was evident at day 1 (P < 0.001) and steadily increased with time, and this was associated with the loss of E-cadherin mRNA expression (Figure 4, A and C). We then examined whether TGF-β–induced fibrotic effects on TEC can be blocked by Smad7. As shown in Figures 4, A(i) and C(i), and 5A, TGF-β–induced low levels of endogenous Smad7 mRNA and protein expression in a time-dependent manner, being significant at day 3. Strikingly, the addition of Dox for 24 h before the addition of TGF-β–termed day 0) induced marked Smad7 mRNA and protein expression in a time-dependent and/or dose-dependent manner (Figures 4, B(i) and C(i), and 5A). When overexpression of Smad7 was induced, TGF-β–induced TEC transdifferentiation into ECM-producing myofibroblasts was significantly inhibited. This was demonstrated by inhibition of de novo α-SMA mRNA and protein synthesis, the maintenance of E-cadherin expression, and inhibition of collagen mRNA and protein synthesis (Figures 4, B and C, and 5). The suppressive effect of TGF-β–induced TEC-myofibroblast transformation and ECM production by Smad7 was time-dependent (Figure 4, B and C) and dose-dependent (Figure 5). These results were further supported by two-color immunostaining in which cells expressing Smad7 (induced by Dox) did not express the myofibroblast marker (α-SMA*)

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**Figure 2. Inhibition of Smad2 nuclear localization and phosphorylation by doxycycline (Dox)–induced overexpression of Smad7 in TEC.**

(A) Western blot analyses show that Dox induces Smad7 overexpression by NRK52E TEC at 24 h in a dose-dependent manner. (B) Western blot analyses show that Dox-induced overexpression of Smad7 inhibits TGF-β–induced Smad2 phosphorylation (30 min) by TEC in a dose-dependent manner. (C) Western blot analyses show that Dox-induced expression of Smad7 inhibits TGF-β (10 ng/ml)–induced Smad2 phosphorylation by TEC in a time-dependent manner. (D) Western blot analyses show that Dox-induced overexpression of Smad7 does not alter TGF-β (10 ng/ml)–induced total Smad2 protein levels. (E) Reverse transcriptase-PCR (RT-PCR) shows that Dox-induced overexpression of Smad7 does not alter TGF-β (10 ng/ml)–induced Smad2 mRNA expression. (F) Immunocytochemical analyses show that Dox-induced overexpression of Smad7 inhibits TGF-β (10 ng/ml for 30 mins)–induced p-Smad2 nuclear localization (dark black nucleus) by TEC in a dose-dependent manner (Magnification, ×400). Nucleus was counterstained with hematoxylin, and each bar represents the mean ± SD for groups of five experiments. Data in (A through E) represent an example for four experiments. **P < 0.01; ***P < 0.001 compared with Dox 0.
versely correlated with the number of α-SMA⁺ cells (Figure 6D).

Discussion

In this study, we have established a Dox-inducible Smad7 expressing normal rat tubular epithelial cell line (NRK52E), which provides a valuable model for the in vitro study of the intracellular mechanism of TGF-β in renal fibrosis. Using this model, we have found that TGF-β activates Smad2 in renal TEC in association with ECM production and myofibroblast transformation and that overexpression of Smad7 blocks this process.

TGF-β has long been considered to play a pathogenic role in renal fibrosis. The recent discovery of the TGF-β signaling pathway, Smads, allows us to delineate the exact mode of TGF-β action in this process. It is well documented that TGF-β binds to TβRII, resulting in the activation of TβRI, which
causes the phosphorylation of specific Smad proteins, called R-Smads, including Smad2 and Smad3, that initiate TGF-β signaling transduction (18-21). On the other hand, activated TβRII also induces the activation of inhibitory Smad7 that function to antagonize TGF-β signaling by preventing TGF-β receptor–mediated phosphorylation of Smad2 and Smad3 (22,23) and by increasing ubiquitin-mediated degradation of the TGF-β type I receptor itself (24). In this study, we found that the fibrotic effects of TGF-β on renal TEC, in terms of collagen production and myofibroblast transformation, may signal through the activation of Smad2. Indeed, blockade of Smad2 activation by inducing overexpression of its inhibitor, Smad7, results in inhibition of TGF-β–induced TEC-myofibroblast transformation and collagen production. The tight association between Smad2 activation and de novo synthesis of α-SMA (a marker of myofibroblasts) and collagen types I, III, and IV mRNA and protein indicates that the phosphorylated Smad2 may act on the target genes to regulate TEC to become collagen-producing myofibroblasts at the transcriptional level when it is translocated into the nucleus. This is further demonstrated by the finding that overexpression of Smad7 inhibits TGF-β–induced Smad2 phosphorylation and nuclear localization, subsequent TEC–myofibroblast transformation, and collagen matrix production. Thus, activation of Smad2 may be required for TGF-β–induced fibrotic effects on TEC. This also implies that activation of Smad2 may be one intracellular mechanism by which TGF-β mediates renal fibrosis. These observations are consistent with the recent finding that TGF-β induces collagen matrix in human mesangial cells by a process involving the activation of Smad proteins (13,14).

It is also possible that the activation of Smad3, another R-Smad protein, may be required for TGF-β–induced fibrotic process in TEC. Indeed, TGF-β–induced Smad3 nuclear localization was also evident in this study (data not shown). Activation of Smad3 has been shown to promote TGF-β–induced α2(I) collagen gene expression by human mesangial cells, which is enhanced by Sp1 (14). There are some functional differences between Smad2 and Smad3. Smad3 is involved in activation of the Smad3 gene promoter (25), whereas Smad2 has a functional role in TGF-β1-induced the matrix metalloproteinase MMP-2 (26). Smad3-deficient mice show accelerated wound healing and die from chronic inflammation (27,28) and colon carcinomas between 4 and 6 mo of age (29), whereas Smad2-deficient mice are embryonic lethal (30). However, the functional role of Smad3 in TGF-β–induced TEC–myofibroblast transformation and collagen production by TEC remains to be defined.

Smad7 and Smad6 belong to an inhibitory subfamily of Smads that block both TGF-β and bone morphogenetic protein signaling, whereas Smad6 is able to inhibit bone morphogenetic protein signaling and partially inhibit TGF-β signaling (22,23,31,32). Endogenous Smad7 is rapidly induced by TGF-β and, in turn, downregulates TGF-β signaling through a negative feedback loop to prevent R-Smad from phosphorylation, an early step of TGF-β signaling (11,12,31). Smad7 may act by competition for binding of R-Smad2/3 to TβRI, thereby blocking Smad2 and Smad3 from interacting with the receptor after TGF-β stimulation (11,12,31,33), or by increasing ubiquitin-mediated degradation of TGF-β receptor (24). It has been shown that Smad7, but not Smad6, is a TGF-β–induced attenuator of Smad2/3–mediated inhibition of embryonic morphogenesis (34,35). Overexpression of Smad7, but not Smad6, inhibits TGF-β–induced heme oxygenase-1 by human TEC (36). Expression of Smad7 transgene also blocks Smad2 phosphorylation induced by bleomycin in mouse lung, and gene transfer of Smad7, but not Smad6, prevents bleomycin-induced lung fibrosis (37). In this study, we have investigated the negative regulatory role of Smad7 in the profibrotic effects of TGF-β on renal TEC using an improved Tet–inducible system in which the expression of Smad7 is under the tight control of
Dox. We found that TGF-β induced endogenous Smad7 expression, but that the low grade of endogenous Smad7 expression was unable to overcome the fibrogenic effect of TGF-β on renal TEC associated with the activation of R-Smads. We found, however, that inducing overexpression of Smad7 transgene by addition of Dox blocked the activation of Smad2 and

Figure 6. Double immunocytochemical analyses demonstrate that Dox-induced overexpression of Smad7 inhibits TGF-β–induced TEC-myofibroblast transformation by TEC in a dose-dependent manner. A Dox-inducible m2-Smad7–expressing NRK52E TEC was stimulated with TGF-β1 (10 ng/ml) for 5 d in the absence (A) or presence (B and C) of Dox and stained with mAbs to α-SMA (myofibroblasts, red) and m2 (Smad7, blue). (A) TGF-β induced marked TEC-myofibroblast transformation (red). (B through D) Dox-induced overexpression of m2-Smad7 (blue) prevents TEC-myofibroblast transformation (red) in a dose-dependent manner as demonstrated in Dox 0.5 μg/ml (B), Dox 2 μg/ml (C), and quantitative data (D), showing that an increase in Smad7 expression (dotted line) inversely correlates with TGF-β–induced α-SMA⁺ cells (solid line) in a dose-dependent fashion. ** P < 0.01 and *** P < 0.001 compared with Dox 0. Data represent the mean ± SD for five experiments. Magnification, ×250 in A through C.
prevented the TGF-β-induced TEC-myofibroblast transformation and stimulation of collagen types I, III, and IV mRNA and protein synthesis. Thus, overexpression of Smad7 is able to alter the physiopathologic balance between R-Smads and I-Smads in TEC in response to TGF-β and blocks TGF-β-induced fibrosis.

Although inhibition of Smad2 and Smad3 may block TGF-β-mediated fibrosis, recent studies in Smad2 and Smad3 knockout mice have demonstrated that absence of Smad2 and Smad3 may also impair the immune system and embryonic development (27–30). In contrast, overexpression of Smad7 may also cause cell death through apoptosis (15,38). Therefore, it is critical to maintain a physiologic balance between R-Smads and I-Smads when attempting to target TGF-β signaling. In this study, the degree of expression of Smad7 could be controlled by varying the concentrations of Dox. These in vitro data implicate that it may be more safe and advantageous to use a Dox-regulated Smad7, rather than naked Smad7 gene alone, to prevent or treat renal fibrosis in vivo.

TGF-β is a major mediator in renal fibrosis and acts to both regulate the transdifferentiation of TEC into myofibroblasts and to stimulate their synthesis of ECM proteins (4–10,39,40). In this study, we demonstrate that the fibrogenic effect of TGF-β in TEC involves activation of Smad2 and that overexpression of Smad7 can block Smad2 activation and the fibrogenic process. Thus, this study demonstrates the complex interplay between R-Smads (Smad2) and I-Smads (Smad7) in TGF-β-induced tubular fibrogenesis.

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An incorrect figure was published in the June 2002 issue of *JASN* (Figure 1 in Li JH, Zhu HJ, Huang XR, Lai KN, Johnson RJ, Lan HY: Smad7 inhibits fibrotic effect of TGF-β on renal tubular epithelial cells by blocking Smad2 activation. *J Am Soc Nephrol* 13: 1464–1472, 2002). The correct Figure 1 is printed below.

*Figure 1. TGF-β1 induces Smad2 nuclear localization and phosphorylation.* (A) Immunocytochemistry demonstrates that TGF-β1 (10 ng/ml) induces phosphorylated Smad2 (p-Smad2) nuclear localization (dark black nucleus) on tubular epithelial cells (TEC) in a time-dependent manner (×400). Nucleus was counterstained with hematoxylin. Each bar represents mean ± SD for groups of five experiments. (B) Western blot analyses reveal that TGF-β1 (10 ng/ml) induces p-Smad2 in both a time- and dose (30 min)-dependent manner. Data represent an example for four experiments. **P < 0.01, ***P < 0.001 compared with medium control (0).