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Role of Smad4 on TGF-β–induced extracellular matrix stimulation in mesangial cells

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Role of Smad4 on TGF-β–induced extracellular matrix stimulation in mesangial cells.

Background. The best characterized signaling pathway employed by transforming growth factor-β (TGF-β) is the Smad pathway; however, its role in matrix production in mesangial cells is unclear. We focused on Smad4, as Smad4 is essential for the activation of Smad-dependent target genes.

Methods. To investigate the function of Smad4 in extracellular matrix (ECM) production, we generated several stably transfected mesangial cell lines (MMC) that have a deletion in the linker region (Smad4ΔM4: Δ275-322) or have a deletion in MH1 of Smad4 (Smad4N4: Δ1-136). The ECM genes, α1 type I collagen (COL1A1), plasminogen activator inhibitor-1 (PAI-1) and fibronectin (FN) were assessed in wild-type mesangial cells and stably transfected Smad4-DN cell lines in the absence and presence of TGF-β.

Results. As compared to wild-type MMC that had a 10.8-fold stimulation of TGF-β–induced p3TP-Lux activity, MMC stably transfected with Smad4ΔM4 and Smad4N4 had only a 2.0-fold and 1.3-fold stimulation, respectively, indicating that they had dominant-negative effects on TGF-β signaling. Basal and TGF-β–induced COL1A1 expression in Smad4 dominant-negative cells were dramatically reduced to very low levels. The early (2 hours) TGF-β–induced PAI-1 mRNA expression was inhibited; however, the sustained (24 to 48 hours) TGF-β–induced expression was not affected in Smad4 dominant-negative cells. For FN, TGF-β–induced expression was maintained in Smad4-dominant negative cells.

Conclusion. These results indicate that Smad4 is essential for basal and TGF-β–induced COL1A1 expression, and contributes to the early, but not sustained TGF-β–induced PAI-1 expression in mesangial cells. However, TGF-β–induced FN expression is independent of Smad4. In conclusion, Smad4 has a discriminate effect in mediating specific ECM molecules stimulated by TGF-β in mesangial cells.

It is now well established that the multifunctional cytokine, transforming growth factor-beta (TGF-β) plays a critical role in the development of a variety of progressive kidney diseases, including diabetic nephropathy [1–3]. TGF-β induces renal fibrosis by stimulating the production of extracellular matrix (ECM), including type I, III, and IV collagen, fibronectin (FN), laminin, proteoglycans [4], and inhibiting the activity of enzymes that degrade matrix proteins via plasminogen activator inhibitor-1 (PAI-1) [5]. Although the role of TGF-β in progressive fibrosis is accepted, the signaling pathway by which TGF-β stimulates matrix accumulation in kidney cells is not clear.

TGF-β exerts its diverse effects by binding to two types of receptors with serine/threonine kinase activity. The ligand first binds to the type II receptor, which consequently activates the type I receptor by direct phosphorylation. The activated type I receptor then phosphorylates receptor-regulated Smads (R-Smads), such as Smad2 and Smad3. After R-Smads are phosphorylated by a type I receptor, they form heteromers with Smad4, common pathway Smad (Co-Smad), and then translocate into the nucleus where the complex activates transcription of specific genes [6–8]. TGF-β–targeted genes are induced either by direct binding of Smad complexes to the promoter, or by association of Smad complexes with other DNA binding proteins, such as FAST1 [9] and coactivators such as p300/CREB binding protein (CBP) [10]. Smad6 and Smad7, which are structurally more distantly related to the other Smads, act as inhibitory Smads by antagonizing the ligand-specific activity [11, 12].

R-Smads and Smad4 are homologous in their amino and carboxyl terminals, called the Mad homology domain (MH) 1 and 2, respectively. These domains are connected by a proline-rich linker region with variable length and amino acid sequence. Direct DNA binding is mediated through the MH1 domain, whereas the MH2 domain up-regulates transcriptional activity [6–8]. A C-terminal part of the Smad4 linker region, which is called Smad activation domain (SAD) (amino acids 274 to 321), is not conserved in other Smads and is essential for the signaling activity of Smad4 presumably through stabilization of oligomer structures with R-Smads or through

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binding with other proteins with transcriptional activity, p300/CBP [10].

The role of the Smad pathway is critical for mediating TGF-β–induced genes such as cyclin-dependent kinase inhibitors $p15^{INK4B}$ and $p21^{CIP1/WAF1}$, c-fos, and Smad7 [13]; however, the role of Smads in matrix stimulation is controversial [13–16]. For example, Smad4-deficient adenocarcinoma cells did not demonstrate stimulation of FN or PAI-1 in response to TGF-β [17], whereas in other studies with the same cell type [15] or in Smad4-deficient fibroblasts [16] TGF-β–induced FN stimulation was not impaired. The basis for these prior discrepant results has not been clarified. With respect to collagens, TGF-β–induced collagen proteins were not altered in Smad4-deficient fibroblasts [16]; however, individual types of collagens were not evaluated. Smad3 has been found to be involved in TGF-β–induced stimulation of the α 2 chain of type I collagen (COL1A2) in mesangial cells [18, 19]; however, dependence of Smad3 on other matrix molecules has not been evaluated. To date, no studies have examined the role of Smad4 in mediating matrix stimulation in mesangial cells.

The use of dominant-negative forms of transcription factors has proven to be a helpful way to study gene regulation in cultured cells [20]. To investigate the function of the Smad-pathway in ECM production, we generated several stably transfected mesangial cell lines that have a deletion in the linker region (Smad4ΔM4) or have a deletion in MH1 of Smad4 (Smad4N4). In this study, we examined α 1 type 1 collagen (COL1A1), PAI-1, and FN production in wild-type mesangial cells and stably transfected Smad4 dominant-negative cell lines.

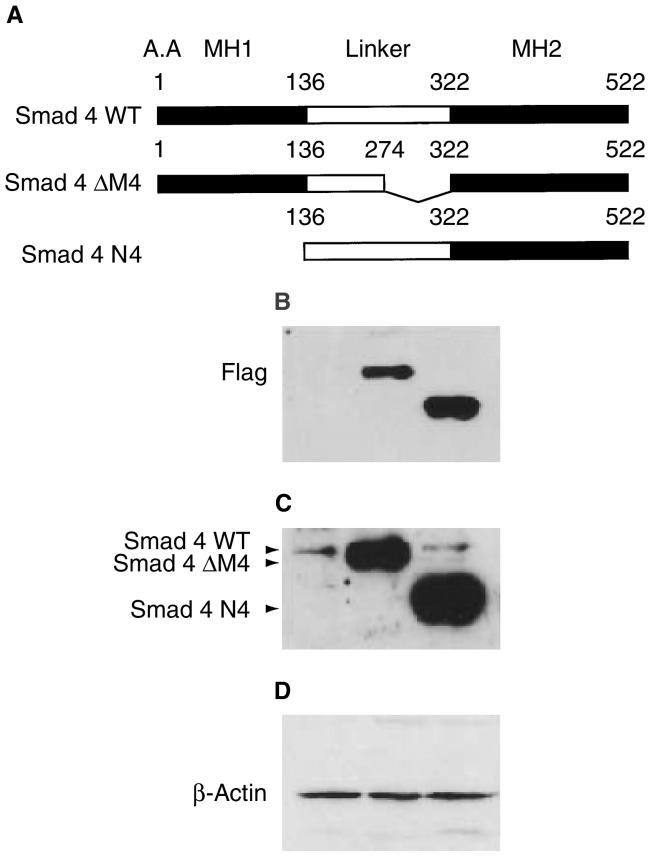

Fig. 1. Stable expression of Smad4ΔM4 and Smad4N4 mutant proteins in murine mesangial cells (MMC). A schematic representation of Smad4 deletion constructs based on the known sequence homology domains of the Smad family of proteins (*A*). MH1 and MH2 domains that are conserved between Smads are shown, respectively. This schema is adapted from [17]. MMC were transfected with Smad4ΔM4 and Smad4N4 followed by selection in 800 μg/mL of geneticin. To demonstrate the expression of Flag-tagged Smad4 dominant-negative constructs, 20 μg of total cellular protein was resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to nitrocellulose membrane, and subjected to Western analysis using antibody for Smad4 (*B*) and the Flag epitope (*C*). The position of Flag-tagged Smad4 dominant-negative proteins are indicated by the arrow. WT is wild-type.

METHODS

Cell culture

A murine mesangial cell line (MMC) was used. Cells were originally isolated from kidneys of SJL/J (H-2) normal mice and transformed with noncapsid forming SV-40 virus to establish a permanent cell line [21]. The cells exhibit many phenotypic features of differentiated mesangial cells, including positive staining for desmin, vimentin, and collagen types I and IV. The use of a transformed cell line was required as the process of stable transformation would require multiple passages and lead to loss of differentiated characteristics in nontransformed cells. Cells were maintained at 37°C in a humidified incubator with 5% CO_2/95% air and propagated in Dulbecco's modified Eagle's medium (DMEM) (Gibco, BRL, Grand Island, NY, USA) containing 100 mg/dL D-glucose, 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L supplemental glutamine. Cells were passaged every 72 hours by light trypsinization. Experiments were initiated by resting cells for 24 hours in 0.5% FCS. In some experiments, cells were treated with recombinant human TGF-β1 (R&D Systems, Minneapolis, MN, USA) at 5 or 10 ng/mL. Cells were harvested after a period of 2, 4, 24, and 48 hours as specifically indicated.

Establishment of stable dominant-negative cell lines

The dominant-negative constructs, 3′ Flag-tagged Smad4ΔM4 and Smad4N4, were cloned into pcDNA3.1 vector, as indicated in Figure 1 and described previously [10, 17]. They were kindly provided by M. de Caestecker (Division of Nephrology, Vanderbilt University, Nashville, TN, USA). As a control for the effects of transfection, the empty vector pcDNA 3.1 (Invitrogen, Carlsbad, CA, USA) was also utilized to generate stably transfected cells. For transfection of Smad4ΔM4 and Smad4N4, sub-

confluent MMC were transfected with 10 μg of DNA per 100 mm dish using the polyamine transfection reagent SuperFect (QIAGEN, Inc., Chatsworth, CA, USA), according to the manufacturer's protocol. Stable transfectants were selected and maintained in media containing 800 μg/mL geneticin (Mediatech, Inc., Herndon, VA, USA). Individual clones were isolated from pools of geneticin-resistant cells by ring cloning.

Transcriptional response assays

The p3TP-Lux luciferase reporter is a widely used bioassay to determine responsiveness to TGF-β [22]. The p3TP-Lux construct has a 31 nucleotide, activator protein-1 (AP-1) site-containing a region of the collagenase promoter, concatemerized 5′ to an –400 nucleotide region of the PAI-1 promoter followed by 70 bp of the adenovirus E4 promoter. Cells were seeded to 50% confluence in six-well plates and incubated in medium containing 0.5% FCS for 24 hours. MMC were transiently transfected using SuperFect (QIAGEN, Inc.) with 1 μg/well of the p3TP-Lux reporter along with pcDNA3.1-LacZ vector (generously provided by S. Sasaki, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA) as an internal control for transfection efficiency. After 3 hours, cells were washed and allowed to recover for 24 hours in growth medium. Cells were serum starved overnight prior to the addition of TGF-β1 (10 ng/mL) for a further 24 hours. Luciferase activity was determined in the cell lysate using the Luciferase Assay System (Promega, Madison, WI, USA), and a Dynatech Laboratories ML3000 luminometer (Dynatech Laboratories, Chantilly, VA, USA). Activities were normalized on the basis of β-galactosidase expression from pcDNA3.1-LacZ vector in all luciferase reporter experiments. All experiments were repeated at least three times with similar results. All assays were performed in triplicate, and represented as mean ± SEM of three independent transfections.

Western blot analysis

For detection of Flag-tagged Smad4 deletion mutant proteins, cells were prepared as described previously [23] and total cellular protein was quantitated using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Total cellular protein (20 μg) was resolved on 10% sodium dodecyl sulfate-polyacramide gel electrophoresis (SDS–PAGE) gels, transferred to nitrocellulose membrane (BioRad Laboratories), and subjected to Western analysis using either a polyclonal antibody to the Flag epitope (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and monoclonal antibodies to Smad4 (Transduction Laboratory, Lexington, KY, USA). Immunocomplexes were detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA). Immunoreactive bands were detected by enhanced chemiluminescence as described above and equal loading of proteins was confirmed based on immunoblotting with an antibody against β-actin (Sigma Chemical Co., St. Louis, MO, USA).

Northern analysis

To assess whether TGF-β1–induced COL1A1, PAI-1, and FN mRNA expression were affected by Smad4 dominant-negative proteins, MMC were treated with TGF-β1 (5 ng/mL) for indicated time points and washed with ice-cold phosphate-buffered saline (PBS). Initial studies were performed with 5 and 10 ng/mL of TGF-β1. As the results were equivalent with respect to matrix stimulation at both doses, 5 ng/mL was chosen for the repeated analyses. Total RNA was isolated using TRI reagent (MRC, Inc., Cincinnati, OH, USA). Total RNA (15 mg) was electrophoresed through a 1.2% agarose gel with 0.67 mol/L formaldehyde and transferred onto nylon membrane (NEN Research Products, Boston, MA, USA) by the capillary method and ultraviolet cross-linked. The probe for mouse COL1A1 [24], PAI-1 [24], and FN [25] has been described previously. Hybridization and washing conditions were performed as described previously [26]. To standardize for loading, membranes were stripped and reprobed with an 18S cDNA probe. Densitometric analysis was performed as described previously [23] and mRNA levels were calculated relative to 18S. Additionally, in experiments with pooled clones of stably transfected cells, mRNA levels were quantitated via phosphoimager and the data expressed in relation to 18S.

Statistical analysis

Data are presented as mean ± SEM. Means were compared using either a two-sided paired *t* test for comparisons within the group or a two-sided independent *t* test between the groups. Bonferroni's correction for multiple comparisons was applied. When necessary (i.e., standard deviations between the independent measurements were different) the nonparametric, Mann-Whitney U test was used. A value of $P < 0.05$ was considered significant. Instat™ Statistical Software for the PC© 1990 GraphPAD Software, Version 1.14 was used for the analysis.

RESULTS

Expression of Flag and Smad4 proteins in geneticin-resistant cell lines

To generate cell lines, which stably expressed deletion mutant forms of Smad4, two different kinds of deletion mutants of Smad4 were used. The schematic constructs of each mutant are shown in Figure 1A. As the Smad4 mutants are not affected in their R-Smad binding site, it is not expected that the dominant-negative mutants would interfere with Smad2/3 phosphorylation or translocation

into the nucleus. After transfection with Smad4ΔM4 and Smad4N4, stable transfectants were selected and maintained in media containing geneticin (800 μg/mL). To confirm overexpression of mutant Smad4, we examined the expression of the exogenous Flag-tagged Smad4 protein using an antibody against the Flag epitope (Fig. 1). As shown in Figure 1B, expression of the exogenous Flag-tagged Smad4 could only be detected in transfected MMC. Additionally, it is apparent that mutant Smad4 is in excess of endogenous Smad4 in the stably transfected mutant cell lines (Fig. 1C). We chose the cell line that had the highest expression of Flag-tagged Smad4 protein from 10 to 15 stably transfected clones.

Stable expression of Smad4ΔM4 and Smad4N4 reduce TGF-β–induced transcriptional responses

To determine whether these cell lines, which expressed Flag-tagged Smad4 mutant protein, have dominant-negative effects on TGF-β–mediated gene induction, we performed the transcriptional activity assay using p3TP-Lux reporter construct. As shown in Figure 2, stimulation with TGF-β was capable of inducing activation of p3TP-Lux in wild-type MMC. Wild-type MMC had a 10.8-fold (10.78 ± 1.97) increase of p3TP-Lux activity by TGF-β1 (10 ng/mL) stimulation compared to basal level (arbitrarily set as 1, $P < 0.05$). In contrast, the Smad4ΔM4 cells had only a 2.0-fold increase (1.96 ± 0.34, 18% of wild-type) and Smad4N4 cells had only a 1.3-fold increase (1.32 ± 0.08, 15% of wild-type) with TGF-β stimulation. Similar results were also obtained with 5 ng/mL of TGF-β1 (data not shown). These results indicate that both Smad4 deletion mutants clearly had dominant-negative effects on TGF-β–induced transcriptional activity of p3TP-Lux.

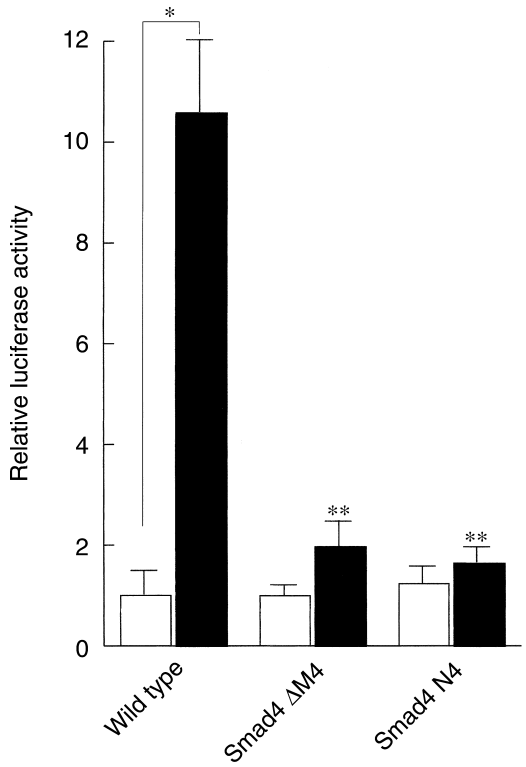

Fig. 2. Expression of Smad4ΔM4 and Smad4N4 proteins abolished transforming growth factor-β (TGF-β) stimulated transcriptional response. Wild-type murine mesangial cells (MMC) and MMC stably transfected with Smad4ΔM4 and Smad4N4 constructs were cotransfected transiently with 1 μg of p3TP-Lux and 0.5 μg of pcDNA3.1-LacZ as described in the **Methods** section. After 24 hours of starvation, cells were incubated in the absence (□) or presence of TGF-β1 (10 ng/mL) (■) for an additional 24 hours. Cells were lysed and luciferase activity was determined using the Promega's Luciferase Assay System according to the manufacturer's instructions. Luciferase activity is expressed as the ratio of specific luciferase activity divided by the β-galactosidase activity of the cotransfected vector. Data are shown as the mean ± SEM of triplicates from three separate experiments. Wild-type MMC without TGF-β at time 0 is arbitrarily set as 1. *$P < 0.05$ vs. wild-type without TGF-β1; **$P < 0.05$ vs. wild-type with TGF-β1.

The basal and TGF-β–stimulated expression of COL1A1 is dependent on Smad4

To examine whether the expression of COL1A1 with TGF-β stimulation was altered in MMC transfected with Smad4ΔM4 and Smad4N4, we analyzed the expression of steady-state COL1A1 mRNA using Northern blot. As shown in Figure 3, in wild-type MMC, the expression of COL1A1 stimulated with TGF-β1 was significantly increased by 1.7-fold at 24 hours and 2.1-fold at 48 hours compared to basal levels, respectively ($P < 0.05$). Overexpression of both Smad4ΔM4 and Smad4N4 DN proteins led to reduction of basal COL1A1 expression at baseline (wild-type, 1.00; Smad4ΔM4, 0.52 ± 0.04; $P < 0.01$; Smad4N4, 0.37 ± 0.15; $P < 0.05$), at 24 hours (wild-type, 1.40 ± 0.06; Smad4ΔM4, 0.66 ± 0.14; $P < 0.01$; Smad4N4, 0.68 ± 0.20; $P < 0.05$), and at 48 hours (wild-type, 1.06 ± 0.13; Smad4ΔM4, 0.27 ± 0.12; $P < 0.01$; Smad4N4, 0.30 ± 0.08; $P < 0.01$). TGF-β–stimulated COL1A1 expression did not show any increase as compared to basal levels in the Smad4 DN cell lines either at 24 hours or 48 hours. This result indicates that both basal and TGF-β–induced COL1A1 expression is dependent on Smad4.

The early, but not sustained PAI-1 stimulation by TGF-β is partly dependent on Smad4

Northern analysis of PAI-1 mRNA was evaluated in wild-type MMC and Smad4 dominant-negative MMC. Expression of PAI-1 mRNA in wild-type MMC was maximally stimulated 2 hours after TGF-β1 addition (15.96 ± 0.36, $P < 0.005$ vs. without TGF-β1 at 2 hours) (Fig. 4). This early peak was significantly attenuated in both Smad4ΔM4 and Smad4N4 dominant-negative cell lines compared to wild-type (Smad4ΔM4, 1.94 ± 0.34, $P < 0.001$; Smad4N4, 9.28 ± 1.28, $P < 0.05$ vs. wild-type with TGF-β1 at 2 hours). At 4 hours, TGF-β–induced PAI-1 mRNA was significantly inhibited only in Smad4ΔM4

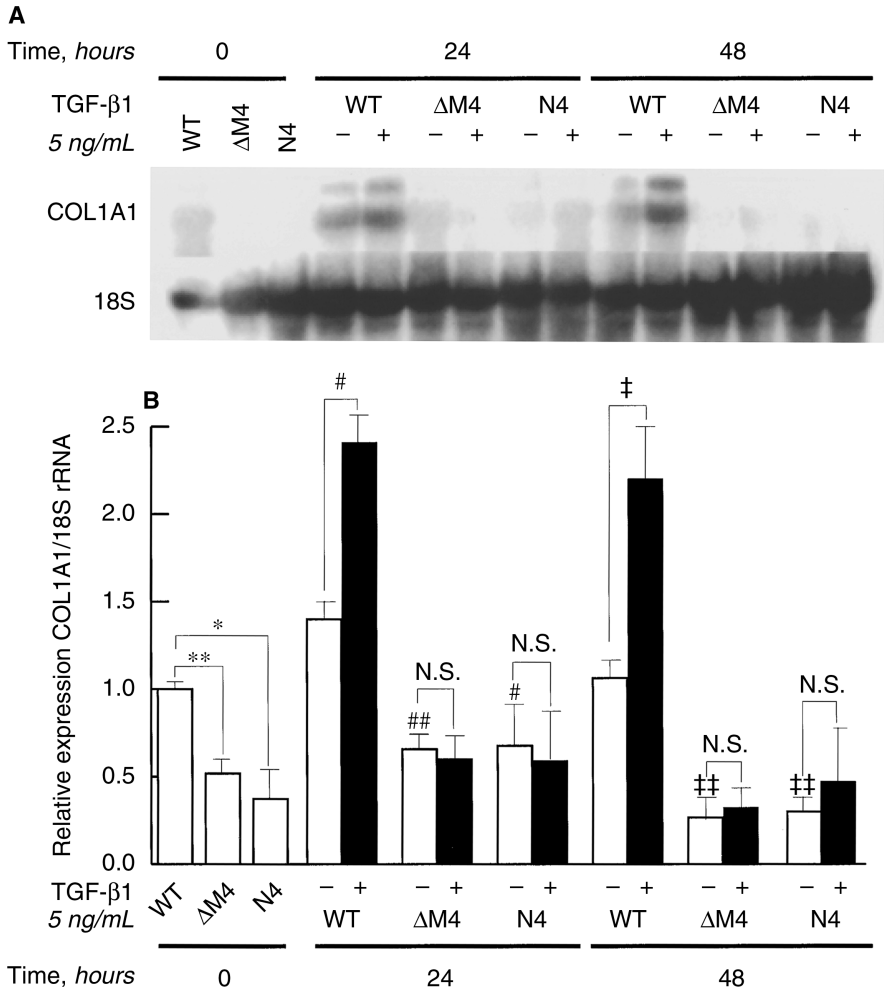

Fig. 3. The expression of α1 type I collagen (COL1A1) is dependent on Smad4. (*A*) Representative Northern blot analysis of COL1A1 expression in total RNA lysates from murine mesangial cells (MMC) [wild-type (WT)], Smad4ΔM4 (ΔM4), and Smad4N4 (N4) dominant-negative cell lines exposed to 5 ng/mL of transforming growth factor-β1 (TGF-β1) for the indicated time periods. The membranes were stripped and reprobed for 18S RNA levels to normalize for RNA loading. (*B*) Quantitative data show the relative expression levels of COL1A1 measured by densitometry after normalization for 18S from three independent experiments. Bar graphs are shown as mean ± SEM expressed relative to the expression in untreated wild-type MMC. Wild-type MMC without TGF-β at time 0 is arbitrarily set as 1. Symbols are: (□), −TGF-β; and (■), +TGF-β. $*P < 0.05$; $**P < 0.01$ vs. wild-type at time 0; $\#P < 0.05$; $\#\#P < 0.01$ vs. wild-type without TGF-β1 at 24 hours; $‡P < 0.05$; $‡‡P < 0.01$ vs. wild-type without TGF-β1 at 48 hours. N.S. is not significant.

dominant-negative cell line (2.40 ± 0.08, $P < 0.005$ vs. without TGF-β1). In contrast to the early peak, the sustained stimulation of PAI-1 expression with TGF-β1, at 24 hours and 48 hours, was maintained in MMC transfected with Smad4ΔM4 and Smad4N4, to similar levels as compared with wild-type MMC. Basal level of PAI-1 mRNA was essentially the same at all time points evaluated.

To verify that the effects of Smad4 dominant-negative were not unique to single clones, pools of three clones that showed high expression of Smad4 dominant-negative Flag were also assessed. To control for transfection, MMC cells were stably transfected with an empty vector. As shown in Figure 5, the effects of TGF-β1 to stimulate COL1A1 and PAI-1 at 24 and 48 hours are robust in stably transfected empty vector cells. The Smad4 dominant-negative mutant cells had marked reduction of basal and TGF-β–stimulated COL1A1 expression, whereas PAI-1 stimulation was not affected. At the early time point (Fig. 6), PAI-1 stimulation is attenuated in pooled Smad4 DN cells. As expected, there is no induction of COL1A1 with TGF-β1 at 2 hours and 4 hours (Fig. 6).

Expression of FN stimulated with TGF-β is independent of Smad4

To determine if TGF-β–induced FN was dependent on Smad4, Northern blot analysis was performed. As shown in Figure 7, TGF-β1 treatment of each cell line resulted in a significant increase of FN at 48 hours. At 48 hours, TGF-β–induced FN mRNA in MMC transfected with Smad4ΔM4 (3.48- ± 0.07-fold increase, $P < 0.05$) and Smad4N4 (2.51- ± 0.15-fold increase, $P < 0.05$) was similar to that in wild-type MMC (1.90- ± 0.04-fold increase as compared to without TGF-β, $P < 0.05$). Basal expression level of FN mRNA tended to be lower in Smad4 dominant-negative cells.

DISCUSSION

This study examined the role of the Smad pathway in TGF-β regulation on ECM molecules in mouse mesangial cells using dominant-negative inhibition of Co-Smad, Smad4. Since mice homozygous for the Smad4 mutation die at embryonic day (E) 7.5 [27], transfection

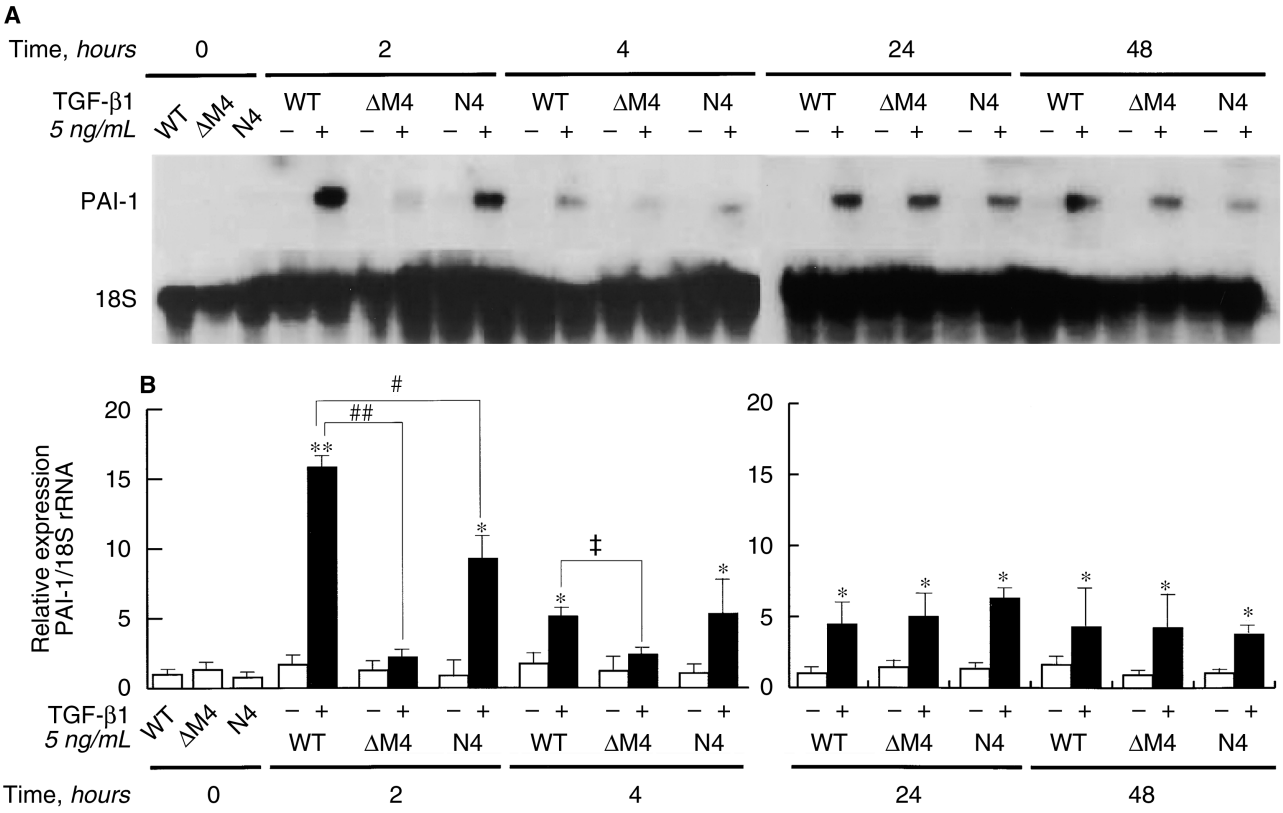

Fig. 4. The early, but not sustained, plasminogen activator inhibitor-1 (PAI-1) stimulation by transforming growth factor-β (TGF-β) is partly dependent on Smad4. (*A*) Representative Northern blot analysis of PAI-1 mRNA expression in total RNA lysates from murine mesangial cells (MMC) [wild-type (WT)], Smad4ΔM4 (ΔM4), and Smad4N4 (N4) dominant-negative cell lines exposed to 5 ng/mL of TGF-β1 for the indicated time periods. (*B*) Bar graph shows the relative expression level of PAI-1 mRNA measured by densitometry after normalization for 18S from three independent experiments. Data are shown as mean ± SEM expressed relative to the expression in untreated wild-type MMC. Wild-type MMC without TGF-β at time 0 is arbitrarily set as 1. Symbols are: (□), −TGF-β; and (■), +TGF-β. $*P < 0.05$; $**P < 0.005$ vs. without TGF-β1; $\#P < 0.05$; $\#\#P < 0.001$ vs. wild-type with TGF-β1 at 2 hours; ‡ $P < 0.005$ vs. wild-type with TGF-β1 at 4 hours.

of Smad4-DN constructs is a useful approach to study the gene regulation of ECM molecules in individual tissue-specific cultures, such as glomerular mesangial cells. In the present study, we established mouse mesangial cell lines stably transfected with the Smad4-deletion mutant constructs, Smad4ΔM4 and Smad4N4, and demonstrated a dominant-negative effect on TGF-β–inducible transcriptional activity in these cell lines. Despite the almost complete inhibition of TGF-β–induced p3TP-Lux activity, there was a discriminate role of Smad4 in COL1A1, PAI-1, and FN expression at the basal and TGF-β–stimulated levels.

With regard to COL1A1, we demonstrated that the COL1A1 expression was completely dependent on Smad4 at the basal level and after stimulation with TGF-β (Fig. 4). This is the first description on the role of Smad4 on murine COL1A1 gene expression. Our studies are discrepant from results obtained with murine embryonic fibroblasts derived from Smad4 knockout mice [16]. In this study, TGF-β–induced collagen proteins were similarly stimulated in wild type and Smad4-deficient fibroblasts; however, discrete collagen types and their α chains were not individually assessed. As we find a complete Smad4 dependence on both basal and TGF-β–stimulated COL1A1 expression, it is likely that specific α chains of collagen isoforms are differentially regulated by Smad4. Subsequent studies to assess each individual α chain from each collagen isoform will be required to resolve this issue. Apart from Smads, the extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways have been implicated in TGF-β stimulation of COL1A1. TGF-β–induced COL1A1 was dependent on the p38 MAPK pathway in rat mesangial cells using a p38 inhibitor [28] and TGF-β–induced COL1A1 expression and promoter activity were inhibited by both ERK inhibitor and dominant-negative form of ERK in human mesangial cells [29].

The mechanism by which Smad4 regulates COL1A1 expression is unclear. There are several reports that TGF-β stimulates the *COL1A2* gene through the Smad-dependent pathway by inducing binding of a Smad-containing complex to a Smad-binding element (SBE) [18].

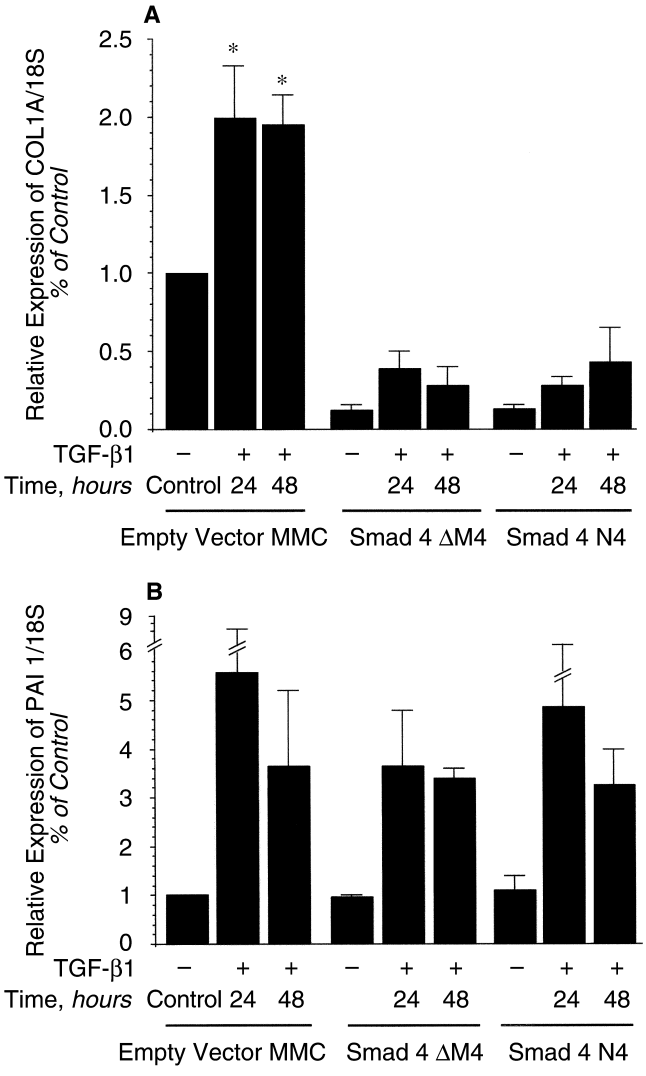

Fig. 5. Smad4 dominant-negative cells have suppression of α1 type I collagen (COL1A1) (*A*) but no effect on delayed stimulation of plasminogen activator inhibitor-1 (PAI-1) (*B*) in mixed clones. Pooled clones of Smad4ΔM4 (ΔM4) and Smad4N4 (N4) stably transfected cells were assessed for basal and transforming growth factor-β1 (TGF-β1)–stimulated expression of COL1A1 and PAI-1. Murine mesangial cells (MMC) with stable transfection with an empty vector were used as control. Similar to data shown in Figures 3 and 4, there is marked inhibition of basal and TGF-β–stimulated levels of COL1A1 at 24 hours and 48 hours in Smad4 dominant-negative cells. Basal levels of PAI-1 are not affected in Smad4 dominant-negative cells and there is no decrease in TGF-β1 stimulation of PAI-1 at 24 hours and 48 hours. The data show the relative expression levels of COL1A1 measured by phosphoimager after normalization for 18S from three independent experiments. Bar graphs are shown as mean ± SEM expressed relative to the expression in untreated MMC. Empty vector transfected MMC without TGF-β (Control) is arbitrarily set as 1. $*P < 0.05$ vs. control.

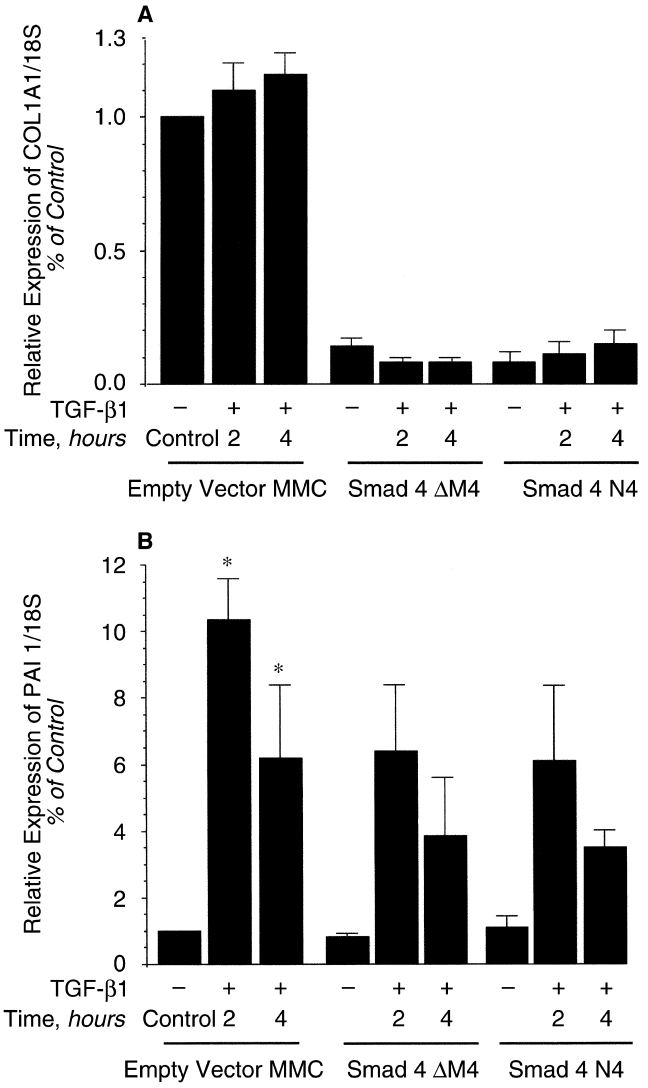

Fig. 6. The effect of early stimulation by transforming growth factor-β1 (TGF-β1) on α1 type I collagen (COL1A1) (*A*) and plasminogen activator inhibitor-1 (PAI-1) (*B*) expression in empty vector and mixed clones of Smad4 dominant-negative cells. TGF-β1 has no effect on COL1A1 at the early time points tested. As noted previously, basal levels of COL1A1 are suppressed in pooled clones of Smad4ΔM4 and SmadN4 stably transfected cells at these time points. TGF-β1 markedly stimulates PAI-1 at the early time points, whereas there is a reduction of PAI-1 stimulation in the pooled clones of Smad4ΔM4 and SmadN4 stably transfected cells. The data show the relative expression levels of COL1A1 and PAI-1 measured by phosphoimager after normalization for 18S from three independent experiments. Bar graphs are shown as mean ± SEM expressed relative to the expression in untreated murine mesangial cells (MMC). Empty vector transfected MMC without TGF-β (Control) is arbitrarily set as 1. $*P < 0.05$ vs. control.

An SBE (CAGACA sequence) in the *COL1A2* promoter recognized by Smad3/4 has been identified [30], but no comparable element has been identified within the 5′ flanking region, the promoter or the first intron of human *COL1A1* [30, 31] or the murine promoter of *COL1A1* (unpublished observation, K.T.). However, Smads may regulate TGF-β–induced COL1A1 expression without directly binding to DNA regulatory elements. For example, the TGF-β–responsive element of the *p21* gene does not contain a CAGA box; however, Smad proteins may transactivate the Sp1-dependent p21 promoter possibly by functionally interacting with Sp1 [32]. A 220 bp region of the transcription start site of human *COL1A1* contains sequences sufficient for its

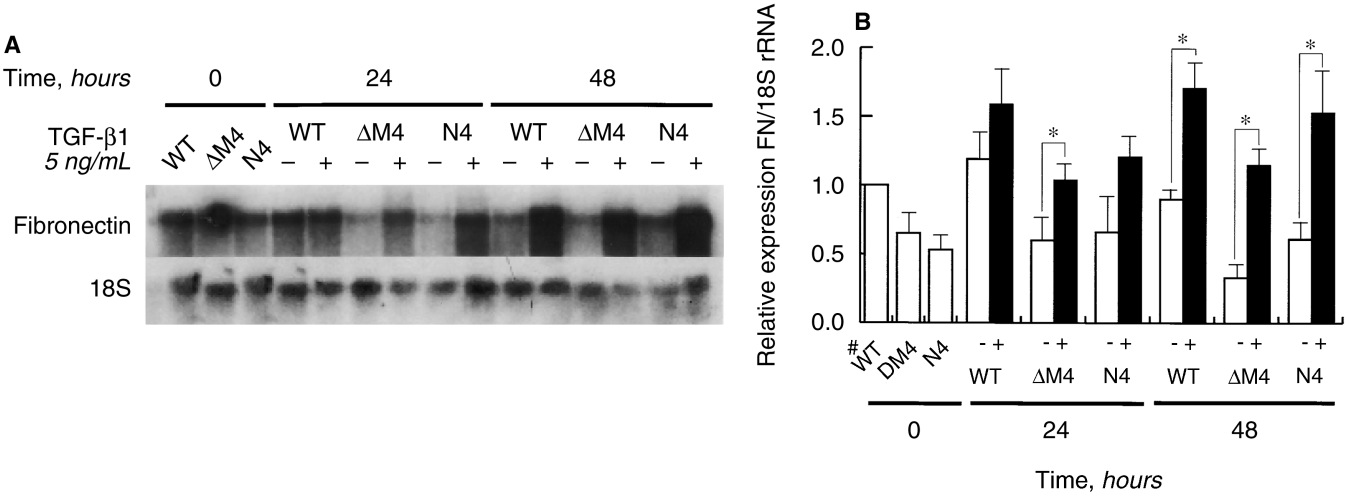

Fig. 7. Transforming growth factor-β (TGF-β)–induced fibronectin expression of fibronectin is independent of Smad4. (*A*) Representative Northern blot analysis of fibronectin in murine mesangial cells (MMC) [wild-type (WT)], Smad4ΔM4 (ΔM4) and Smad4N4 (N4) dominant-negative cell lines exposed to 5 ng/mL of TGF-β1 for the indicated time periods. Total RNA (15 μg) was electrophoresed, transferred onto nylon membrane, and probed with a murine probe for fibronectin. The membranes were stripped and reprobed for 18S rRNA levels to normalize for RNA loading. (*B*) Bar graph shows the relative expression level of fibronectin mRNA measured by densitometry after normalization for 18S. Data are shown as mean ± SEM from three independent experiments and expressed relative to the expression in untreated wild-type MMC. Wild-type MMC without TGF-β at time 0 is arbitrarily set as 1. Symbols are: (□), −TGF-β; and (■), +TGF-β. $^*P < 0.05$ vs. without TGF-β1.

basal transcription and is also sufficient for its inducibility by TGF-β [31]. Two partially conserved glucocorticoid (GC)-rich sequences in this region of the *COL1A1* promoter are functional binding sites for Sp1 [33, 34]. More recently, a homologous region in the rat *COL1A1* promoter, which is flanked by GC-rich Sp1 binding sites, was found to be involved in TGF-β1 stimulation via binding of CCAAT binding factor (CBF) [35]. The binding of CBF to this site is also important for basal *COL1A1* promoter activity [35, 36]. These reports suggest that Smads may form complexes with Sp1 and possibly CBF to play an important role in mediating basal and TGF-β–induced COL1A1 expression without binding to the *COL1A1* promoter directly [18, 32, 33, 35].

In the case of PAI-1, there was Smad4 dependence for the TGF-β–induced PAI-1 expression at the early period (2 to 4 hours), but no dependence at the sustained period (24 to 48 hours) (Fig. 5). Based on prior studies, it appears that Smad2 and Smad3 are critical and necessary factors for TGF-β–induced PAI-1 expression at the early time points (5 hours) [13]. However, the role of Smad4 in PAI-1 expression with TGF-β stimulation has revealed conflicting results [14–16]. Sirard et al [16] identified Smad4-dependent (−730) and -independent (−580) elements in the *PAI-1* promoter sequence. Datta, Blake, and Moses [37] suggest that the *PAI-1* promoter is induced by TGF-β primarily by two mechanisms. First, the *PAI-1* promoter is strongly activated by TGF-β–induced binding of a Smad3/Smad4 containing nuclear complex to the CAGA box. Second, the proximal promoter (−82 and –40), containing two Sp1 sites, mediates moderate induction through TGF-β–induced interaction between Smad3/Smad4 and Sp1, independent of Smad/DNA binding. Both these mechanisms may be required for full induction of the PAI-1 promoter. In addition, the results of the present study indicates that only the early expression of TGF-β is partly dependent on Smad4 and the sustained expression is completely independent of Smad4. The complex, time-dependent role of Smad4 in TGF-β–induced PAI-1 stimulation may help to explain the divergent results from prior studies.

TGF-β–induced FN stimulation was not altered in the Smad4 dominant-negative cell lines transfected with either the Smad4ΔM4 or the Smad4N4 construct (Fig. 7). This result is consistent with prior observations that the Smad pathway was dispensable for TGF-β–induced FN synthesis. In Smad4-deficient cell lines, such as MDM-MB 468, BxPC3, and SW480.7 cell line [15], Smad4-deficient fibroblasts [16] and Smad2- and Smad3-deficient fibroblasts [13], TGF-β–induced FN stimulation was maintained. It is unclear which TGF-β signaling pathway is critical for FN production in mesangial cells. Hocevar, Brown, and Howe [15] demonstrated that TGF-β–induced FN expression was dependent on Smad-independent c-Jun NH2 terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway. TGF-β has been shown to activate JNK/SAPK in a number of cell types [38]; however, not in mesangial cells [28]. We have previously reported that TGF-β stimulates the protein kinase A signaling pathway and that protein kinase A activation contributed to TGF-β stimulation of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) phosphorylation and *FN* gene expression [39]. The p38 MAPK pathway also has been

shown to mediate TGF-β signaling [38] and lead to activation of activating transcription factor (ATF-2) [40] and CREB [41]. In this regard, TGF-β may up-regulate *FN* gene expression in mesangial cells in a p38 MAPK/ATF/CREB–dependent manner.

Smad4 is an essential component mediating the activation of Smad-dependent target genes [6–8] and Smad4 is the only Co-Smad thus far identified in mammals, whereas two Smad4-like molecules have been isolated in *Xenopus* and *Caenorhabditis elegans* [42, 43]. It is currently not known whether Smad4 is absolutely required for all the signals in the Smad pathways in mammals. In addition to our studies, recent observations have identified a number of TGF-β actions that are Smad4 independent [13, 15, 16]. This raises the possibility that as yet unidentified Co-Smads could mediate certain TGF-β responses in the absence of Smad4 or that Smad2/3 may work independently of Smad4. With respect to progressive kidney fibrosis, further understanding of the signaling pathways involved in mediating each of the matrix proteins regulated by TGF-β would lead to a focused therapeutic approach at defined stages of disease.

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REFERENCES

1. Blobe GC, Schiemann WP, Lodish HF: Role of transforming growth factor beta in human disease. *N Engl J Med* 342:1350–1358, 2000
2. Sharma K, McGowan TA: TGF-beta in diabetic kidney disease: Role of novel signaling pathways. *Cytokine Growth Factor Rev* 11:115–123, 2000
3. Ziyadeh FN, Hoffman BB, Han DC, *et al*: Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal anti-transforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci USA* 97:8015–8020, 2000
4. Ignotz RA, Endo T, Massague J: Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-beta. *J Biol Chem* 262:6443–6446, 1987
5. Laiho M, Saksela O, Andreasen PA, Keski-Oja J: Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-beta. *J Cell Biol* 103:2403–2410, 1986
6. Miyazono K, ten Dijke P, Heldin CH: TGF-beta signaling by Smad proteins. *Adv Immunol* 75:115–157, 2000
7. Massague J: How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 1:169–178, 2000
8. Massague J, Chen YG: Controlling TGF-beta signaling. *Genes Dev* 14:627–644, 2000
9. Labbe E, Silvestri C, Hoodless PA, *et al*: Smad2 and Smad3 positively and negatively regulate TGF beta-dependent transcription through the forkhead DNA-binding protein FAST2. *Mol Cell* 2:109–120, 1998
10. de Caestecker MP, Yahata T, Wang D, *et al*: The Smad4 activation domain (SAD) is a proline-rich, p300-dependent transcriptional activation domain. *J Biol Chem* 275:2115–2122, 2000
11. Imamura T, Takase M, Nishihara A, *et al*: Smad6 inhibits signalling by the TGF-beta superfamily. *Nature* 389:622–626, 1997
12. Nakao A, Afrakhte M, Moren A, *et al*: Identification of Smad7, a TGF beta-inducible antagonist of TGF-beta signalling. *Nature* 389:631–635, 1997
13. Piek E, Ju WJ, Heyer J, *et al*: Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. *J Biol Chem* 276:19945–19953, 2001
14. Datto MB, Frederick JP, Pan L, *et al*: Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. *Mol Cell Biol* 19:2495–2504, 1999
15. Hocevar BA, Brown TL, Howe PH: TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *EMBO J* 18:1345–1356, 1999
16. Sirard C, Kim S, Mirtsos C, *et al*: Targeted disruption in murine cells reveals variable requirement for Smad4 in transforming growth factor beta-related signaling. *J Biol Chem* 275:2063–2070, 2000
17. de Caestecker MP, Hemmati P, Larisch-Bloch S, *et al*: Characterization of functional domains within Smad4/DPC4. *J Biol Chem* 272:13690–13696, 1997
18. Poncelet AC, Schnaper HW: Sp1 and Smad proteins cooperate to mediate transforming growth factor-beta 1-induced alpha 2(I) collagen expression in human glomerular mesangial cells. *J Biol Chem* 276:6983–6992, 2001
19. Poncelet AC, de Caestecker MP, Schnaper HW: The transforming growth factor-beta/SMAD signaling pathway is present and functional in human mesangial cells. *Kidney Int* 56:1354–1365, 1999
20. Greenwel P, Tanaka S, Penkov D, *et al*: Tumor necrosis factor alpha inhibits type I collagen synthesis through repressive CCAAT/enhancer-binding proteins. *Mol Cell Biol* 20:912–918, 2000
21. Wolf G, Haberstroh U, Neilson EG: Angiotensin II stimulates the proliferation and biosynthesis of type I collagen in cultured murine mesangial cells. *Am J Pathol* 140:95–107, 1992
22. Wrana JL, Attisano L, Carcamo J, *et al*: TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* 71:1003–1014, 1992
23. Sharma K, Ziyadeh FN, Alzahabi B, *et al*: Increased renal production of transforming growth factor-beta1 in patients with type II diabetes. *Diabetes* 46:854–859, 1997
24. Islam M, Burke JF, Jr, McGowan TA, *et al*: Effect of anti-transforming growth factor-beta antibodies in cyclosporine-induced renal dysfunction. *Kidney Int* 59:498–506, 2001
25. Sharma K, Jin Y, Guo J, Ziyadeh FN: Neutralization of TGF-beta by anti-TGF-beta antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in STZ-induced diabetic mice. *Diabetes* 45:522–530, 1996
26. Sharma K, Ziyadeh FN: Renal hypertrophy is associated with upregulation of TGF-beta 1 gene expression in diabetic BB rat and NOD mouse. *Am J Physiol* 267:F1094–F1101, 1994
27. Sirard C, de la Pompa JL, Elia A, *et al*: The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev* 12:107–119, 1998
28. Chin BY, Mohsenin A, Li SX, *et al*: Stimulation of pro-alpha(1)(I) collagen by TGF-beta(1) in mesangial cells: Role of the p38 MAPK pathway. *Am J Physiol Renal Physiol* 280:F495–F504, 2001
29. Hayashida T, Poncelet AC, Hubchak SC, Schnaper HW: TGF-beta 1 activates MAP kinase in human mesangial cells: A possible role in collagen expression. *Kidney Int* 56:1710–1720, 1999
30. Chen SJ, Yuan W, Lo S, *et al*: Interaction of smad3 with a proximal smad-binding element of the human alpha2(I) procollagen gene promoter required for transcriptional activation by TGF-beta. *J Cell Physiol* 183:381–392, 2000
31. Jimenez SA, Varga J, Olsen A, *et al*: Functional analysis of human alpha 1(I) procollagen gene promoter. Differential activity in colla-

gen-producing and -nonproducing cells and response to transforming growth factor beta 1. *J Biol Chem* 269:12684–12691, 1994
32. Moustakas A, Kardassis D: Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members. *Proc Natl Acad Sci USA* 95:6733–6738, 1998
33. Zhang W, Ou J, Inagaki Y, *et al*: Synergistic cooperation between Sp1 and Smad3/Smad4 mediates transforming growth factor beta1 stimulation of alpha 2(I)-collagen (COL1A2) transcription. *J Biol Chem* 275:39237–39245, 2000
34. Chen SJ, Yuan W, Mori Y, *et al*: Stimulation of type I collagen transcription in human skin fibroblasts by TGF-beta: Involvement of Smad 3. *J Invest Dermatol* 112:49–57, 1999
35. Lindahl GE, Chambers RC, Papakrivopoulou J, *et al*: Activation of fibroblast procollagen alpha 1(I) transcription by mechanical strain is transforming growth factor-beta-dependent and involves increased binding of CCAAT-binding factor (CBF/NF-Y) at the proximal promoter. *J Biol Chem* 277:6153–6161, 2002
36. Maity SN, Golumbek PT, Karsenty G, de Crombrugghe B: Selective activation of transcription by a novel CCAAT binding factor. *Science* 241:582–585, 1988
37. Datta PK, Blake MC, Moses HL: Regulation of plasminogen activator inhibitor-1 expression by transforming growth factor-beta-induced physical and functional interactions between Smads and Sp1. *J Biol Chem* 275:40014–40019, 2000
38. Hanafusa H, Ninomiya-Tsuji J, Masuyama N, *et al*: Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. *J Biol Chem* 274:27161–27167, 1999
39. Wang L, Zhu Y, Sharma K: Transforming growth factor-beta1 stimulates protein kinase A in mesangial cells. *J Biol Chem* 273:8522–8527, 1998
40. Jiang Y, Chen C, Li Z, *et al*: Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *J Biol Chem* 271:17920–17926, 1996
41. Iordanov M, Bender K, Ade T, *et al*: CREB is activated by UVC through a p38/HOG-1-dependent protein kinase. *EMBO J* 16:1009–1022, 1997
42. Masuyama N, Hanafusa H, Kusakabe M, *et al*: Identification of two Smad4 proteins in *Xenopus*. Their common and distinct properties. *J Biol Chem* 274:12163–12170, 1999
43. Howell M, Itoh F, Pierreux CE, *et al*: *Xenopus* Smad4beta is the co-Smad component of developmentally regulated transcription factor complexes responsible for induction of early mesodermal genes. *Dev Biol* 214:354–369, 1999