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Inhibitory Smads and TGF-β Signaling in Glomerular Cells

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Abstract. Smad6 and Smad7 are inhibitory SMADs with putative functional roles at the intersection of major intracellular signaling networks, including TGF-β, receptor tyrosine kinase (RTK), JAK/STAT, and NF-κB pathways. This study reports differential functional roles and regulation of Smad6 and Smad7 in TGF-β signaling in renal cells, in murine models of renal disease and in human glomerular diseases. Smad7 is upregulated in podocytes in all examined glomerular diseases (focal segmental glomerulosclerosis [FSGS], minimal-change disease [MCD], membranous nephropathy [MNP], lupus nephritis [LN], and diabetic nephropathy [DN]) with a statistically significant upregulation in "classical" podocyte-diseases such as FSGS and MCD. TGF-β induces Smad7 synthesis in cultured podocytes and Smad6 synthesis in cultured mesangial cells. Although Smad7 expression inhibited both Smad2- and Smad3-mediated TGF-β signaling in podocytes, it inhibited only Smad3 but not Smad2 signaling in mesangial cells. In contrast, Smad6 had no effect on TGF-β/Smad signaling in podocytes and enhanced Smad3 signaling in mesangial cells. These data suggest that Smad7 is activated in injured podocytes *in vitro* and in human glomerular disease and participates in negative control of TGF-β/Smad signaling in addition to its pro-apoptotic activity, whereas Smad6 has no role in TGF-β response and injury in podocytes. In contrast, Smad6 is upregulated in the mesangium in human glomerular diseases and may be involved in functions independent of TGF-β/Smad signaling. These data indicate an important role for Smad6 and Smad7 in glomerular cells *in vivo* that could be important for the cell homeostasis in physiologic and pathologic conditions.

The TGF-β signaling system controls an array of cellular responses to various forms of chronic glomerular injury, including extracellular matrix accumulation, proliferation, hypertrophy, and podocyte apoptosis (1-5). Increased expression of TGF-β isoforms and components of the intracellular signaling system is described in most models of glomerular disease and progressive human disease, irrespective of the underlying etiology (6). Together, these studies implicate the TGF-β pathway as the key molecular signaling cascade involved in mediating cellular pathomechanisms resulting in glomerulosclerosis and interstitial fibrosis.

The SMAD family of intracellular proteins consists of several functionally and structurally defined subgroups (reviewed in references 7 and 8). Receptor-activated Smads (R-Smads) are characterized by conserved N-terminal and C-terminal major homology domains, MH1 and MH2, respectively. In this subfamily, Smad1, Smad5, Smad8 act as positive signal transducers activated by interaction with ligand-activated bone morphogenetic protein (BMP) receptors, whereas Smad2 and Smad3 act in the TGF-β/activin receptor pathway. R-Smad activation depends on their C-terminal serine phosphorylation by cognate receptor kinases, followed by complex formation with another type of Smad, common partner Smad4, and nuclear translocation and transcriptional activation of target genes. Smad6 and Smad7 lack N-terminal MH1 domain and are the only members of the SMAD subfamily of inhibitory Smads (I-Smads).

I-Smads are induced at a transcriptional level by TGF-β and BMP and may inhibit intracellular signaling emanating from ligand-activated BMP receptors and/or TGF-β receptors, providing a mechanism for negative feedback regulation (9). At physiologic concentrations, Smad6 may selectively inhibit BMP receptor signaling, whereas Smad7 inhibits TGF-β/Activin receptor signaling. Smad7 may function as a general negative regulator of TGF-β receptor signaling, inasmuch as Smad7 mediates both autoinhibitory feedback and downregulation of TGF-β signaling by major opposing pathways, including Jak1/Stat1 (10) and the NF-κB pathway (11). Although I-Smads may inhibit R-Smads by forming stable interactions with activated type I receptors, thus preventing phosphorylation/activation of R-Smads by receptor kinase (9,12), recent reports define Smad7 as an adaptor in an E3 ubiquitin-ligase complex that targets the TGF-β receptor for degradation (13,14). Additional activities of I-Smads may include transcriptional repressor function (Smad6) (15) and pro-apoptotic functions (1,16-18). Here we report differential functional roles and regulation of Smad6 and Smad7 in TGF-β signaling in renal cells, in murine models of renal disease, and in human

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glomerular diseases. We could identify podocytes and mesangial cells in the mouse glomerulum as cell types expressing Smad6 and Smad7 under physiologic conditions and demonstrate different kinetics in murine podocytes and murine mesangial cells after stimulation with TGF-β *in vitro*. We can demonstrate that Smad7 inhibits Smad2- and Smad3-dependent signaling in both cell types with a preference for Smad3-dependent signaling in mesangial cells. In contrast, Smad6 has only an incomplete inhibitory effect on Smad3 in podocytes. *In vivo*, in a mouse model for podocyte-defects (CD2AP-knockout) and a model for inflammatory immunocomplex-mediated glomerular disease (NZB/NZW-F1), we observed upregulation of Smad7 and downregulation of Smad6 in podocytes and upregulation of Smad6 in the mesangium. These data are supported by a blinded study on human biopsies, indicating a regulation of Smad6 and Smad7 in the same pattern in different human glomerular diseases. Taken together, these data indicate an important role for Smad6 and Smad7 in glomerular cells *in vivo* that could be important for the cell homeostasis in physiologic and pathologic conditions. Furthermore, Smad6, which is induced in mesangial cells by TGF-β, shows no significant inhibitory effect on TGF-β function but could be involved in modulation of inflammatory and fibrogenetic responses of mesangial cells.

Materials and Methods

Animals

For murine experimental models, we used 6-wk-old wild-type C57BL/6 mice; 12- and 24-wk-old NZB/NZW mice developing immune-mediated lupus-like glomerular lesions (19) and 2- and 4-wk-old CD2AP −/− (knockout) and +/+ (wild-type) littermate mice, which develop renal lesions similar to progressive primary focal segmental glomerulosclerosis (FSGS) in humans (20). Animals were sacrificed by carbon dioxide inhalation plus cervical dislocation as approved by our animal protocol, and the kidneys were fixed in 10% formalin before standard processing and embedding for histology as described in Schiffer *et al.* (1).

Cell Culture

Cultivation of conditionally immortalized mouse podocytes was performed as reported by Mundel and Kriz (21). To propagate podocytes, cells were cultivated on type I collagen (Becton Dickinson, Bedford, MA) at 33°C in the presence of 20 U/ml mouse recombinant γ-interferon (Sigma, St. Louis, MO) (permissive conditions) to enhance expression of a thermosensitive T antigen. To induce differentiation, podocytes were maintained at 37°C without γ-interferon, resulting in absence of thermosensitive T antigen (non-permissive conditions). Murine mesangial cells (MMC) were obtained from Dr. Fuad Ziyadeh (22) and cultured in RPMI or DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin, and glutamine.

Immunodetection of Smad6 and Smad7 In Situ

Immunoperoxidase labeling and immunofluorescence labeling were performed as described previously (1). In brief, kidney tissue was formalin fixed and paraffin embedded. Sections were processed according to a routine histologic procedure (Xylene, 2 × 5 min; ethanol, 5 min each of 100%, 95%, 70%, 50%). After rehydration, antigen retrieval by microwave treatment was carried out by heating the sections three times for 3 min in a 0.1 M sodium citrate buffer (pH 6). Unspecific binding of antibodies was blocked with Tris-buffered saline (TBS)–1% bovine serum albumin (BSA)–5% normal goat serum (NGS) for 30 min at room temperature. Smad6 was detected using a rabbit polyclonal antibody raised against a peptide sequence specific for Smad6 (Zymed, San Francisco, CA). Smad7 was detected by using affinity-purified rabbit antibody raised against the peptide, KAF of C-terminal end of Smad7 (23). Antibodies were applied overnight at 4°C in a 1:5 dilution, followed by three washes with TBS. For identification of podocytes, tissue was double-stained with monoclonal mouse-anti synaptopodin (24). Antigen-antibody interaction was visualized by using a peroxidase labeling kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA), or by using FITC- or Cy3-conjugated secondary antibodies for immunofluorescence detection (Jackson ImmunoResearch, West Grove, PA).

Clinical Renal Biopsy Samples

Serial sections (3 μm) of paraffin-embedded renal biopsy samples from adult subjects in a random historic collection (I.SD Roberts, John Radcliffe Hospital, Oxford, UK) were obtained from I.S.D.R. Pre-diagnosed cases included: (*1*) immune complex–mediated diseases, including idiopathic membranous nephropathy with nephrotic-range proteinuria (MNP) and lupus nephritis WHO class IV or higher with proteinuria (LN); (*2*) diseases with primary podocyte defects, including primary focal segmental glomerulosclerosis (FSGS) with nephrotic-range proteinuria (4.5 to 11 g/d) and hypertension, and idiopathic minimal-change disease (MCD) with nephrotic-range proteinuria between 5 to 19.5g/d; and (*3*) diabetic nephropathy (DN) with proteinuria between 2 to 6.8 g/d, including two cases with diabetes mellitus type I, three cases with type II, and one case of diabetes after pancreatectomy for chronic pancreatitis.

Western Blot Analyses

Whole cell protein lysates in RIPA buffer (0.1% SDS, 1% Triton X-100, 1% Na-deoxycholate, 150 mM NaCl, 10 mM Tris, pH 7.2) were derived from podocytes or MMC left either untreated or treated with TGF-β (5 ng/ml) for 3 hr following overnight serum-starvation. Aliquots (80 μg) were resolved by SDS-gel electrophoresis and transferred to nitrocellulose (Osmonics, Westborough, MA). Membranes were probed with anti-Smad7, anti-Smad6 (Zymed, San Francisco, CA), or anti-tubulin (Sigma, St. Louis, MO) for protein loading control as described (1). C-terminal phosphorylation of Smad2 was detected using a phospho-Smad2–specific rabbit polyclonal antibody as described (25). Antigen-antibody complexes were visualized using enhanced chemiluminescence reagents according to the manufacturer's protocol (Pierce, Rockford, IL).

Adenoviral Gene Transfer

Adenoviruses encoding LacZ, Flag-Smad6, or Flag-Smad7 were used to infect podocytes and mesangial cells. The stock maintenance in 293T cells and the establishment of titers is described by Nakao *et al.* (26). Optimal infection conditions were established using a β-galactosidase–encoding virus and staining of fixed monolayers with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal); for the Flag-Smad6- and Flag-Smad7-encoding virus, the Flag-expression was detected by western blotting. Transduction efficiencies were typically in excess of 90%. For routine infections, the cells were seeded at a density of 5×10^4 cells/cm^2 in a 100 mm tissue culture plates. The next day the culture medium was changed to RPMI containing 5% FBS 1 h before infection. Cells were incubated with 200 multiplicity

of infection (MOI) for 12 h. Cells were then washed and incubated in 0.2% FBS for 12 h and stimulated with TGF-β1 (5 ng/ml) or left untreated as indicated.

Cell Transfection Studies

The different SMADs were cloned into the expression plasmid pCDNA3.1 (Invitrogen Corp., San Diego, CA) with an additional FLAG-epitope-tag. Cells were seeded on six-well dishes and cotransfected with the reporter constructs SBE-4 (palindromic Smad-binding element) as specific readout for Smad3-mediated (27) and ARE/Fast-1 (transcription factor forkhead activin signal transducer-1 [Fast-1] acts as a co-factor for Smad2) as readout for Smad2-mediated promotor activation (for review see references 28 and 29) linked to the luciferase gene and with the pRSV-Gal (Promega, Madison, WI) to normalize for transfection efficiency using either Superfect or Effectene-Reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. Luciferase/galactosidase activities were determined using assay kits from Promega. Luciferase activity was measured using an analytical luminescence laboratory luminometer. To correct for differences in transfection efficiency, luciferase units were normalized for galactosidase activity in the same lysate. The normalized readings were expressed as folds of induction compared with empty vector readings (pCDNA3.1).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays (EMSA)

For nuclear extract preparation, the cells were harvested by lysing in an NP-40 containing sucrose buffer (0.32 M sucrose, 10 nM Tris HCl [pH 8.0], 3 mM $CaCl_2$, 2 mM MgOAc, 0.1 mM EDTA, 0.5% NP-40, 1 mM DTT). After washing, the nuclei were suspended in a hypotonic low-salt buffer (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 25% glycerol, 1% NP-40, 0.5 mM DTT). Then a high-salt buffer (with 800 mM KCl) was added slowly. Nuclear extracts were incubated on a shaker at 4°C for 45 min and cleared from debris and nuclear envelopes retaining genomic DNA. All buffers contained protease inhibitor mix (Roche Diagnostics GmbH, Mannheim, Germany) and okadaic acid (Calbiochem, Canada) at concentrations recommended by the manufacturer. EMSA were performed as described previously (30) using nuclear extracts from LacZ-infected, AdSmad6-infected, or AdSmad7-infected podocytes and MMC either untreated or treated with TGF-β1 (5 ng/ml) for 45 min. Complementary oligonucleotides containing the Smad binding element (SBE) sequence were γ-^{32}P-end labeled by T4 polynucleotide kinase reaction and annealed. SBE-probe (50,000 cpm) was incubated with 5 μg of nuclear extract in binding buffer (10 mM Tris-HCl [pH 8.0], 150 mM KCl, 0.5 mM EDTA, 0.1% Triton-X100, poly(dI-dC), 12.5% glycerol, and 1 mM DTT) with or without preincubation for 10 min with a 50-fold molar excess of unlabeled annealed competitor. For antibody interference studies (supershift analyses), the nuclear extracts were preincubated with goat polyclonal anti-Smad3 (sc-6202x; Santa Cruz Biotechnology Inc., Santa Cruz, CA). DNA-binding complexes were separated by nondenaturing 4% polyacrylamide gel electrophoresis at room temperature and visualized by autoradiography.

Results

Expression of Inhibitory Smads in Normal and Diseased Mouse Glomeruli

To identify cellular patterns of expression of Smad6 and Smad7 proteins in normal glomeruli, we used specific antibodies for immunodetection of I-Smads together with the podocyte-specific marker synaptopodin in normal mouse kidneys. Both Smad6 and Smad7 showed co-localization with synaptopodin in a fraction of synaptopodin-positive podocytes (Figure 1A, white arrowheads). In addition, Smad6 and Smad7 expression was detectable in synaptopodin-negative, endocapillary/mesangial cells (Figure 1A, yellow arrowheads). Preincubation of Smad7 antibody with the respective immunogenic blocking peptide completely abolished the observed staining patterns, confirming specificity of anti-Smad7 staining (data not shown). No blocking peptide is available for Smad6; therefore, we incubated the tissue sections with rabbit IgG in the same concentration, which resulted in no specific staining pattern (data not shown). Next, we used mice carrying targeted deletions of the Cd2ap gene (CD2AP −/−) and NZB/W-F1 mice as well-characterized murine models for nonimmune-mediated and immune-mediated glomerular diseases, respectively. CD2AP −/− mice develop primary glomerular and subsequent tubulointerstitial lesions, closely resembling defects characteristic of progressive primary FSGS in humans (31). NZB/W-F1 mice develop a lupus-like nephritis with immune complex and complement deposition. They show no renal phenotype at 12 wk, and the disease develops around 20 wk with heavy proteinuria leading to renal failure and death at 24 to 28 wk (19). Numbers and staining intensity of Smad7-positive podocytes was increased at 2 wk (early stage) and further increased at 4 wk (advanced stage) in CD2AP −/− compared with littermate CD2AP +/+ mice (Figure 1B, lower panels). In contrast, Smad6 expression was reduced or absent in podocytes but significantly increased in a mesangial expression pattern in 4-wk-old CD2AP −/− compared with CD2AP +/+ (Figure 1B, upper panel). Similar patterns of cell type-dependent I-Smad isoform switching were observed during the course of lupus-like nephritis in NZB/NZW-F1 mice (Figure 1C). Our results demonstrate that both primary podocyte injury (CD2AP −/−) and immune-mediated glomerular injury (NZB/NZW-F1) induced Smad7 and reduced Smad6 expression in podocytes, whereas Smad6 expression was strikingly increased in mesangial compartments in both glomerular injury models.

Smad7 Protein is Induced in Podocytes in Humans with FSGS and MCD, and Smad6 Protein Is Suppressed in Podocytes in Humans with Lupus Nephritis and Diabetic Nephropathy

On the basis of these observations and previously reported observations (1) from different experimental models of progressive glomerular disease, we wished to validate whether Smad6 and Smad7 proteins were differentially regulated in podocytes in a random series of biopsies representing human glomerular diseases (see Materials and Methods for details). Immunoperoxidase labeling was performed as shown in Figures 1B and 1C, and image and statistical analyses were performed blindly without prior knowledge of any clinical information or pathologic diagnoses. On average in normal kidney, 7.1 (± 0.95 SD) podocytes/glomerular section and 5.7 (± 0.75 SD) podocytes/glomerular section were positive for Smad6 and Smad7, respectively (Figure 2). There was a trend

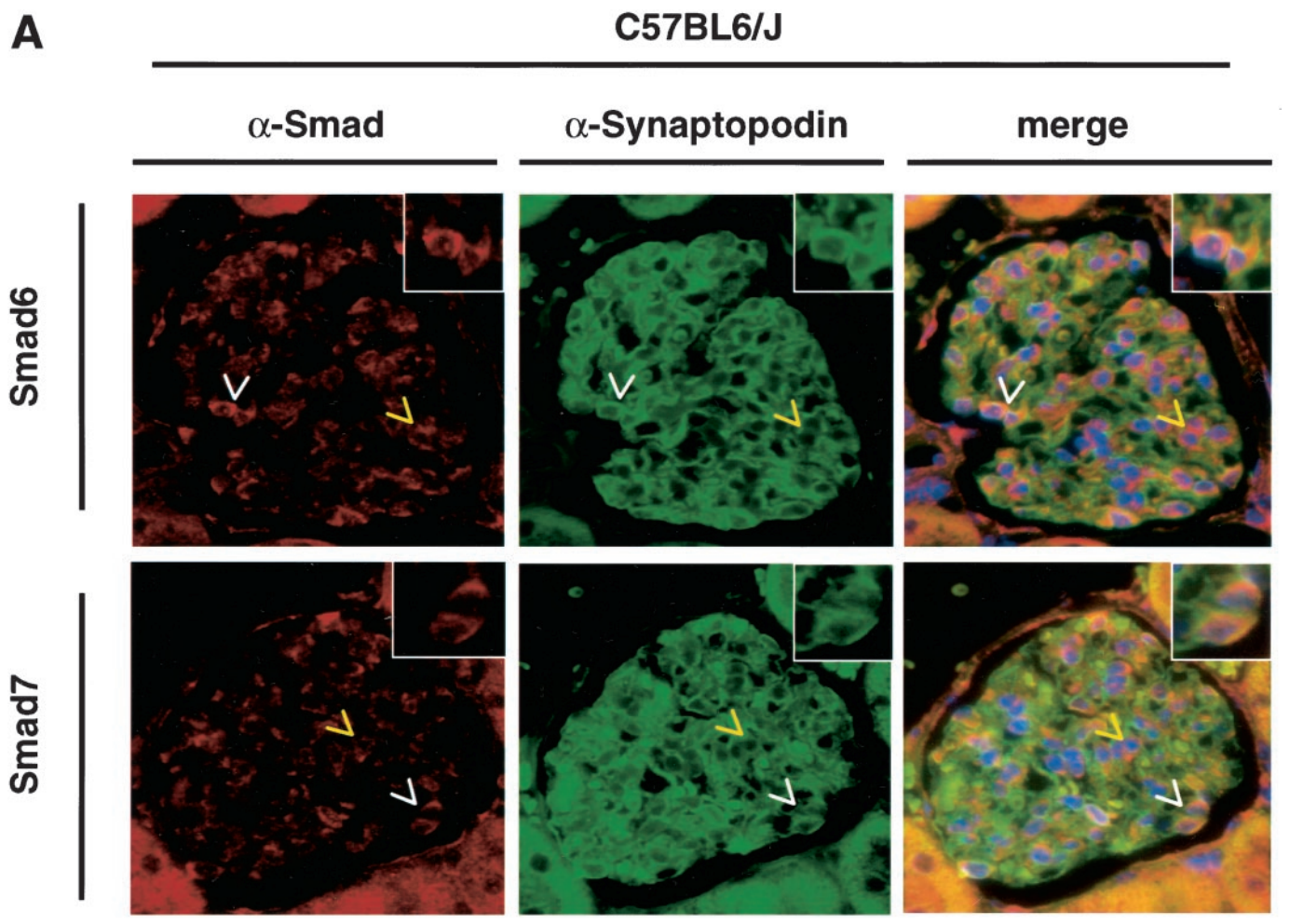


Figure 1. Expression of inhibitory Smad6 and Smad7 overlaps in normal and differs in diseased mouse glomeruli. (A) Indirect immunofluorescence double-labeling on sections of normal 6-wk-old C57BL6/J mouse kidney using mouse-monoclonal anti-synaptopodin antibody together with FITC-conjugated secondary goat anti-mouse antibody, and rabbit polyclonal anti-Smad6 (upper panels) or anti-Smad7 (lower panels) together with Cy3-conjugated secondary anti-rabbit-antibody, respectively. DAPI staining was used for visualization of cell nuclei. White open arrows depict synaptopodin-positive podocytes labeled with Smad6 or Smad7 (insert in upper right corner). Yellow open arrows depict synaptopodin-negative endocapillary cells labeled with Smad6 or Smad7, respectively. (B) Smad6 (upper panels) and Smad7 (lower panels) immunoperoxidase labeling of kidney sections from CD2AP +/+ and CD2AP −/− littermates at 2 wk and 4 wk of age, respectively. Black arrows indicate podocytes stained positive. (C) Smad6 (upper panels) and Smad7 (lower panels) immunoperoxidase labeling of kidney sections from 12-wk-old and 24-wk-old NZB/NZW-F1 mice. Black open arrows depict positively stained podocytes.

toward decreased Smad6 expression in podocytes in all disease groups, whereas suppression of Smad6 was statistically significant in lupus nephritis (4.03 ± 1.91 SD; $P < 0.05$) and diabetic nephropathy (4.43 ± 1.19 SD; $P < 0.05$) compared with normal kidney. In contrast, Smad7 tended to be induced in podocytes in all disease groups, and the increased expression achieved statistical significance in FSGS (8.7 ± 2.3 SD; $P < 0.05$) and MCD (11.4 ± 4.5 SD; $P < 0.05$) compared with normal kidney, respectively. Mesangial expression was semiquantitatively scored as reduced, equal, or enhanced compared with normal specimen. There was no reduced expression of Smad6 and Smad7 compared with normal tissue and no clear upregulation of Smad7 but a trend toward increased Smad6 expression in the mesangial/endocapillary compartment (Table 1). These data directly validate randomly selected clinical biopsy material our observations obtained from animal models (CD2AP −/− [this report]; TGF-β1 transgenic mice [1], and NZB/NZW [this report]).

TGF-β Induces Smad7 Protein Expression in Podocytes and Smad6 Protein Expression in Mesangial Cells In Vitro

TGF-β has been shown to induce transcription of the Smad6 gene (M Bitzer and EP Böttinger, unpublished data) and of the Smad7 gene in various nonrenal cell types through Smad3/Smad4–dependent signaling (30,32). Although it is well documented that mesangial expression of TGF-β isoforms and cognate signaling receptors is increased in most glomerular diseases in humans and numerous experimental models, increased TGF-β expression in podocytes has recently been

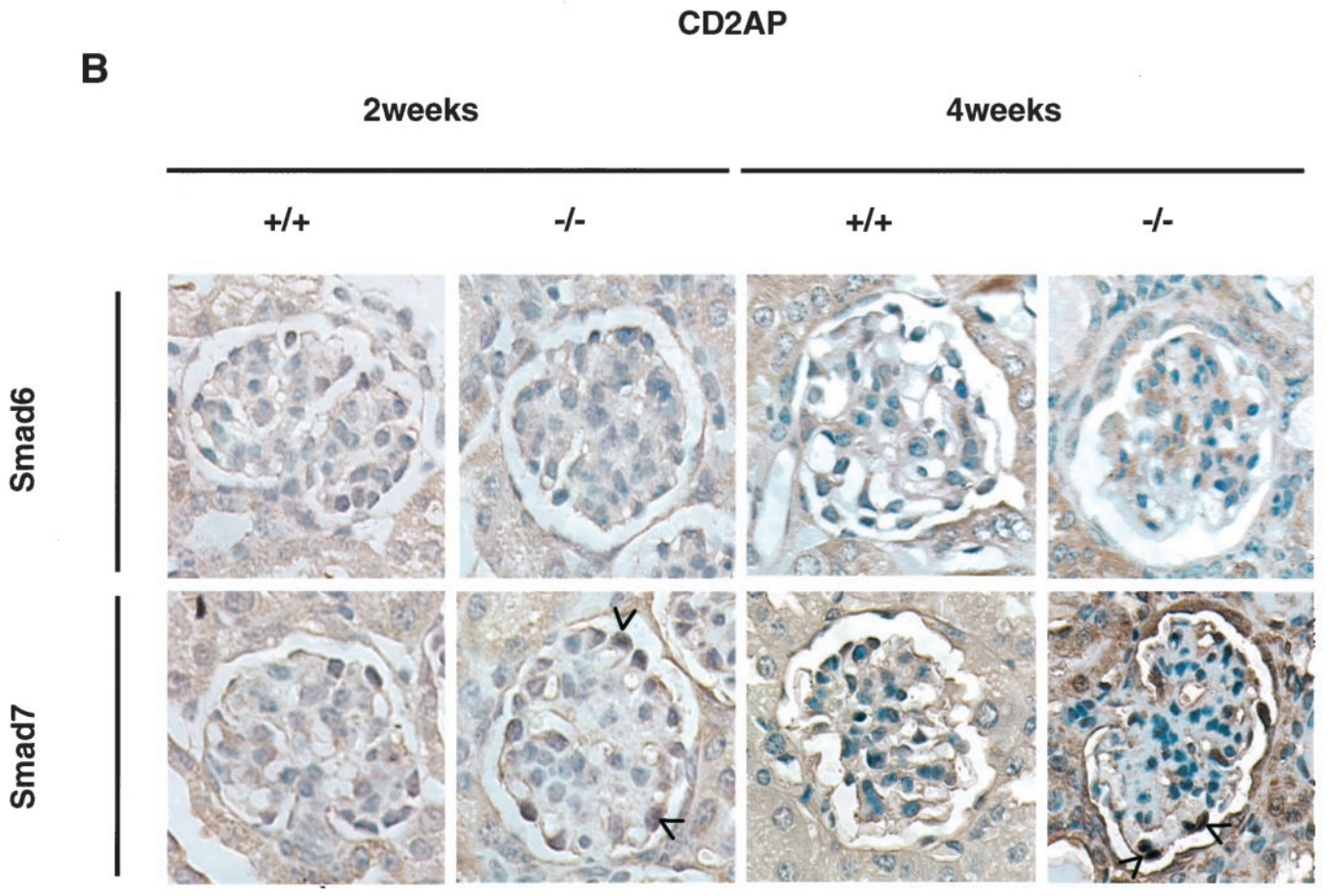
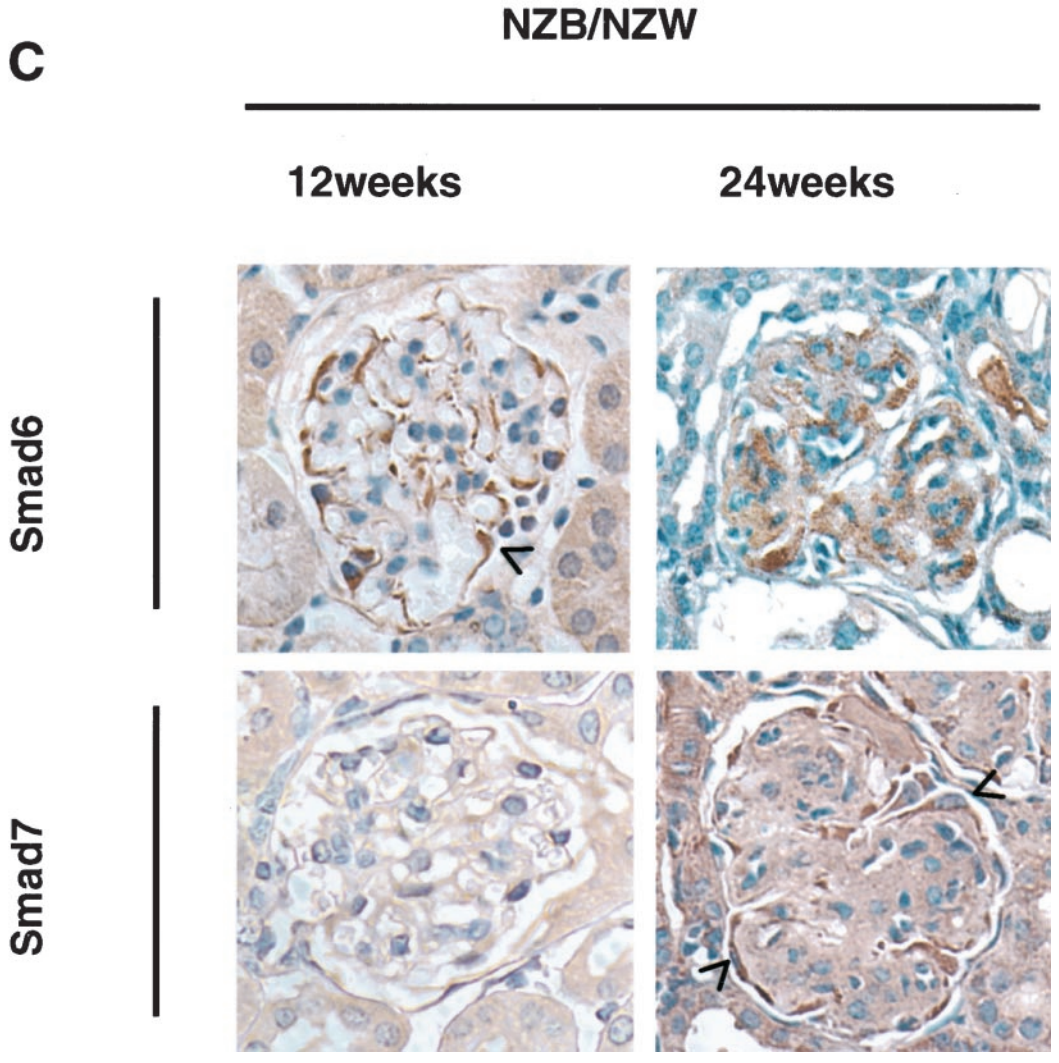


Figure 1. (Continued)

reported in nephrotoxic nephritis (33). To examine whether TGF-β regulates Smad6 and/or Smad7 directly in cultured murine podocytes and mesangial cells, we performed Western blot analyses using anti-Smad6 or anti-Smad7 antibodies. Smad6 protein was absent in untreated or TGF-β–treated podocytes, and TGF-β increased baseline expression of Smad6 in MMC (Figure 3). In contrast, Smad7 was absent in untreated cells and strongly induced by TGF-β in podocytes, whereas TGF-β did not affect baseline Smad7 expression in MMC (Figure 3). Thus, TGF-β selectively regulates Smad7 expression in podocytes and Smad6 expression in mesangial cells in culture. These results are consistent with our *in vivo* observations and indicate that increased expression of Smad7 in podocytes and of Smad6 in endocapillary/mesangial cells may be associated with TGF-β activity, depending on the etiology of glomerular injury.

Smad6 Does Not Inhibit Activation of Smad2-Dependent or Smad3-Dependent Signaling by TGF-β in Podocytes and Mesangial Cells

Although Smad6 is primarily considered a mediator of negative autoinhibition of BMP receptor and BMP-Smad signaling via Smad1, Smad5, and Smad9, it may also inhibit TGF-β receptor and TGF-β–Smad signaling via Smad2 and Smad3 (34-37). To begin to examine a potential functional relevance of increased Smad6 expression in mesangium of glomerular injury models in TGF-β/SMAD signaling, we used biochemical and transient transcriptional assays to selectively assess the effect of Smad6 on either Smad2 pathway and target promoter activation or Smad3 pathway and target promoter activation. Overexpression of Smad6 in podocytes or mesangial cells (MMC) using established adenoviral gene transfer methodology (26) had no inhibitory effect on C-terminal serine phosphorylation of Smad2 induced by TGF-β, when compared with AdLacZ control adenoviral transduction (Figure 4A). An additional band of unknown significance of slightly higher molecular weight than phospho-Smad2 appeared specifically in TGF-β–treated and AdSmad6-transduced podocytes (Figure 4A, asterisk). Adenoviral Flag-Smad6 expression levels were verified by immunoblotting with anti-FLAG (Figure 4A). Because C-terminal phosphoserine-specific anti-Smad3 antibodies are currently not available, we employed EMSA with oligonucleotide probes containing an SBE sequence to examine Smad3 activation (27,30). Presence of Smad3 in TGF-β–inducible DNA-protein binding complexes (Figure 4B, lanes 3 and 13) was verified using anti-Smad3 antibody interference (Figure 4B, lanes 5 and 15). Adenoviral expression of Smad6 had no effect on TGF-β–induced Smad3/4 binding complexes in MMC (Figure 4B, compare lanes 13 and 18) and partially



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inhibited complex formation in podocytes (Figure 4B, compare lanes 3 and 8). Next, podocytes or MMC were transiently co-transfected with either SBE4-Luc (27), a Smad3/4-specific luciferase reporter construct, or ARE-Luc/Fast1, a Smad2-specific luciferase reporter assay system (28,29), together with control plasmid (empty vector pcDNA3) or test expression plasmids (pSmad6, pSmad7). pSmad6 co-transfection appeared to increase activation by TGF-β of SBE4-Luc ($P < 0.05$) and ARE-Luc ($P = 0.09$) in MMC compared with control pcDNA3, whereas it had no significant effect on these transcriptional responses in podocytes (Figure 4C). Thus, our biochemical and transcriptional assays consistently demonstrate that overexpression of Smad6 does not inhibit Smad2 or Smad3 activation by TGF-β in podocytes and MMC.

Table 1. Mesangial expression of Smad6 and Smad7 in human biopsies[a]

	FSGS ($n = 7$)	MCD ($n = 6$)	MNP ($n = 8$)	LN ($n = 6$)	DN ($n = 6$)
Smad6 expression equal to normal	4	2	1	2	3
Smad6 expression enhanced	3	4	7	4	3
Smad7 expression equal to normal	3	2	4	5	3
Smad7 expression enhanced	4	4	4	1	3

[a] Immunohistological staining for Smad6 and Smad7 was performed on a random series of human glomerular diseases. The staining was semiquantitatively ranked for equal or enhanced expression compared to normal specimen. Numbers represent cases in each group. FSGS, focal segmental glomerulosclerosis; MCD, minimal-change disease; MNP, membranous nephropathy; LN, lupus nephritis; DN, diabetic nephropathy.

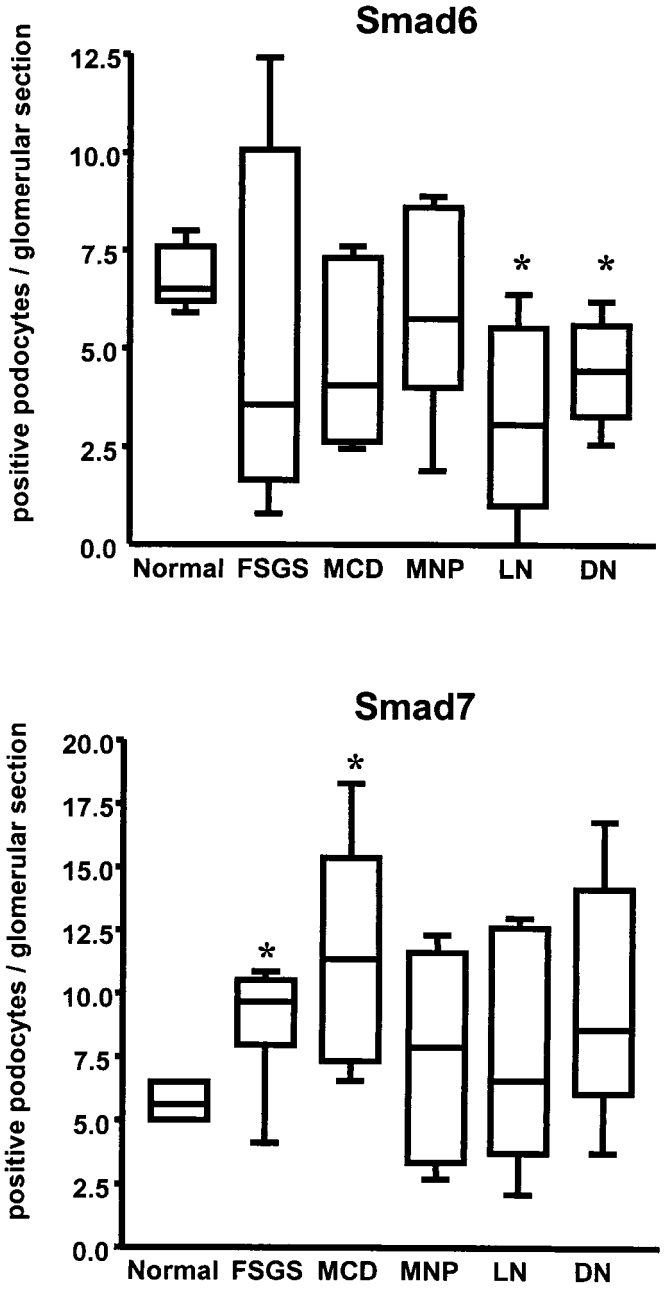

Figure 2. Boxplot statistical analyses of semiquantitative podocyte immunolabelings for Smad6 and Smad7 in human glomerular diseases. Boxes indicate 50% of the data (25th to 75th percentile) with the median number (center lines), and the whiskers indicate maximum and minimum value of Smad6-positive (upper panel) or Smad7-positive podocytes per glomerular section (lower panel) in normal adult human kidney cortex (Normal; $n = 4$), and renal biopsy sections representing primary focal segmental glomerulosclerosis (FSGS; $n = 7$), minimal-change disease (MCD; $n = 6$), membranous nephropathy (MNP, $n = 8$), lupus nephritis (LN; $n = 6$), and diabetic nephropathy (DNP; $n = 6$). Biopsy sections with at least eight glomeruli were scored, and scores from all glomeruli and all biopsies in each group were included in statistical disease group comparisons. * indicates disease groups with statistically significant differences ($P < 0.05$) of number of stained podocytes per glomerular section in disease groups compared with normal kidney.

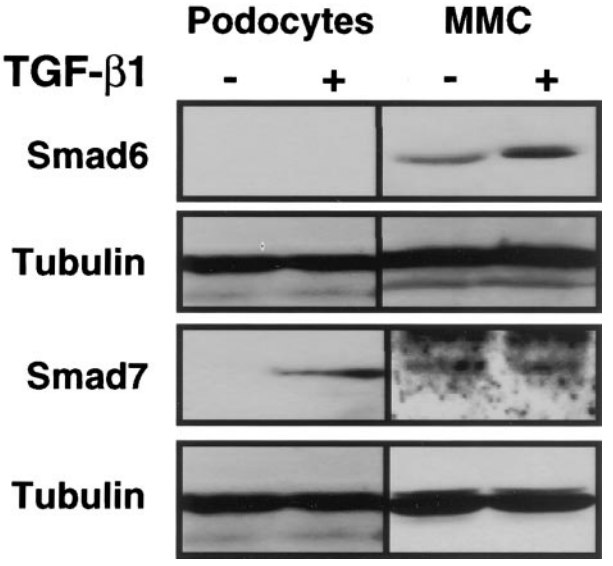

Figure 3. Immunoblot of Smad6 and Smad7 protein in murine podocytes and mouse mesangial cells. Mesangial cells (MMC) and podocytes were left untreated (−) or treated (+) with TGF-β1 (5 ng/ml) for 3 hr before cell lysis. Protein loading control is represented by tubulin staining. Immunoblot shown is representative for two independent experiments.

Smad7 Inhibits Both Smad2-Dependent and Smad3-Dependent TGF-β Signaling in Podocytes, and It Inhibits Smad3-Dependent but not Smad2-Dependent TGF-β Signaling in MMC

Using the identical assay systems as described in the previous paragraph (Figure 4, A through C), we examined the effects of increased Smad7 expression on TGF/SMAD signaling. Adenoviral gene transfer of Smad7 completely blocked C-terminal serine phosphorylation of Smad2 by TGF-β in podocytes, whereas it only partially reduced Smad2 phosphorylation in MMC (Figure 4A). TGF-β–induced Smad3/4 DNA-binding complex formation was completely abolished by adenoviral expression of Smad7 in both podocytes (Figure 4C, lane 10 *versus* lane 3) and MMC (Figure 4C, lane 20 *versus* lane 13). Although adenoviral Smad7 expression completely prevented Smad3/4-dependent transcriptional activation of SBE4-Luc induced by TGF-β in podocytes and MMC, Smad2-dependent ARE-Luc activation was completely blocked in podocytes but not in MMC (Figure 4C). These data demonstrate that Smad7 is an efficient inhibitor of both Smad2- and Smad3-dependent TGF-β signaling and transcriptional activation in podocytes. In contrast, Smad7 is a selective inhibitor of Smad3-dependent TGF-β signaling but not of Smad2-dependent signaling in MMC.

Discussion

Little is known about inhibitory I-Smads, Smad6 and Smad7, in renal biology and pathophysiology. Our studies are providing new insights into functional roles and regulation of I-Smads in glomerular cells, experimental models of glomerular disease in mice, and common human glomerular diseases. On the basis of our results, we propose a new working model for differential roles of

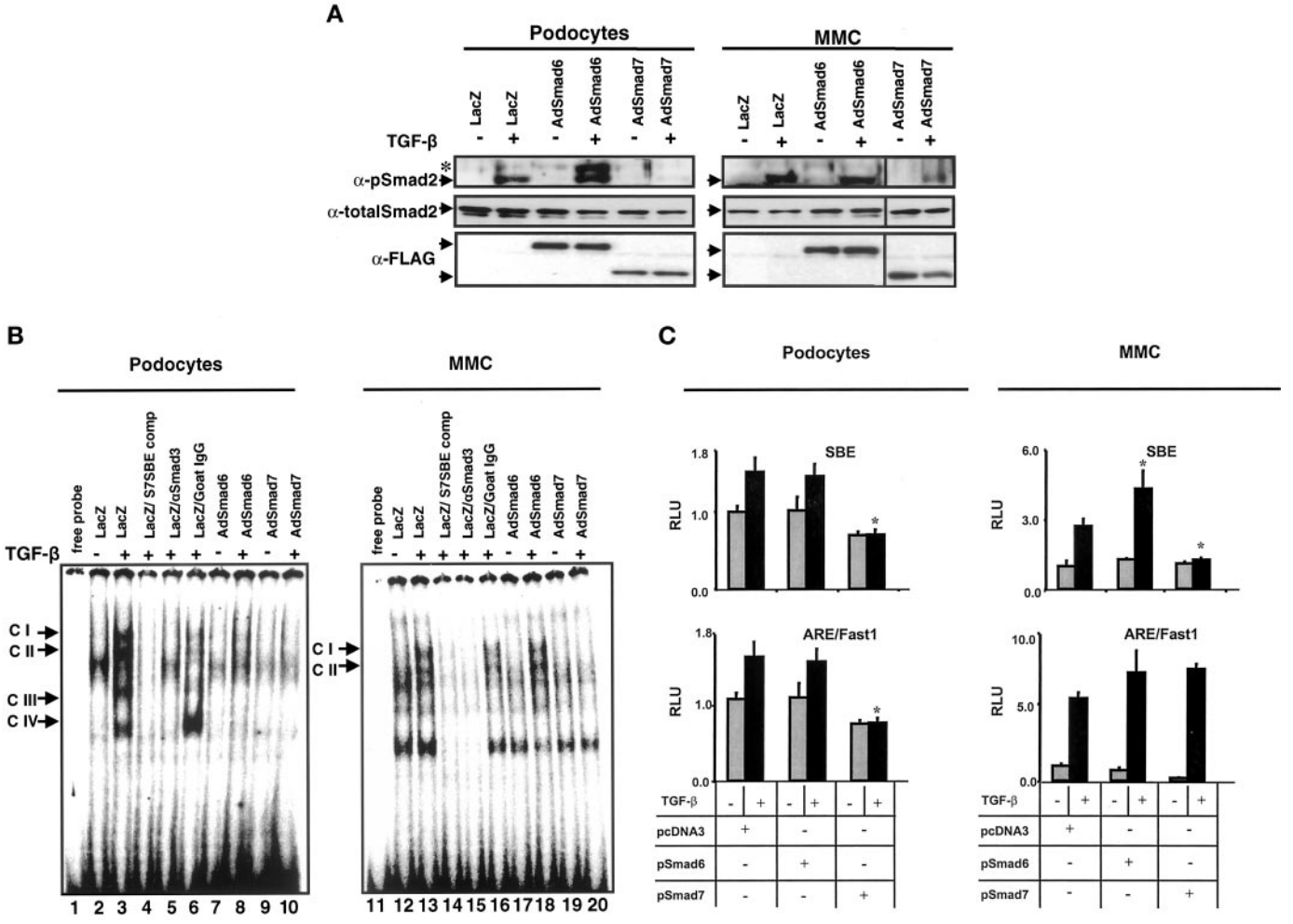

Figure 4. Effects of Smad6 and Smad7 overexpression on Smad2- or Smad3-mediated signaling in podocytes and mesangial cells. (A) Immunoblot demonstrating Smad2-phosphorylation in podocytes and mesangial cells after overexpression of Smad6 and Smad7 and after treatment with TGF-β1. Podocytes and mesangial cells were infected with AdLacZ, AdSmad6, or AdSmad7 as indicated, and protein was harvested 15 min after treatment with or without TGF-β1 as indicated. Membranes were probed with an antiserum specific for phosphorylated Smad2. (B) Electrophoretic mobility shift assay (EMSA) comparing the effects of Smad6 and Smad7 on the TGF-β–inducible Smad binding protein complex (SBC) after TGF-β treatment in podocytes and mesangial cells. For proof of specificity, a goat polyclonal anti-Smad3 antibody or goat-IgG was added before the S7SBE-probe. (C) Influence of TGF-β1 on SBE4- and ARE/Fast-1-luciferase reporter activation in renal cells co-transfected with control empty vector construct pcDNA3 or pSmad6 or pSmad7. Histograms show mean ± SD of normalized luciferase activity (RLU) of representative triplicate transfection experiments in conditionally immortalized podocytes and MMC. Readings are expressed as fold induction compared with empty vector readings (pCDNA3). Cells were co-transfected with expression plasmids and treated with TGF-β1 as indicated. Gray bars represent results in untreated cells, and black bars represent results in cells treated with TGF-β1 (5 ng/ml).

Smad6 and Smad7 in glomerular cells exposed to major types of injury *in vivo*, including nonimmune and podocyte-dependent etiologies and immune-mediated injury. Smad7 synthesis is consistently induced in podocytes in animal models and human glomerular disease. It has distinct functional roles in podocytes, including efficient inhibition of TGF-β–activated Smad2- and Smad3–dependent signal transduction and transcriptional activation (this report), and enhancement of caspase-independent apoptosis (1). We have previously shown that Smad7 induces apoptosis in podocytes through inhibition of anti-apoptotic NF-κB signaling, while it did not inhibit apoptosis induced by TGF-β through p38/MAPK signaling (1,17). Together, these results suggest that (*1*) Smad7 may selectively inhibit Smad2/Smad3-dependent but not Smad2/3-independent TGF-β signaling and (*2*) the pro-apoptotic TGF-β activity is mediated through Smad2/3-independent signaling in podocytes. Together with evidence of regulation of Smad7 synthesis by major pathways such as NF-κB, Jak/Stat, shear stress, and Erk/MAPK (reviewed in reference 38) and its emerging role in targeting signaling proteins for ubiquitination and degradation (13,39), these observations indicate an important multifunctional role for Smad7 well beyond negative regulation of TGF-β/Smad signaling in podocyte pathophysiology. It will be important to decipher the molecular determinants of Smad7 multifunctionality.

This study was designed to examine general regulatory mechanisms of TGF-β/Smad signaling that act upstream of

functionally defined groups of target genes, including extracellular matrix genes, cell cycle and apoptosis regulators, etc. Thus, we show here that overexpression of Smad7 inhibits Smad3-dependent TGF-β signaling in mesangial cells and podocytes. Poncelet *et al.* (40) reported that Smad3 may be required for stimulation of type I collagen gene expression by TGF-β1 in human mesangial cells; therefore, we speculate that the lack of inducible expression of inhibitory Smad7 in mesangial cells observed in our study *in vitro* and *in vivo* may permit enhanced fibrogenic activities of TGF-β selectively in mesangial cells. In contrast, the observed induction of Smad7 in podocytes *in vitro* and *in vivo* may limit fibrogenic TGF-β/Smad3 signaling and may shift TGF-β signaling activities to promote apoptotic responses in podocytes. Thus, it is possible that differential cell type–specific regulation of Smad7 synthesis in glomerular cells may specify fibrogenic (mesangial cells) or proapoptotic (podocytes) TGF-β responsiveness in glomerular disease. It follows from our discussion that a yet unidentified putative signaling mechanism may exist specifically in mesangial cells, which may suppress Smad7 synthesis in response to TGF-β activation.

In contrast with Smad7, we find no evidence for an inhibitory role of Smad6 in activation of Smad2- or Smad3-dependent signaling by TGF-β in podocytes. In addition, Smad6 expression appears to be reduced in podocytes in murine models and human glomerular disease. Whether downregulation of Smad6 in podocytes is significant and its potential functional role will require further investigation. In contrast, our results provide considerable evidence for a role of Smad6 in mesangial injury and/or pathophysiology. First, there is strong upregulation of Smad6 observed in endocapillary/mesangial compartments in murine models (Figure 1C) and a trend toward increased Smad6 in human glomerular disease (Table1). Second, Smad6 is induced by TGF-β in mesangial cells. Third, expression of Smad6 in mesangial cells consistently and significantly enhanced Smad3–dependent transcriptional activity induced by TGF-β. Interestingly, bone morphogenetic protein 7 (BMP7) is an essential regulator of kidney development (41) that is known to oppose TGF-β signaling (42) and ameliorate renal fibrogenesis induced by ureteral obstruction (43). Smad6 is an efficient inhibitor of BMP signaling; it is therefore possible that TGF-β induces Smad6 in mesangial cells to downmodulate negative cross-regulation by BMP-7, resulting in indirect enhancement of TGF-β/Smad signaling in the injured mesangium. Further work is required to test this intriguing hypothesis.

In summary, we provide evidence for cell type–dependent differential roles of Smad6 and Smad7 in regulation of TGF-β signaling in glomerular pathobiology. Although Smad7 may function as signaling switch, inhibiting TGF-β/Smad2/3–dependent transcriptional responses and promoting pro-apoptotic activities by TGF-β in podocytes, Smad6 may enhance transcriptional responses induced by TGF-β via Smad2 and/or Smad3 in mesangial cells, perhaps by mediating negative crosstalk with opposing BMP signaling pathways.

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