Signaling Mechanism of TGF-β1 in Prevention of Renal Inflammation: Role of Smad7

Wansheng Wang,* Xiao R. Huang,* Allen G. Li,† Fang Liu,* Jin-Hua Li,* Luan D. Truong,*‡ Xiao J. Wang,† and Hui Y. Lan*

*Departments of Medicine and Pathology, Baylor College of Medicine, Houston, Texas; †Department of Dermatology, Oregon Health & Science University, Portland, Oregon; and ‡Department of Pathology, The Methodist Hospital, Houston, Texas

TGF-β has been shown to play a critical role in anti-inflammation; however, the signaling mechanisms of TGF-β in anti-inflammatory response remains largely unclear. This study reported that mice that overexpress latent TGF-β1 on skin are protected against renal inflammation in a model of obstructive kidney disease and investigated the signaling mechanism of TGF-β1 in inhibition of renal inflammation *in vivo* and *in vitro*. Seven days after urinary obstruction, wild-type mice developed severe renal inflammation, including massive T cell and macrophage infiltration and marked upregulation of IL-1β, TNF-α, and intercellular adhesion molecule-1 (all $P < 0.001$). Surprising, renal inflammation was prevented in transgenic mice. This was associated with an increase in latent TGF-β1 in circulation (a 10-fold increase) and renal tissues (a 2.5-fold increase). Further studies showed that inhibition of renal inflammation in TGF-β1 transgenic mice was also associated with a marked upregulation of renal Smad7 and IκBα and a suppression of NF-κB activation in the diseased kidney (all $P < 0.01$). These *in vivo* findings suggested the importance of TGF-β–NF-κB cross-talk signaling pathway in regulating renal inflammation. This was tested *in vitro* in a doxycycline-regulated Smad7-expressing renal tubular cell line. Overexpression of Smad7 was able to upregulate IκBα directly in a time- and dose-dependent manner, thereby inhibiting NF-κB activation and NF-κB–driven inflammatory response. In conclusion, latent TGF-β may have protective roles in renal inflammation. Smad7-mediated inhibition of NF-κB activation *via* the induction of IkBα may be the central mechanism by which latent TGF-β prevents renal inflammation.

J Am Soc Nephrol 16: 1371–1383, 2005. doi: 10.1681/ASN.2004121070

The TGF-β family, including TGF-β1, TGF-β2, and TGF-β3, is a group of pleiotropic secreted cytokines with unique and potent anti-inflammatory and immunoregulatory properties (1). They have a broad spectrum of biologic functions and act on a large variety of cell types. Several experimental and human studies reveal an important role for TGF-β in renal fibrosis (2), which is crucial for the progression to ESRD. Mice with overexpression of active TGF-β1 develop severe renal damage with progressive renal fibrosis (3). It is interesting that blockade of TGF-β by anti–TGF-β antibodies alleviates renal fibrosis in diabetic nephropathy in mice and puromycin aminonucleoside nephropathy in rats but worsens albuminuria and proteinuria in these disease models (4,5), suggesting a complex role of TGF-β, beyond its profibrotic effects, in the progression of renal diseases. We speculated that these observations might be associated with protective roles of circulating latent TGF-β in renal inflammation. Thus, blockade of circulating TGF-β with a neutralizing TGF-β antibody enhances proteinuria in these disease models. However, there is lack of evidence to support this hypothesis. The signaling mechanisms that are responsible for the potential anti-inflammatory effects of TGF-β are yet largely unknown. Although the role of TGF-β in fibrosis has been firmly established, little attention has been paid to the circulating levels of this cytokine in the disease conditions. TGF-β functions in both autocrine and paracrine manners to regulate cell proliferation, apoptosis, differentiation, chemotaxis, extracellular matrix production, cell migration, and immune response (6). Mice with targeted disruption of TGF-β1 display no gross abnormalities at birth but succumb to wasting associated with multifocal inflammation at approximately day 20 after birth (7). Systemic administration of TGF-β greatly alleviates the inflammatory response in streptococcus cell wall–induced erosive arthritis and prevents the relapse of autoimmune encephalomyelitis, insulitis, and systemic lupus erythematosus in the MRL/lpr/lpr murine model (8–11), strongly suggesting the anti-inflammatory effect of circulating TGF-β.

Although it is known that TGF-β plays an important role in inflammatory responses, the underlying signaling mechanisms by which TGF-β exerts its anti-inflammatory function remain largely unclear. Because mice that overexpress the bioactive form of hepatic TGF-β1 exhibit progressive renal injury associated with a highly increased circulating active TGF-β1 (12), our study sought to test the hypothesis that the increased circulat-

Received December 10, 2004. Accepted February 16, 2005.

Published online ahead of print. Publication date available at www.jasn.org.

Address 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

ISSN: 1046-6673/1605-1371

ing latent form of TGF-β1 may have protective roles in renal inflammation. This was investigated in a progressive model of obstructive kidney disease in mice that overexpress latent TGF-β1 in the circulation and in the skin. Furthermore, this study explored the underlying signaling mechanism of TGF-β in anti-inflammation in the kidney disease.

Materials and Methods

K5.TGFβ1^{wt} TGF-β1 Transgenic Mice

The approximately 1.6-kb full-length wild type (Wt) human TGF-β1 cDNA was inserted into the bovine K5 expression vector (13). The K5.TGF-β1 plasmid then was microinjected into the pronuclei of mouse embryos that were obtained from strain ICR female mice. After birth, transgenic mice were genotyped by PCR analysis of their tail DNA using primers specific for human TGF-β1 cDNA, the sequence being forward 5′-TCTGCTGAGGAGGCTCAAGTT and reverse 5′-ACCTCGGCGGCCGGTAG. The product size is 271 bp. All TGF-β transgenic mice develop psoriasis-like skin lesions at the age of 2 to 6 mo (14).

Obstructive Kidney Disease Model

A progressive kidney disease model was induced in both Wt and K5.TGFβ1^{wt} transgenic mice (15 to 20 g body wt, 3 mo of age) by left ureter ligation as described previously (unilateral ureteral obstruction [UUO]) (15). Groups of six mice were killed on day 7 after the ligation. The experimental procedures were approved by Animal Experimental Committee at Baylor College of Medicine. Kidney tissue samples were collected for histology, immunohistochemistry, Western blot, real-time PCR, and quantitative ELISA analyses.

Measurement of TGF-β1 in Plasma and Renal Tissues

TGF-β1 levels in plasma and renal tissues including the active form, the latency-associated protein (LAP), and total TGF-β1 were analyzed quantitatively by the commercial ELISA kits (R&D System Inc., Minneapolis, MN), according to the manufacturer's instructions. Briefly, protein samples were acidified with 1 N HCl and neutralized with 1.2 N NaOH/0.5 M HEPES to assay for the amount of total (the sum of latent and active) TGF-β1. The concentration of active TGF-β1 protein was analyzed on samples that were not acidified, whereas the levels of latent TGF-β1 protein were measured using a specific anti-LAP antibody.

Cell Culture

To test the hypothesis that TGF-β negatively regulates inflammatory response by inducing its negative signaling regulator, Smad7, we used a doxycycline-regulated Smad7-expressing tubular cell line (TEC). This cell line was established with a well-characterized normal rat kidney TEC line (NRK52E) by stably transfecting a flag-tagged (M2) Smad7 gene, as described previously (16). Smad7 transgene expression by TEC was tightly controlled by the addition of doxycycline (0 to 2 μg/ml) in the culture medium. After an 18-h induction of Smad7, proinflammatory cytokines IL-1β (10 ng/ml) and TNF-α (10 ng/ml) were added into the cells for 0 to 24 h and the effects of Smad7 on IL-1β, TNF-α, and intercellular adhesion molecule-1 (ICAM-1) expression and on the activation of NF-κB were examined.

Western Blot Analysis

Protein from kidney tissues and cultured cells was extracted with RIPA lysis buffer (1% Nonidet P-40, 0.1% SDS, 1 mM PMSF, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, and 1 mM sodium fluoride in PBS). After protein concentrations were determined, 20 μg of the protein was mixed with an equal amount of 2× SDS loading buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, and 0.2% bromophenol blue) for Western blot analysis. Briefly, samples were heated at 99°C for 5 min and then transferred to a polyvinylidene difluoride membrane. Nonspecific binding to the membrane was blocked for 1 h at room temperature with 5% BSA in Tris-buffered saline buffer (TBST; 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20). The membranes then were incubated overnight at 4°C with primary antibodies against Smad7 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Flag-M2 (Sigma, St. Louis, MO), and NF-κB p50 or p65 (Santa Cruz); IκBα (Santa Cruz); phospho-IκBα (Cell Signaling, Beverly, MA); and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon Inc., Temecula, CA). After being washed extensively, the membranes then were incubated with horseradish peroxidase–conjugated secondary antibody for 1 h at room temperature in 1% BSA/TBST. The signals were visualized by an enhanced chemiluminescence system (Amersham, Piscataway, NJ).

Real-Time PCR

Total kidney RNA was isolated using the RNeasy kit, according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). The cDNA was synthesized as described previously (17). Real-time PCR was run with the Opticon real-time PCR machine (MJ Research Inc., Waltham, MA). The specificity of real-time PCR was confirmed *via* routine agarose gel electrophoresis and Melting-curve analysis. Housekeeping gene GAPDH was used as an internal standard. The primers used in this study are as follows: Smad7, forward 5′-GTGCTATGTCGCTCTGGACTTTGA and reverse 5′-ATGAAAGATGGCTGGAAGAGGGTC; ICAM-1, forward 5′-TCAGGTATCCATCCATCCCAGAGA and reverse 5′-AGCTCATCTTTCAGCCACTGAGTC; IL-1β, forward 5′-CTTCAGGCAGGCAGTATCACTCAT and reverse 5′-TCTAATGGGAACGTCACACACCAG; TNF-α, forward 5′-CATGAGCACAGAAAGCATGATCCG and reverse 5′-AAGCAGGAATGAGAAGAGGCTGAG; TGF-β, forward 5′-CAACAATTCCTGGCGTTACCTTGG and reverse 5′-GAAAGCCCTGTATTCCGTCTCCTT; GAPDH, forward 5′-TGCTGAGTATGTCGTGGAGTCTA and reverse 5′-AGTGGGAGTTGCTGTTGAAATC.

Transient Transfection and Promoter Activity Assay

Doxycycline-regulated Smad7-expressing NRK52E cells were transiently transfected by the Lipofectamine (Invitrogen Inc., Carlsbad, CA) with the specific NF-κB responsive promoter 241RMI-SEAP (secreted alkaline phosphatase) plasmid (gift from Dr. Bharat B. Aggarwal, Department of Molecular Oncology, University of Texas MD Anderson Cancer Center) (18). A control plasmid, pCMV-βGal (Clontech, Palo Alto, CA), was co-transfected into the cells for transfection efficiency. The transfection procedure was carried out according to the manufacturer's instruction. The SEAP and β-galactosidase activities were analyzed by SEAP reporter gene assay kit and β-gal reporter gene assay kit, respectively (Roche Inc., Indianapolis, IN), according to the manufacturer's instructions. NF-κB SEAP activity was reported as the SEAP activity normalized to β-gal activity.

Electrophoretic Mobility Shift Assay

Nuclear proteins from cultured NRK52E cells were isolated as described previously (19). Aliquots of 5 μg of nuclear extracts were incubated with a 19-bp double-strand oligonucleotide probe that contained consensus sequence that binds to NF-κB/c-Rel homodimeric and heterodimeric complexes (Santa Cruz) in binding buffer for 30 min at 4°C. The probe is end-labeled with ^{32}P-dATP by using T4 polymerase (Invitrogen Inc.) according to the manufacturer's instructions. After the binding reaction, the samples were loaded on a 5% native polyacryl-

amide gel and run for 3 to 4 h at 4°C. The gel then was dried and developed on an x-ray film.

Histology and Immunohistochemistry

Changes in renal morphology were examined in methyl Carnoy's-fixed, paraffin-embedded tissue sections (4 μm) stained with hematoxylin and eosin or periodic acid-Schiff. Infiltration of $CD3^+$ T cells and macrophages; expression of IL-1β, TNF-α, ICAM-1; and activation of NF-κB p50/p65 were determined by three-layer immunohistochemistry with mAb to macrophages (M170); $CD3^+$ T cells (OKT3); and rabbit polyclonal antibodies to IL-1β, TNF-α, ICAM-1, p65, and p50 NF-κB subunits. mAb were generated in our laboratory from standard cell lines (M170 and OKT3; gifts from Dr. David Nikolic-Paterson, Monash University, Melbourne, Australia), whereas polyclonal rabbit antibodies were purchased from Santa Cruz Biotechnology. Immunostaining for $CD3^+$ T cells, macrophages, IL-1β, TNF-α, and ICAM-1 was performed in 2% paraformaldehyde-lysine-periodate–fixed frozen sections as described previously (20), whereas detection of NF-κB/p50 and p65 subunits was performed in methyl Carnoy's fixed paraffin sections using a microwave-based antigen retrieval technique (21). After being developed with 3,3-diaminobenzidine, sections were counterstained with hematoxylin. Tubulointerstitial NF-κB activated cells, infiltrating $CD3^+$ T cells, and macrophages were counted and expressed as positive cells per cm^2 as described previously (20). The quantification of IL-1β, TNF-α, and ICAM-1 expression was analyzed by using Optima program, and the results were expressed as percentage of positive area examined.

Statistical Analyses

Data obtained from this study are expressed as the mean ± SEM. Statistical analyses were performed using one-way ANOVA from GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

Results

TGF-β1 Transgenic Mice Have an Elevated Level of Latent TGF-β1 in Plasma and Renal Tissues but Exhibit a Normal Renal Function and Histology

The K5.TGFβ1^{wt} transgenic mice were generated successfully in the ICR background mice, as demonstrated by the genotypic PCR (Figure 1A). These mice displayed psoriasis-like skin lesions with overexpression of TGF-β1 in keratinocytes (14). Cir-

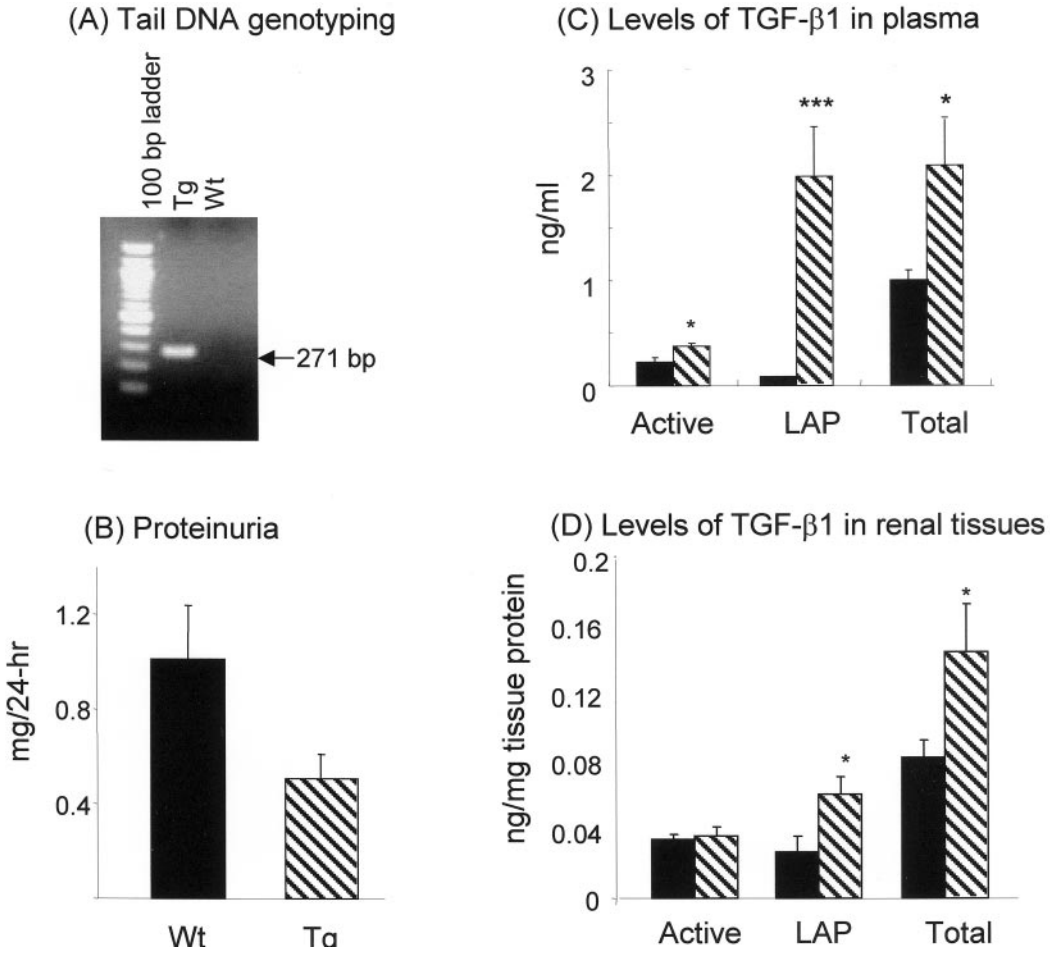

Figure 1. Pertinent features of K5.TGFβ1^{wt} transgenic (Tg) mice. (A) DNA from the tails of K5.TGFβ1^{wt} Tg mice is amplified with specific primers showing the 271-bp delivered gene. (B) Circulating levels of active, latency-associated protein (LAP), and total TGF-β1 in Tg mice are significantly higher than that of wild-type (Wt) mice (ng/ml). (C) The local renal levels of active, LAP, and total TGF-β1. Both LAP and total TGF-β1 in the kidney tissues are significantly higher in Tg mice than in Wt mice. (D) The 24-h urinary protein excretion is within normal levels in both Tg and Wt mice (<1 mg/24 h). ▨, Tg mice; ■, Wt mice; each bar represents the mean ± SEM for groups of six mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *versus* Wt mice.

culating levels of latent TGF-β1 were significantly elevated in the transgenic mice when compared with the Wt mice (a 10-fold increase; $P < 0.001$), and this was associated with a 1.7-fold increase ($P < 0.05$) in the levels of circulating active TGF-β1 (Figure 1C). In the kidney, the levels of total and latent TGF-β1 but not the active form of TGF-β1 were elevated in the transgenic mice when compared with the Wt mice (two- and three-fold increases; $P < 0.05$, respectively; Figure 1D). However, TGF-β transgenic mice exhibited a normal renal function as determined by a normal level of 24-h urine protein excretion (Figure 1B) and histology (Figure 2, A and B).

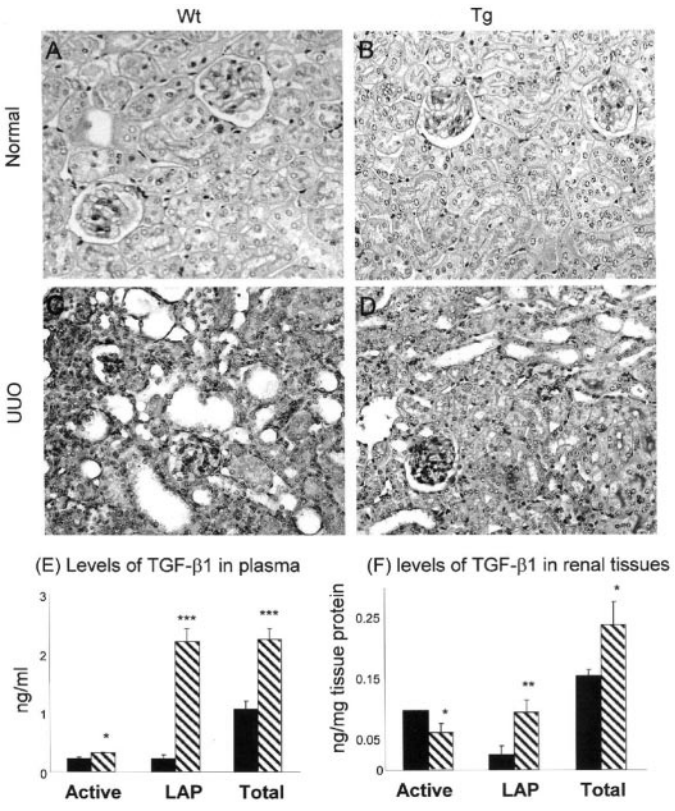

Figure 2. Histologic features of kidneys of K5.TGFβ1^{wt} Tg and Wt mice in normal and obstructive kidney. (A and B) Kidneys from both K5.TGFβ1^{wt} Tg and Wt mice show normal renal histology. (C) A representative obstructive kidney from a Wt mouse shows severe renal damage, including interstitial fibrosis, tubular atrophy, and mononuclear cell infiltration, at day 7 after urethral ligation. (D) A representative obstructive kidney from a Tg mouse shows relatively normal histology at day 7 after the ureter ligation. (E) Circulating levels of total TGF-β1, active form of TGF-β1, and LAP in both Tg and Wt mice at day 7 after induction of obstructive kidney disease. (F) The local renal levels of active, LAP, and total TGF-β1 in both Tg and Wt mice at day 7 after induction of obstructive kidney disease. Note that active TGF-β1 within the diseased kidney is significantly increased in Wt mice but not in Tg mice. ▨, Tg mice; ■, Wt mice; each bar represents the mean ± SEM for groups of six mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *versus* Wt mice. Tissue sections are stained with periodic acid-Schiff. Magnification, ×200.

TGF-β1 Transgenic Mouse Are Protected against Renal Histologic Damage and Leukocytic Infiltration in Obstructive Kidney Disease

Seven days after the left ureteral ligation, Wt mice developed moderate to severe tubulointerstitial damage, including focal tubular atrophy, mononuclear inflammatory cell infiltration, and fibrosis. In contrast, TGF-β1 transgenic mice exhibited a relatively normal renal histology (Figure 2, C and D). The circulating levels of active, latent (LAP), and total TGF-β1 remained unchanged in both Wt and transgenic mice after the ureteral ligation (Figure 2E). However, Wt mice exhibited a significant increase in active TGF-β1 within the diseased kidney compared with the TGF-β1 transgenic mice in which a latent form of TGF-β1 remained dominant (Figure 2F). As shown in Figure 3, B and E, Wt mice developed severe renal inflammation as demonstrated by a marked increase in the number of T cells and macrophages in the tubulointerstitium, which was prevented in TGF-β1 transgenic mice (Figure 3, C and F). Quantitative analysis showed that T cell and macrophage infiltration in the tubulointerstitium was significantly inhibited in TGF-β1 transgene mice (Figure 3, G and H).

Upregulation of Inflammatory Cytokines and Adhesion Molecule in Obstructive Kidney Is Prevented in TGF-β Transgenic Mice

It has been well accepted that inflammatory cytokines and adhesion molecules, such as IL-1β, TNF-α, and ICAM-1, play an important role in the pathogenesis of kidney diseases (22,23). As shown in Figure 4, real-time PCR demonstrated that Wt mice exhibited a marked increase in mRNA levels of IL-1β, TNF-α, and ICAM-1 in the diseased kidney (all $P < 0.001$), which was significantly inhibited in TGF-β1 transgenic mice. Similar results were also obtained at the protein level by immunohistochemistry, as shown in Figure 5.

NF-κB Activation in Obstructive Kidney Is Inhibited in TGF-β1 Transgenic Mice

NF-κB, a critical transcriptional factor for controlling inflammatory response, has been shown to play a central role in inflammatory diseases, including kidney diseases (24). As shown in Figure 6, immunohistochemistry revealed that the NF-κB/p65 subunit (Figure 6, E and H) but not the p50 subunit (Figure 6, B and G) was markedly activated in Wt mice as evidenced by its nuclear location in the areas of severe tubulointerstitial damage. In contrast, activation of the NF-κB/p65 subunit was abrogated in TGF-β1 transgenic mice (Figure 6, F and H).

Smad7 Is Upregulated in Both Normal and Diseased Kidney in TGF-β1 Transgenic Mice

We then investigated the potential mechanism by which TGF-β transgenic mice exhibited a protective role in renal inflammation. It has been reported that Smad7 is capable of inhibiting transcriptional activity of NF-κB (25), and it is well documented that Smad7 is induced by TGF-β1 and acts as a negative regulator of the TGF-β1 signaling pathway (26).

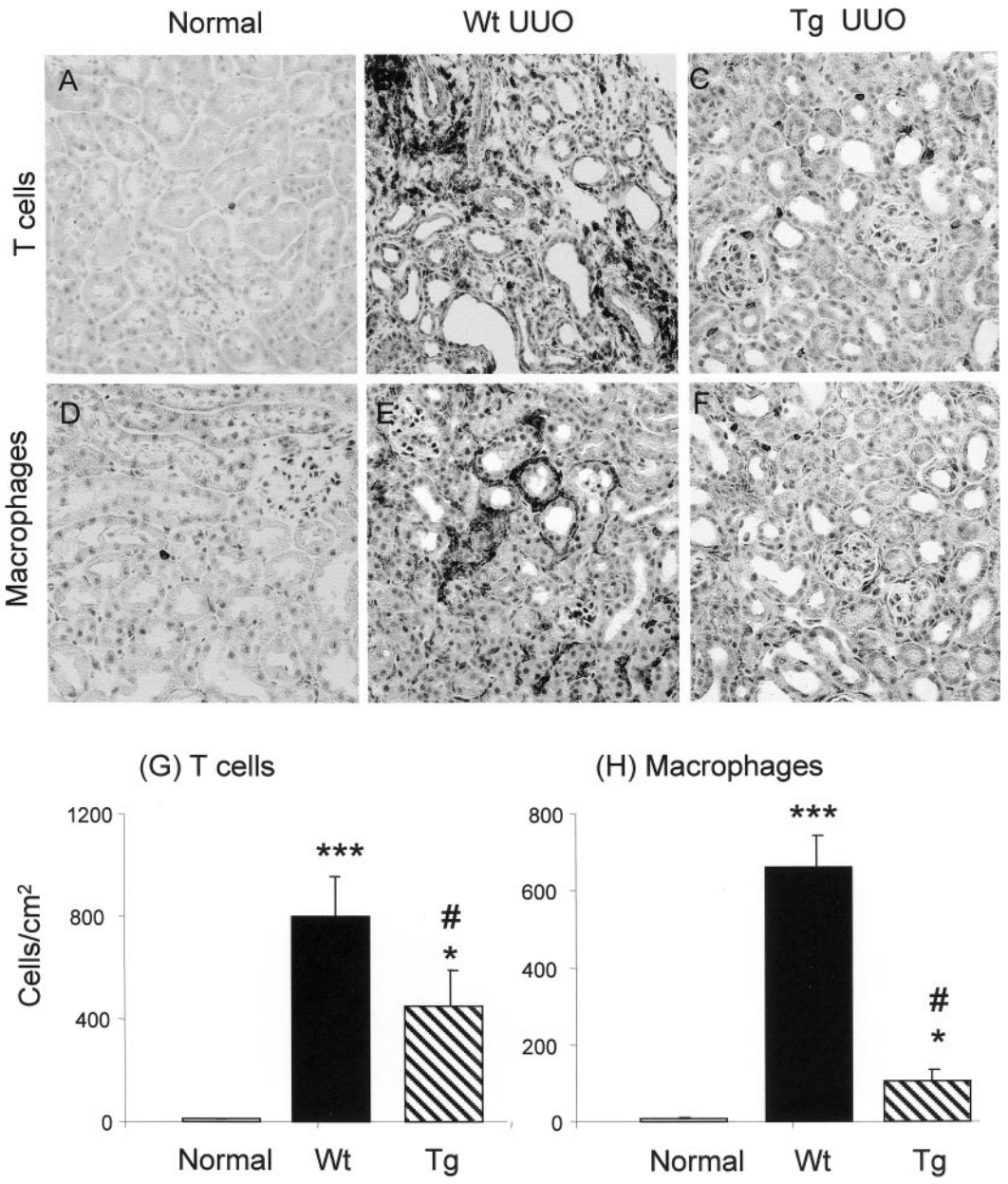

Figure 3. Immunohistochemistry shows that leukocytic infiltration is markedly decreased in the kidney of K5.TGFβ1^{wt} Tg mice. (A and D) Normal Wt kidneys stained for CD3$^+$ T cells (A) or macrophages (D). (B and E). Obstructive kidneys from Wt mice show extensive infiltration of CD3$^+$ T cells (B) or macrophages (E). (C and F) Obstructive kidneys from the K5.TGFβ1^{wt} Tg mice show a few CD3$^+$ T cell (C) or macrophage infiltration in tubulointerstitium. (G and H). Quantification of leukocytic infiltration in the unilateral ureteral obstruction (UUO) model: □, normal control; ■, Wt UUO; ▨, Tg UUO. Each bar represents the mean ± SEM for a group of six mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *versus* normal control; #$P < 0.05$ *versus* Wt UUO.

Hence, we hypothesized that prevention of renal inflammation in TGF-β1 transgenic mice may be associated with upregulation of Smad7. As shown in Figure 7, both real-time PCR and Western blot analyses revealed that, compared with Wt mice, a marked increase in renal Smad7 mRNA and protein was found in both normal and diseased kidney in the TGF-β1 transgenic mice. This observation poses the question of whether upregulation of renal Smad7 contributes to the prevention of renal NF-κB activation and inflammatory responses in obstructive kidney disease, which is investigated further below.

Upregulation of Smad7 Suppresses IL-1β– or TNF-α–Induced NF-κB Activation and Inflammatory Response In Vitro

It is known that TGF-β1 can induce Smad7 expression (27). Activation of the NF-κB pathway has been shown to play a critical role in renal inflammation (24). Thus, we hypothesized that upregulation of renal Smad7 may block NF-κB–mediated renal inflammation. This hypothesis was tested in a doxycycline-regulated Smad7-expressing normal rat kidney tubular epithelial cell line (NRK52E) in which Smad7 expression is tightly regulated by the concentrations of doxycycline (16). As

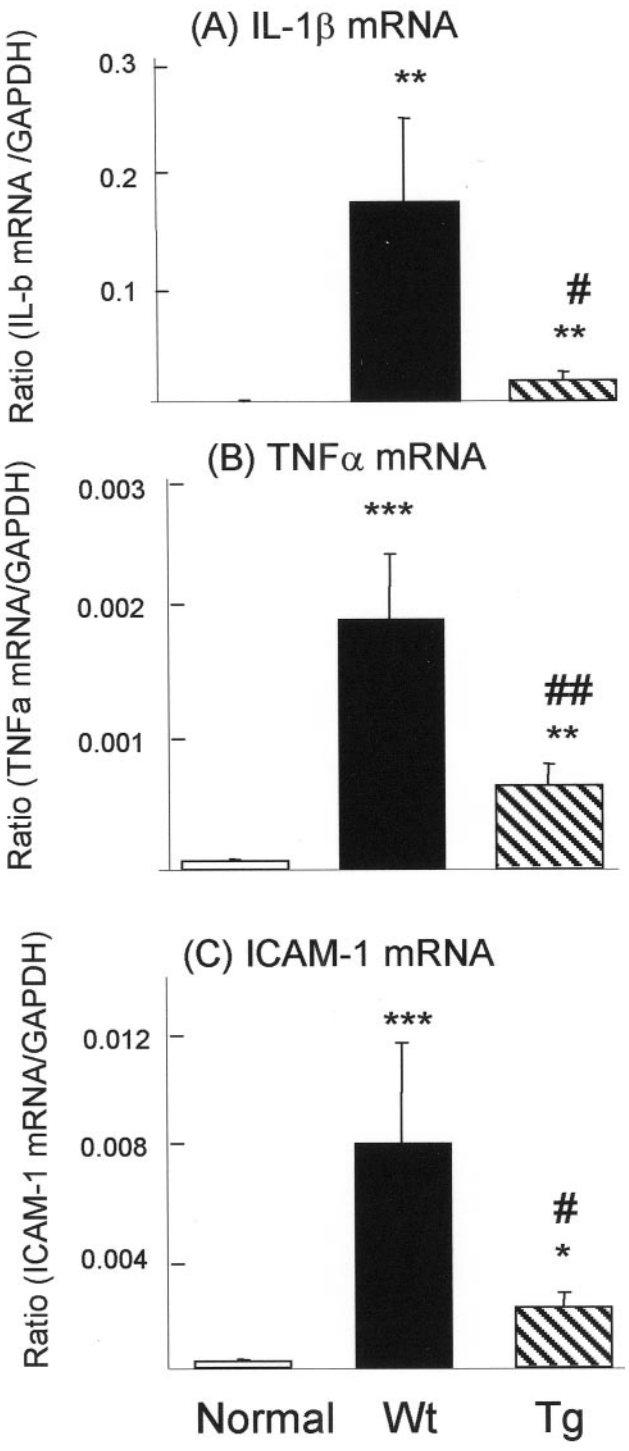

Figure 4. Real-time PCR shows IL-1β, TNF-α, and intercellular adhesion molecule-1 (ICAM-1) mRNA expression in the K5.TGFβ1^{wt} Tg and Wt UUO mice kidneys. Total renal RNA extracted from normal control (□), Wt UUO (■), and K5.TGFβ1^{wt} Tg UUO (▨) were reverse-transcribed and subjected to real-time PCR for IL-1β (A), TNF-α (B), and ICAM-1 (C). Each bar represents the mean ± SEM for a group of six mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* normal control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* Wt UUO.

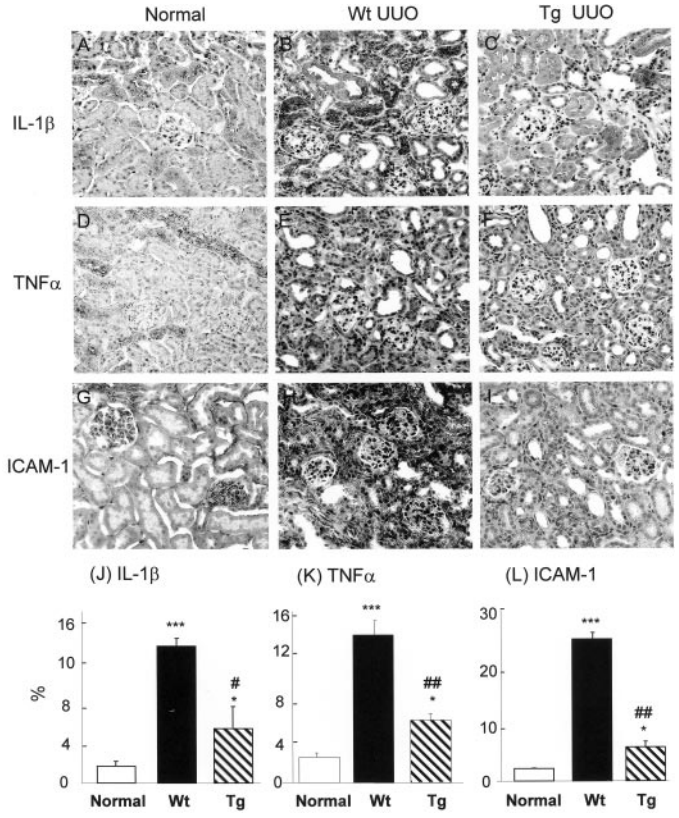

Figure 5. Immunohistochemistry shows that upregulation of IL-1β, TNF-α, and ICAM-1 expression is prevented in the K5.TGFβ1^{wt} Tg mice. (A through C) IL-1β, TNF-α, and ICAM-1 expression in the normal Wt control. (D through F) IL-1β, TNF-α, and ICAM-1 expression in the Wt UUO mice. (G through I) IL-1β, TNF-α, and ICAM-1 expression in the K5.TGFβ1^{wt} Tg UUO mice. (J through L) Semiquantitative analysis of IL-1β, TNF-α, or ICAM-1 expression in the kidney tissues (percentage of positive staining) using Optimum Imaging Analysis System as described in the Materials and Methods section: □, normal control; ■, Wt UUO; ▨, Tg UUO. Each bar represents the mean ± SEM for a group of six mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* normal control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* Wt UUO.

shown in Figure 8A, two-colored immunohistochemistry demonstrated that cells that were cultured in the presence of doxycycline substantially induced Smad7 expression. Importantly, cells with marked Smad7 expression completely blocked IL-1β–induced NF-κB/p65 nuclear translocation, whereas cells without or with minimal Smad7 expression exhibited NF-κB/p65 nuclear location. This is demonstrated further by Western blot analysis of the nuclear protein. Addition of doxycycline completely inhibited IL-1β– or TNF-α–induced NF-κB/p65 but not p50 nuclear translocation (Figure 8B), indicating that upregulation of Smad7 is capable of inhibiting IL-1β or TNF-α–mediated NF-κB activation. This observation was confirmed further by electrophoretic mobility shift assay. As shown in Figure 8C, cells that were cultured with doxycycline suppressed NF-κB DNA binding activity in response to IL-1β and TNF-α stimulation.

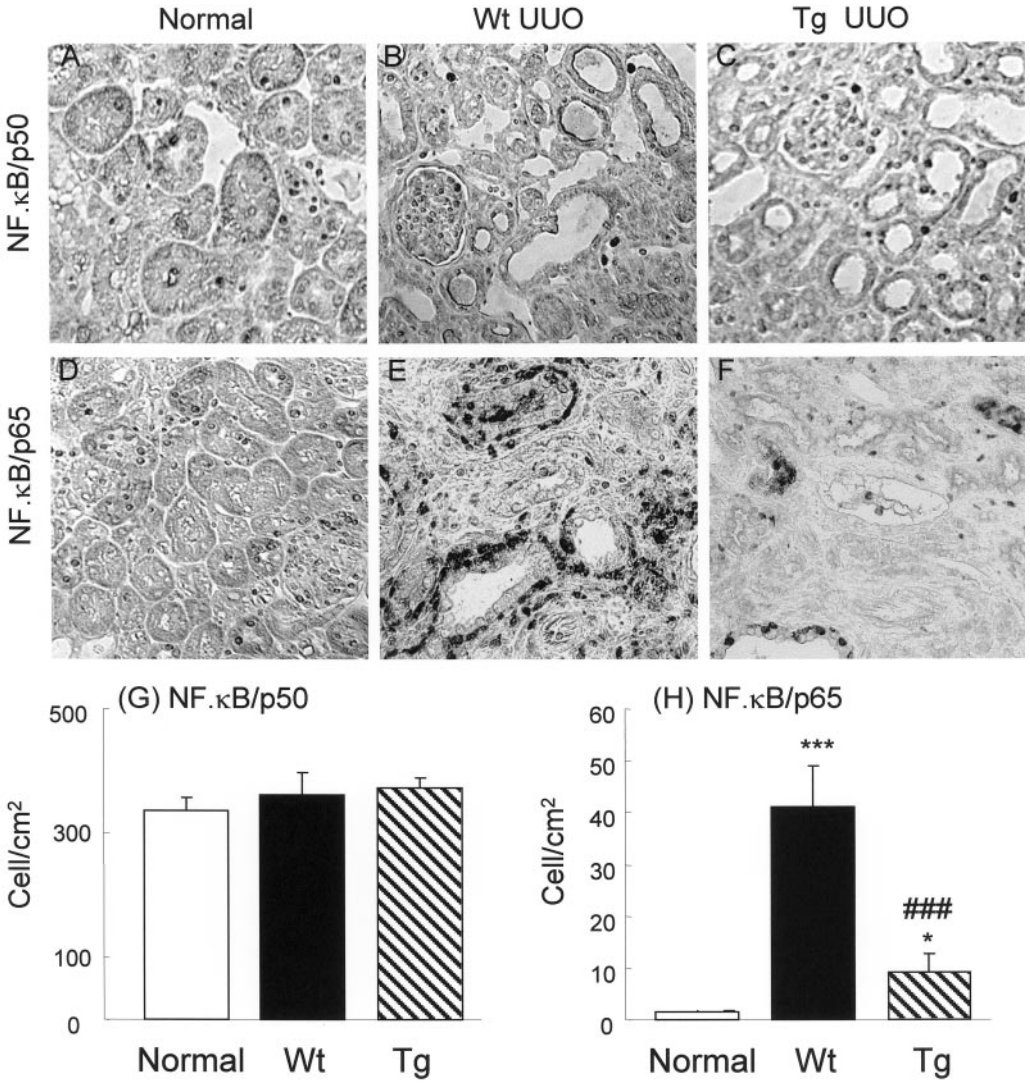

Figure 6. Immunohistochemistry shows that NF-κB/p65 activation is prevented in the K5.TGFβ1^{wt} Tg UUO mice. Activation of NF-κB/p50 or p65 subunits is identified as nuclear staining with antibodies (dark black). (A and D) NF-κB/p50 or NF-κB/p65 nuclear location in the normal Wt control. (B and E) NF-κB/p50 or NF-κB/p65 nuclear location in the Wt UUO mice. (C and F) NF-κB/p50 or NF-κB/p65 nuclear location in the K5.TGFβ1^{wt} Tg UUO mice. (G and H) Semiquantitative analysis of NF-κB/p50 or NF-κB/p65 nuclear location: □, normal control; ■, Wt UUO; ▨, Tg UUO. Each bar represents the mean ± SEM for a group of six mice. *$P < 0.05$, ***$P < 0.001$ *versus* normal control; $^{\#\#\#}P < 0.001$ *versus* Wt UUO.

The effect of overexpression of Smad7 on the transcriptional activities of NF-κB was investigated further in this doxycycline-regulating Smad7-expressing tubular cell line by transient transfection with an NF-κB reporter, 241RMI-SEAP (18). As shown in Figure 9, A and B, doxycycline-induced Smad7 expression significantly inhibited IL-1β– or TNF-α–induced NF-κB promoter SEAP activities. This was associated with inhibition of IL-1β–induced, NF-κB–driven proinflammatory cytokine (IL-1β and TNF-α) and adhesion molecule (ICAM-1) expression (Figure 9, C through E).

Upregulation of IκBα by Smad7 Is a Key Mechanism by Which TGF-β1 Suppresses NF-κB Activation and Renal Inflammation In Vitro *and in Obstructive Kidney Disease*

Next we studied the signaling mechanism by which overexpression of Smad7 inhibits NF-κB activation. NF-κB is inactivated by binding to its inhibitor, IκBα, which traps NF-κB in the cytoplasm and prevents its nuclear translocation. We hypothesized that induction of IκBα by Smad7 may be a key mechanism of anti-inflammatory effects of TGF-β1. Results shown in Figure 10 demonstrated that doxycycline induced Smad7 expression by tubular cells in a time- and dose-dependent manner. This was tightly associated with an increase in IκBα expression (Figure 10, A and B). However, overexpression of Smad7 did not alter the levels of IκBα phosphorylation/degradation induced by IL-1β and TNF-α (Figure 10C). These observations were tested further in the diseased kidney in both Wt and TGF-β1 transgenic animals. A marked increase in renal Smad7 in TGF-β1 transgenic mice was associated with a substantial upregulation of IκBα and inhibition of IκBα phosphorylation (p-IκBα). In contrast, Wt mice showed a

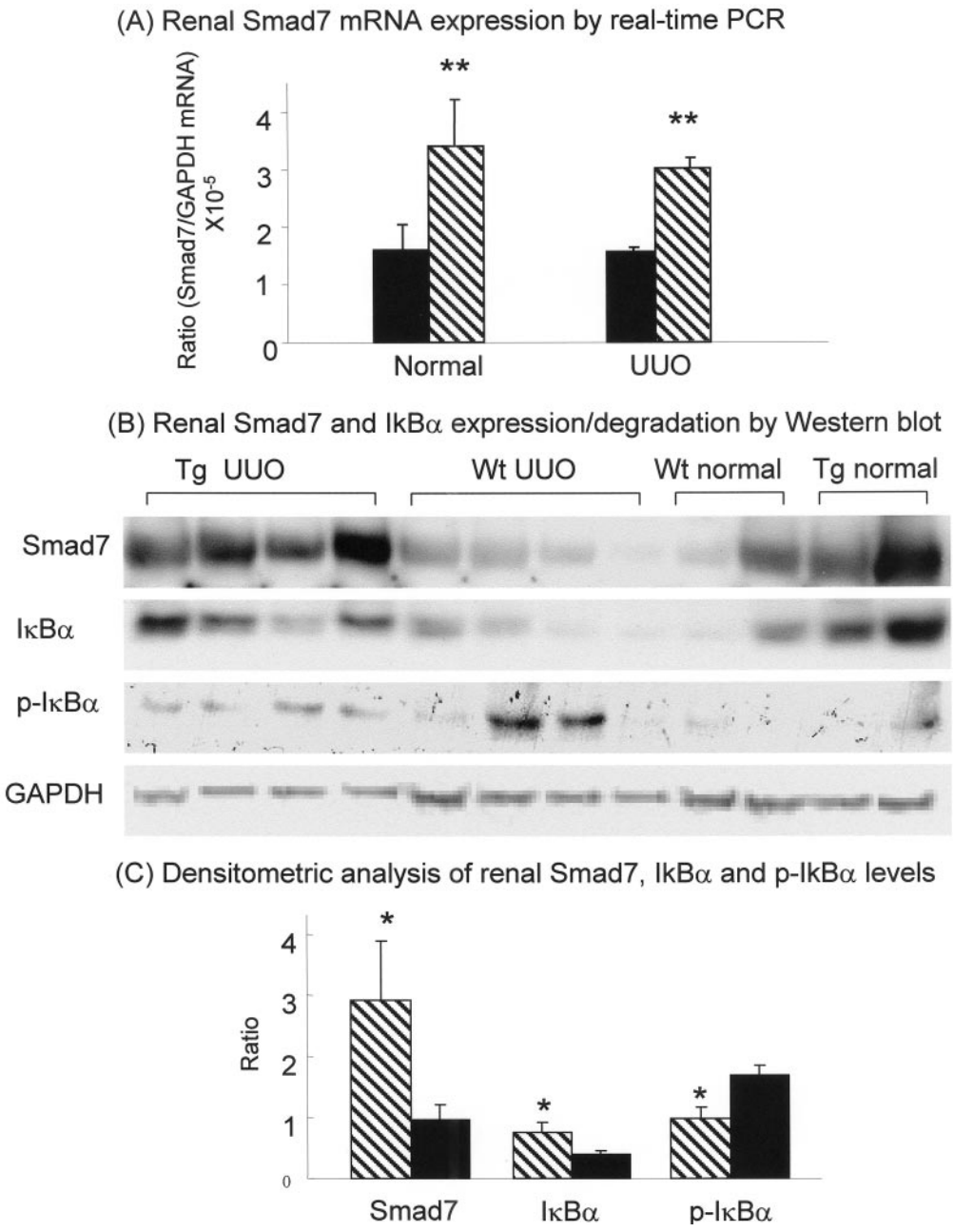

Figure 7. Expression of renal Smad7 and IκBα and phosphorylation of IκBα in both normal and diseased kidney in Wt and K5.TGFβ1^{wt} Tg mice by real-time PCR and Western blot analyses. (A) Total RNA extracted from renal tissues of Wt (■) or K5.TGFβ1^{wt} Tg mice (▨) was reverse-transcribed and subjected to real-time PCR for the quantification of Smad7 mRNA levels, normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Each bar represents the mean ± SEM for a group of six mice. $^{**}P < 0.01$ *versus* Wt. (B) Total proteins extracted from the renal tissue of Wt or K5.TGFβ1^{wt} Tg mice were subject to Western blot analysis of Smad7, total IκBα, and p-IκBα. Each lane represents result from one mouse kidney. (C) Densitometric analysis of renal Smad7, IκBα, and p-IκBα levels in the Wt UUO (■) or K5.TGFβ1^{wt} Tg mice UUO (▨). The band density has been normalized to GAPDH. Each bar represents the mean ± SEM. $^{*}P < 0.05$ *versus* Wt.

decreased renal Smad7 in the obstructive kidney, which was associated with the reduction of renal IκBα and an increase in p-IκBα (Figure 7, B and C).

Discussion

We report here that increased circulating latent TGF-β has protective roles in renal inflammation in obstructive kidney disease induced in TGF-β1 transgenic mice. Upregulation of renal Smad7 may be a central mechanism of the anti-inflammatory effects of TGF-β1 in this disease model. Induction of IκBα by Smad7, thereby inhibiting NF-κB activation and NF-κB–driven inflammatory response, may be a key signaling pathway whereby TGF-β1 exerts its anti-inflammatory properties *in vivo* and *in vitro*. Thus, this study demonstrates the

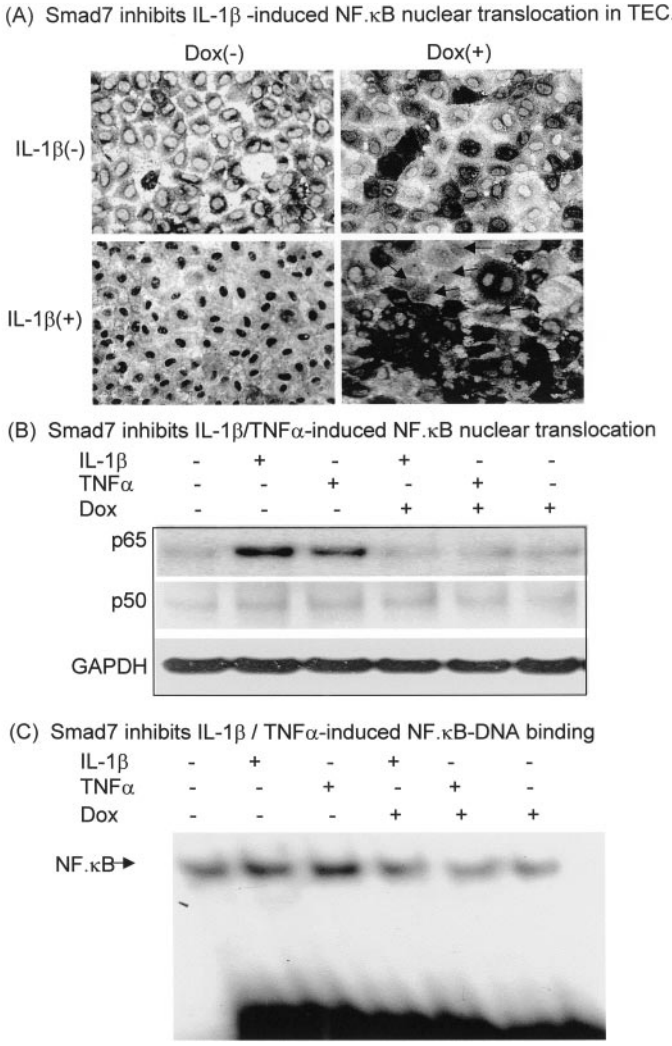

Figure 8. Overexpression of Smad7 inhibits IL-1β– and TNF-α–induced NF-κB/p65 activation in a doxycycline-regulated Smad7-expressing NRK52E TEC line. (A) Two-color immunohistochemistry demonstrates that doxycycline-induced (2 μg/ml) overexpression of Smad7 (cytoplasmic black) by TEC abolishes IL-1β–induced (10 ng/ml) NF-κB/p65 subunit nuclear translocation (black nucleus) at 30 min. Note that all TEC expressing Smad7 (cytoplasmic black) induced by doxycycline are negative for nuclear NF-κB/p65 staining, but those with absence of Smad7 show NF-κB/p65 nuclear translocation (arrows) in response to IL-β. (B) Western blot analysis of nuclear proteins shows that addition of doxycycline (Dox; 1 μg/ml) for 18 h blocks IL-1β– (10 ng/ml) or TNF-α–induced (10 ng/ml) NF-κB/p65 nuclear location at 10 min. Note that NF-κB/p50 is not activated after IL-1β or TNF-α stimulation. (C) Electrophoretic mobility shift assay shows that the addition of Dox (1 μg/ml for 18 h) inhibits IL-1β– (10 ng/ml) or TNF-α–induced (10 ng/ml) NF-κB DNA binding activity at 10 min. Data represent three independent experiments.

protective roles of circulating latent TGF-β in inflammatory kidney disease and delineates the signaling mechanism of TGF-β in anti-inflammation.

TGF-β1 is produced and secreted *in vivo* as a latent complex, consisting of mature dimeric TGF-β1, LAP, and a latent TGF-β binding protein (LTBP). LAP binds to the N-terminal of TGF-β, rendering TGF-β latent, thereby preventing TGF-β from binding to its receptor, whereas LTBP-1 binds the LAP-TGF-β complex and prevents TGF-β from interacting with local matrix protein. TGF-β must be liberated from LAP to become activated. The mechanisms involved in the liberation of TGF-β from LAP include plasmin, thrombospondin-1, reactive oxygen species, and acid (28,29). TGF-β is a pleiotropic cytokine with significant anti-inflammatory and immunosuppressive properties. Mice that are deficient in TGF-β1 develop a lethal multiorgan inflammation at 3 wk of age (7). TGF-β inhibits the production of proinflammatory cytokines from macrophages and T cells (30,31). Administration of TGF-β attenuates autoimmune diseases, including collagen-induced arthritis (32), allergic encephalomyelitis (33), and experimental colitis (34). Various experimental approaches have shown convincingly the importance of LAP-TGF-β as targeting molecules of inflammation and immune diseases. Indeed, $CD4^+$ T helper cells engineered to produce latent TGF-β1 reverse allergen-induced airway hyperactivity and inflammation, which is associated with an increase in the levels of activated TGF-β (35). T cells that are engineered to produce latent TGF-β1 downregulate Th1-mediated autoimmune and Th2-mediated allergic inflammatory processes (36). $CD4^+CD25^-$ T cells that express LAP on the surface suppress $CD4^+$CD45RB high-induced colitis by a TGF-β–dependent mechanism (37). All of these studies suggest that overexpression of latent TGF-β1 on immune effector cells produces protective roles in inflammation and immune-mediated diseases. In this study, transgenic expression of the latent TGF-β1 in epidermal keratinocytes also resulted in prevention of renal inflammation in obstructive kidney disease, which was associated with a significant increase in the circulating levels of a latent form of TGF-β1. This finding further demonstrates that latent TGF-β1 derived from nonimmune cells also plays a protective role in inflammatory response in the kidney disease.

A most significant finding in our study is that upregulation of renal Smad7 may be a central mechanism whereby TGF-β1 transgenic mice are protected against renal injury. It is now clear that TGF-β signals through two downstream proteins, Smad2 and Smad3, to induce Smad7 expression, which in turn inhibits TGF-β signaling (27). Thus, overexpression of Smad7 can block TGF-β–induced renal fibrosis *in vivo* and *in vitro* (15,16). Beyond the antifibrotic effect of Smad7 in renal fibrosis, we have now shown that upregulation of Smad7 can also inhibit renal inflammation. This is consistent with the previous report that overexpression of Smad7 in T cells prevents cell-mediated renal injury in anti–glomerular basement membrane glomerulonephritis and overexpression of Smad7 within the kidney inhibits renal inflammation in remnant kidney disease (38,39). Therefore, upregulation of Smad7 may be a key mechanism by which TGF-β exerts its protective role in renal fibrosis and inflammation.

Although it is now clear that Smad7 acts by inhibition of Smad2/3 activation to exert its antifibrotic effect of TGF-β, it remains unknown the signaling mechanism by which Smad7 prevented renal inflammation in obstructive kidney disease. It

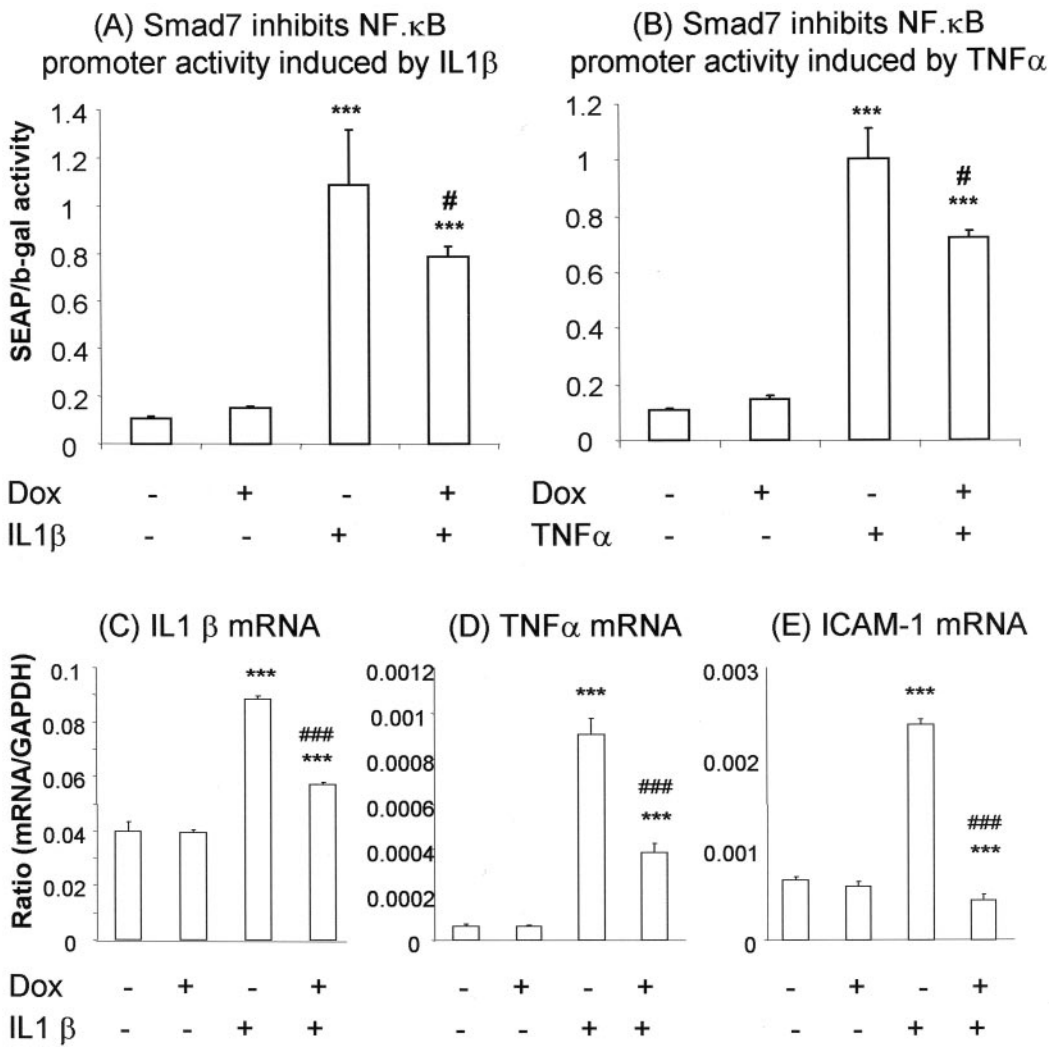

Figure 9. Overexpression of Smad7 inhibits NF-κB transcriptional activity and the IL-1β, TNF-α, ICAM-1 mRNA expression induced by IL-1β in a dose- and time-dependent manner in a Dox-regulated Smad7 expressing NRK52E TEC cell line. (A and B) NF-κB reporter assay (241RMI-SEAP). Dox-regulated Smad7-expressing NRK52E TEC are transfected with 241RMI-SEAP (NF-κB responsive construct), and pCMV-βGal with lipofectamine and Smad7 expression is induced by 1 μg/ml Dox for 18 h. Cells then are stimulated with IL-1β (10 ng/ml; A) or TNF-α (10 ng/ml; B) for 24 h for the analysis of SEAP and β-galactosidase activity. (C through E) Real-time PCR. Results show that Dox-induced (1 μg/ml for 18 h) overexpression of Smad7 blocks IL-1β–induced (10 ng/ml) mRNA expression of IL-1β (C), TNF-α (D), and ICAM-1 (E) at 3 h. Each bar represents the mean ± SEM for three independent experiments. $^{***}P < 0.001$ *versus* basal control; $^{\#}<0.05$, $^{\#\#\#}<0.001$ *versus* IL-1β or TNF-α treatment group.

is well accepted that NF-κB is a key transcriptional factor to regulate a variety of inflammatory responses (24). NF-κB is composed of p50 and p65 subunits, among which p65 is a potent transcriptional activator, strongly promoting inflammatory reaction in kidney diseases (40). In this study, marked activation of p65, not p50, was closely correlated with the renal inflammation, consistent with the previous observation in experimental diabetic nephropathy (41). A putative NF-κB regulatory site has been found in the mouse Smad7 promoter, suggesting a functional link between the NF-κB and Smad7 (42). In our study, by using a doxycycline-regulated Smad7-expressing tubular epithelial cell line, we were able to show that doxycycline-induced overexpression of Smad7 substantially suppresses NF-κB activation as demonstrated by inhibition of IL-1β– and TNF-α–induced NF-κB/p65 nuclear translocation, NF-κB transcriptional activity (reporter assay), and NF-κB DNA binding activity. These findings are consistent with the previous reports that induction of Smad7 inhibits transcriptional activity of NF-κB in MDCK cells and in conditionally immortalized mouse podocytes (25,43).

It is well established that NF-κB activation is regulated by its inhibitor, IκBα. Under basal conditions, IκBα binds to NF-κB p50/p65 subunits to prevent their nuclear translocation. Once IκBα is phosphorylated or degraded, p50/p65 subunits become activated and translocate into the nucleus to activate the target genes. TGF-β is able to induce IκBα expression (44); however, its intracellular mechanism remains unclear. In our study, we demonstrated that Smad7 was able to induce directly the ex-

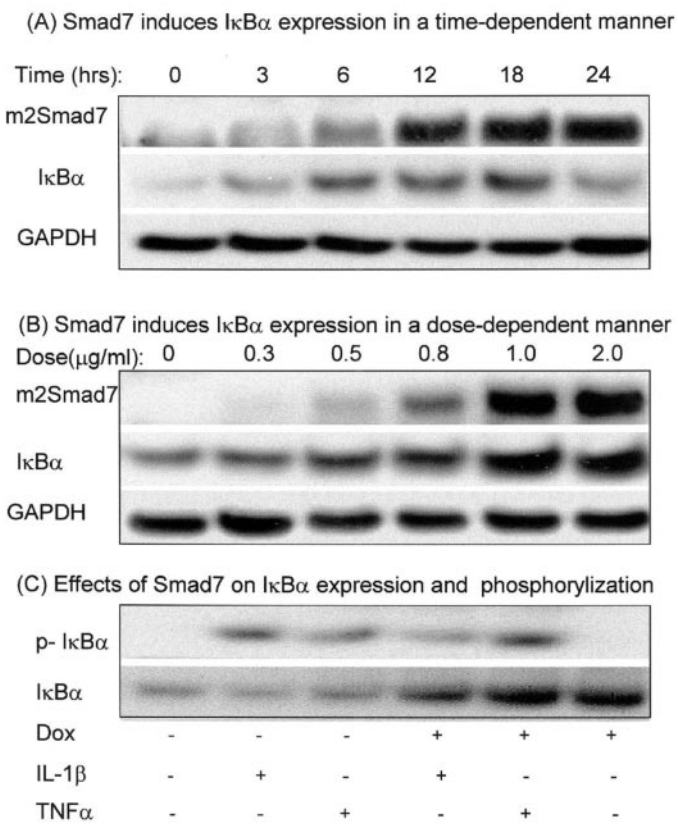

Figure 10. Western blot analysis shows that Dox-induced overexpression of Smad7 increases IκBα expression by TEC in a dose- and time-dependent manner. Dox-induced Flag-tagged M2 Smad7 transgene expression is determined with the anti-M2 antibody. (A) Dox (1 μg/ml) induces Smad7 and IκBα expression in TEC in a time-dependent manner. (B) Addition of Dox for 18 h induces Smad7 and IκBα expression in TEC in a dose-dependent manner in TEC. (C) Dox-induced (1 μg/ml) overexpression of Smad7 upregulates IκBα but does not alter the phosphorylation levels of IκBα induced by IL-1β (10 ng/ml) and TNF-α (10 ng/ml) at 10 min. Data represent three independent experiments.

pression of IκBα. This suggests that TGF-β may act by stimulating Smad7 to induce IκBα expression, thereby preventing NF-κB from activation. This *in vitro* finding was supported further by the *in vivo* finding. Upregulation of renal Smad7 in TGF-β1 transgenic mice was associated with an increase in renal IκBα expression, resulting in suppression of NF-κB activation and NF-κB–driven inflammatory response in the diseased kidney (Figures 4 through 6). It was noted that doxycycline-induced overexpression of renal Smad7 could not prevent IκBα phosphorylation (Figure 10C) and produced a modest effect on inhibition of NF-κB promoter activity (Figure 9) induced by high concentrations of IL-1β (10 ng/ml) and TNF-α (10 ng/ml) *in vitro*. In contrast, in TGF-β transgenic mice, increased renal Smad7 was associated with a significant inhibition of IκBα phosphorylation, which was associated with the low expression levels of IL-1β and TNF-α and significant inhibition of NF-κB activation and NF-κB–dependent renal information (Figures 3 through 7). This discrepancy between *in vitro* and *in vivo* may be associated with the difference in the levels of Smad7 and proinflammatory cytokines in the cell culture system and in the diseased kidney in TGF-β transgenic mice. In addition, it is possible that latent TGF-β1 may act *via* different mechanisms other than Smad7 to regulate NF-κB activation or that Smad7 functions differently *in vivo* in the kidney or *in vitro* in cultured tubular cells. Nevertheless, inhibition of IκBα phosphorylation in the diseased kidney may be one mechanism by which increased latent TGF-β1 prevents NF-κB–driven renal inflammation.

An interesting observation in this study is that although K5.TGFβ1^{wt} transgenic mice were protected against renal inflammation, they developed inflammatory skin lesions, including epidermal hyperproliferation, massive infiltration of neutrophils, T lymphocytes, and macrophages (14). The discrepancy between kidney and skin lesions may be associated with the local levels of latent and active TGF-β1. The latent TGF-β1 can function to inhibit bioactivities of active TGF-β1 *in vitro* and *in vivo* (45,46). Administration of LAP-TGF-β1 completely reverses suppression of early proliferative response in the remnant liver in mice with overexpression of a bioactive form of TGF-β1 and prevents skin fibrosis in a murine model of scleroderma graft-*versus*-host disease (46,47). In this study, K5.TGFβ1^{wt} transgenic mice developed severe skin inflammation at the site where latent TGF-β1 was massively produced. It is likely that the excessive latent TGF-β1 may prevent biologic functions of TGF-β1 in anti-inflammatory response, resulting in massive macrophage and T cell infiltration and production of proinflammatory cytokines and chemokines (14). In addition, the distinct action of TGF-β1 on various tissue types may partially account for the discrepancy in the skin and the diseased kidney in K5.TGFβ1^{wt} mice. K5.TGFβ1^{wt} transgenic mice also secrete latent TGF-β1 from the skin, resulting in a significant increase in the levels of latent TGF-β in circulation (a 10-fold increase) and renal tissues (a two-fold increase compared with Wt mice). The latent TGF-β1 can be activated by plasmin and thrombospondin-1 within the circulation at certain levels, resulting in a moderate activation of circulating TGF-β1 (a 1.7-fold increase compared with Wt mice). It is highly possible that the persistent high levels of latent *versus* low levels of active forms of TGF-β1 within circulation and renal tissues may counterregulate each other to balance the biologic effects of TGF-β1 on extracellular matrix production and inflammatory response in the kidney by upregulating renal Smad7. Thus, an excessive latent TGF-β1 in the disease status may be in favor of the anti-inflammatory effect of TGF-β. In contrast, normal Wt mice have equal levels of latent and active TGF-β1 in both circulation and renal tissue; however, active form of TGF-β1 was significantly increased in the diseased kidney (a three-fold increase compared with the latent form of TGF-β1). This was associated with a marked reduction of renal Smad7 and the development of severe renal inflammation in obstructive kidney. Thus, upregulation of Smad7 within the normal and diseased kidney in K5.TGFβ1^{wt} transgenic mice may be a key mechanism responsible for prevention of renal inflammation in response to high levels of latent TGF-β1 in obstructive kidney

disease. However, several fundamental questions regarding latent *versus* active TGF-1 in induction of renal Smad7 expression remain unsolved: (*1*) Why is an increase in latent TGF-β1 but not an active form of TGF-β1 associated with upregulation of renal Smad7? (*2*) Does latent TGF-β1 act to stimulate Smad7 expression *via* the conventional TGF-β/Smad signaling pathway or an alternative pathway independent of Smad? Although the latent TGF-β1 has been shown to counterregulate bioactivities of active TGF-β1 *in vitro* and *in vivo* (45,46), the mechanisms are yet unclear. These questions are complicated but will require further investigation because they are potentially important for understanding the roles of latent *versus* active TGF-β1 in renal inflammation.

In summary, we demonstrated that increased circulating and local levels of latent TGF-β1 could prevent renal inflammation in a murine model of obstructive kidney disease in K5.TGF$\beta1^{wt}$ transgenic mice. Upregulation of renal Smad7, which inhibits NF-κB activation by inducing IκBα expression, might be the central mechanism by which K5.TGF$\beta1^{wt}$ transgenic mice are protected against renal inflammation. In contrast to the finding that overexpression of an active hepatic TGF-β1 largely elevates circulating bioactive TGF-β1 and results in severe renal damage (3), the renoprotective effects of latent TGF-β1 on obstructive kidney disease suggest that latent TGF-β may have therapeutic potential for kidney diseases.

Acknowledgments

This study was supported by grants from the National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases (P50 DK64233 and R01DK062828) and Genzyme Renal Innovations Program to H.Y.L. and a grant from National Institutes of Health/National Cancer Institute (CA79998) to X.J.W.

References

1. Letterio JJ, Roberts AB: Regulation of immune responses by TGF-beta. *Annu Rev Immunol* 16: 137–161, 1998
2. Border WA, Noble NA: TGF-beta in kidney fibrosis: A target for gene therapy. *Kidney Int* 51: 1388–1396, 1997
3. Kopp JB, Factor VM, Mozes M, Nagy P, Sanderson N, Bottinger EP, Klotman PE, Thorgeirsson SS: Transgenic mice with increased plasma levels of TGF-beta 1 develop progressive renal disease. *Lab Invest* 74: 991–1003, 1996
4. Ziyadeh FN, Hoffman BB, Han DC, Iglesias-De La Cruz MC, Hong SW, Isono M, Chen S, McGowan TA, Sharma K: Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci U S A* 97: 8015–8020, 2000
5. Ma LJ, Jha S, Ling H, Pozzi A, Ledbetter S, Fogo AB: Divergent effects of low versus high dose anti-TGF-beta antibody in puromycin aminonucleoside nephropathy in rats. *Kidney Int* 65: 106–115, 2004
6. Roberts AB: Molecular and cell biology of TGF-beta. *Miner Electrolyte Metab* 24: 111–119, 1998
7. Yaswen L, Kulkarni AB, Fredrickson T, Mittleman B, Schiffman R, Payne S, Longenecker G, Mozes E, Karlsson S: Autoimmune manifestations in the transforming growth factor-beta 1 knockout mouse. *Blood* 87: 1439–1445, 1996
8. Chen W, Jin W, Cook M, Weiner HL, Wahl SM: Oral delivery of group A streptococcal cell walls augments circulating TGF-beta and suppresses streptococcal cell wall arthritis. *J Immunol* 161: 6297–6304, 1998
9. Jin YX, Xu LY, Guo H, Ishikawa M, Link H, Xiao BG: TGF-β1 inhibits protracted-relapsing experimental autoimmune encephalomyelitis by activating dendritic cells. *J Autoimmun* 14: 213–220, 2000
10. Piccirillo CA, Chang Y, Prud'homme GJ: TGF-β1 somatic gene therapy prevents autoimmune disease in nonobese diabetic mice. *J Immunol* 161: 3950–3956, 1998
11. Raz E, Dudler J, Lotz M, Baird SM, Berry CC, Eisenberg RA, Carson DA: Modulation of disease activity in murine systemic lupus erythematosus by cytokine gene delivery. *Lupus* 4: 286–292, 1995
12. Sanderson N, Factor V, Nagy P, Kopp J, Kondaiah P, Wakefield L, Roberts AB, Sporn MB, Thorgeirsson SS: Hepatic expression of mature transforming growth factor beta 1 in transgenic mice results in multiple tissue lesions. *Proc Natl Acad Sci U S A* 92: 2572–2576, 1995
13. He W, Li AG, Wang D, Han S, Zheng B, Goumans MJ, Ten Dijke P, Wang XJ: Overexpression of Smad7 results in severe pathological alterations in multiple epithelial tissues. *EMBO J* 21: 2580–2590, 2002
14. Li AG, Wang D, Feng XH, Wang XJ: Latent TGFβ1 overexpression in keratinocytes results in a severe psoriasis-like skin disorder. *EMBO J* 23: 1770–1781, 2004
15. Lan HY, Mu W, Tomita N, Huang XR, Li JH, Zhu HJ, Morishita R, Johnson RJ: Inhibition of renal fibrosis by gene transfer of inducible Smad7 using ultrasound-microbubble system in rat UUO model. *J Am Soc Nephrol* 14: 1535–1548, 2003
16. Li JH, Zhu HJ, Huang XR, Lai KN, Johnson RJ, Lan HY: Smad7 inhibits fibrotic effect of TGF-beta on renal tubular epithelial cells by blocking Smad2 activation. *J Am Soc Nephrol* 13: 1464–1472, 2002
17. Wang W, Tzanidis A, Divjak M, Thomson NM, Stein-Oakley AN: Altered signaling and regulatory mechanisms of apoptosis in focal and segmental glomerulosclerosis. *J Am Soc Nephrol* 12: 1422–1433, 2001
18. Manna SK, Mukhopadhyay A, Van NT, Aggarwal BB: Silymarin suppresses TNF-induced activation of NF-kappa B, c-Jun N-terminal kinase, and apoptosis. *J Immunol* 163: 6800–6809, 1999
19. Timchenko NA, Wilde M, Iakova P, Albrecht JH, Darlington GJ: E2F/p107 and E2F/p130 complexes are regulated by C/EBPα in 3T3–L1 adipocytes. *Nucleic Acids Res* 27: 3621–3630, 1999
20. Lan HY, Bacher M, Yang N, Mu W, Nikolic-Paterson DJ, Metz C, Meinhardt A, Bucala R, Atkins RC: The pathogenic role of macrophage migration inhibitory factor in immunologically induced kidney disease in the rat. *J Exp Med* 185: 1455–1465, 1997
21. Lan HY, Mu W, Nikolic-Paterson DJ, Atkins RC: A novel, simple, reliable, and sensitive method for multiple immunoenzyme staining: Use of microwave oven heating to block antibody crossreactivity and retrieve antigens. *J Histochem Cytochem* 43: 97–102, 1995
22. Timoshanko JR, Kitching AR, Iwakura Y, Holdsworth SR, Tipping PG: Leukocyte-derived interleukin-1β interacts

with renal interleukin-1 receptor I to promote renal tumor necrosis factor and glomerular injury in murine crescentic glomerulonephritis. *Am J Pathol* 164: 1967–1977, 2004
23. Okada S, Shikata K, Matsuda M, Ogawa D, Usui H, Kido Y, Nagase R, Wada J, Shikata Y, Makino H: Intercellular adhesion molecule-1-deficient mice are resistant against renal injury after induction of diabetes. *Diabetes* 52: 2586–2593, 2003
24. Barnes PJ, Karin M: Nuclear factor-kappaB: A pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 336: 1066–1071, 1997
25. Lallemand F, Mazars A, Prunier C, Bertrand F, Kornprost M, Gallea S, Roman-Roman S, Cherqui G, Atfi A: Smad7 inhibits the survival nuclear factor kappaB and potentiates apoptosis in epithelial cells. *Oncogene* 20: 879–884, 2001
26. Zhao J, Shi W, Chen H, Warburton D: Smad7 and Smad6 differentially modulate transforming growth factor beta-induced inhibition of embryonic lung morphogenesis. *J Biol Chem* 275: 23992–23997, 2000
27. Afrakhte M, Moren A, Jossan S, Itoh S, Sampath K, Westermark B, Heldin CH, Heldin NE, ten Dijke P: Induction of inhibitory Smad6 and Smad7 mRNA by TGF-beta family members. *Biochem Biophys Res Commun* 249: 505–511, 1998
28. Lyons RM, Gentry LE, Purchio AF, Moses HL: Mechanism of activation of latent recombinant transforming growth factor beta 1 by plasmin. *J Cell Biol* 110: 1361–1367, 1990
29. Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, Rifkin DB, Sheppard D: The integrin alpha v beta 6 binds and activates latent TGF beta 1: A mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 96: 319–328, 1999
30. Werner F, Jain MK, Feinberg MW, Sibinga NE, Pellacani A, Wiesel P, Chin MT, Topper JN, Perrella MA, Lee ME: Transforming growth factor-beta 1 inhibition of macrophage activation is mediated via Smad3. *J Biol Chem* 275: 36653–36658, 2000
31. Cottrez F, Groux H: Regulation of TGF-beta response during T cell activation is modulated by IL-10. *J Immunol* 167: 773–778, 2001
32. Chernajovsky Y, Adams G, Triantaphyllopoulos K, Ledda MF, Podhajcer OL: Pathogenic lymphoid cells engineered to express TGF beta 1 ameliorate disease in a collagen-induced arthritis model. *Gene Ther* 4: 553–559, 1997
33. Cautain B, Damoiseaux J, Bernard I, van Straaten H, van Breda Vriesman P, Boneu B, Druet P, Saoudi A: Essential role of TGF-beta in the natural resistance to experimental allergic encephalomyelitis in rats. *Eur J Immunol* 31: 1132–1140, 2001
34. Fuss IJ, Boirivant M, Lacy B, Strober W: The interrelated roles of TGF-beta and IL-10 in the regulation of experimental colitis. *J Immunol* 168: 900–908, 2002
35. Hansen G, McIntire JJ, Yeung VP, Berry G, Thorbecke GJ, Chen L, DeKruyff RH, Umetsu DT: CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. *J Clin Invest* 105: 61–70, 2000
36. Thorbecke GJ, Umetsu DT, deKruyff RH, Hansen G, Chen LZ, Hochwald GM: When engineered to produce latent TGF-beta1, antigen specific T cells down regulate Th1 cell-mediated autoimmune and Th2 cell-mediated allergic inflammatory processes. *Cytokine Growth Factor Rev* 11: 89–96, 2000
37. Oida T, Zhang X, Goto M, Hachimura S, Totsuka M, Kaminogawa S, Weiner HL: CD4+CD25- T cells that express latency-associated peptide on the surface suppress CD4+CD45RB high-induced colitis by a TGF-beta-dependent mechanism. *J Immunol* 170: 2516–2522, 2003
38. Kanamaru Y, Nakao A, Mamura M, Suzuki Y, Shirato I, Okumura K, Tomino Y, Ra C: Blockade of TGF-beta signaling in T cells prevents the development of experimental glomerulonephritis. *J Immunol* 166: 2818–2823, 2001
39. Ng YY, Hou CC, Wang W, Huang XR, Lan HY: Blockade of NF-kappaB activation and renal inflammation by ultrasound-mediated gene transfer of Smad7 in rat remnant kidney. *Kidney Int Suppl* 94: S83–S91, 2005
40. Guijarro C, Egido J: Transcription factor-kappa B (NF-kappa B) and renal disease. *Kidney Int* 59: 415–424, 2001
41. Lee FT, Cao Z, Long DM, Panagiotopoulos S, Jerums G, Cooper ME, Forbes JM: Interactions between angiotensin II and NF-kappaB-dependent pathways in modulating macrophage infiltration in experimental diabetic nephropathy. *J Am Soc Nephrol* 15: 2139–2151, 2004
42. Nagarajan RP, Chen F, Li W, Vig E, Harrington MA, Nakshatri H, Chen Y: Repression of transforming-growth-factor-beta-mediated transcription by nuclear factor kappaB. *Biochem J* 348: 591–596, 2000
43. Schiffer M, Bitzer M, Roberts IS, Kopp JB, ten Dijke P, Mundel P, Bottinger EP: Apoptosis in podocytes induced by TGF-beta and Smad7. *J Clin Invest* 108: 807–816, 2001
44. Azuma M, Motegi K, Aota K, Yamashita T, Yoshida H, Sato M: TGF-beta1 inhibits NF-kappaB activity through induction of IkappaB-alpha expression in human salivary gland cells: A possible mechanism of growth suppression by TGF-beta1. *Exp Cell Res* 250: 213–222, 1999
45. Miller DM, Ogawa Y, Iwata KK, ten Dijke P, Purchio AF, Soloff MS, Gentry LE: Characterization of the binding of transforming growth factor-beta 1, -beta 2, and -beta 3 to recombinant beta 1-latency-associated peptide. *Mol Endocrinol* 6: 694–702, 1992
46. Bottinger EP, Factor VM, Tsang ML, Weatherbee JA, Kopp JB, Qian SW, Wakefield LM, Roberts AB, Thorgeirsson SS, Sporn MB: The recombinant proregion of transforming growth factor beta1 (latency-associated peptide) inhibits active transforming growth factor beta1 in transgenic mice. *Proc Natl Acad Sci U S A* 93: 5877–5882, 1996
47. Zhang Y, McCormick LL, Gilliam AC: Latency-associated peptide prevents skin fibrosis in murine sclerodermatous graft-versus-host disease, a model for human scleroderma. *J Invest Dermatol* 121: 713–719, 2003