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Original Article

Smad2 and Smad3 play differential roles in the regulation of matrix deposition-related enzymes in renal mesangial cells

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Abstract: Glomerulosclerosis, the very common feature of the end-stage renal disease is characterized by excessive extracellular matrix (ECM) deposition, especially by mesangial matrix deposition in glomeruli. Transforming growth factor-beta1-Smad (TGF-β1-Smad) signaling and connective tissue growth factor (CTGF) play important roles in the development of glomerulosclerosis. Together with membrane-type-1 matrix metalloproteinase (MT1-MMP) and tissue inhibitor of metalloproteinase-2 (TIMP-2), matrix metalloproteinase-2 (MMP-2) is responsible for the mesangial matrix degradation. In the present study we aim to investigate the distinct contributions of Smad2 and Smad3 to the expressions of MMP-2, MT1-MMP, TIMP-2 and CTGF in response to TGF-β1 treatment in an in vitro culture model of HBZY-1 (rat glomerular mesangial cells). RNA interference was used to achieve selective and specific knockdown of Smad2 and Smad3. Cellular MT1-MMP, MMP-2, TIMP-2 and CTGF were assessed by Western blot. Their corresponding mRNA expressions were also assessed by real time RT-PCR. TGF-β1 treatment induced a fibrotic response with decreased expressions of MMP-2 and MT1-MMP while increased expressions of TIMP-2 and CTGF. The downregulation of MMP-2 and MT1-MMP induced by TGF-β1 were Smad2-dependent, whereas the upregulation of CTGF were Smad3-dependent. Increases in TIMP-2 expression were dependent on both Smad2 and Smad3 and were abolished by combined knockdown of both Smad2 and Smad3. In conclusion, we have demonstrated the distinct roles of Smad2 and Smad3 in TGF-β1-induced expression of CTGF and the key enzymes responsible for mesangial matrix deposition in glomerular mesangial cells. This can be of therapeutic value in designing targeted anti-fibrotic therapies for glomerulosclerosis.

Keywords: TGF-β1, CTGF, MMP-2, TIMP-2, MT1-MMP, Smad2, Smad3, glomerular mesangial cell

Introduction

Transforming growth factor-beta1 (TGF-β1) has been identified as the most potent pro-fibrogenic cytokine. The pathogenesis of renal fibrosis, characterized by an excessive accumulation and deposition of extracellular matrix (ECM) components, is mediated primarily by TGF-β1 activation [1]. Glomerular mesangial cells (GMCs) contribute to the development of renal fibrosis, in particular to glomerulosclerosis development, by increased synthesis of ECM components and decreased ECM degradation, a process mediated by matrix metalloproteinases (MMPs) in response to TGF-β1 [2-4]. Evidences from a number of studies have indicated that MMP-2, tissue inhibitor of metalloproteinase-2 (TIMP-2) as well as the membrane-type-1 MMP (MT1-MMP), all secreted by GMCs, were involved in the development of glomerulosclerosis [5-8]. TGF-β1 exerts its pro-fibrotic effects by direct mechanisms and indirectly by induction of secondary mediators such as connective tissue growth factor (CTGF). The accumulated data support the idea that CTGF may play a pivotal role both in glomerulosclrosis and in renal tubulointerstitial fibrosis and it may serve as an essential therapeutic target for renal fibrosis [9-11]. TGF-β1 exerts its pro-fibrotic effects through its complicated signaling networks. Evidence from in vivo and in vitro studies suggests that the predominant signaling pathway responsible for pro-fibrotic effects of TGF-β1 in the glomerular mesangium is the

Smad (Sma and Mad protein) signaling pathway [4, 12, 13]. Previous studies identified a requirement for Smad signaling in the induction of CTGF, which act as a downstream mediator of TGF-β1 in renal fibrogenesis [14, 15]. Some evidences also indicate that Smad signaling pathway is involved in the mesangial matrix deposition [12, 16].

It is now well established that binding of TGF-β1 to its receptor II (TβRII) results in phosphorylation of the TGF-β receptor type I (TβRI)-kinase, which in turn phosphorylates the two receptor-regulated Smads (Smad2 and Smad3). Subsequently, phosphorylated Smad2 and Smad3 bind to the common Smad4 and form the Smad complex, which translocates into the nucleus, where they regulate target gene transcription in a cell-type-specific manner through interaction with other transcription factors, co-activators and co-repressors [17, 18]. In spite of marked similarity in their structure, accumulating evidence over the last few years has suggested that Smad2 and Smad3 have distinct roles in TGF-β1-induced cellular responses [19-21]. Previous studies performed in Smad3 null mice and renal or non-renal cells with Smad3 knockdown suggested Smad3 may be a critical mediator in TGF-β-induced profibrotic responses [4, 19, 20, 22]. However the role of Smad2 in the kidney diseases, especially in glomerular diseases remains largely unknown due to the unavailability of Smad2 knockout mice because of the embryonic lethality [23]. Furthermore, the role of Smad3 in TGF-β1-induced dysregulation of renal mesangial matrix degradation in GMCs has not previously been investigated. Here we have investigated the differential role of Smad2 and Smad3 in expressions of MMP-2, TIMP-2, MT1-MMP and CTGF in response to TGF-β1 in rat GMCs with selective knockdown of Smad2 or Smad3 by using RNA interference.

Materials and methods

Cell culture

Rat glomerular mesangial cells (HBZY-1) were purchased from China Center for Type Culture Collection (Wuhan, Hubei, China). The HBZY-1 cells were cultured in DMEM (Dulbecco's modified Eagle's medium/F12 medium (1:1, v/v) containing 5% (v/v) fetal-calf serum (Gibco BRL Life Technologies, Beijing, China) in the absence of any antibiotics and growth factors. Cells were used in the exponential growth phase in all the experiments.

siRNA transfections

Three siRNAs targeting Smad2 and Smad3 respectively were designed by Wuhan Genesil Biotechnology. The most effective Smad2 and Smad3 siRNAs were chosen according to the knockdown effect of Smad2 and Smad3 evaluated by western blot assays in our preliminary studies. Sequences for Smad2 and Smad3 siRNAs are as follows: Smad2 siRNA, Sense: 5' GCCGAGUGCCUAAGUGAUAtt 3', Anti-sense: 5' UAUCACUUAGGCACUCGGCtt 3'; Smad3 siRNA, Sense 5' GCUUUGAGGCUGUCUACCAtt 3', Anti-sense 5' UGGUAGACAGCCUCAAAGCtt 3'. The transfection was performed following the protocol described in our previously published paper [24]. Briefly, HBZY-1 cells were transfected at about 50-60% confluence with siRNAs specific to Smad2, Smad3 or nontargeting siRNAs using FuGENE6 Transfection Reagent (Promega). After 24 hours of transfection, cells were treated with either vehicle (0.1% BSA) or TGF-β1 (2 ng/ml) for 48 h under serum-free conditions.

Quantitative real-time RT-PCR

The total RNA of HBZY-1 cells were extracted with RNAiso Plus (TaKaRa, Biotechnology, Dalian, China). Single-stranded cDNA was synthesized by using a PrimeScript TM RT reagent Kit (TaKaRa). Quantitative real-time PCR assay was performed on the Mx3005P (Stratagene, Cedar Creek, TX, USA) using SYBR Premix Ex Taq TM II (TaKaRa). The sequences of primers are as follows: MMP2, forward 5'-AGGGCACCTCTTACAACAGC-3', reverse 5'-CCCGGTCATAATCCTCGGTG-3'; TIMP2, forward 5'-AGATCACACGCTGCCCTATG-3', reverse 5'-TGGTGCCCATTGATGCTCTT-3'; MT1-MMP, forward 5'-ACGATGAAGGCTATGAGGCG-3', reverse 5'-CTTCCTCCGAACATTGGCCT-3'; CTGF, forward 5'-TAGCTGCCTACCGACTGGAA-3', reverse 5'-CTTAGAACAGGCGCTCCACT-3'; 18S rRNA, forward 5'-CATTCGAACGTCTGCCCTATC-3', reverse 5'-CCTGCTGCCTTCCTTGGA-3'. The specific products of PCR were normalized to 18S rRNA.

Western blot assay

Protein extraction and western blot were conducted as previously described [24]. Proteins

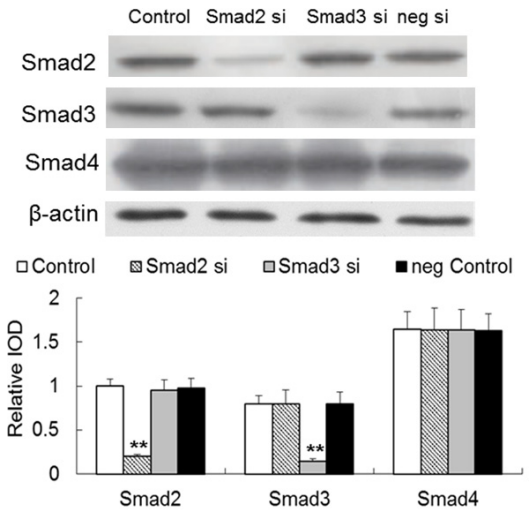

Figure 1. Smad2 and Smad3 siRNA treatment results in selective and specific knockdown of respective Smad proteins. HBZY-1 cells were transfected with Smad2 or Smad3 siRNA for 24 hours. Western blot assay showed the effects of Smad2 or Smad3 siRNA on Smads protein expression. Smad2 si: transfection with Smad2 siRNA; Smad3 si: transfection with Smad3 siRNA; neg si: transfection with non-targeting control siRNA. The data obtained from at least three individual experiments are expressed as means ± SEM. **P < 0.01 vs. control. (IOD: Integral Optical Density).

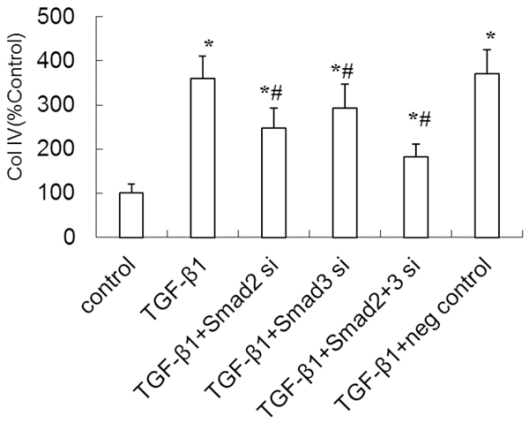

Figure 2. Smad2- or Smad3-knockdown reduces TGF-β1-induced soluble collagen IV in GMCs cells. HBZY-1 cells were treated with either TGF-β1 (2 ng/ml) or vehicle (0.1% BSA) for 48 h after 24 h transfection with Smad2 and/or Smad3 siRNA. Soluble collagen IV in cell culture supernatants was detected by ELISA at the end of the cell culture period. (n = 6; *P < 0.05 vs. control group; #P < 0.05 vs. TGF-β1 group).

were detected with primary antibodies to Smad2, Smad3 (Cell Signaling, Boston, MA, USA), Smad4, MMP-2, TIMP-2, MT1-MMP and CTGF (Abcam, Hong Kong, China).

Enzyme-linked immunosorbent assay

At the end of the experimental incubation period the cell culture supernatants were collected and cleared by centrifugation. Soluble collagen type IV was quantified using a competitive sandwich ELISA kit according to the manufacturer's protocol (Shanghai Yihan Biochemistry Technology, Shanghai, China).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) with Student-Newman-Keuls test. A value of $P < 0.05$ was considered significant.

Results

Smad2 and Smad3 siRNA treatment results in selective and specific knockdown of respective Smad proteins

Smad2 and Smad3 siRNA treatment resulted in approx. 80% reduction in band density of respective Smad proteins in HBZY-1 cells after 24 hours of transfection. The knockdown of respective Smad proteins by targeting siRNA was specific, as non-targeting control siRNA did not affect either of the receptor-regulated Smads. The knockdown was also selective, as Smad3 protein levels were reduced by Smad3 siRNA, but not by Smad2 siRNA. Similarly, Smad2 protein levels were reduced by Smad2 siRNA and not by Smad3 siRNA. Neither of the transfections had any effect on Smad4 protein levels (**Figure 1**).

Smad2- or Smad3-knockdown reduces TGF-β1-induced soluble collagen IV in GMCs cells

TGF-β1 treatment for 48 h resulted in more than 3-fold increased soluble collagen IV in HBZY-1 cell culture supernatants. Smad2-knockdown and Smad3-knockdown attenuated the increased collagen IV level in cell supernatants by approx. 30% and 20% respectively. The rise in collagen IV induced by TGF-β1 was significantly reduced but not completely prevented by the treatment with both Smad2 and Smad3 siRNA (**Figure 2**).

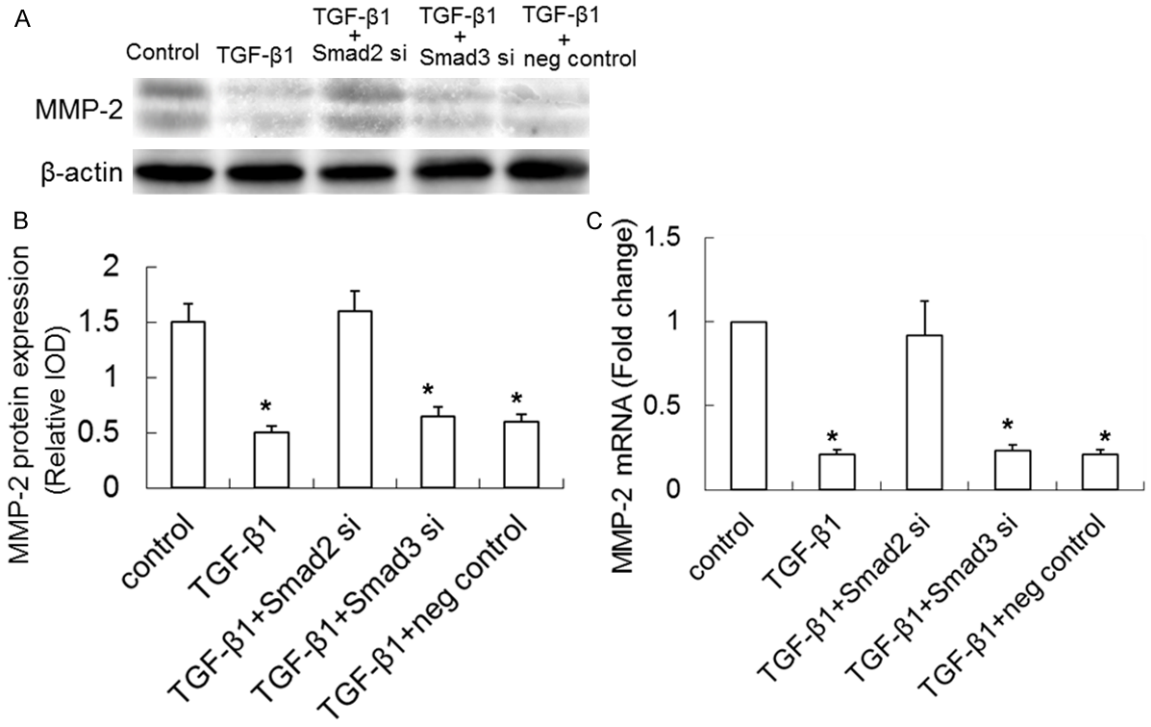

Figure 3. Down-regulation of MMP-2 in GMCs in response to TGF-β1 treatment is Smad2-dependent. HBZY-1 cells were treated with either TGF-β1 (2 ng/ml) or vehicle (0.1% BSA) for 48 h after 24 h transfection with Smad2 or Smad3 siRNA. A: Representative image of western blot for MMP-2 is shown. β-Actin served as loading control. B: Quantification of the results of western blot for MMP-2. C: The results of quantitative real-time PCR for MMP-2. The data obtained from at least three individual experiments are expressed as means ± SEM. *P < 0.05 vs. control.

Down-regulation of MMP-2 in GMCs in response to TGF-β1 treatment is Smad2-dependent

TGF-β1 treatment for 48 h resulted in a about 60-80% decrease in MMP-2 expression at both mRNA level and protein level in HBZY-1 cells. The TGF-β1-induced down-regulation of MMP-2 was markedly prevented by Smad2 knockdown, but not by Smad3 knockdown (**Figure 3**). These results demonstrate that down-regulation of MMP-2 induced by TGF-β1 in GMCs cells is critically dependent on Smad2.

Down-regulation of MT1-MMP in GMCs in response to TGF-β1 treatment is Smad2-dependent

TGF-β1 treatment for 48 h resulted in dramatic reduction in MT1-MMP expression in HBZY-1 cells, both at protein level (approx. 70%) and at mRNA level (approx. 80%). This reduction was markedly inhibited in the presence of Smad2 siRNA. However Smad3 knockdown had no effect on the TGF-β1-induced reduction in MT1-MMP expression (**Figure 4**). These results demonstrate that reduction of MT1-MMP induced by TGF-β1 in GMCs is Smad2-dependent.

Up-regulation of TIMP-2 in response to TGF-β1 in GMCs is dependent on both Smad2 and Smad3

TGF-β1 treatment for 48 h resulted in 3-4-fold increases in TIMP-2 expression at both mRNA level and protein level in HBZY-1 cells. Smad2 or Smad3 knockdown did not prevent the induction of TIMP-2 independently. About 60% TGF-β1-induced TIMP-2 was attenuated by Smad2 knockdown, whereas approx. 40% expression of TIMP-2 was attenuated by Smad3 knockdown. As inhibition of either Smad2 or Smad3 resulted in partial reduction of TGF-β1-induced TIMP-2, we next investigated the effect of simultaneous knockdown of both Smad2 and Smad3 on TGF-β1-induced TIMP-2 expression. Treatment with both Smad2 and Smad3 siRNA resulted in complete inhibition of TGF-β1-induced TIMP-2 in HBZY-1 cells (**Figure 5**). We conclude from these experiments that TGF-

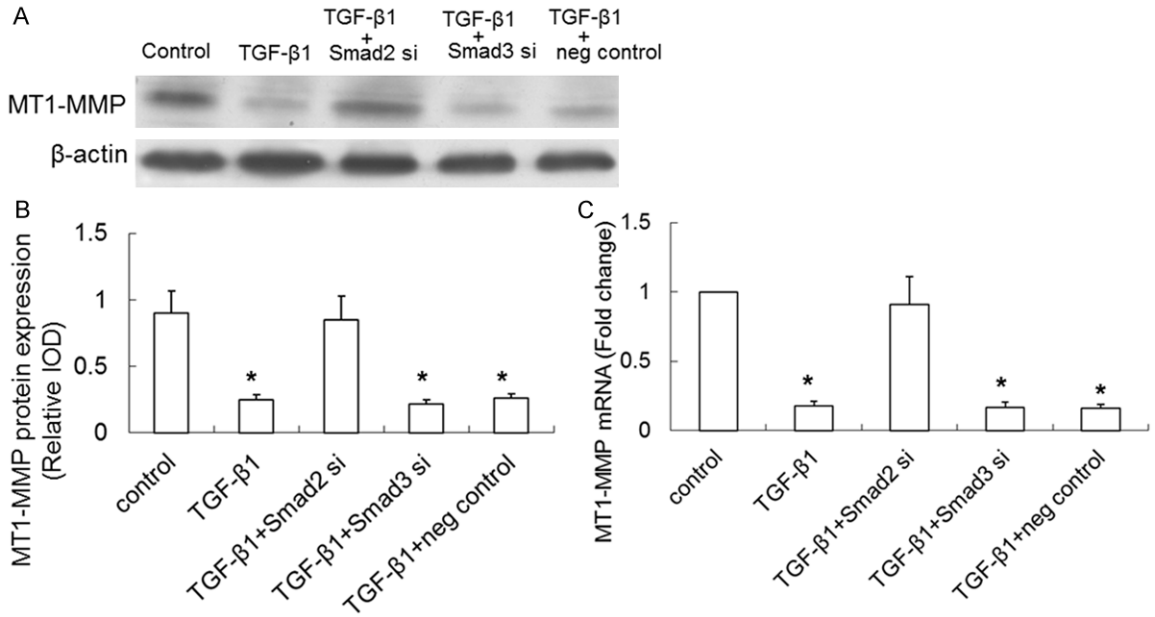

Figure 4. Down-regulation of MT1-MMP in GMCs in response to TGF-β1 treatment is Smad2-dependent. HBZY-1 cells were treated with either TGF-β1 (2 ng/ml) or vehicle (0.1% BSA) for 48 h after 24 h transfection with Smad2 or Smad3 siRNA. A: Representative image of western blot for MT1-MMP is shown. β-Actin served as loading control. B: Quantification of the results of western blot for MT1-MMP. C: The results of quantitative real-time PCR for MT1-MMP. The data obtained from at least three individual experiments are expressed as means ± SEM. *P < 0.05 vs. control.

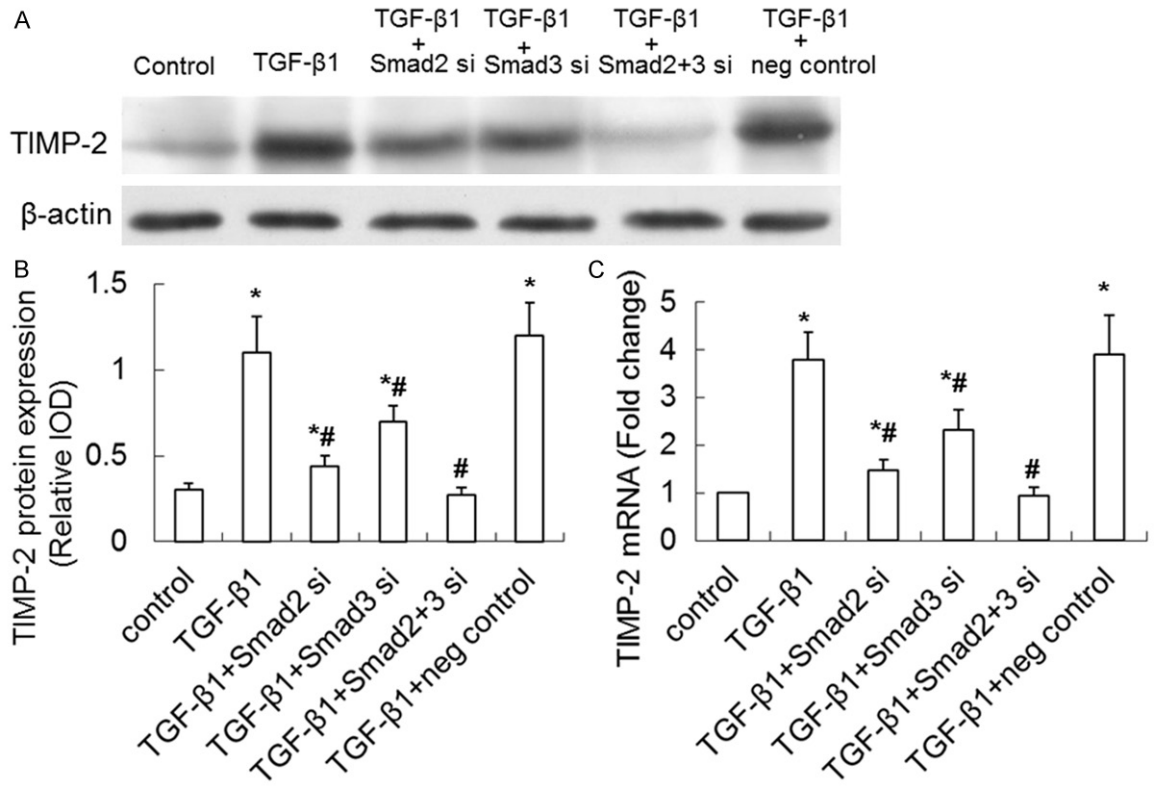

Figure 5. Up-regulation of TIMP-2 in response to TGF-β1 in GMCs is dependent on both Smad2 and Smad3. HBZY-1 cells were treated with either TGF-β1 (2 ng/ml) or vehicle (0.1% BSA) for 48 h after 24 h transfection with Smad2 and/or Smad3 siRNA. A: Representative image of western blot for TIMP-2 is shown. β-Actin served as loading

control. B: Quantification of the results of western blot for TIMP-2. C: The results of quantitative real-time PCR for TIMP-2. The data obtained from at least three individual experiments are expressed as means ± SEM. *P < 0.05 vs. control; #P < 0.05 vs. TGF-β1 group.

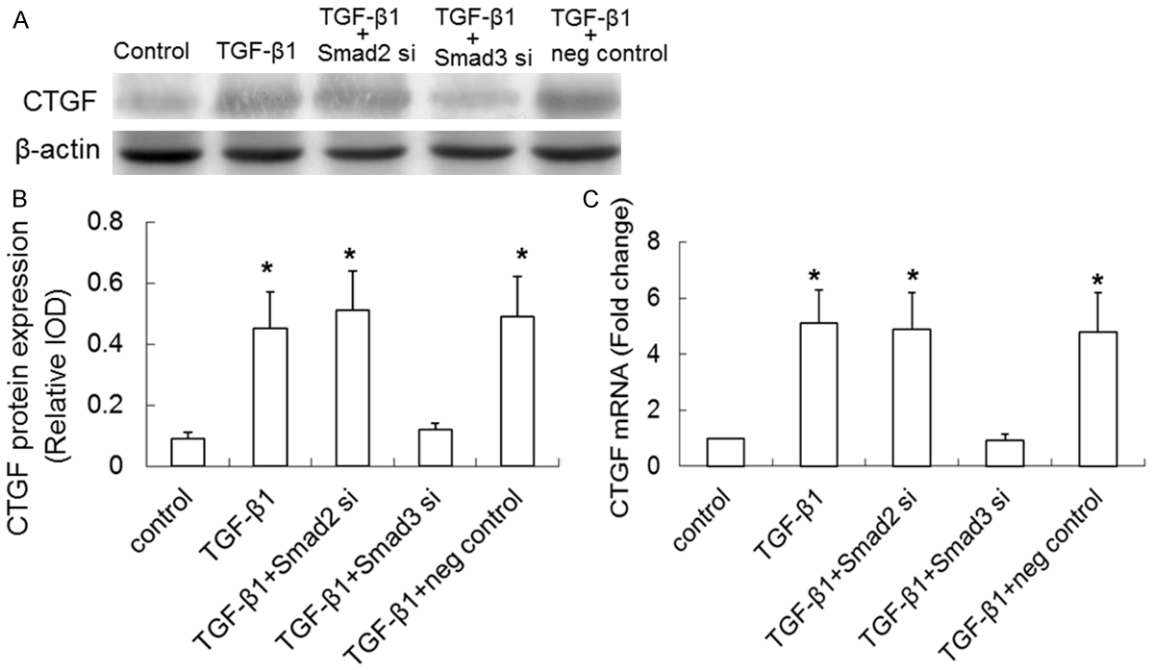

Figure 6. Up-regulation of CTGF in response to TGF-β1 in GMCs is dependent on Smad3. HBZY-1 cells were treated with either TGF-β1 (2 ng/ml) or vehicle (0.1% BSA) for 48 h after 24 h transfection with Smad2 or Smad3 siRNA. A: Representative image of western blot for CTGF is shown. β-Actin served as loading control. B: Quantification of the results of western blot for CTGF. C: The results of quantitative real-time PCR for CTGF. The data obtained from at least three individual experiments are expressed as means ± SEM. *P < 0.05 vs. control.

β1-induced TIMP-2 in GMCs involves both Smad2 and Smad3 signaling through parallel pathways.

Up-regulation of CTGF in response to TGF-β1 in GMCs is dependent on Smad3

TGF-β1 treatment for 48 h resulted in significant induction of (about 4-5-fold) CTGF mRNA and protein in HBZY-1 cells. This induction was markedly attenuated in the presence of Smad3 siRNA. However, Smad2 knockdown shows no effect on TGF-β1-induced CTGF expression. These results demonstrate that induction of CTGF with TGF-β1 in GMCs is dependent on Smad3 and not on Smad2 (**Figure 6**).

Discussion

It is well established that glomerulosclerosis, the very common feature of the end-stage renal disease is characterized by excessive ECM deposition, especially by mesangial matrix deposition in glomeruli. During the long period of glomerulosclerosis development the mesangium is usually the first to react to injurious glomerular events and is often the last to return to normal after the pathological insult has ceased and repair mechanisms have been activated. Repairing mesangial damage represents a fundamental process needed for restoring glomerular function [25]. GMCs have been identified as the main producer of mesangial matrix and its regulating factors, which contribute critically to the structural and functional integrity of the glomerulus [26]. Thus, understanding the role of GMCs in the regulation of mesangial matrix might be crucial for the development of novel and effective therapeutic strategies for kidney diseases. In this study we focus on the distinct role of TGF-β-Smad signaling pathway in the regulation of mesangial matrix degradation. TGF-β1 mediates mesangial matrix deposition by stimulating ECM production while inhibiting its degradation [27]. MMP-2 (also called gelati-

nase A or collagenase IV), the predominant MMP synthesized by GMCs, is responsible for the mesangial matrix degradation. MMP-2 is secreted as a zymogen (proMMP-2) and is activated post-translationally on the cell surface by MT1-MMP. The activation of proMMP-2 is regulated by a complex mechanism involving formation of a trimolecular complex with MT1-MMP and TIMP-2 [28]. TIMP-2 plays a dual role in the regulation of MMP-2 activation, functioning both to promote and to inhibit the activation process in a concentration-dependent manner [28]. TIMP-2 bridges the interaction between the MMP-2 zymogen and MT1-MMP such that at low TIMP-2 concentration an adjacent TIMP-2-free MT1-MMP can effectively activate proMMP-2. However, at high TIMP-2 concentration, all of the cell surface MT1-MMP undergoes complex formation with TIMP-2, thereby inhibiting proMMP-2 activation. Previously altered expressions of MMP-2, TIMP-2 and MT1-MMP in a variety of glomerular diseases, in both human beings and experimental animal models or cultured cells, have been reported [5-8]. While studies suggested Smad pathway is present and functional in GMCs and that it can mediate TGF-β-stimulated ECM expression, the role of Smads in mesangial matrix degradation is still not clear [1, 10].

In the present study siRNA was used to achieve selective knockdown of Smad2 and Smad3. This approach enabled us to study the role of endogenous Smad proteins in TGF-β1-induced cellular responses in GMCs. First, we confirmed that either Smad2 or Smad3 has significant effect on ECM deposition of mesangium due to Smad2 and/or Smad3 siRNA remarkably cutting down the secreted Col IV induced by TGF-β1 in GMCs, although these siRNA treatments do not abolish the increase of Col IV completely. We next investigated the regulation of the key proteolytic enzymes (MMP-2, TIMP-2 and MT1-MMP) responsible for ECM degradation in GMCs. TGF-β1 treatment for 48 h resulted in a significant decrease in MMP-2 expression as well as the expression of its activator, MT1-MMP in GMCs. This decrease was almost completely prevented by Smad2 knockdown. However, Smad3 knockdown had a minimal, or no effect on down-regulation of MMP-2 and MT1-MMP. Data come from experiments performed on high glucose-stimulated mesangial cells and estrogen-responsed mesangial cells also revealed an antiparallel relationship between MMP-2 and Smad2 or between MT1-MMP and Smad2 [29, 30]. These evidences demonstrate that Smad2 is essential for the down-regulation of MMP-2 and MT1-MMP in response to TGF-β1 in GMCs. In contrast to the decreased expression of MMP-2 and MT1-MMP, TGF-β1 treatment for 48 h resulted in dramatic (3-4 fold) induction of TIMP-2 in GMCs. The roles of Smad2 and Smad3 in the regulation of TIMP-2 appeared more complex. Inhibition of either Smad2 or Smad3 only resulted in partial reduction of TGF-β1-induced TIMP-2 expression, while treatment with both Smad2 and Smad3 siRNA resulted in complete inhibition of TGF-β1-induced TIMP-2 expression. These results suggest that TGF-β1-induced TIMP-2 expression in GMCs involves both Smad2 and Smad3 signaling through parallel pathways. Our results might account for the facts that the expression of TIMP-2 going down with reduced transcription and phosphorylation of Smad2 or Smad3 in mesangial cells and hepatic stellate cells [16, 31]. Consistently with the previously published documents on glomerulosclerosis [1, 17, 32], our results further confirm the idea that TGF-β-Smad contributes to the development of glomerulosclerosis not only by increasing ECM synthesis but also by hampering the degradation of ECM.

CTGF was identified as a downstream mediator of TGF-β in progressive renal disease, where it modulates matrix accumulation, cell migration and reorganization of the actin cytoskeleton, paralleling pathogenic alterations to the mesangium during the progression of nephropathy [9, 33]. Given the crucial role which CTGF plays in TGF-β-induced pro-fibrotic effects, significant effort has been directed toward understanding the regulation of CTGF expression driven by TGF-β1. Lakos et al. have previously reported that fibroblasts derived from Smad3 knockout mice show marked attenuation of TGF-β1-induced CTGF mRNA expression [34]. In agreement with Lakos' and the other previous experimental data from Smad2/3-knockdown proximal-tubule epithelial cells [34], we found that TGF-β1-induced CTGF in GMCs was Smad3-dependent. However the interaction of CTGF with TGF-β1 is controversial, and the exact mechanisms of the regulation of CTGF remain unclear. Gressner et al. reported that it is through STAT3 pathway, not Smad2 (and

Smad1/3) pathway that interleukin-6 inhibit CTGF expression driven by TGF-β1 in hepatocytes [35]. These conflicting results might be explained by the crosstalk between TGF-β1-Smad pathway and non-Smad pathways [36]. The crosstalk also exists between CTGF and TGF-β1. In contrast to the Smad3-dependent induction, CTGF interestingly causes a clear inhibition of TGF-β1-dependent Smad2 and Smad3 phosphorylation and reporter gene expression in renal mesangial cells [37].

In summary, our data demonstrate that the down-regulation of MMP-2 and MT1-MMP are critically dependent on Smad2 and the induction of CTGF is Smad3-dependent, while TIMP-2 induction involves both Smad2 and Smad3 in GMCs. These results suggest that TGF-β-Smad may play an important role in the regulation of mesangial matrix degradation process. Our study contributes to a deep comprehension over the pathogenesis of glomerulosclerosis and provides a novel therapeutic strategy for glomerulosclerosis.

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Disclosure of conflict of interest

None.

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