Smad3 mediates ANG II-induced hypertensive kidney disease in mice

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Liu Z, Huang XR, Lan HY. Smad3 mediates ANG II-induced hypertensive kidney disease in mice. Am J Physiol Renal Physiol 302: F986–F997, 2012. First published January 11, 2012; doi:10.1152/ajprenal.00595.2011.—Although Smad3 is a key mediator for fibrosis, its functional role and mechanisms in hypertensive nephropathy remain largely unclear. This was examined in the present study in a mouse model of hypertension induced in Smad3 knockout (KO) and wild-type (WT) mice by subcutaneous angiotensin II infusion and in vitro in mesangial cells lacking Smad3. After angiotensin II infusion, both Smad3 KO and WT mice developed equally high levels of blood pressure. However, disruption of Smad3 prevented angiotensin II-induced kidney injury by lowering albuminuria and serum creatinine (P < 0.01), inhibiting renal fibrosis such as collagen type I and IV, fibronectin, and α-SMA expression (all P < 0.01), and blocking renal inflammation including macrophage and T cell infiltration and upregulation of IL-1β, TNF-α, and monocyte chemotactic protein-1 in vivo and in vitro (all P < 0.001). Further studies revealed that blockade of angiotensin II-induced renal transforming growth factor (TGF)-β1 expression and inhibition of Smurf2-mediated degradation of renal Smad7 are mechanisms by which Smad3 KO mice were protected from angiotensin II-induced renal fibrosis and NF-κB-driven renal inflammation in vivo and in vitro. In conclusion, Smad3 is a key mediator of hypertensive nephropathy. Smad3 promotes Smurf2-dependent ubiquitin degradation of renal Smad7, thereby enhancing angiotensin II-induced TGF-β/Smad3-mediated renal fibrosis and NF-κB-driven renal inflammation. Results from this study suggest that inhibition of Smad3 or overexpression of Smad7 may be a novel therapeutic strategy for hypertensive nephropathy.

Increasing evidence shows that angiotensin II (ANG II) is a key mediator of hypertensive nephropathy and mediates progressive kidney injury by stimulating renal fibrosis and inflammation (26, 27). Many studies have shown that blockade of ANG II with angiotensin-converting enzyme inhibitors and/or angiotensin AT1 receptor blockers is capable of protecting against and slowing down the progression of kidney injury in patients with hypertension and diabetes (10, 20). It is well accepted that ANG II mediates kidney injury by stimulating transforming growth factor (TGF)-β1 expression (2, 36). In vitro, addition of ANG II is able to induce TGF-β1 and its type II receptor expression by tubular epithelial cells (37, 38). In patients with diabetes and hypertension, the renoprotective effect of anti-ANG II treatment is associated with inhibition of TGF-β1 production (8, 30, 32), suggesting a close link between ANG II and TGF-β1 in the pathogenesis of chronic kidney disease (CKD). This is further demonstrated by the ability of TGF-β blockade to attenuate proteinuria and renal injury in experimental rat models of remnant kidney disease (18), Dahl salt-sensitive rats (7), and diabetic nephropathy (1). However, the role and mechanisms of TGF-β1 in ANG II-mediated CKD including hypertensive nephropathy remain largely unclear.

It is well established that TGF-β1, after binding to its receptors, activates the downstream mediators, called Smad2 and Smad3, to exert its biological effects, which are negatively regulated by Smad7 via a negative feedback mechanism (31). In addition to TGF-β1, ANG II can also activate Smad signaling to induce fibrosis via TGF-β-dependent and -independent mechanisms (25, 34, 39). The latter involves the activation of ERK/p38 MAP kinases (25, 34, 39), suggesting the importance of the ANG II-TGF-β/Smad signaling cross talk pathway in CKD. Indeed, Smad2 and Smad3 are highly activated in the diseased kidney from patients with hypertension and diabetes (21, 34). Overexpression of Smad7 is capable of blocking Smad2/3 activation and progressive renal fibrosis including epithelial-myoﬁbroblast transition (EMT) in a rat model of remnant kidney disease and in tubular epithelial cells in response to ANG II (11, 39, 40), demonstrating a critical role for the Smad pathway in ANG II-induced renal fibrosis.

In the context of renal fibrosis, it is generally accepted that Smad3 is pathogenic (15, 29), while Smad2 is renal protective because conditional deletion of Smad2 from the kidney largely enhances Smad3-mediated renal fibrosis in a mouse model of obstructive nephropathy (23). The distinct role of Smad2 and Smad3 in renal fibrosis is also demonstrated by the finding that deletion of Smad3, but not Smad2, from either vascular or renal tubular epithelial cells prevents ANG II- or advanced glycation end products-induced fibrosis, including connective tissue growth factor (CTGF) expression and EMT (6, 34, 39, 40). However, the functional role of Smad3 in ANG II-induced hypertensive nephropathy remains unexplored. This was examined in the present study in a mouse model of hypertension induced by subcutaneous ANG II infusion in Smad3 knockout (KO) mice and in vitro in Smad3 KO mesangial cells. In addition, mechanisms through which Smad3 mediates ANG II-induced renal fibrosis and inflammation were investigated.

Materials and Methods

Mouse model of ANG II-induced hypertension. Littermate Smad3 KO and wild-type (WT) male mice. congenic to the C57BL/6 strain, were used in this study (41). Hypertensive renal injury was induced by subcutaneous infusion of ANG II at a dose of 1.46 mg kg⁻¹ day⁻¹ for 28 days via osmotic minipumps as described previously (12). Blood pressure was measured by the tail-cuff method using the CODA noninvasive blood pressure system (Kent Scientific, Torrington, CT) in conscious mice according to the manufacturer’s instructions. Kidney tissue samples were collected at day 28 for histology and immunohistochemistry. For Western blot and real-time PCR analyses, renal cortical tissues were collected by carefully removing the renal pelvis and medullar tissues. The experimental procedures were approved by
the Animal Experimental Committee at The Chinese University of Hong Kong.

Renal function and proteinuria. Serum creatinine levels were determined using Stanbio creatinine kits (Stanbio Laboratory, Boerne, TX), as recommended by the manufacturer. Twenty-four-hour urine samples were collected before and weekly after ANG II infusion for microalbuminuria assay. Microalbuminuria was detected by a competitive ELISA according to the manufacturer’s protocol. Results of mouse microalbuminuria are expressed as micrograms protein per milligram urine creatinine.

Histology and immunohistochemistry. Changes in renal morphology were examined in methyl Carnoy’s fixed, paraffin-embedded tissue sections (4 μm) stained with periodic acid-Schiff (PAS). Immunostaining of paraffin sections was performed using a microwave-based antigen-retrieval technique. Antibodies used in this study included monocyte chemoattractant protein (MCP)-1, IL-1β, TNF-α, TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA); fibronectin (DAKO, Carpinteria, CA); collagen I and IV (Southern Technology, Birmingham, AL); rat anti-mouse monoclonal antibody to macrophages (F4/80, Serotec, Oxford, UK); and rabbit polyclonal antibodies to CD3+ T cells. All slides were counterstained with hematoxylin.

Expression of IL-1β, TNF-α, and MCP-1 and accumulation of collagen I in the entire cortical tubulointerstitium (cross section of the renal cortex) was determined by quantitative Image-Pro plus software (Media Cybernetics, Bethesda, MD), while the number of F4/80+ and CD3+ cells in the tubulointerstitium was counted under high-power fields (×40) by means of a 0.0625-mm² graticule fitted in the eyepiece of the microscope as previously described (4, 23). In addition, to quantitatively evaluate renal pathology, deposition of PAS-stained ECM within the cortex and collagen IV and fibronectin in the glomerular area was analyzed by Image-Pro plus software as previously described (4).

Western blot analysis. Renal tissues were collected for Western blot analysis as previously described (4, 23). Briefly, after the protein was transferred onto a nitrocellulose membrane, the membrane was incubated overnight with primary antibodies against phospho-IκBα (ser32), phospho-NF-κB/p65 (ser276), phospho-Smad3 (s423-425, Cell Signaling Technology, Danvers, MA); IκBα, NF-κB/p65, Smad7, TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA); fibronectin (DAKO, Carpinteria, CA); collagen I and IV (Southern Technology, Birmingham, AL); rat anti-mouse monoclonal antibody to macrophages (F4/80, Serotec, Oxford, UK); and rabbit polyclonal antibodies to CD3+ T cells. All slides were counterstained with hematoxylin.

Fig. 1. Smad3 knockout (KO) mice are protected against ANG II-induced renal injury. A: systolic blood pressure. B: urinary albumin excretion (UAE). C: serum creatinine. D: histological damage [periodic acid-Schiff (PAS)-stained sections]. E: quantitative analysis of PAS-stained sections. Note that ANG II-induced increase in UAE, serum creatinine, glomerular hypercellularity, and ECM deposition is prevented in Smad3 KO mice despite hypertension. PAS staining also shows that disruption of Smad3 prevents ANG II-induced glomerular arterial thickening (arrow) in Smad3 wild-type (WT) mice. Arrows indicate glomerular arterioles. Values are means ± SE for at least 8 mice. Sections are counterstained with hematoxylin. Magnification: ×400. *P < 0.05, **P < 0.01 compared with saline (SL) control mice. ##P < 0.01, ###P < 0.001 compared with ANG II-infused Smad3 WT mice or as indicated.

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Fig. 2. Smad3 KO mice are protected against ANG II-induced renal fibrosis in a mouse model of hypertension. A: immunohistochemical staining of collagen I. B: Western blot analysis for collagen I (Col-I), fibronectin (FN), and α-smooth muscle actin (SMA) expression. C: quantitative analysis of collagen I in immunohistochemically stained sections. D–F, quantitative real-time PCR analysis for collagen I mRNA, fibronectin mRNA, and α-SMA mRNA expression. Note that while ANG II induces a moderate to severe renal fibrosis in Smad3 WT mice, Smad3 KO mice have normal levels of all fibrosis markers examined, despite hypertension. Values are means ± SE for groups of 8 mice. Sections are counterstained with hematoxylin. Magnification: ×200. *P < 0.05, **P < 0.01, ***P < 0.001 compared with saline control mice. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with ANG II-infused Smad3 WT mice.
Smurf2 (Santa Cruz Biotechnology); Smad3 (06-920, Upstate Biotechnology, Lake Placid, NY); α-smooth muscle actin (SMA; Sigma, St. Louis, MO); collagen I (Southern Technology); and GAPDH (Chemicon, Temecula, CA), followed by the LI-COR IRDye 800-labeled secondary antibodies (Rockland Immunocoretics, Gilbertsville, PA) in the dark for 1 h at room temperature. Signals were scanned and visualized by Odyssey Infrared Imaging System (LiCor, Lincoln, NE). The ratio of the protein interest was subjected to GAPDH and was densitometrically analyzed by Image J software (NIH, Bethesda, MD).

Real-time PCR analysis. Renal mRNA expression was quantitatively analyzed by real-time PCR with primers against mouse mRNA

Fig. 3. Smad3 KO mice are protected against ANG II-induced collagen type IV and fibronectin accumulation in a mouse model of hypertension. A: immunohistochemical staining of collagen IV. B: immunohistochemical staining of fibronectin. C: quantitative analysis of glomerular collagen IV in both glomeruli and tubulointerstitium. Note that while ANG II induces a moderate to severe renal fibrosis in Smad3 WT mice, Smad3 KO mice exhibit normal levels of both collagen IV and fibronectin accumulation, despite hypertension. Values are means ± SE for groups of 8 mice. Sections are counterstained with hematoxylin. Magnification: ×200. **P < 0.01, ***P < 0.001 compared with saline control mice. ##P < 0.01, ###P < 0.001 compared with ANG II-infused Smad3 WT mice.

Fig. 4. Deletion of Smad3 prevents ANG II-induced CD3+ T cell and F4/80+ macrophage infiltration in the kidney. A: CD3+ T cells. B: F4/80+ macrophages. C: quantitative analysis of CD3+ T cells and F4/80+ macrophages. Compared with Smad3 WT mice in which ANG II induces many CD3+ and F4/80+ cell infiltration within the kidney, Smad3 KO mice have a few T cells and macrophages infiltrating the tubulointerstitium. Values are means ± SE for at least 8 mice. Sections are counterstained with hematoxylin. Magnification: ×200. ***P < 0.001 compared with saline control mice. ###P < 0.001 compared with ANG II-infused Smad3 WT mice.
of IL-1β, TNF-α, MCP-1, fibronectin, Smad7, TGF-β1, collagen I, α-SMA, and GAPDH as described previously (4, 12, 23), whereas the primers for Smurf2 are as follows: forward 5'-GCTGCTTTGTG-GATGAGAAT-3', reverse 5'-CCTGCTGCGTTGCTCTTTGT-3'. Reaction specificity was confirmed by melting curve analysis. The housekeeping gene GAPDH was used as an internal standard, and the ratio of the mRNA examined to GAPDH was calculated and is expressed as means ± SE.

**In vitro study of Smad3 WT and KO mesangial cells.** Mesangial cells were isolated from both Smad3 WT and KO mice and were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (GIBCO, Carlsbad, CA). Mesangial cells were characterized as vimentin positive but CD31 and cytokeratin or nephrin negative. Cells at passages 3–5 were used for studies. Briefly, cells were serum starved for 16 h, followed by treatment with ANG II (1 μmol/l) for periods of 3, 6, 12, 24, and 72 h to study the mechanisms of fibrosis and inflammation.

**Statistical analyses.** Data obtained from this study were expressed as means ± SE. Statistical analyses were performed using one-way ANOVA, followed by a Newman-Keuls posttest using Prism 5.0 (GraphPad Software, San Diego, CA).

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**Fig. 5. Deletion of Smad3 prevents ANG II-induced renal TNF-α, IL-1β, and MCP-1 expression detected by immunohistochemistry and quantitative real-time PCR.**

**A:** TNF-α. **B:** IL-1β. **C:** MCP-1. Note that Smad3 KO mice are protected from ANG II-induced upregulation of proinflammatory cytokines and MCP-1 expression in the glomerulus and the tubulointerstitium, largely in tubular epithelial cells. Values are means ± SE for groups of 8 mice. Sections are counterstained with hematoxylin. Magnification: ×200. *P < 0.05, ***P < 0.001 compared with saline control mice. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with ANG II-infused Smad3 WT mice.
RESULTS

Smad3 KO mice are protected against ANG II-induced renal injury independent of blood pressure. As shown in Fig. 1A, Smad3 WT and KO mice treated with saline showed normal systolic blood pressure but developed hypertension with equal levels of elevated systolic blood pressure after ANG II infusion. However, significantly higher levels of urinary albumin excretion and serum creatinine were developed in Smad3 WT mice but not in Smad3 KO mice (Fig. 1, B and C). Histologically, chronic ANG II infusion caused glomerular and vascular hypercellularity and increased ECM deposition within the mesangium and glomerular arterioles in Smad3 WT mice, which was blunted in Smad3 KO mice (Fig. 1, D and E).

Smad3 KO mice are protected against renal fibrosis and inflammation in a mouse model of ANG II-induced hypertension. As shown in Figs. 2 and 3, immunohistochemistry, Western blotting, and quantitative real-time PCR analyses detected that compared with the saline-treated mice, chronic ANG II infusion significantly increased collagen I, collagen IV, α-SMA, and fibronectin mRNA and protein expression in kidney tissues of Smad3 WT mice. In contrast, mice lacking Smad3 were protected from ANG II-induced renal fibrosis, showing normal levels of collagen type I and IV, fibronectin, and α-SMA mRNA and protein expression (Figs. 2 and 3). Chronic ANG II infusion also resulted in the development of moderate renal inflammation with greater numbers of CD3+ T cells and F4/80+ macrophages infiltrating the tubulointerstitium in Smad3 WT mice (Fig. 4). This was accompanied by a marked upregulation of TNF-α, IL-1β, and MCP-1 as demonstrated by immunohistochemistry and real-time PCR (Fig. 5). In contrast, disruption of Smad3 prevented ANG II-induced renal inflammation with a few CD3+ and F4/80+ cells with-

![Graphs and figures](http://ajprenal.physiology.org/)

**Fig. 6.** Disruption of Smad3 from mesangial cells inhibits ANG II-induced fibrosis in vitro. A: quantitative real-time PCR analysis of collagen I, α-SMA, and fibronectin mRNA expression in response to ANG II (1 μM). B: Western blot analysis of collagen I, fibronectin, and α-SMA protein expression in response to ANG II (1 μM). Values are means ± SE for at least 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared with negative control (0). #P < 0.05, ###P < 0.001 compared with ANG II-treated Smad3 WT cells.
out upregulation of TNF-α, IL-1β, and MCP-1 in Smad3 KO mice (Figs. 4 and 5).

Disruption of Smad 3 inhibits ANG II-induced renal fibrosis and inflammation in vitro. The functional role of Smad3 in ANG II-induced renal fibrosis and inflammation was also determined in the primary culture of mesangial cells. Real-time PCR and Western blot analysis detected that addition of ANG II induced a marked upregulation of collagen I, fibronectin, and α-SMA expression in Smad3 WT cells (Fig. 6A and B). In contrast, cells null for Smad3 were protected from ANG II-induced fibrosis responses (Fig. 6, A and B). Similarly, quantitative real-time PCR analysis also detected that disruption of Smad3 from mesangial cells prevented ANG II-induced inflammatory responses, including IL-1β, TNF-α, and MCP-1 expression (Fig. 7).

Smad3 mediates ANG II-induced renal fibrosis via the positive feedback mechanism of TGF-β/Smad signaling. We then investigated the mechanism by which Smad3 KO mice were protected against ANG II-mediated renal fibrosis in vivo and in vitro. Because ANG II mediates renal fibrosis by stimulating TGF-β1 expression (2, 36–38), we thus hypothesized that ANG II-induced TGF-β1 expression may be Smad3 dependent. As shown in Fig. 8, A and B, ANG II infusion induced a marked upregulation of TGF-β1 mRNA and protein, which was associated with strong activation of Smad3 signaling within the diseased kidneys in Smad3 WT mice. In contrast, deletion of Smad3 resulted in abrogation of ANG II-induced upregulation of TGF-β1 and activation of Smad3 (Fig. 8, A and B). Similarly, in vitro ANG II induced TGF-β1 expression and Smad3 activation were also blunted in Smad3 KO mesangial cells (Fig. 8, C and D). These data confirm the hypothesis that Smad3 is essential for ANG II-induced TGF-β1 expression during renal fibrosis.

Because activation of Smad2/3 is negatively regulated by Smad7 and Smad7 is degraded during renal fibrosis via the E3 ligase Smurfl2-dependent mechanism (31, 40), we then examined the hypothesis that ANG II may act by stimulating Smad3 to amplify Smad signaling by causing Smad7 degradation in vivo and in vitro. As shown in Fig. 9, A–C, and 10, A–C, in Smad3 WT mice and mesangial cells, ANG II-induced activation of Smad signaling was associated with a marked upregulation of Smurfl2 mRNA and protein expression but degradation of Smad7 protein, although Smad7 mRNA remained upregulated. In contrast, deletion of Smad3 abolished ANG II-induced Smurfl2 mRNA and protein expression in vivo and in vitro. Interestingly, although deletion of Smad3 prevented ANG II-induced upregulation of renal Smad7 mRNA, levels of renal Smad7 protein remained high (Figs. 9 and 10, A–C). These results revealed the critical role for the Smad3-mediated Smurfl2-dependent Smad7 degradation pathway in ANG II-induced progressive renal fibrosis.

Smad3 mediates ANG II-induced renal inflammation by enhancing NF-κB signaling via the Smurfl2-dependent ubiquitin degradation of Smad7 in vivo and in vitro. We next investigated the mechanism through which disruption of Smad3 inhibits renal inflammation. Because Smad7 is able to induce IκBα expression to inhibit NF-κB-driven renal inflammation (16, 35), we examined whether Smad3-mediated loss of renal Smad7 contributes to NF-κB-driven renal inflammation in response to ANG II in vivo and in vitro. Western blot analysis detected that ANG II-mediated Smurfl2-dependent degradation of renal Smad7 was also associated with degradation of IκBα and activation of NF-κB signaling, as evidenced by a marked increase in levels of phosphorylated IκBα and NF-κB/p65 in Smad3 WT, but not in Smad3 KO mice (Fig. 9, D and E). Similarly, in vitro, deletion of Smad3 impaired ANG II-induced NF-κB signaling by preventing Smurfl2-mediated Smad7 protein degradation (Fig. 10, D and E).

**DISCUSSION**

In the present study, we found that mice null for Smad3 were protected against ANG II-induced nephropathy, including the development of albuminuria, an increase in serum levels of creatinine, and progressive renal fibrosis and inflammation, despite hypertension. Results from this study provided evidence for an essential role of Smad3 in the pathogenesis of hypertensive nephropathy and insights into the mechanisms of ANG II-mediated renal fibrosis and inflammation.
A novel and significant finding in the present study was the identification of two positive regulatory pathways through which Smad3 mediates ANG II-induced renal fibrosis. First, ANG II acted by stimulating Smad3 to induce TGF-β expression because mice and mesangial cells lacking Smad3 gene were protected against ANG II-induced TGF-β1 expression in vivo and in vitro. Once TGF-β1 is produced, it activates Smad3 again to mediate renal fibrosis via the TGF-β-dependent Smad signaling pathway. Identification of the ANG II-Smad3-TGF-β-Smad3 amplification loop in renal fibrosis may well explain the previous findings.

Fig. 8. Disruption of Smad3 impairs ANG II-induced TGF-β/Smad signaling in vivo and in vitro. A: immunohistochemistry and quantitative real-time PCR analysis of renal TGF-β1 expression. B: representative Western blots of phospho-Smad3 in kidney tissues. C: real-time PCR analysis of TGF-β mRNA expression from Smad3 KO and WT mesangial cells stimulated with ANG II (1 μM). D: Representative western blots of phospho-Smad3 from ANG II (1 μM)-stimulated mesangial cells. Results show that deletion of Smad3 inhibits ANG II-induced upregulation of TGF-β1 and abolishes Smad3 signaling in vivo and in vitro. Values are means ± SE for groups of 8 mice of at least 3 independent experiments in vitro. ** P < 0.01, *** P < 0.001 compared with the saline control mice or negative controls in vitro (0). ### P < 0.001 compared with ANG II-infused Smad3 WT mice or cells. Sections are counterstained with hematoxylin. Magnification: ×200.
findings that ANG II induced TGF-β1 to mediate renal fibrosis and that anti-TGF-β treatment attenuated ANG II-induced renal injury (1, 2, 7, 8, 18, 30, 36–38). Because ANG II is capable of activating Smad2/3 via the early ERK/p38 MAPK-Smad cross talk pathway, followed by a late TGF-β-dependent mechanism (25, 34, 39), it is highly possible that ANG II may stimulate the early activation of Smad3 via the ERK/p38 MAPK-Smad cross talk pathway to induce TGF-β1 expression, which activates Smad3 again via the TGF-β-dependent pathway. Therefore, impaired ANG II-induced TGF-β-dependent and independent Smad signaling may be a mechanism by which mice and cells lacking Smad3 were protected from ANG II-induced renal fibrosis in vivo and in vitro. We have previously reported that deletion of Smad3, but not Smad2 from vascular smooth muscle cells and renal tubular epithelial cells is capable of inhibiting ANG II-induced vascular ECM production and tubulointerstitial fibrosis including EMT via TGF-β1 and CTGF-dependent mechanisms (34, 39, 40). In the present study, we added new information that Smad3 also plays an important role in ANG II-mediated glomerulosclerosis as demonstrated by the findings that mice and mesangial cells lacking Smad3 were able to protect ANG II-induced ECM production and accumulation in vivo and in vitro, revealing an essential role for Smad3 in ANG II-mediated renal fibrosis under hypertensive conditions.
We also found that activation of the Smurf2-mediated Smad7 degradation pathway was required for Smad3-mediated renal fibrosis in response to ANG II in vivo and in vitro. This was supported by the findings that ANG II-mediated renal fibrosis was associated with upregulation of Smurf2 but degradation of Smad7 protein in Smad3 WT, not in Smad3 KO, mice and cells. It is well recognized that Smad7 is an inhibitory Smad that negatively regulates Smad2 and Smad3 activation by its negative feedback mechanism (31). Smad7 acts as an adaptor protein to recruit E3 ubiquitin ligases such as Smurf2 and arkadia to the TGF-β receptor complex and then target their degradation through the proteasomal-ubiquitin degradation pathway (14). Once Smad7 is degraded, activation of Smad2 and Smad3 and renal fibrosis is enhanced. This is clearly demonstrated by a recent finding that upregulation of renal Smurf2 causes an ubiquitin-dependent degradation of renal Smad7, resulting in enhanced TGF-β/Smad signaling and progressive renal fibrosis (9). In contrast, blockade of the AT1 receptor or knockdown of Smurf2 is capable of inhibiting ANG II-induced Smad7 degradation and Smad3-mediated EMT(40).

Fig. 10. Disruption of Smad3 from mesangial cells impairs ANG II-induced Smurf2-mediated degradation of renal Smad7 protein and inhibits ANG II-activated NF-κB signaling in vitro. A: real-time PCR analysis of Smurf2 mRNA expression. B: real-time PCR analysis of Smad7 mRNA expression. C: representative Western blots and quantitative analysis for Smurf2 and Smad7 protein levels. D: representative Western blots and quantitative analysis for phospho-IκBα (P-IκBα) and total IκBα levels. E: representative Western blots and quantitative analysis for phospho-NF-κB/p65 (P-p65) and total NF-κB/p65 (p65) protein levels. Results show that deletion of Smad3 inhibits ANG II (1 μM)-induced Smurf2 upregulation and Smad7 protein degradation, thereby preventing IκBα from ubiquitin degradation and activation of NF-κB/p65 signaling in mesangial cells. Values are means ± SE for at least 3 independent experiments. **P < 0.01, ***P < 0.001 compared with negative controls (0). #P < 0.05, ##P < 0.01, ###P < 0.001 compared with ANG II-treated Smad3 WT cells.
All these studies reveal the critical role of the Smurf2-mediated Smad7 degradation pathway in Smad3-mediated ANG II-induced fibrosis. Thus overexpression of Smad7 inhibits, but disruption of Smad7 enhances, ANG II-induced Smad3-mediated renal fibrosis and EMT in vitro (39, 40) and in a number of animal models including obstructive nephropathy (5, 17), remnant kidney disease (11, 40), and diabetic nephropathy (4). In addition, Smurf2 is also capable of degrading Smad2 and Smad transcriptional corepressors such as Ski, SnoN, and TG-interacting factor in obstructive nephropathy (33). Because Smad 2 and all these Smad corepressors are renoprotective (23, 33), targeting them for degradation by Smurf2 may be an additional mechanism for ANG II-induced, Smad3-mediated renal injury in vivo and in vitro.

Activation of the Smurf2-dependent Smad7-ubiquitin degradation pathway may also contribute to enhancing ANG II-induced, NF-κB-mediated renal inflammation. It is well recognized that ANG II acts by stimulating the NF-κB signaling pathway to mediate renal inflammation (19, 28). Our recent study also found that activation of NF-κB is negatively regulated by Smad7 because Smad7 is able to induce IκBα, an inhibitor of NF-κB, to inhibit IL-1β- and TNF-α-induced inflammatory responses in vitro (35). Furthermore, gene transfer of Smad7 into the inflamed kidney cannot inhibit NF-κB-mediated renal inflammation in remnant kidney disease (24), autoimmune crescentic glomerulonephritis (13), and diabetic nephropathy (4), demonstrating an important role for the Smad7-NF-κB cross talk pathway in negatively regulating renal inflammation. Evidence for the anti-inflammatory role of Smad7 also comes from recent studies in mouse models of diabetic and obstructive nephropathy in which mice lacking the Smad7 gene had higher levels of NF-κB activation and developed more severe kidney inflammation (4, 5). Therefore, impaired Smurf2-mediated Smad7 degradation, thereby preventing ANG II-induced NF-κB signaling, may be a key mechanism by which Smad3 KO mice were protected against ANG II-induced renal inflammation in vivo and in vitro.

Consistent with the previous studies in obstructive nephropathy and ANG II-induced cardiac remodeling (12, 29), inhibition of MCP-1-dependent macrophage infiltration may be another mechanism by which Smad3 KO mice were protected from ANG II-induced renal inflammation. It is known that MCP-1 is a direct target gene of TGF-β1/Smad3 (22). Therefore, deletion of Smad3 impairs ANG II-induced MCP-1 expression and inhibits macrophage accumulation in this and other studies in Smad3 KO mice (12, 29).

In conclusion, the present study demonstrates that Smad3 plays an essential role in the pathogenesis of ANG II-induced hypertensive nephropathy. Smad3 mediates renal fibrosis by promoting angiotensin II-induced TGF-β/Smad signaling. Enhanced Smurf2-dependent ubiquitin degradation of renal Smad7 may be a major mechanism by which Smad3 mediates ANG II-induced renal fibrosis and inflammation. Results from this study suggest that targeting Smad3 or overexpression of Smad7 may be a novel therapeutic strategy for hypertensive nephropathy.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: Z.L. and X.R.H. performed experiments; Z.L. and X.R.H. analyzed data; Z.L., X.R.H., and H.Y.L. interpreted results of experiments; Z.L. and X.R.H. prepared figures; Z.L. and H.Y.L. drafted manuscript; Z.L., X.R.H., and H.Y.L. approved final version of manuscript; H.Y.L. provided conception and design of research; H.Y.L. edited and revised manuscript.

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