# Disruption of Smad4 impairs TGF-β/Smad3 and Smad7 transcriptional regulation during renal inflammation and fibrosis *in vivo* and *in vitro*

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**The mechanism by which TGF-β regulates renal inflammation and fibrosis is largely unclear; however, it is well accepted that its biological effects are mediated through Smad2 and Smad3 phosphorylation. Following activation, these Smads form heteromeric complex with Smad4 and translocate into the nucleus to bind and regulate the expression of target genes. Here we studied the roles of Smad4 to regulate TGF-β signaling in a mouse model of unilateral ureteral obstruction using conditional Smad4 knockout mice and in isolated Smad4 mutant macrophages and fibroblasts. Disruption of Smad4 significantly enhanced renal inflammation as evidenced by a greater CD45$^+$ leukocyte and F4/80$^+$ macrophage infiltration and upregulation of IL-1β, TNF-α, MCP-1, and ICAM-1 in the obstructed kidney and in IL-1β-stimulated macrophages. In contrast, deletion of Smad4 inhibited renal fibrosis and TGF-β1-induced collagen I expression by fibroblasts. Further studies showed that the loss of Smad4 repressed Smad7 transcription, leading to a loss of functional protein. This, in turn, inhibited IκBα expression but enhanced NF-κB activation, thereby promoting renal inflammation. Interestingly, deletion of Smad4 influenced Smad3-mediated promoter activities and the binding of Smad3 to the COL1A2 promoter, but not Smad3 phosphorylation and nuclear translocation, thereby inhibiting the fibrotic response. Thus, Smad4 may be a key regulator for the diverse roles of TGF-β1 in inflammation and fibrogenesis by interacting with Smad7 and Smad3 to influence their transcriptional activities in renal inflammation and fibrosis.**

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It has been known for long that transforming growth factor (TGF)-β1 has a distinct role in regulating renal inflammation and fibrosis.[1–4] Although recent discovery of TGF-β/Smad signaling has delineated the signaling mechanism of TGF-β1 in many physiopathological processes including development, inflammation and immunity, tissue repair and fibrogenesis, and cancer development, the mechanism by which TGF-β diversely regulates the responses of renal inflammation and fibrosis remains largely unclear. It is now well accepted that after binding to its receptors, TGF-β1 exerts its biological effects through the activation of its downstream mediators, Smad2 and Smad3, by phosphorylation. Further, the activated Smad2/3 form heteromeric complex with Smad4, a common Smad, and translocate into the nucleus to bind and regulate the expression of targeted genes, which is negatively regulated by an inhibitory Smad, namely Smad7.[5] Emerging evidence has shown that Smad3 can transcriptionally bind the DNA sequences of the target gene and has an important role in tissue repair and fibrosis under various pathological conditions in the skin, liver, lung, heart, and kidney.[6–10] In contrast, Smad2 is protective in renal fibrosis by counter-regulating Smad3 signaling.[11] However, the functional role of Smad4 in the pathogenesis of inflammation and fibrosis under pathological conditions remains largely unknown. This may be attributed to the unavailability of Smad4 knockout (KO) mice due to the early embryonic lethality.[12] Recent studies using conditional Smad4 KO mice have demonstrated that specific deletion of Smad4 from the heart impairs cardiogenesis or causes cardiac hypotrophy and failure,[13,14] whereas deletion of Smad4 from the skin results in delayed wound healing and enhanced inflammation-associated carcinogenesis.[15] However, the functional role of Smad4 in other diseased organs such as kidney inflammation and fibrosis remains unexplored and, more importantly, the underlying mechanisms of Smad4 in regulating inflammation and fibrosis are not fully understood. Considering the central position of Smad4 in TGF-β/Smad signal transduction, the present study tested the hypothesis that Smad4 may be a key regulator of TGF-β signaling and may have an important regulatory role in renal

inflammation and fibrosis. This hypothesis was examined *in vitro* in fibroblasts and macrophages and *in vivo* in a mouse model of unilateral ureteral obstructive (UUO) kidney disease, in which Smad4 was conditionally deleted from the kidney, as well as kidney fibroblasts and peritoneal macrophages using the Cres-Loxp technology. In addition, the underlying mechanisms by which Smad4 regulates renal inflammation and fibrosis were also determined.

## RESULTS

### Generation and characterization of conditional Smad4 KO mice and cells

Conditional Smad4 KO mice (Smad4ff/KspCre) were generated by crossing the Smad4 flox/flox mice (Smad4ff) with the kidney-specific promoter (Cadherin-16)-driven Cre mice (KspCre) in which Smad4 was specifically deleted from the kidney tubular epithelial cells. As shown in Figure 1a, PCR revealed that transgenic expression of Cre recombinase (235 bp) in the Smad4ff mouse induced a substantial deletion of the floxed *Smad4* gene (420 bp) by the Cre recombination as defined by the deleted allele (500 bp). This result was further confirmed by real-time PCR and western blot analysis that ~50% of Smad4 mRNA and protein was deleted from kidneys of conditional Smad4 KO mice (Figure 1b and c). Immunohistochemically, compared with the Smad4ff mice in which Smad4 was highly expressed by all kidney cells, Smad4ff/KspCre mice exhibited a marked deletion of Smad4 from most tubular epithelial cells, but not glomerular cells (Figure 1d), because of the presence of active KspCre in kidney tubular epithelial cells only. *In vitro*, compared with the control Smad4ff cells, western blot analysis revealed that transfection of adenovirus Cre substantially deleted the Smad4 from peritoneal macrophage (Figure 1e) and kidney fibroblast (Figure 1f) that were isolated from Smad4ff mice.

### Deletion of Smad4 enhances renal inflammation in a mouse model of the UUO kidney and in interleukin-1β-stimulated macrophages

Histologically, there was no detectable abnormality of kidney histology in conditional Smad4 KO mice, which was further confirmed by no evidence of renal inflammation and fibrosis detected by immunohistochemistry, real-time PCR, and

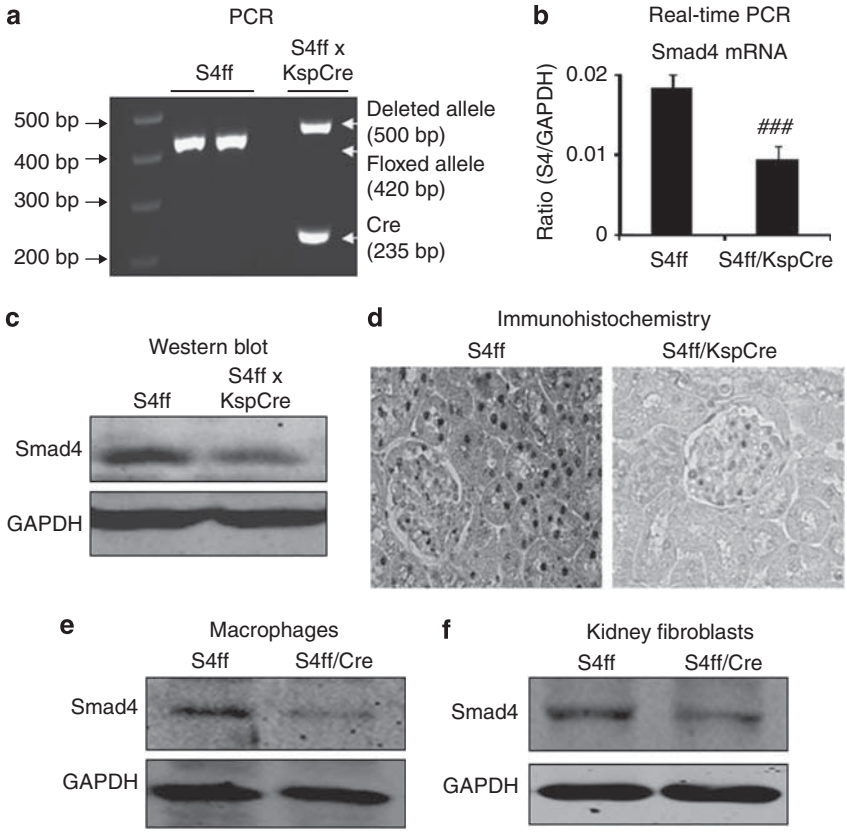


**Figure 1 | Characterization of conditional Smad4 knockout mice, peritoneal macrophages, and kidney fibroblasts.** (**a**) PCR demonstrates that the Cre recombination (235 bp) results in the Smad4-floxed gene (*S4ff*, 420 bp) being partly deleted from the kidney (500 bp). (**b**, **c**) Real-time PCR and western blot analysis show a deletion of Smad4 mRNA and protein from the S4ff/KspCre mouse kidney. (**d**) Immunohistochemistry detects that Smad4 is highly expressed by all kidney cell types (cytoplasm and nuclear staining) in Smad4ff mice and is specifically deleted from most of the kidney tubular epithelial cells in S4ff/KspCre mice. (**e**, **f**) Western blot analysis shows that Smad4ff macrophages and kidney fibroblasts cells infected with adenovirus Cre results in a substantial deletion of Smad4. Data represent mean ± s.e.m. for groups of six to eight mice or for four independent experiments *in vitro*. $^{###}P < 0.001$ vs. Smad4ff mice. Original magnifications × 400 (**d**). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

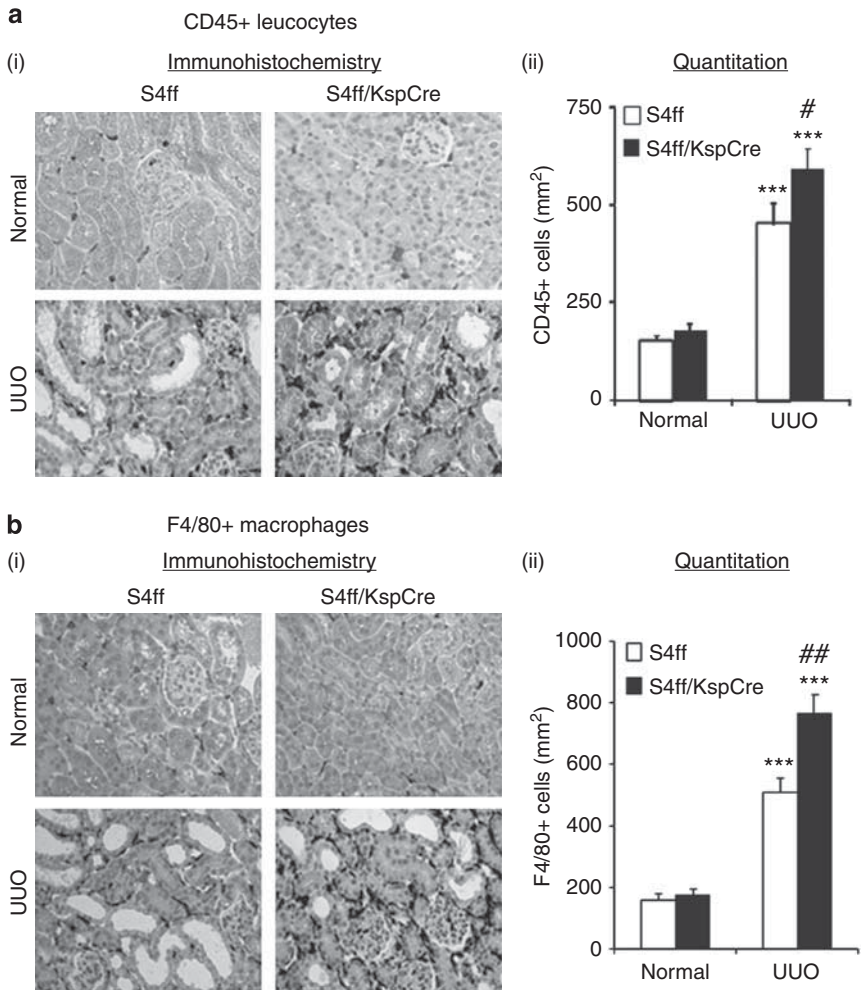


**Figure 2 | Disruption of Smad4 from the kidney promotes inflammatory cell infiltration in a mouse model of unilateral ureteral obstruction (UUO).** (**a**) CD45+ leukocytes; (**b**) F4/80+ macrophages. Compared with the Smad4ff mouse, disruption of Smad4 from S4ff/KspCre mice largely enhances tubulointerstitial inflammation by CD45+ leukocytes and F4/80+ macrophages. Data are expressed as mean ± s.e.m. for groups of six to eight mice. $^{***}P<0.001$ vs. normal; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. UUO kidneys of Smad4ff mice. Original magnifications × 400.

western blot analysis (Figures 2–4). However, the UUO kidney with conditional deletion of Smad4 from tubular epithelial cells significantly enhanced tubulointerstitial CD45+ leukocyte and F4/80+ macrophage infiltration when compared with the control Smad4ff mice (Figure 2). Further studies by immunohistochemistry and real-time PCR revealed that enhanced inflammatory cell infiltration in the UUO kidney of Smad4 KO mice was associated with a marked upregulation of proinflammatory cytokines such as interleukin-1β and tumor necrosis factor (TNF)-α (Figure 3), chemokine monocyte chemotactic protein-1 (MCP-1), and adherent molecules (intercellular adhesion molecule-1; Figure 4). These *in vivo* findings were also confirmed by *in vitro* study in peritoneal macrophages. As shown in Figure 5a and b, real-time PCR showed that the addition of interleukin-1β was able to induce inflammatory response such as interleukin-1β and intercellular adhesion molecule-1 mRNA expression in a time- and dosage-dependent manner, peaking at 1 h with an optimal dose of 10 ng/ml. Interestingly, disruption of Smad4 in macrophages not only enhanced interleukin-1β-induced inflammatory responses but also largely blocked the inhibitory effect of TGF-β1 on interleukin-1β-induced upregulation of TNF-α, interleukin-1β, MCP-1, and intercellular adhesion molecule-1 mRNA expression (Figure 5c–f), revealing a necessary role for Smad4 in anti-inflammatory property of TGF-β1.

### Disrupted Smad4 impairs Smad7 transcriptional regulation in nuclear factor κB activation *in vivo* and *in vitro*

We then examined mechanisms whereby deletion of Smad4 enhanced inflammatory responses *in vivo* and *in vitro*. We have previously shown that Smad7 is a negative regulator of TGF-β signaling in renal inflammation, which inactivates the nuclear factor κB (NF-κB) pathway via induction of IκBα, an inhibitor of NF-κB signaling.[16] In the present study, we found that enhanced renal inflammation in Smad4 KO mice was

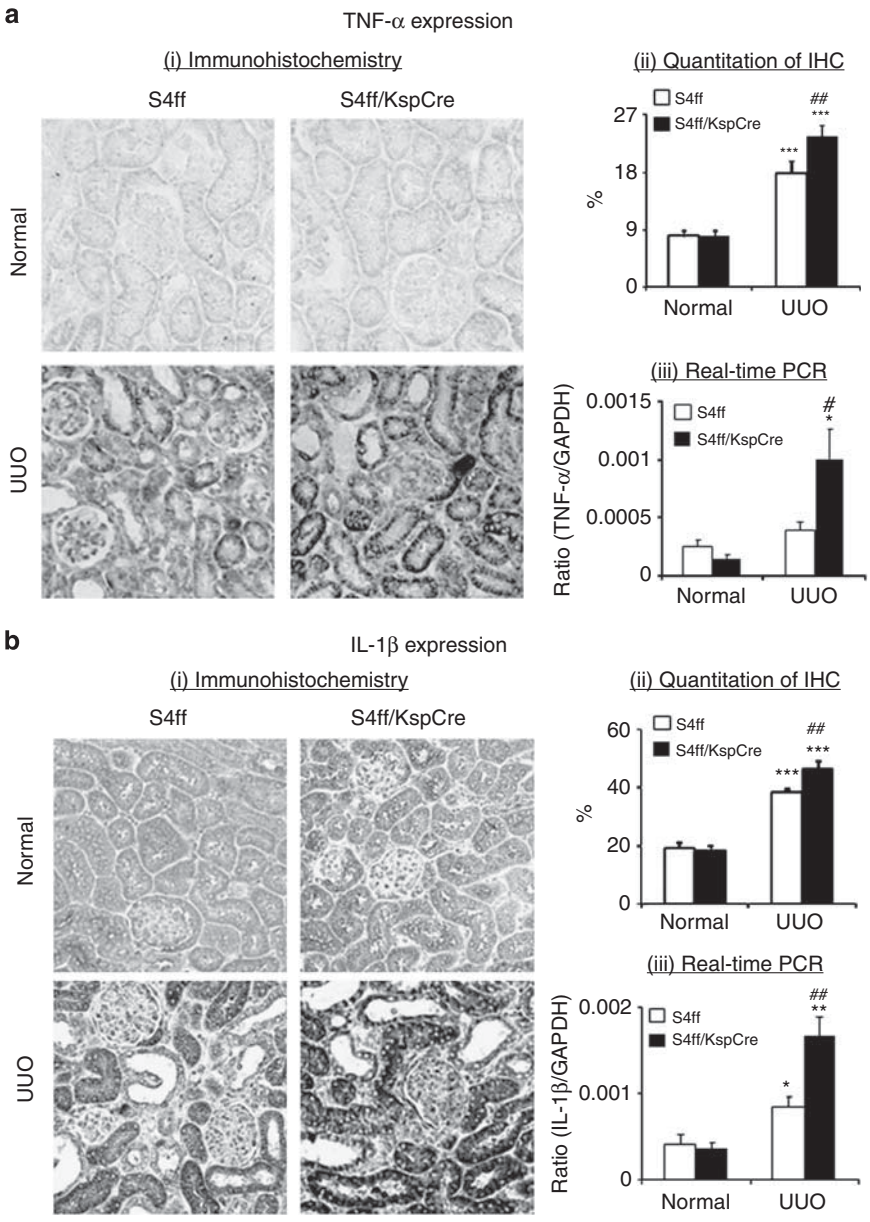


**Figure 3 | Disruption of Smad4 from the kidney enhances expression of proinflammatory cytokines (tumor necrosis factor (TNF)-α and interleukin (IL)-1β) in the unilateral ureteral obstructive (UUO) kidney.** (**a**) TNF-α expression; (**b**) IL-1β expression. (i) Immunohistochemistry (IHC), (ii) quantitative data of IHC, (iii) real-time PCR for TNF-α or IL-1β mRNA expression. Compared with the Smad4ff mouse, disruption of Smad4 from S4ff/KspCre mice largely enhances TNF-α and IL-1β mRNA and protein expression. Data are expressed as the mean ± s.e.m. for groups of six to eight mice. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. normal; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Smad4ff UUO mice. Original magnifications × 400. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

associated with a loss of Smad7 in both mRNA and protein levels (Figure 6a), which was accompanied by decreased IκBα expression while increasing phospho-IκBα, thereby resulting in activation of NF-κB/p65 as demonstrated by enhanced phosphorylation and nuclear translocation of NF-κB/p65 subunit in tubulointerstitium (Figure 6b and c). Further *in vitro* studies in peritoneal macrophages found that deletion of Smad4 resulted in abrogation of TGF-β1-induced Smad7 mRNA and protein expression and promoter activity (Figure 7a), which was associated with reduced IκBα expression and increased phosphorylation of both IκBα (P-IκBα) and NF-κB/p65 (P-p65), and NF-κB response secreted alkaline phosphatase (SEAP) promoter activities (Figure 7b and c).

### Disruption of Smad4 inhibits renal fibrosis in a mouse model of UUO and *in vitro* in TGF-β1-stimulated fibroblasts

As TGF-β/Smad signaling has been shown to have a key role in renal fibrosis,[1–4] we next investigated whether deletion of Smad4 from the kidney and fibroblasts has an impact on fibrogenesis. In contrast to renal inflammatory response,

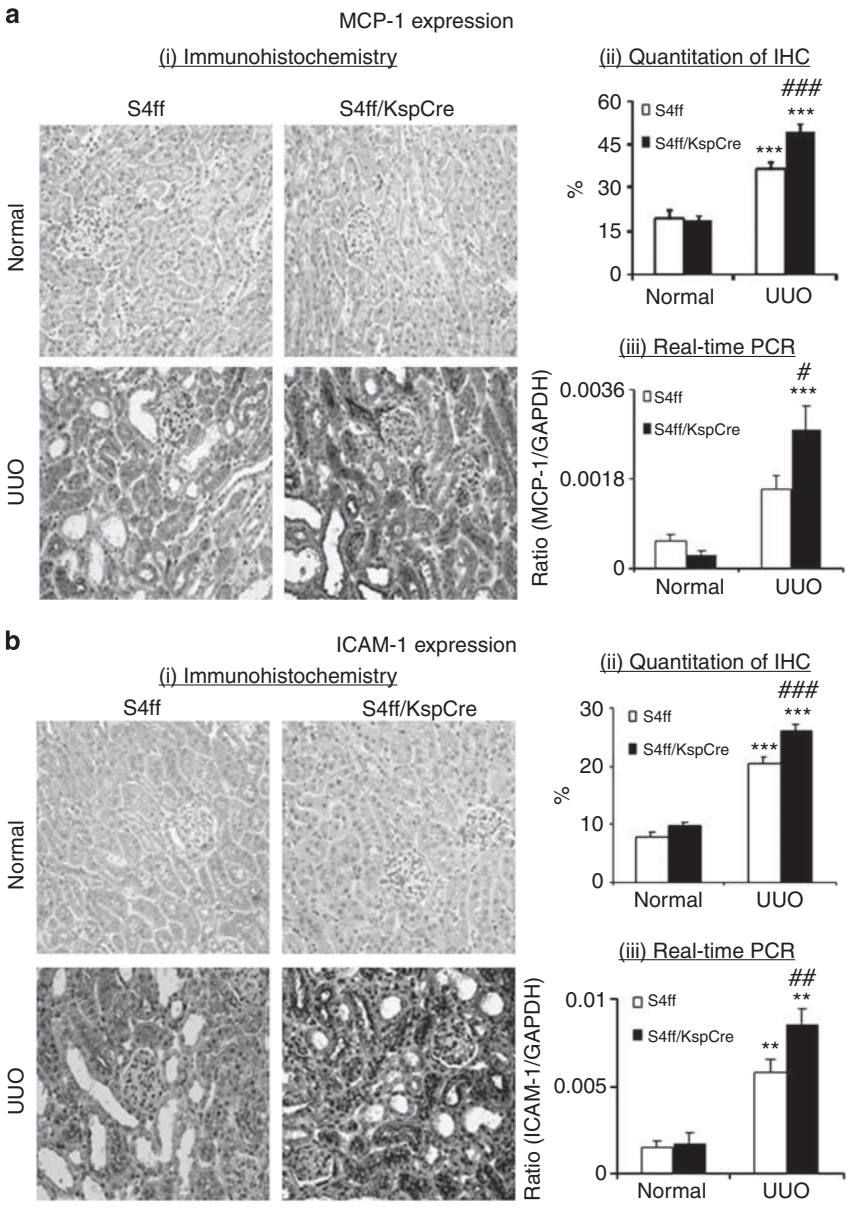


**Figure 4 | Disruption of Smad4 from the kidney enhances the expression of monocyte chemotactic protein-1 (MCP-1) and intercellular adhesion molecule (ICAM)-1 in the unilateral ureteral obstructive (UUO) kidney.** (**a**) MCP-1 expression; (**b**) ICAM-1 expression. (i) Immunohistochemistry, (ii) quantitative data of immunohistochemistry (IHC), (iii) real-time PCR for MCP-1 or ICAM-1 mRNA expression. Compared with the Smad4ff mouse, disruption of Smad4 from S4ff/KspCre mice largely enhances MCP-1 and ICAM-1 mRNA and protein expression. Data are expressed as the mean ± s.e.m. for groups of six to eight mice. $^{**}P<0.01$, $^{***}P<0.001$ vs. normal; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Smad4ff UUO mice. Original magnifications × 200. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

deletion of Smad4 from the kidney significantly reduced the severity of tubulointerstitial fibrosis in the UUO kidney as demonstrated by Masson's trichrome staining (Figure 8). Immunohistochemistry, real-time PCR, and western blot analysis also revealed that collagen I expression within the fibrotic kidney was also significantly inhibited in conditional Smad4-deficient mice when compared with the Smad4-floxed mice (Figure 9a–e). Similar results were also found in TGF-β1-stimulated kidney fibroblasts in which disrupted Smad4 significantly blocked TGF-β1-induced collagen I mRNA and protein expression as determined by real-time PCR and western blot analysis (Figure 9f and g).

### Disrupted Smad4 impairs Smad3 transcriptional activities, but does not alter Smad2/3 signaling in response to TGF-β1 *in vitro*

To explore the mechanism by which deletion of Smad4 inhibits renal fibrosis, we examined TGF-β1-induced Smad2/3 signaling in the UUO kidney and in kidney fibroblasts in response to TGF-β1. Unexpectedly, disruption of Smad4 from the kidney did not alter the activation level of Smad2/3, as demonstrated by Smad3 phosphorylation and phospho-Smad2/3 nuclear translocation by western blot analysis and immunohistochemistry (Figure 10a and b). This was further demonstrated *in vitro* that disrupted Smad4 from kidney

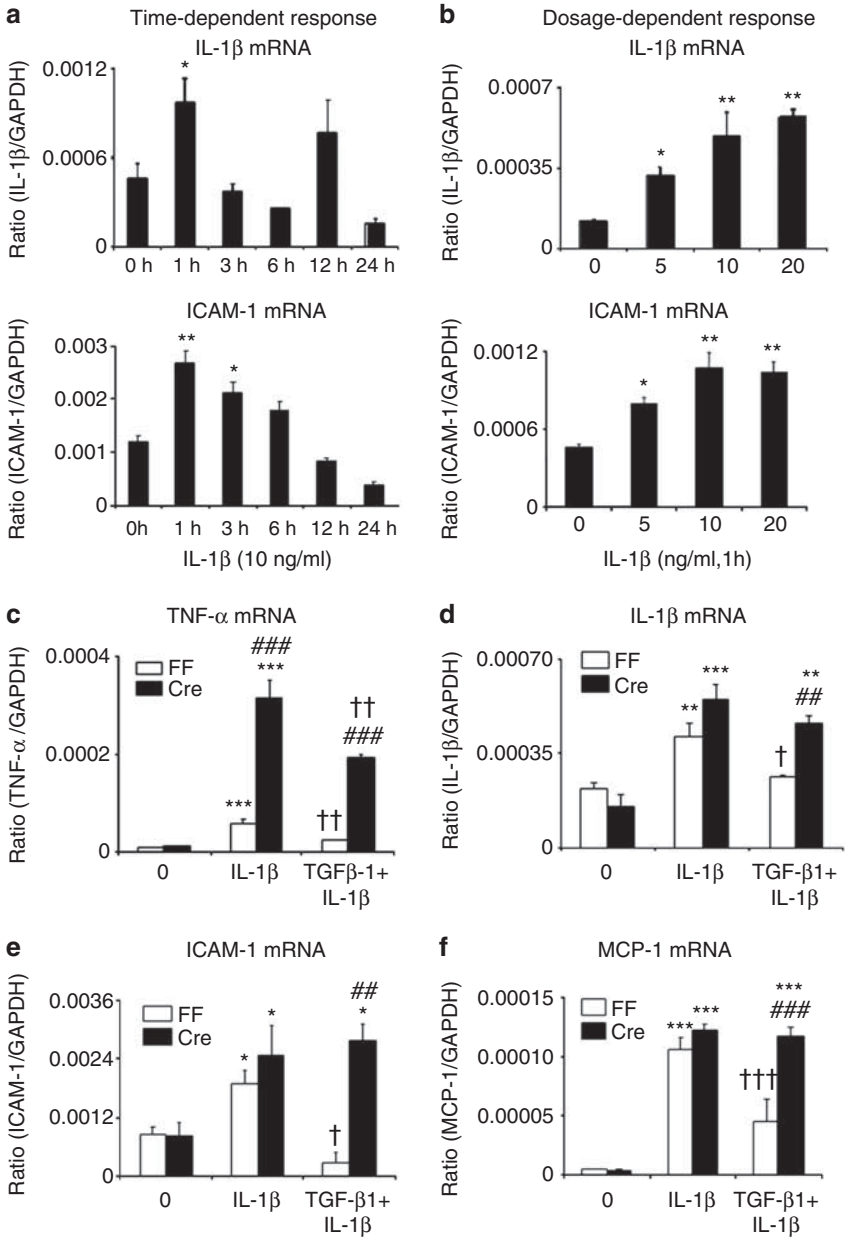


**Figure 5 | Disruption of Smad4 prevents the anti-inflammatory effect of transforming growth factor (TGF)-β1 on interleukin-1β-stimulated peritoneal macrophages *in vitro*.** (**a**) A time-dependent response after interleukin-1β (IL-1β) stimulation (10 ng/ml). (**b**) A dosage-dependent response (1 h after IL-1β stimulation). (**c**–**f**) The effect of TGF-β1 on interleukin-1β-induced mRNA expression of tumor necrosis factor (TNF)-α (**c**), IL-1β (**d**), intercellular adhesion molecule (ICAM)-1 (**e**), and MCP-1 (**f**). Real-time PCR detects that disruption of Smad4 from macrophages prevents the inhibitory effect of TGF-β1 on interleukin-1β-induced inflammatory response. Macrophages were pretreated with TGF-β1 (2 ng/ml) for 12 h, followed by addition of interleukin-1β (10 ng/ml) for 1 h as described in Materials and Methods. Data are expressed as mean ± s.e.m. for four independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs. normal (time or dose 0); $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Smad4ff cells; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs. interleukin-1β treatment only. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCP-1, monocyte chemotactic protein-1.

fibroblasts did not influence the levels of Smad3 phosphorylation in response to TGF-β1 (Figure 10c).

We then investigated whether deletion of Smad4 influenced Smad3 transcriptional activities by examining the Smad3/4 promoter activities and the binding of Smad3 to COL1A2. Surprisingly, conditional deletion of Smad4 from kidney fibroblasts did prevent TGF-β1-induced Smad3/4 promoter activities and the binding of Smad3 to COL1A2, as determined by the chromatin immunoprecipitation assay (Figure 11).

Next, we determined whether deletion of Smad4 has an impact on Smad-independent signaling mechanisms by examining the extracellular signal-regulated protein kinase (ERK)1/2 phosphorylation. As shown in Figure 12, disrupted Smad4 did not alter the increased phosphorylation levels of ERK1/2 in the UUO kidney when compared with

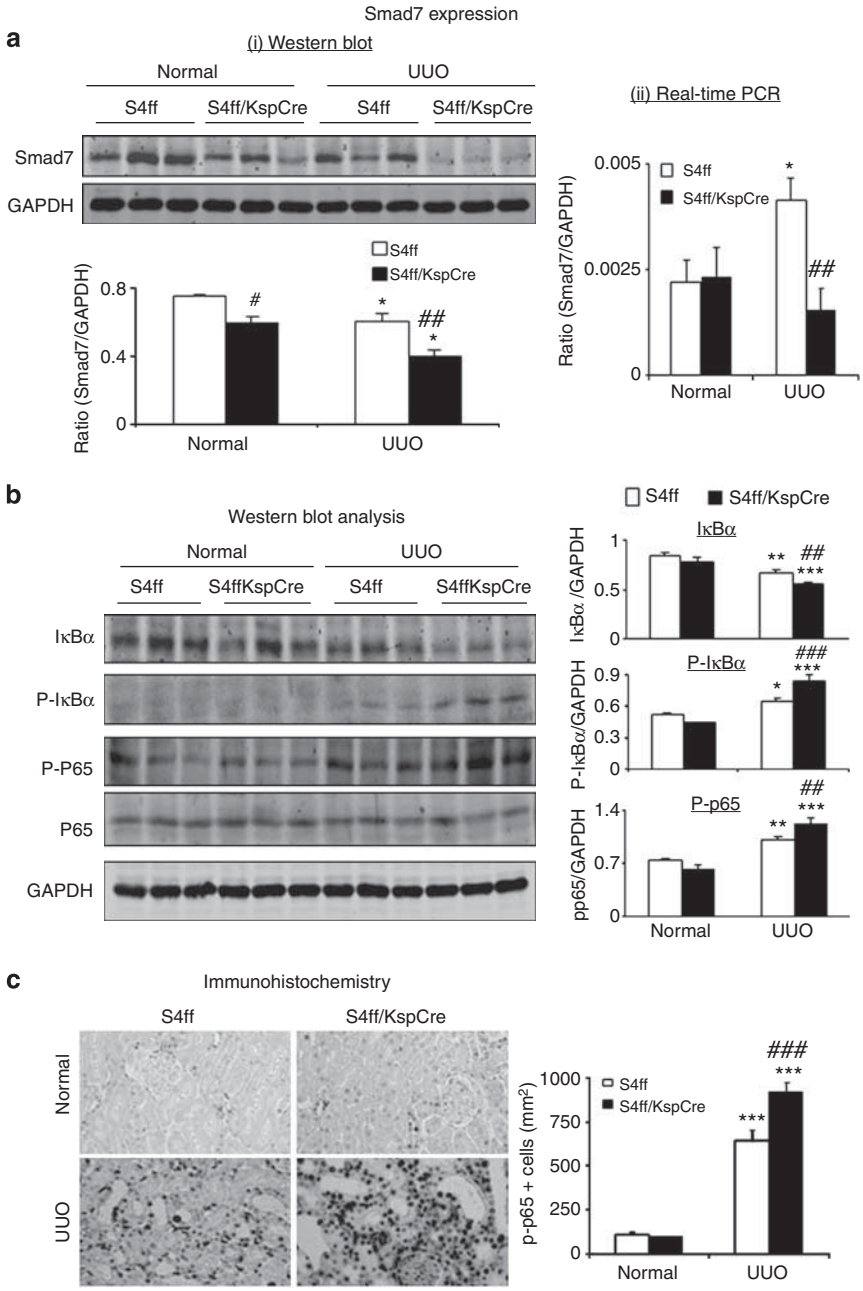


**Figure 6 | Disruption of Smad4 inhibits Smad7 and IκBα expression, which results in increasing IκBα degradation and nuclear factor κB (NF-κB) activation in the unilateral ureteral obstructive (UUO) kidney.** (**a**) Real-time PCR and western blot analysis detects that disrupted Smad4 enhances a loss of Smad7 mRNA and protein expression in the UUO kidney. (**b**) Western blot analysis shows that deletion of Smad4 inhibits IκBα expression but increases its phosphorylation (P-IκBα), resulting in enhanced NF-κB/p65 phosphorylation (P-p65) in the UUO kidney. (**c**) Immunohistochemistry reveals that disruption of Smad4 enhances phospho-NF-κB/p65 nuclear translocation in the UUO kidney. Data are expressed as mean ± s.e.m. for groups of six to eight mice. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. normal; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Smad4ff UUO mice. Original magnifications × 400 (**c**). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Smad4-floxed mice, although a transient increase in phospho-ERK1/2 was observed in conditional KO kidney fibroblasts at 15 min after addition of TGF-β1.

## DISCUSSION

Emerging evidence shows that TGF-β1 functions by stimulating its downstream mediators Smad2 and Smad3 to exhibit its biological activities including inflammation and fibrosis. Although Smad4 has been known as a common Smad in the signal transduction pathway of the TGF-β family, its functional role and mechanisms in TGF-β1-regulated inflammatory and fibrosis responses remain largely unclear. In the present study, we found that disruption of Smad4 from the kidney resulted in largely enhanced renal

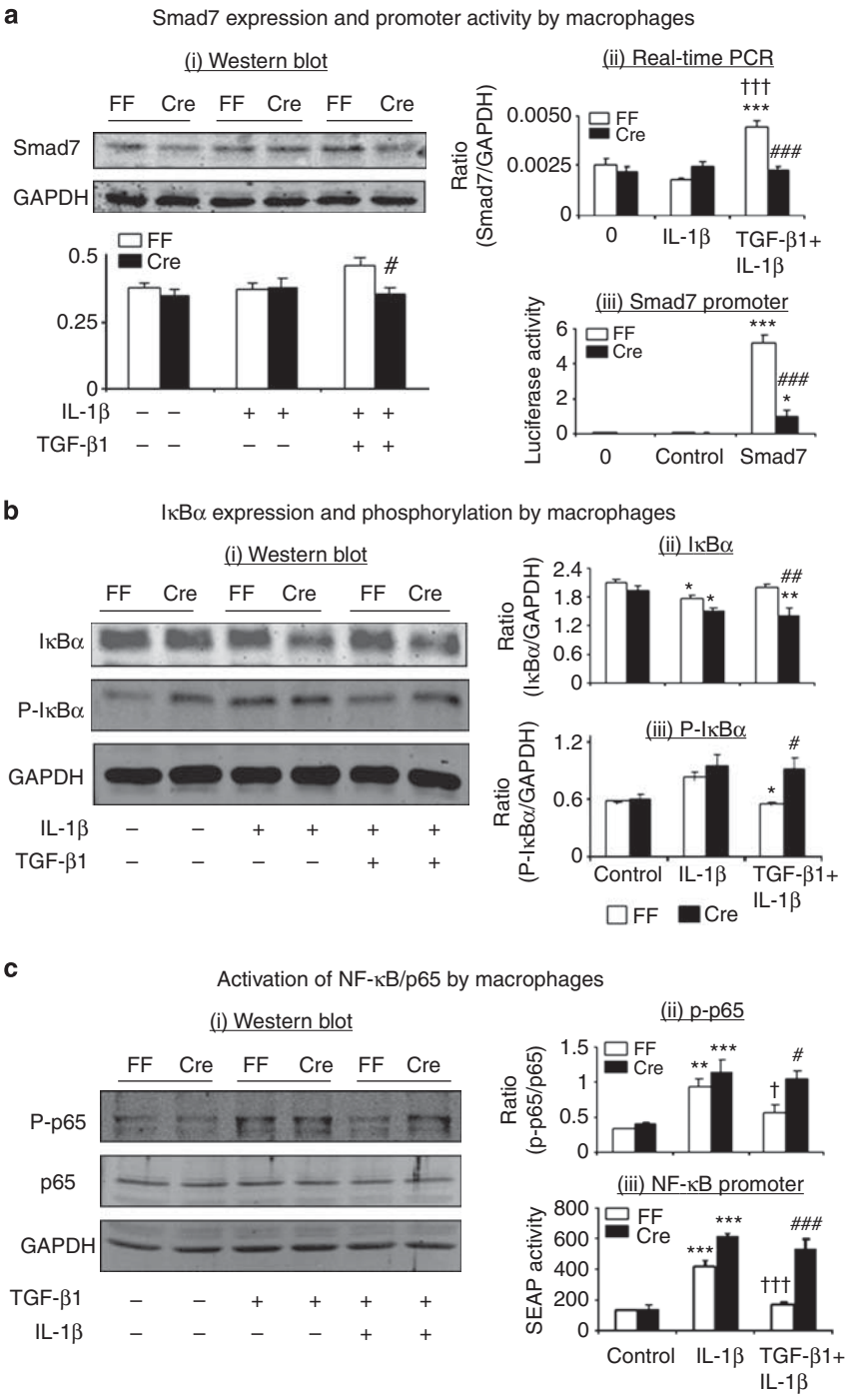


**Figure 7 | Disruption of Smad4 inhibits transforming growth factor (TGF)-β1-induced Smad7 expression and promoter activity, but promotes loss of IκBα while enhancing interleukin-1β-stimulated IκBα degradation and nuclear factor κB (NF-κB) activation in macrophages *in vitro*.** (**a**) Real-time PCR, western blot analysis, and promoter assay detect that disrupted Smad4 enhances a loss of Smad7 mRNA and protein expression and promoter activity in response to TGF-β1 (2 ng/ml) in peritoneal macrophages. (**b**, **c**) Western blot analysis shows that deletion of Smad4 reduces IκBα expression and results in a loss of inhibitory effect of TGF-β1 on interleukin-1β (10 ng/ml)-stimulated IκBα degradation (P-IκBα) and NF-κB/p65 phosphorylation (P-p65) by macrophages. Furthermore, the NF-κB SEAP promoter assay also shows that disrupted Smad4 prevents the inhibitory effect of TGF-β1 on interleukin-1β-induced NF-κB SEAP promoter activities. Data are expressed as mean ± s.e.m. for three to four independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Smad4 FF macrophages; $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$ vs. interleukin-1β treatment only. Cre, Smad4 Cre; FF, Smad4ff; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEAP, secreted alkaline phosphatase.

inflammation including CD45+ leukocyte and F4/80+ macrophage infiltration and upregulation of proinflammatory cytokines, chemokines, and adhesion molecules in a mouse model of UUO kidney. In contrast, deletion of Smad4 inhibited progressive renal fibrosis such as collagen matrix expression. All these findings *in vivo* were also further evident

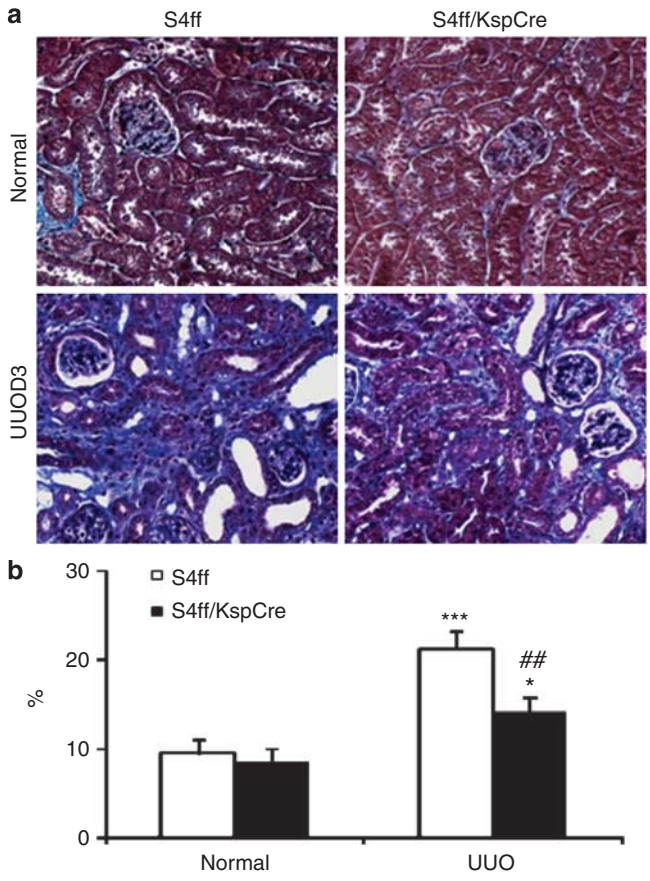


**Figure 8 | Disruption of Smad4 from the kidney inhibits renal fibrosis in a mouse model of unilateral ureteral obstruction (UUO).** (**a**) Masson's trichrome staining; (**b**) quantitative analysis. Data reveal that disrupted Smad4 significantly inhibits collagen matrix deposition in the obstructed kidney. Data are expressed as mean ± s.e.m. for groups of five mice *in vivo*. $^{*}P<0.05$, $^{***}P<0.001$ vs. normal; $^{\#\#}P<0.01$ vs. Smad4ff UUO mice. Original magnifications × 200 (**a**).

*in vitro* that deletion of Smad4 from macrophages or fibroblasts resulted in a loss of suppressive effect of TGF-β1 on interleukin-1β-stimulated inflammation, while inhibiting TGF-β1-mediated renal fibrosis. Taken together, results from the present study demonstrated that Smad4 not only served as a common Smad in signal transduction of the TGF-β family, but also had an essential role in anti-inflammation and fibrogenesis under pathological conditions.

A new and most significant finding in this study was that impaired Smad7 transcriptional regulation in inhibition of NF-κB signaling was a mechanism by which disrupted Smad4 enhanced renal inflammation in the UUO kidney and interleukin-1β-stimulated macrophages. It has been shown that Smad7 transcription is regulated by TGF-β1 through direct binding of Smad3 and Smad4 to the Smad7 promoter.[17] Studies in Smad2 and Smad3 KO mouse embryonic fibroblasts and Smad4-null MD-MBA-468 cell line confirm that Smad3 and Smad4, instead of Smad2, are necessary for the induction of the Smad7 promoter reporter gene in response to TGF-β.[18] Thus, disrupted Smad4 resulted in a loss of Smad7 expression transcriptionally and impaired Smad7 promoter activities functionally, as found in the present study. Consistent with the previous findings that Smad7 is capable of inducing IκBα expression, an inhibitor of NF-κB, thereby inhibiting NF-κB-driven renal inflammation,[16,19] the present findings that disruption of Smad4 promoted NF-κB-dependent inflammation *in vivo* and *in vitro* by downregulating IκBα expression in a Smad7-dependent manner added new evidence that Smad4 may be a key regulator in TGF-β/Smad7-mediated inhibition of NF-κB-driven inflammation. This novel finding was further supported by the ability of disrupted Smad4 to prevent the inhibitory effect of TGF-β1 on interleukin-1β-induced inflammatory response in macrophages.

Interestingly, in contrast to enhanced renal inflammation, disruption of Smad4 suppressed renal fibrosis in the UUO kidney and TGF-β1-stimulated fibroblasts. This observation was consistent with a previous study that deletion of Smad4 from mesangial cells inhibited extracellular matrix deposition induced by TGF-β1.[20] More importantly, we found that inhibition of renal fibrosis by deleting Smad4 was associated with impaired Smad3 transcriptional activities, instead of Smad2/3 activation. It is now well accepted that TGF-β1 is a key mediator of fibrosis by causing phosphorylation of Smad2 and Smad3, which forms a complex with Smad4 and then translocates to nuclei to bind the target genes.[21,22] However, the present study found that disrupted Smad4 did not alter phosphorylation levels of Smad2/3 nor phospho-Smad2/3 nuclear translocation in the UUO kidney and TGF-β1-stimulated fibroblasts, suggesting that Smad4 may not be required for either TGF-β1-induced phosphorylation of Smad2/3 or phospho-Smad2/3 nuclear translocation. This finding is consistent with a previous study in Smad4-null cancer cell lines in which deletion of Smad4 does not prevent nuclear translocation of Smad2 and Smad3.[23] In contrast, we found that inhibition of renal fibrosis in conditional Smad4 KO mice and fibroblasts was associated with impaired transcriptional activity of Smad3 as evidenced by blocking TGF-β-induced Smad3/4 promoter activity and the binding of Smad3 to the COL1A2 promoter. It is known that Smad3-binding sequences are located in the promoter regions of COL1A2, COL2A1, COL3A1, COL5A1, COL6A1, and COL6A3;[24–26] thus, deletion of Smad4 may influence the binding activity of Smad3 to the collagen promoter, thereby inhibiting TGF-β-induced fibrotic response.

In the present study, we also found that deletion of Smad4 had minimal effect on activation of ERK1/2 because disrupted Smad4 from the kidney did not alter the phosphorylation levels of ERK1/2 in the UUO kidney, although significant but transient phosphorylation levels of ERK1/2 were induced in conditional Smad4 KO kidney fibroblasts in response to TGF-β1 *in vitro*. Indeed, TGF-β1 is capable of activating the ERK pathway to mediate renal fibrosis.[27,28] However, results from the present study implied that although TGF-β1 functions by stimulating both

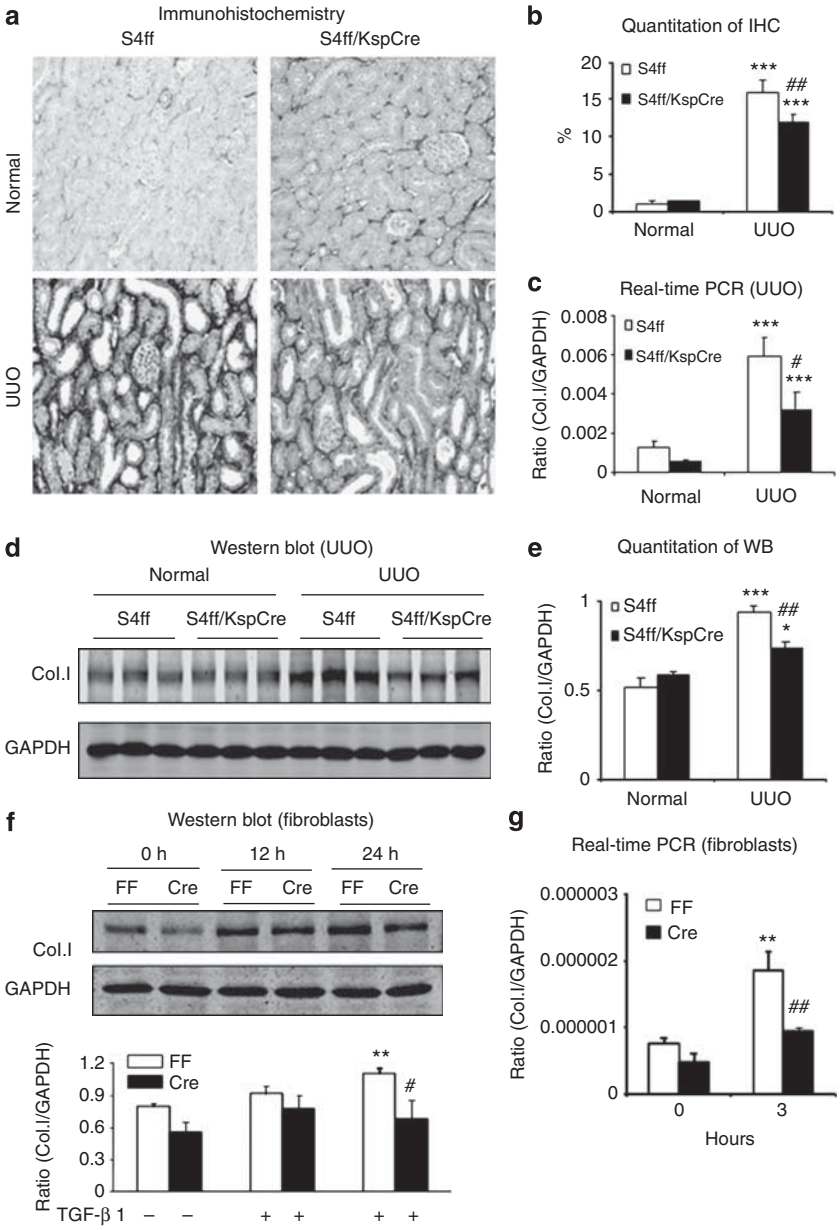


**Figure 9 | Disruption of Smad4 from the kidney and kidney fibroblasts inhibits collagen I (Col.I) matrix expression in a mouse model of UUO and transforming growth factor (TGF)-β1-stimulated kidney fibroblasts *in vitro*.** (**a, b**) Immunostaining and quantitative analysis of col.I. (**c**) Real-time PCR analysis of col.I mRNA expression. (**d, e**) Western blot (WB) and quantitative analysis of col.I protein. (**f**) WB analysis of col.I protein expression from TGF-β1 (2 ng/ml for 24 h)-stimulated kidney fibroblasts. (**g**) Real-time PCR analysis of col.I mRNA expression by kidney fibroblasts in response to TGF-β1 (2 ng/ml). Data show that disrupted Smad4 significantly inhibits col.I mRNA and protein expression in the UUO kidney and TGF-β1-stimulated kidney fibroblasts. Data are expressed as mean ± s.e.m. for groups of six to eight mice *in vivo* and for three to four independent experiments *in vitro*. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. normal or no treatment; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Smad4ff UUO mice or Smad4ff fibroblasts (FF). Original magnifications × 200 (**a**). Cre, S4ff/Cre; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemistry.

Smad-dependent and -independent pathways to exert its biological activities,[22] the Smad-dependent mechanism may be a major pathway leading to renal fibrosis, as disrupted Smad4 significantly attenuated severe tubulointerstitial fibrosis in a mouse model of obstructive nephropathy.

In conclusion, the present study demonstrates that Smad4 may be a key regulator for diverse roles of TGF-β1 in anti-inflammation and fibrogenesis. Smad4 may interact with Smad7 and Smad3 to influence their functional activities transcriptionally in renal inflammation and fibrosis.

## MATERIALS AND METHODS

### Generation of conditional Smad4 KO mice

Smad4 flox/flox mouse (C57B/L6) was crossed with KspCre (C57B/L6) mouse to specifically delete Smad4 from the kidney tubular epithelial cells. The generation and characterization of the Smad4

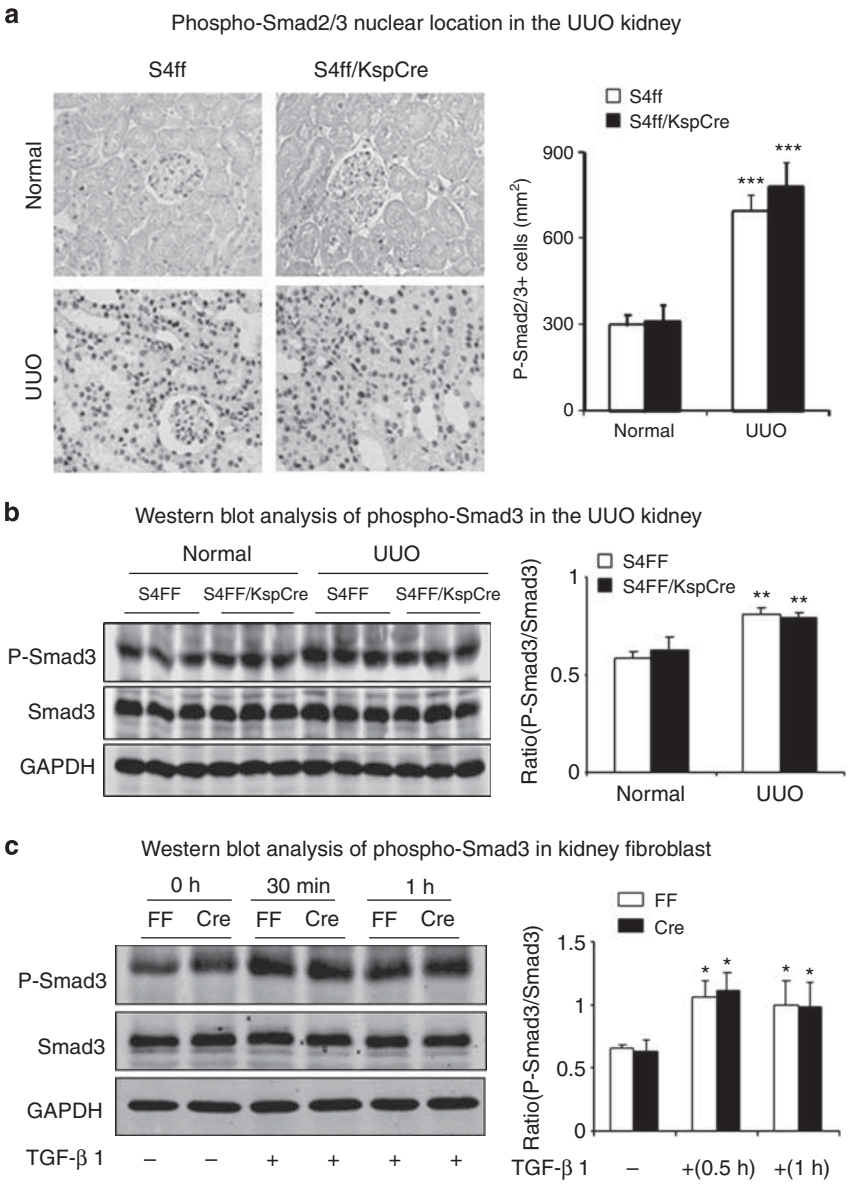


**Figure 10 | Disruption of Smad4 does not alter phosphorylation levels of Smad2/3 and their nuclear translocation in the unilateral ureteral obstructive (UUO) kidney and in transforming growth factor (TGF)-β1-stimulated kidney fibroblasts *in vitro*.** (**a**, **b**) Immunostaining and quantitative analysis of phospho-Smad2/3 nuclear translocation in the UUO kidney. (**b**) Western blot analysis of phospho-Smad3 in the UUO kidney. (**c**) Western blot analysis of phospho-Smad3 in kidney fibroblasts in response to TGF-β1 (2 ng/ml for 30 min and 1 h). Results show that disruption of Smad4 does not alter phosphorylation of Smad2 and Smad3 in the UUO kidney and TGF-β1-stimulated kidney fibroblasts *in vitro*. Data represent mean ± s.e.m. for groups of six to eight mice or for four independent experiments *in vitro*. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. normal or untreated cells. Original magnifications × 200 (**a**). Cre, Smad4ff/Cre; FF, Smad4ff; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

flox mouse and KspCre transgenic mouse are described previously.[29,30] PCR, quantitative real-time PCR, western blotting analysis, and immunohistochemistry were used to identify the specific deletion of Smad4 from the kidney as shown in Figure 1.

### Mouse model of unilateral ureteral obstruction

A mouse model of UUO nephropathy was induced in genetically identical littermates of Smad4ff and S4ff/KspCre mice by the left ureteral ligation as previously described.[31] Groups of six to eight mice were used. Kidney tissue samples were harvested for immunohistochemistry, western blot analysis, and real-time reverse transcription-PCR as previously described.[32] The experimental procedures were approved by the Animal Ethics Committee of Chinese University of Hong Kong.

### Generation of conditional Smad4 KO macrophages and fibroblasts

Peritoneal macrophage was obtained from the Smad4ff mice by 1 h adhesion on the culture plastic disk and determined by immunohistochemistry with more than 90% of F4/80 + . Smad4 FF kidney fibroblast was isolated and characterized as previously described.[33] The isolated cells were cultured with RPMI 1640 containing 15%

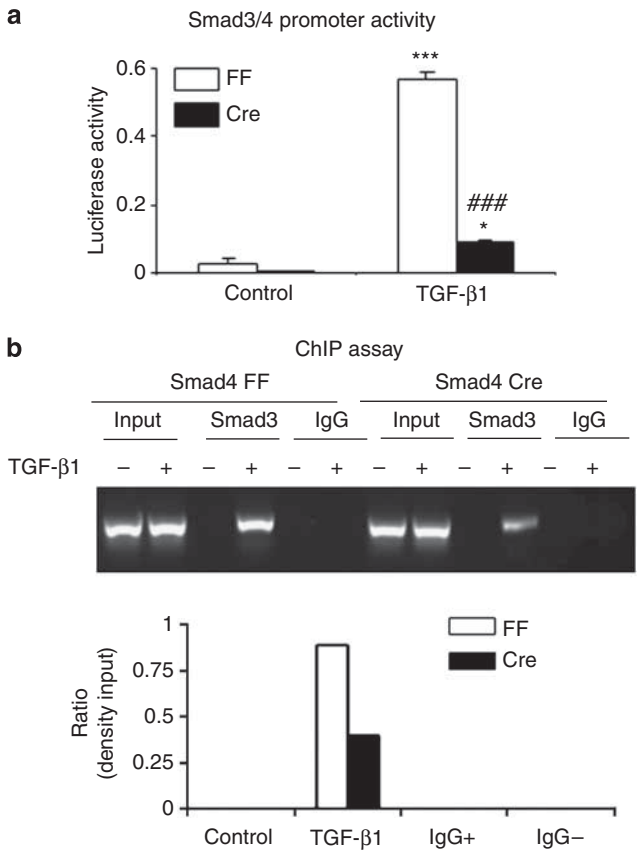


**Figure 11 | Disruption of Smad4 from the kidney fibroblasts abrogates transforming growth factor (TGF)-β1-induced Smad3/4 promoter activities and Smad3 binding to COL1A2 promoter *in vitro*.** (**a**) Promoter assay. TGF-β1 at 2 ng/ml for 24 h largely increases Smad3/4 promoter activity in Smad4ff fibroblasts, which is blocked in those cells with disruption of Smad4. (**b**) Chromatin immunoprecipitation (ChIP) assay shows that deletion of Smad4 largely inhibits TGF-β1 (2 ng/ml)-induced binding of Smad3 to the COL1A2 promoter in kidney fibroblasts. Data represent mean ± s.e.m. for three to four independent experiments. $^{*}P<0.05$, $^{***}P<0.001$ vs. control; $^{###}P<0.001$ vs. Smad4ff fibroblasts (FF). Cre, S4ff/Cre.

fetal bovine serum for three passages, and Smad4 was conditionally deleted by infecting cells with Cre-expressing adenovirus (MOI of 50) as previously described.[34] Cells infected with adenovirus β-gal were used as a control. Smad4 deletion from the cells was determined by western blot analysis using anti-Smad4 antibody.

The characterized conditional Smad4 KO or Smad4ff macrophages were stimulated with recombinant human interleukin-1β (R&D Systems, Minneapolis, MN) at concentrations of 0, 5, 10, and 20 ng/ml for periods of 0, 1, 3, 6, 12, and 24 h for the detection of inflammatory response. To determine a role of Smad4 in anti-inflammatory effect of TGF-β1, macrophages were pretreated with recombinant human TGF-β1 (R&D Systems) for 12 h, followed by the addition of an optimal dose of interleukin-1β (10 ng/ml). Similarly, the role of Smad4 in regulation of TGF-β1-induced fibrosis response was determined in conditional Smad4 KO or Smad4ff kidney fibroblasts by addition of TGF-β1(2 ng/ml) for periods of 0, 3, 12, and 24 h, respectively. Both inflammatory and fibrosis responses were determined at the mRNA level by real-time PCR and at the protein levels by western blot analysis following the protocol as previously described.[16,32,34,35] At least three independent experiments were performed.

### RNA extraction and real-time PCR examination

Total RNA was extracted from kidney tissues and cultured cells using the RNeasy Isolation Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Real-time PCR was performed using the Bio-Rad iQ SYBR Green supermix with Opticon2 (Bio-Rad, Hercules, CA) as previously described.[32] The primers used in this study, including mouse collagen I, MCP-1, interleukin-1β, TNF-α, and glyceraldehyde 3-phosphate dehydrogenase, were described previously.[16,32] Other primers include the following: Smad4 forward: 5′-AGCCGTCCTTACCCACTGAA-3′, reverse: 5′-GGTGG TAGTGCTGTTATGATGGT-3′. The ratio for the mRNA of interest was normalized with glyceraldehyde 3-phosphate dehydrogenase and expressed as the mean ± standard errors of the mean (s.e.m.).

### Masson's trichrome staining and immunohistochemistry

To evaluate the histological damage, Masson's trichrome staining was performed with the 'Trichrome stain kit' (Scy Tek Laboratoris, West Logan, UT) according to the manufacturer's instruction. In addition, immunohistochemistry was performed in paraffin sections to examine renal inflammation and fibrosis using a microwave-based antigen retrieval technique.[35] The antibodies used in this study included MCP-1 (eBioscience, San Diego, CA), TNF-α, interleukin-1β, ICAM-1, F4/80, CD45, phospho-Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA), collagen I (Southern Biotech, Birmingham, AL), and Phospho-p65NF-κB (Cell Signaling Technology, Danvers, MA). Sections were counterstained with hematoxylin after immunostaining. Positive signals were quantitatively analyzed using the quantitative Image Analysis System (AxioVision 4, Carl Zeiss, Jena, Germany) as described previously.[32,35]

### Western blot analysis

Proteins from kidney tissues and cultured cells were extracted with RIPA lysis buffer, and western blot analysis was performed as described previously.[16] After blocking nonspecific binding with 5% BSA (1 h, reverse transcription), membranes were then incubated with the primary antibody against Smad4, total Smad3, collagen I (Southern Biotech), phospho-Smad3, Phospho-p65NF-κB, p65NF-κB, phospho-IκBα, IκBα, total ERK1/2 (Cell Signaling Technology), Smad7, P-ERK1/2 (Santa Cruz Biotechnology), glyceraldehyde 3-phosphate dehydrogenase (Chemicon, Temecula, CA) overnight at 4 °C, followed by treatment with IRDye 800-conjugated secondary antibody (Rockland immunochemicals, Gilbertsville, PA). Signals were captured using the LiCor/Odyssey infrared image system (LI-COR Biosciences, Lincoln, NE). Signal intensities of each western blot band were quantified and analyzed by using the Image J software (NIH, Bethesda, MD, USA).

### Promoter assays

Kidney fibroblasts were transiently transfected with p(CAGA)12-Luc plasmid (Smad3/4-responsive promoter) and macrophages were transfected with Smad7 promoter reporter or the specific NF-κB-responsive promoter 241RMI-SEAP (secreted alkaline phosphatase) plasmid using jetPEI (Polyplus-transfection, New York, NY), following the protocol described previously.[17,36] $PGL_3$ basic plasmid was co-transfected into the cells as control and renilla plasmid for transfection efficiency. The Smad3/4 and Smad7 luciferase activities were analyzed by the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega, San Luis Obispo, CA)

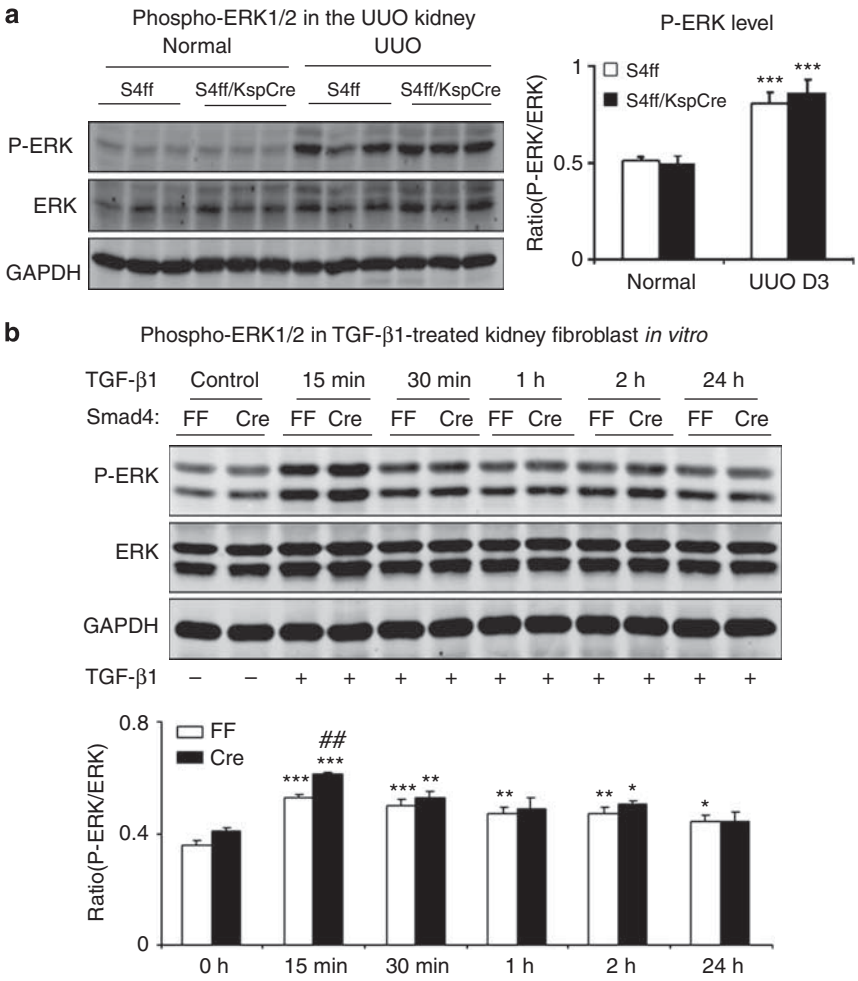


**Figure 12 | Disruption of Smad4 has minimal effects on the activation of extracellular signal-regulated protein kinase (ERK) pathway in the unilateral ureteral obstructive (UUO) kidney and in transforming growth factor (TGF)-β1-stimulated kidney fibroblasts *in vitro*.** (**a**) Western blot analysis of phospho-ERK1/2 in the UUO kidney. (**b**) Western blot analysis of phospho-ERK1/2 in the TGF-β1 (2 ng/ml)-treated kidney fibroblast. Data are expressed as mean ± s.e.m. for groups of six to eight mice *in vivo* and for four independent experiments *in vitro*. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. normal or no treatment; $^{\#\#}P<0.01$ vs. Smad4ff fibroblasts (FF). Cre, S4ff/Cre; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

and were reported as the firefly luciferase activity normalized to renilla luciferase activity, whereas the NF-κB SEAP activities were detected by the SEAP reporter gene assay kit (Roche, Indianapolis, IN) and were reported as the SEAP activity normalized to protein concentration measured with the Lowry protein assay (Bio-Rad). Three independent experiments were performed throughout the study.

### Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation was performed with the transcription factor chromatin immunoprecipitation kit according to the manufacturer's instructions and as previously described.[11] Precipitated DNAs were detected by PCR using specific primers to detect the binding of Smad3 to COL1A2: forward: 5′-AGGCAGGTCTGG GCTTTATT-3′. reverse: 5′-CGTATCCACAAAGCTGAGCA-3′.

### Statistical analysis

Data obtained from this study are expressed as mean ± s.e.m. and were analyzed using one-way analysis of variance, followed by Newman–Keuls *post hoc* test (Prism 5.0 GraphPad Software, San Diego, CA).

### DISCLOSURE

All the authors declared no competing interests.

### ACKNOWLEDGMENTS

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