

Research Paper

Blocking interleukin-6 trans-signaling protects against renal fibrosis by suppressing STAT3 activation

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Abstract

Rationale: Renal fibrosis is the terminal manifestation of chronic and irreversible renal disease. Effective therapies other than dialysis are extremely limited. In this study, we investigated the potential effects of targeting elevated interleukin-6 (IL-6) levels in the treatment of renal fibrosis.

Methods: Fc-gp130 was used to specifically block IL-6 trans-signaling. Unilateral ureteral occlusion (UUO) and ischemia reperfusion (IR) mouse models were constructed to investigate the therapeutic effect of Fc-gp130 on renal fibrosis. The role of IL-6 trans-signaling and phosphorylation of signal transducer and activator of transcription (STAT) 3 in regulating fibroblast accumulation and extracellular matrix protein deposition were evaluated in cell experiments and mouse models.

Results: The kidneys of mice with UUO were found to have elevated soluble IL-6 receptor (sIL-6R) levels in the progression of fibrosis. Fc-gp130 attenuated renal fibrosis in mice, as evidenced by reductions in tubular atrophy and the production of extracellular matrix protein. Blockade of IL-6 trans-signaling with Fc-gp130 also reduced inflammation levels, immune cell infiltration, and profibrotic cytokines expression in renal tissue, with decreased STAT3 phosphorylation and reduced fibroblast accumulation in the renal tissue. In vitro, Fc-gp130 also reduced the phosphorylation of STAT3 induced by transforming growth factor (TGF)- β 1 in fibroblasts. Furthermore, the therapeutic effect of Fc-gp130 was confirmed in a model of acute kidney injury-chronic kidney disease.

Conclusion: Overall, IL-6 trans-signaling may contribute to crucial events in the development of renal fibrosis, and the targeting of IL-6 trans-signaling by Fc-gp130 may provide a novel therapeutic strategy for the treatment of renal fibrosis.

Key words: chronic kidney disease, renal fibrosis, IL-6 trans-signaling, STAT3, Fc-gp130

Introduction

Chronic kidney disease (CKD), including renal interstitial fibrosis, is a worldwide public health problem causing high morbidity and mortality [1]. Renal fibrosis, characterized by fibroblasts activation and extracellular matrix (ECM) deposition, is the terminal manifestation and common irreversible pathway by which CKD progresses to end stage [2]. Renal fibrosis results in trauma of renal parenchyma and loss of renal function [3]. The incidence of end

stage renal disease is rapidly increasing in the prevalence of diabetes and hypertension [4]. Clinically, there are few effective therapies for renal fibrosis other than dialysis. Therefore, the development of new strategies for antifibrotic therapy is required.

Interleukin-6 (IL-6) regulates the innate and adaptive immune responses, and it has become a major target for clinical intervention in many

proinflammatory conditions [5]. IL-6 signaling occurs through two main pathways. In classical pathway, IL-6 binds to the membrane-bound IL-6 receptor (mIL-6R), then recruits signal transducer glycoprotein 130 (gp130) [6]; this leads to its homodimerization, which further activates the Janus kinase (JAK)/Tyk tyrosine kinases and leads to the phosphorylation of signal transducer and activator of transcription (STAT) 3. STAT3 is a transcription factor that regulates many downstream target genes [7]. In trans-signaling pathway, soluble IL-6R (sIL-6R) detected in body fluids binds to IL-6 with comparable affinity to that of membrane-bound IL-6R. The IL-6/sIL-6R complex also activates membrane gp130, causing subsequent phosphorylation of JAK/STAT3. IL-6 is therefore involved in intracellular signal transduction. It is generally believed that the anti-inflammatory activity of IL-6 is mediated by classical signaling, whereas the proinflammatory property is mediated by the trans-signaling pathway [8].

The role of IL-6 in renal fibrosis remains controversial. Elevated plasma levels of IL-6 are commonly observed in CKD patients [9]. However, IL-6 knockout (KO) mice showed comparable expression of ECM proteins in the obstructed kidney compared to that in wild-type (WT) mice [10]. IL-6 classic signaling can protect renal tissue from overshooting immune responses [11]. IL-6 trans-signaling has been shown to play a central role in crescentic nephritis [12]. As a specific antagonist of IL-6 trans-signaling in vivo, soluble gp130 is elevated in many pathological conditions [13, 14]. Gp130 was suggested to be a novel specific antagonist of IL-6 trans-signaling [15]. Fc-linked gp130 was produced, and the homodimer was found more effective. Furthermore, recombinant gp130 is currently in clinical trials [16]. Therefore, soluble gp130, a specific IL-6 trans-signaling inhibitor that does not affect classic signaling, could be an ideal therapeutic drug for renal inflammation [17].

In our previous work, we constructed and purified recombinant Fc-gp130 [18]. The Fc-gp130 homodimer was more effective than monomer gp130 at blocking IL-6 trans-signaling, as each functional IL-6/sIL-6R complex associates with two molecules of gp130 to transduce the intracellular downstream signal. In this study, we aimed to determine whether IL-6 trans-signaling is important in the development of renal fibrosis. Our results show that treatment with Fc-gp130, by interfering with IL-6 trans-signaling, attenuates renal fibrosis, therefore indicating that IL-6 trans-signaling blockade could be a promising and novel therapeutic approach for the treatment of renal fibrosis.

Results

Fc-gp130 attenuated renal fibrosis caused by unilateral ureteral occlusion (UO)

In UO-ligated mice, the levels of sIL-6R in the kidney (Figure 1A), IL-6 and sIL-6R in plasma (Supplementary Figure S1A-B) were significantly increased on day 14 after UO as compared with levels in those receiving sham treatment. In addition, significant increases in IL-6, sIL-6R, mIL-6R, and gp130 in UO kidneys were also observed by day 14 (Figure 1B, Supplementary Figure S1C). It is worth noting that sIL-6R levels in the kidney were elevated from day 3 to day 14, suggesting a role for IL-6 trans-signaling in the progression of renal fibrosis (Figure 1B). To confirm this, we pre-treated mice with Fc-gp130 to investigate whether specific blockade of IL-6 trans-signaling would protect against renal fibrosis. Interestingly, Fc-gp130 treatment in UO mice significantly reduced parenchymal loss and tubular atrophy (Figure 1C), whereas genetic deletion of IL-6 had no substantial protective effect on UO-induced fibrosis. Masson's staining also revealed that UO mice showed fibrotic renal tissue with extensive collagen deposition, whereas Fc-gp130 treatment significantly reduced collagen deposition in UO mice (Figure 1D). Next, we analyzed the effect of Fc-gp130 on the production of major components of the ECM, in UO renal tissue. Immunohistochemical (IHC) staining showed increased accumulation of fibronectin, collagen I, collagen III and vimentin following UO, whereas Fc-gp130-treated mice exhibited reduced ECM content (Figure 1E-F, Supplementary Figure S1D-E). However, there were no significant differences in renal interstitial fibrosis or deposition of ECM between WT and IL-6 KO mice following UO injury. Collagen I and fibronectin proteins were also significantly increased in UO kidneys, and Fc-gp130 treatment significantly reduced collagen I and fibronectin protein levels in the kidneys of UO mice (Figure 3D). Overall, these data suggest that IL-6 trans-signaling promotes renal fibrosis.

Fc-gp130 reduced inflammation in UO mice

Inflammation is an important driver in the progression of renal fibrosis [19, 20]. Upon injury, renal tubular cells produce chemokines to recruit immune cells and inflammatory cytokines to activate myofibroblasts to produce ECM [21]. Therefore, we next evaluated the impacts of Fc-gp130 on the inflammatory responses in UO mice. NF- κ B p65 activation and nuclear translocation in renal tubular epithelial cells were significantly reduced in Fc-gp130-treated UO mice compared with that in

UO mice alone (Figure 2A, Supplementary Figure S2A). IHC analysis also showed that Fc-gp130 inhibited the recruitment of F4/80-positive macrophages (Figure 2B), CD4⁺ (Figure 2C) and CD8⁺ T cells (Figure 2D) in the kidneys of UO mice. In addition, the mRNA levels of proinflammatory mediator, such as tumor necrosis factor- α (*Tnfa*) (Figure 2E), monocyte chemoattractant protein 1

(*Mcp1*) (Figure 2F), and CXC chemokine ligand 16 (*Cxcl16*) (Figure 2G), were also significantly reduced upon Fc-gp130 treatment. The mRNA levels of *Il6* (Figure 2H) and *Tgfb* (Figure 2I) were not significantly reduced by Fc-gp130. Therefore, blockade of IL-6 trans-signaling with Fc-gp130 attenuated renal inflammation in the UO mouse model.

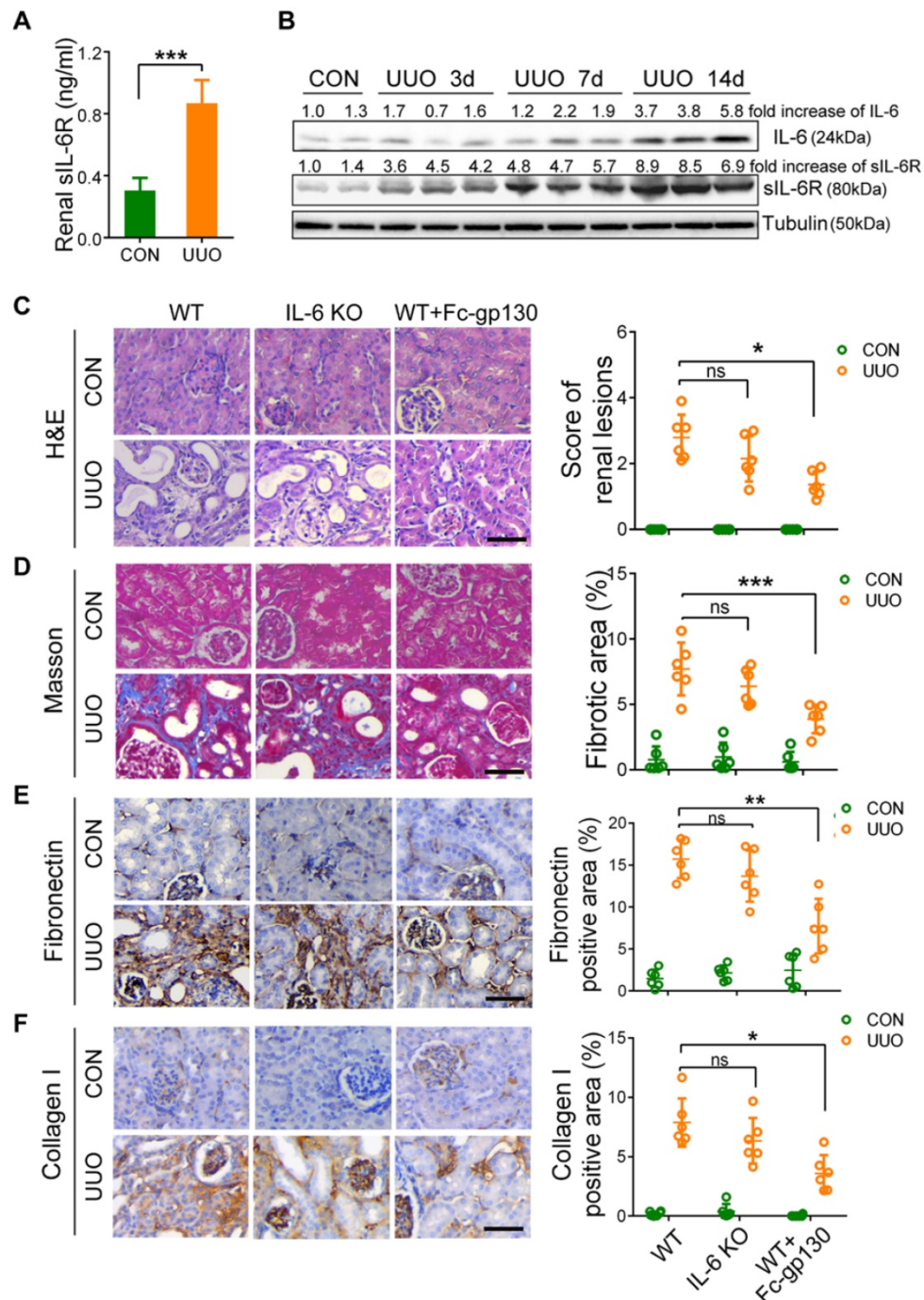


Figure 1. Renal fibrosis was reduced in mice with unilateral ureteral obstruction (UO) treated with Fc-gp130. (A) ELISA of sIL-6R in renal lysates from mice on day 14 after UO and sham control (CON) surgery. (B) Western blotting analysis of IL-6, sIL-6R and tubulin in renal lysates on days 3, 7, and 14 after UO. (C) Samples from WT mice pretreated with or without Fc-gp130 and IL-6 KO C57BL/6 mice were collected on day 14 after UO or CON surgery. Renal sections were stained with H&E for visualizing parenchymal loss and tubular atrophy, and renal lesions were scored. (D) Masson trichrome staining for tubulointerstitial fibrosis and collagen deposition (blue), and quantitative analysis of fibrotic area. (E-F) Immunohistochemistry and quantitative analysis of collagen I (E) and fibronectin (F) content in renal tissue. Scale bar, 50 μ m. Data are means \pm SD, $n = 6$ per group; ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Similar results from three independent experiments are shown.

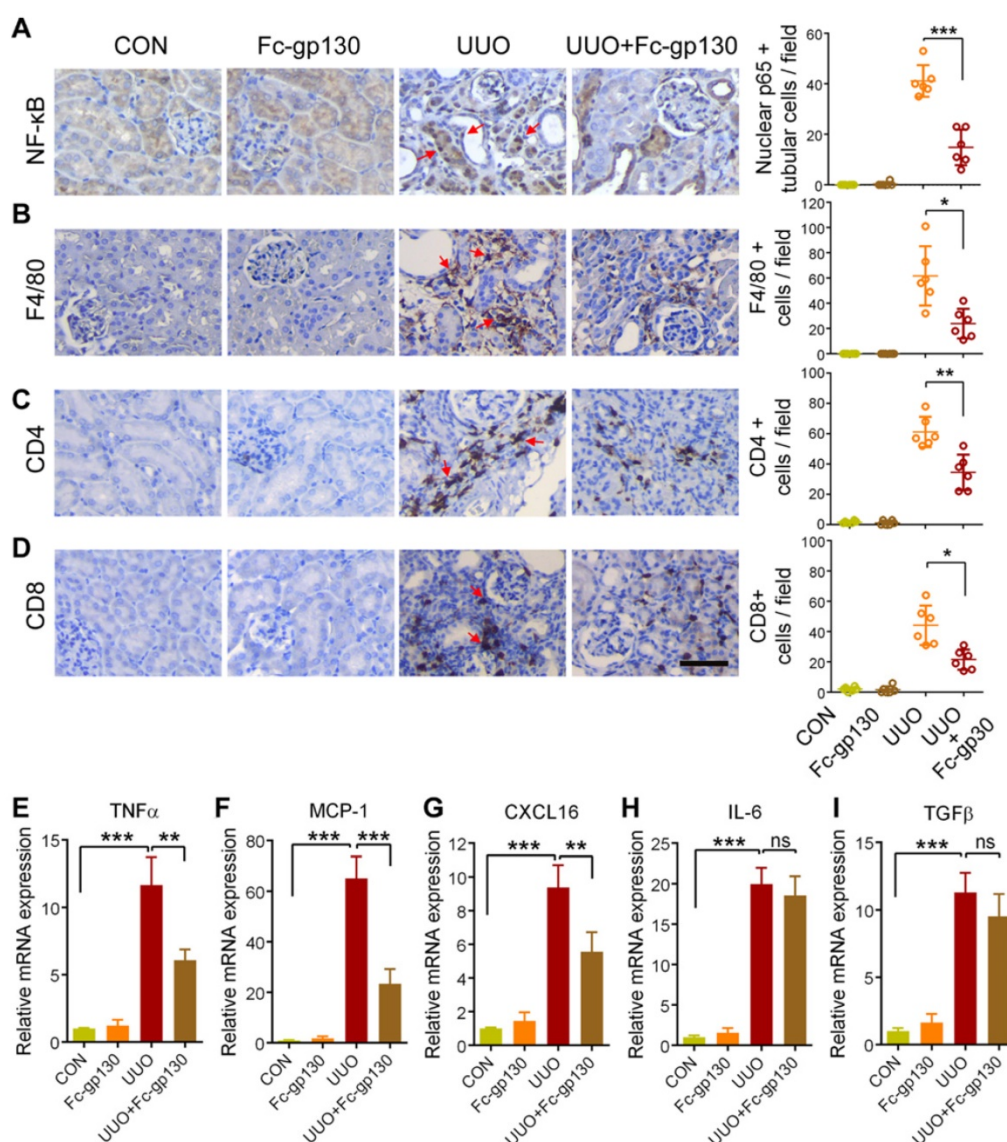


Figure 2. Renal inflammatory responses were reduced in UUO mice treated with Fc-gp130. (A–D) Samples from mice pretreated with or without Fc-gp130 were collected on day 7 after UUO or sham control (CON) surgery. Immunohistochemistry of NF-κB p65 and quantitative analysis of p65 nuclear localization (A). Immunohistochemistry of F4/80 (B), CD4 (C), and CD8 (D) and positive cells in renal tissue. **(E–I)** RT-qPCR analysis of mRNA levels of *Tnfa* (E), *Mcp1* (F), *Cxcl16* (G), *Il6* (H), and *Tgfb* (I). Data are means \pm SD, $n = 6$ per group; ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Similar results from three independent experiments are shown.

Fc-gp130 influenced STAT3 activation in fibroblasts

Immunostaining of α -smooth muscle actin (α -SMA) was used to investigate fibroblast accumulation. In UUO mice, Fc-gp130 treatment diminished fibroblast accumulation, as evidenced by reduced numbers of α -SMA-positive cells (Figure 3A). More specifically, p-STAT3-positive interstitial fibroblasts were abundant in UUO mice but were reduced in number after Fc-gp130 treatment, while p-STAT3-positive tubular epithelial cells were observed in both groups (Figure 3B-1 and 3B-2). The profibrogenic effect of IL-6 trans-signaling depends on the activation of STAT3 [22]. In UUO mice, phosphorylation of STAT3 (p-STAT3) and total STAT3 increased significantly over time, peaking on

day 14 after UUO injury (Supplementary Figure S2B). Western blotting analysis on day 14 renal lysates also revealed that the ratios of p-STAT3/STAT3 and p-Smad3/Smad3, the levels of α -SMA, fibronectin and collagen I were attenuated, and the ratios of p-Smad1/5/8/Smad1 were increased in Fc-gp130-treated UUO mice compared to those in UUO mice alone; however, the ratios of p-Smad2/Smad2 and p-AKT/AKT, the levels of TGF- β , IL-6 and sIL-6R in Fc-gp130-treated and non-treated UUO mice were not significantly different (Figure 3C-D).

In NRK-49F cells, Fc-gp130 attenuated phosphorylation of STAT3 induced by IL-6 plus IL-6R treatment, whereas the p-STAT3 induced by low or high dose of IL-6 alone was not affected (Supplementary Figure S2C). We confirmed that

Fc-gp130 specifically inhibits IL-6 trans-signaling in renal fibroblasts. IL-6 alone did not alter cell proliferation, but stimulation with IL-6 and IL-6R resulted in an increase in proliferation, while Fc-gp130 treatment reduced it (Figure 4A). Cells treated with IL-6 in the absence of sIL6R had a background of phosphorylated STAT3 due to signaling via the membrane bound IL-6R (classical signaling), and IL-6 plus sIL-6R induced phosphorylation of STAT3 (classical and trans-signaling) was significantly suppressed by Fc-gp130 (Figure 4B, Supplementary Figure S2D-E). This suggests that Fc-gp130 reduced fibroblast proliferation and p-STAT3 activated by IL-6 trans-signaling. We confirmed the result by examining the effect of stattic, an inhibitor of STAT3 activation. The cell proliferation activated by IL-6 trans-signaling was reduced by stattic (Figure 4C).

Stattic inhibited both IL-6 induced and IL-6 plus IL-6R induced phosphorylation of STAT3 (Figure 4D). IL-6 treatment induced a slight increase in p-STAT3 in NRK-49F. IL-6 plus sIL-6R induced a prominent increase in p-STAT3 levels, comparable to that observed in fibroblasts stimulated by transforming growth factor (TGF)- β 1 (Figure 4E). As expected, TGF- β 1-induced increases in p-STAT3 and fibronectin levels were significantly attenuated by Fc-gp130 treatment at 6 and 12 h, and p-Smad3 was reduced by Fc-gp130 at 12 h (Figure 4F). Moreover, the levels of p-Smad1/5/8 was increased by Fc-gp130 at 12 h (Figure 4G). Therefore, Fc-gp130 attenuated the activation of renal fibroblasts in response to TGF- β 1 in vitro. These data support the contribution of IL-6 trans-signaling to fibroblast and STAT3 activation in renal fibrosis.

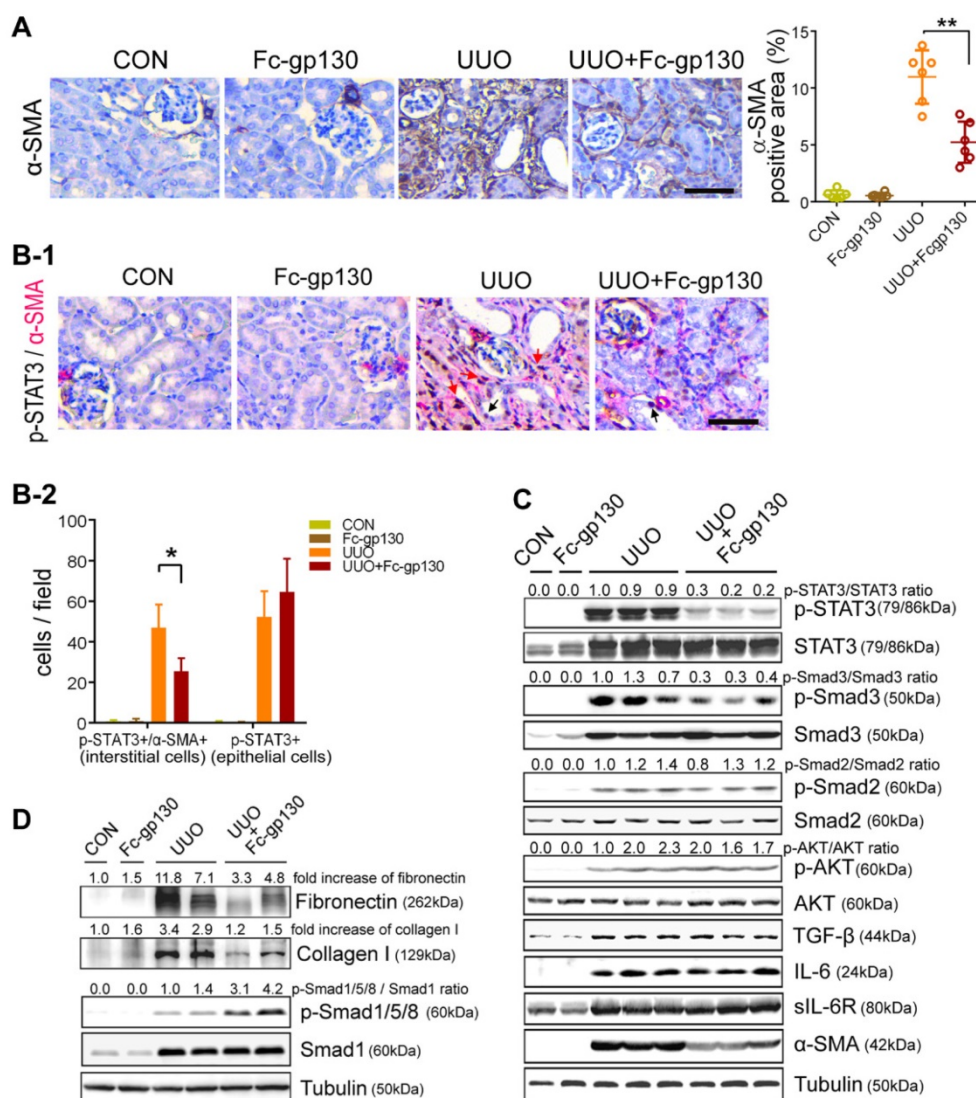


Figure 3. Fc-gp130 suppressed STAT3 activation in renal tissues of UUO mice. (A) Immunohistochemistry and quantitative analysis of α -SMA content in renal tissue. **(B)** Immunohistochemistry of p-STAT3 (brown) in fibroblasts (α -SMA, red) from renal sections. Red arrows denote p-STAT3 and α -SMA co-positive interstitial cells, black arrows denote p-STAT3 positive epithelial cells (B-1), and quantitative analysis of positive cells (B-2). Scale bar, 50 μ m. Data are means \pm SD, $n = 6$ pre group; * $p < 0.05$, ** $p < 0.01$. **(C-D)** Renal tissue samples were collected on day 14 after UUO and Fc-gp130 treatment. Western blotting analysis of phosphorylated (p)-STAT3, total STAT3, p-Smad3, Smad3, p-Smad2, Smad2, p-AKT, AKT, TGF- β , IL-6, sIL-6R, α -SMA (C), Fibronectin, Collagen I, p-Smad1/5/8, Smad1 and tubulin in renal lysates (D). Each experiment was performed at least in triplicate.

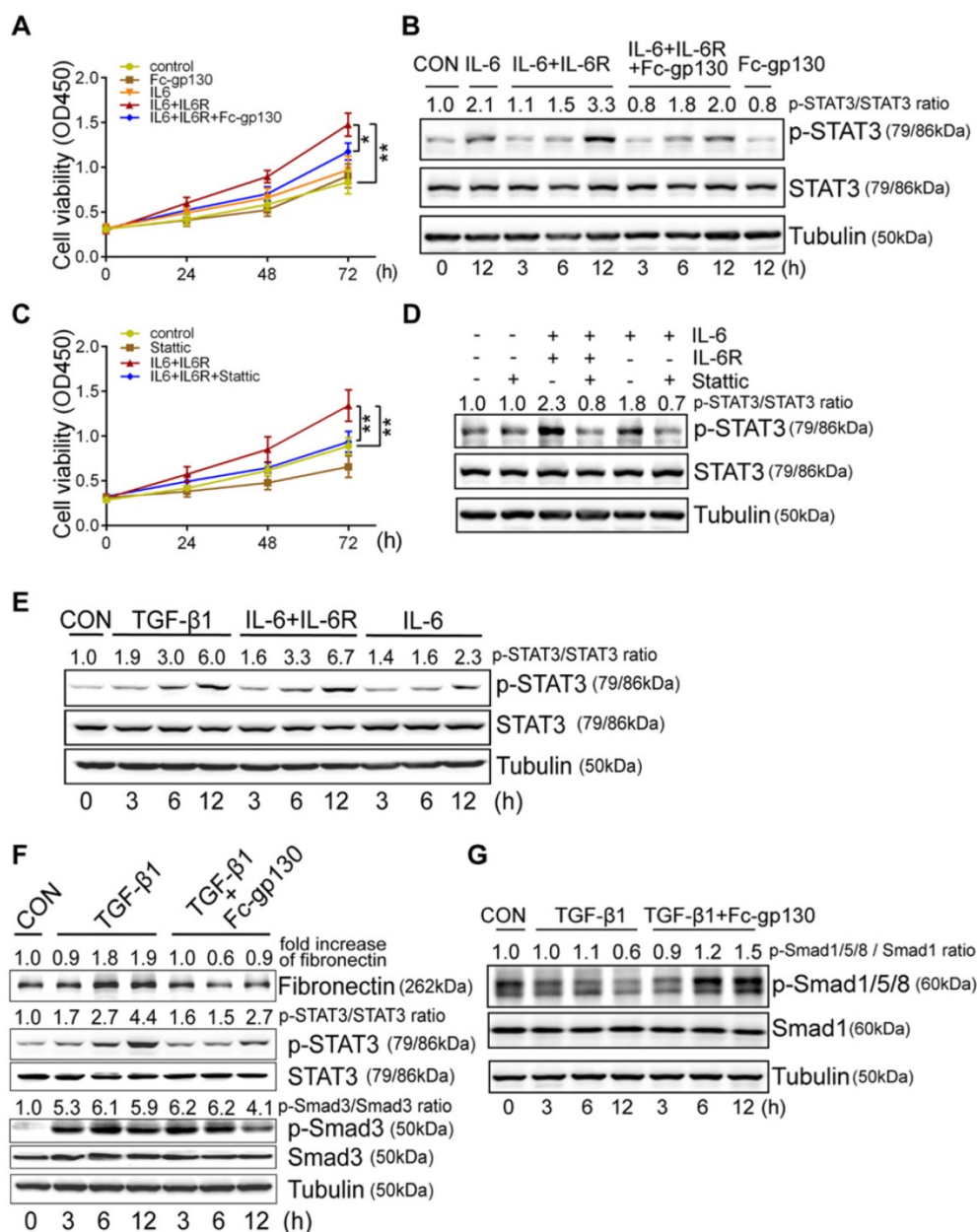


Figure 4. Fc-gp130 inhibited proliferation of fibroblasts by suppressing STAT3 activation. (A-B) NRK-49F cells were incubated with vehicle (CON), IL-6 (50 ng/ml), IL-6R (200 ng/ml) and Fc-gp130 (500 ng/ml) for the indicated durations. Changes of cell proliferation (A), and western blotting analysis of p-STAT3, STAT3 and tubulin (B). **(C-D)** NRK-49F cells were incubated with vehicle (CON), IL-6 (50 ng/ml), IL-6R (200 ng/ml) and Static (2 μM) for the indicated plans and durations. Changes of cell proliferation (C), and western blotting analysis of p-STAT3, STAT3 and tubulin at 12h (D). Data are means ± SD, 6 duplicate samples in each group for (A) and (C); *p < 0.05, ** p < 0.01. **(E)** NRK-49F cells were incubated with vehicle (CON), TGF-β1 (5 ng/ml), IL-6 (50 ng/ml) plus IL-6R (200 ng/ml), or IL-6 only for the indicated durations. Western blotting analysis of p-STAT3, STAT3 and tubulin. **(F-G)** NRK-49F cells were incubated with vehicle (CON), TGF-β1 (5 ng/ml) only, or TGF-β1 (5 ng/ml) plus Fc-gp130 (500 ng/ml) for 3, 6, 12 h. Western blotting analysis of fibronectin, p-STAT3, total STAT3, p-Smad3, total Smad3 (F), p-Smad1/5/8, total Smad1 and tubulin (G). Each experiment was performed at least in triplicate.

Fc-gp130 protected against fibrosis after ischemia reperfusion (IR) injury

To further confirm the protective effect of Fc-gp130, we constructed a different model of renal fibrosis, the acute kidney injury (AKI)-CKD mouse model, which is more consistent with the clinicopathological process. In AKI-CKD model, the levels of sIL-6R and IL-6 in plasma were significantly increased on day 14 after IR injury (the left renal artery was clamped for 45 min) as compared with

levels in those receiving sham control (CON) treatment (Supplementary Figure S3A-B). In addition, significant increases in IL-6, sIL-6R, mL-6R, and gp130 in IR kidneys were also observed by day 14 (Figure 5A, Supplementary Figure S3C). After IR injury, the concentration of blood urea nitrogen (BUN) and creatinine increased rapidly in one day, then return to normal gradually. The concentrations of BUN and creatinine were significantly decreased in Fc-gp130 treated IR group on day 14 and 28

(Supplementary Figure S3D-E). This indicated that Fc-gp130 can accelerate the recovery of IR injured kidneys.

Mice treated with or without Fc-gp130 were exposed to severe renal IR injury and sacrificed at day 42 after injury. Protein levels of fibronectin, α -SMA, p-STAT3, and STAT3 were also significantly increased in IR kidneys, and Fc-gp130 treatment reduced fibronectin, α -SMA, and p-STAT3 levels (Figure 5B). IR mouse sections showed extensive renal damage as compared with that in sham control sections. Fc-gp130 treatment reduced lesions in IR

mice (Figure 5C). On day 42 after IR injury, both glomeruli and interstitial areas between tubules showed significant fibrosis (Figure 5D). IR kidneys showed significantly increased α -SMA, fibronectin, collagen III and vimentin staining (Figure 5E-F, Supplementary Figure S3F-G). Fc-gp130-treated IR mice showed significant reductions in ECM deposition and α -SMA content relative to levels in IR mice alone. These results confirmed that Fc-gp130 attenuates chronic renal fibrosis induced by IR in mice.

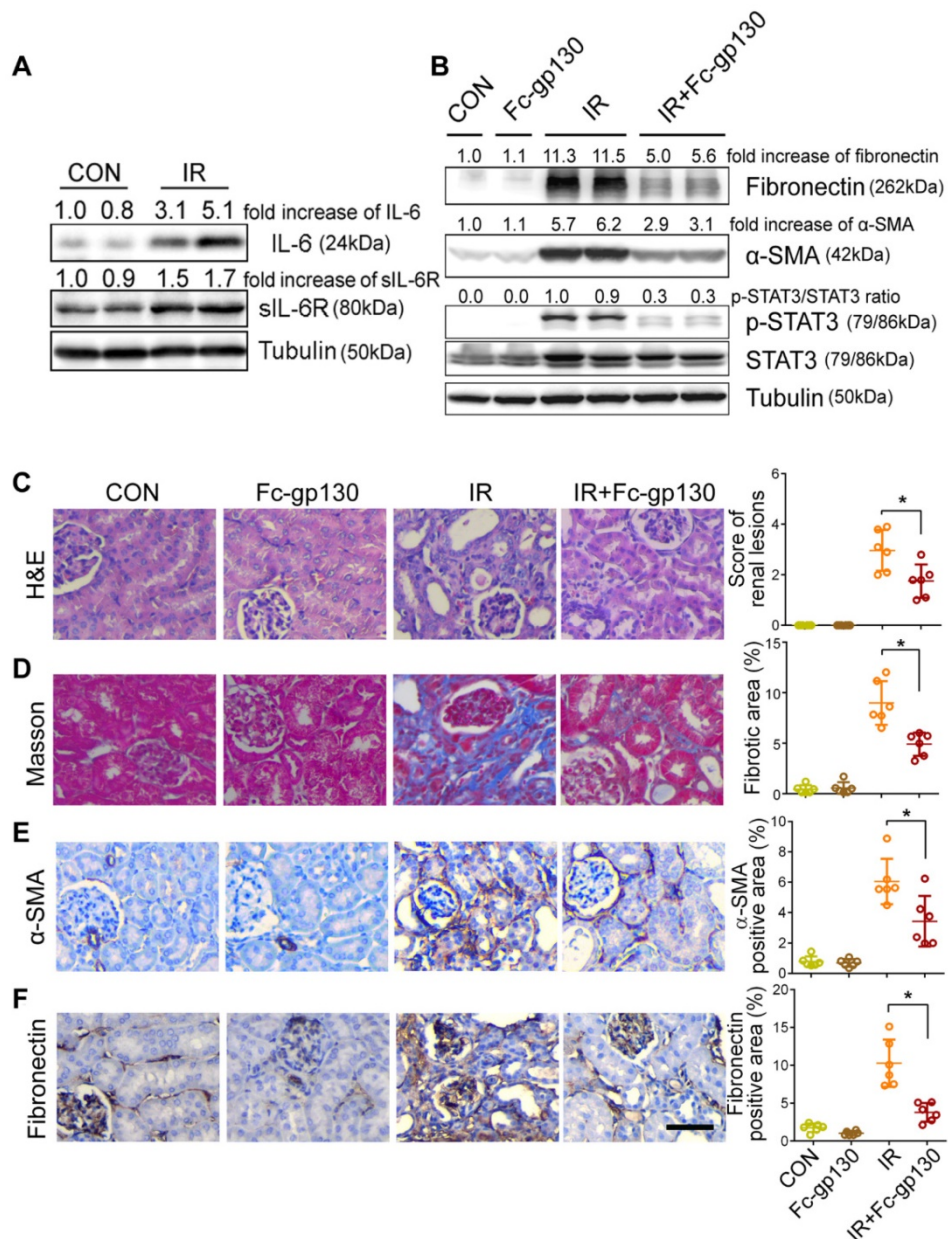


Figure 5. Renal fibrosis was reduced in AKI-CKD mice treated with Fc-gp130. (A) Renal tissue samples were collected on day 14 after renal ischemia reperfusion (IR) or sham control (CON) surgery. Western blotting analysis of IL-6, sIL-6R and tubulin in renal lysates. (B) Mice were treated with vehicle (saline) or Fc-gp130, and samples were collected on day 42 after renal IR or sham control surgery. Western blotting analysis of fibronectin, α -SMA, p-STAT3, STAT3 and tubulin in renal lysates. (C–F) Renal sections were stained with H&E for visualizing parenchymal loss and tubular atrophy, and renal lesions were scored (C). Masson trichrome staining for tubulointerstitial fibrosis and collagen deposition (blue), and quantitative analysis of fibrotic area (D). Immunohistochemistry and quantitative analysis of α -SMA (E) and fibronectin (F) content. Scale bar, 50 μ m. Data are means \pm SD, n = 6 per group; ns, not significant, *p < 0.05. Similar results from three independent experiments are shown.

Discussion

IL-6 trans-signaling, mediated by sIL-6R, has been implicated in various chronic inflammatory conditions: rheumatoid arthritis [23], asthma [24] and neurodegenerative disorders [25], for example. However, its role in renal fibrosis has not yet been elucidated. In this study, we showed that targeting of the IL-6/sIL-6R complex using Fc-gp130 attenuated ECM protein production and inflammation in renal tissues of UUO and AKI-CKD mouse models, suggesting that IL-6 trans-signaling plays an essential role in the development and progression of renal interstitial fibrosis. Therefore, blocking of IL-6 trans-signaling with Fc-gp130 provides a promising new therapeutic strategy for the treatment of renal fibrosis.

Elevated levels of the cytokine IL-6 are linked to renal fibrosis. However, previously, WT and IL-6 KO mice under UUO showed no significant phenotypic differences [10], probably because the effects of ablating both the anti-inflammatory classical signaling and proinflammatory trans-signaling pathways may compensate for each other. Similarly, our results indicated that WT and IL-6 KO mice showed no significant changes in renal tissue fibrosis. In the IL-6 KO mice, the absent of pro-inflammatory trans-signaling probably alleviated fibrosis; but the anti-inflammatory of classic IL-6 signaling is also absent in injured renal tissue, and the increase of other inflammatory processes may aggravate renal fibrosis. Specifically neutralizing the pro-inflammatory IL-6 trans-signaling would not compromise other beneficial activities of IL-6 [26, 27]. In this study, we showed that IL-6 trans-signaling plays a key role in the renal fibrotic process. This is consistent with recent studies in pulmonary and cardiac fibrosis [28, 29]. Soluble gp130 is the natural inhibitor of IL-6 trans-signaling [30]. Using Fc-gp130, an antagonist of IL-6 signaling [31, 32], we specifically blocked IL-6 trans-signaling without interfering with the classical signaling [33, 34]. We speculated that this is the cause for better treatment effect of Fc-gp130. In the construction of this molecule, an IgG Fc fragment was added before the N terminus of gp130, producing a homodimer that is more effective than soluble gp130 [18]. Fc-fused proteins that have been found clinically efficacious include abatacept [35], aflibercept [36], and romiplostim [37]. Fc-linked gp130 is currently in clinical trials [16]. Overall, Fc-gp130 attenuated renal fibrosis in mouse models.

Various cells and molecules are involved in the process of damage repair and reconstruction [38]. IL-6 signaling is not the sole mechanism for renal fibrosis. Complex cross-talk in vivo might contribute to the pro- and anti-inflammatory/regenerative properties

of IL-6 [39]. Cells stained for p-STAT3 were observed in tubular epithelial cells and fibroblasts in obstructed kidneys. Quantitative analysis showed that Fc-gp130 decreased the number of p-STAT3 activated fibroblast, but not the number of p-STAT3-positive tubular epithelial cells. Some studies have implicated STAT3 activation in tubular epithelial cells with the progression of kidney interstitial fibrosis [40]. Previous studies have demonstrated that STAT3 signaling in kidney tubular epithelial cells inhibits cells apoptosis and plays an important protective role in limiting kidney dysfunction after acute injury [41-43]. A previous study demonstrated that a receptor-independent gp130/STAT3 agonist prevented renal fibrosis [44]. That might be because hyperactive STAT3 plays an important role in protecting the kidneys. Classic signaling can be activated by IL-6 alone in cells with membrane-bound IL-6R and gp130. In contrast, virtually all cells express membrane-bound gp130, and a receptor-independent gp130/STAT3 agonist can activate STAT3 in almost all cells. STAT3 is mechanistically involved in the communication between epithelial cells and fibroblasts [45]. Accordingly, activated STAT3 in renal fibroblast is the main driver of renal fibrosis, and tubular epithelial cells probably make a protective contribution.

Activated STAT3 has been found to be an important transcription factor in the pathogenesis of pulmonary and kidney fibrosis [46, 47]. After ligand-induced dimerization of IL-6R, STAT3 binds to associated JAKs and can be phosphorylated. In addition, a recent study reported that STAT3 could be activated by TGF- β and plays an essential role in UUO-induced renal interstitial fibrosis [22]. Therefore, cross-talk between IL-6-STAT3 and TGF- β signaling may exist. The fact that Fc-gp130 treatment affected the phosphorylation of STAT3 further supports this hypothesis. It has also been reported that IL-6-STAT3 signaling enhances the expression of TGF- β 1 to promote fibrosis [48]. However, in our study, we did not observe any reduction in *Tgfb* mRNA levels in Fc-gp130-treated animals. Furthermore, Fc-gp130 treatment inhibited p-STAT3 levels and ECM production in NRK-49F fibroblasts with sufficient TGF- β 1 supplementation in the culture. Fc-gp130 could not only inhibit TGF- β downstream effector p-SMAD3, but upregulate the anti-fibrotic molecules p-SMAD1/5/8, suggesting that Fc-gp130 may interfered with TGF- β signaling through regulation of downstream molecules, independent of an effect on its expression levels. IL-6 trans-signaling plays a major role inflammation [49]. Fc-gp130 also affected the activation of NF- κ B, another key transcription factor in the development of

fibrosis and inflammation. However, the detailed mechanism underlying this cross-talk is unclear, and additional studies are needed to provide further insight.

After injury, inflammatory response and IL-6 signaling are rapidly activated in renal tissue. Due to the fact that the structure and function are affected in damaged and fibrotic kidneys, administered drugs are often not efficiently distributed to the renal target cells [50]. Fc-gp130 treatment in advance could confirm the effective concentration in the body, and timely reduce the inflammation. In present study the Fc-gp130 treatment started one week prior. In fact, the disease process of human renal fibrosis can go on a long time. Fc-gp130 treatment thus may have a therapeutic effect after renal injury or fibrosis occurs. However, the opportunity and methods of Fc-gp130 for human kidney disease need further study. There may be side effects when using systemic Fc-gp130 administration. Stability, immunogenicity, and the lack of absorption are limitations of therapeutic proteins [51]. Therefore, high concentration of Fc-gp130 is required, which might inhibit both classic and trans-signaling of IL-6 and evoke adverse outcomes [52]. Kidney or target cell specific delivery systems might overcome these limitations in fibrosis therapy [53]. Subsequently, specific delivery methods could be explored to improve the targeting of Fc-gp130 in the kidney.

In conclusion, the present study found that specifically neutralizing IL-6 trans-signaling attenuated renal fibrosis in UUO and AKI-CKD mouse models. IL-6 trans-signaling may thus represent a critical therapeutic target for managing fibrosis, and Fc-gp130 may be a candidate therapeutic agent for renal fibrosis.

Materials and Methods

Animals and treatment

Animal experiments were approved by the Ethics Committee of Drum Tower Hospital, Medical School of Nanjing University. WT C57BL/6 mice were purchased from the Animal Core Facility of Nanjing Medical University. IL-6 KO mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME USA). Since males have better consistency and greater susceptibility to IR than females [54], we performed all animal experiments using male mice only. Under specific pathogen-free conditions, male mice (8 weeks old) weighing 20-25 g were housed in standard rodent cages. Mice were divided into four groups for treatment: sham [phosphate-buffered saline (PBS) treatment and sham surgery]; Fc-gp130 (Fc-gp130 treatment and sham

surgery); UUO/IR (PBS treatment and induction of renal fibrosis); UUO/IR+Fc-gp130 (Fc-gp130 treatment and induction of renal fibrosis). Recombinant mouse Fc-gp130 (Vazyme, Nanjing, China) was used to block IL-6 trans-signaling. Fc-gp130 (20 µg/kg/day) [18] or vehicle (PBS) was intraperitoneally injected into mice every other day from one week before the surgery.

UUO model

WT or IL-6 KO mice were anesthetized intraperitoneally with 2% pentobarbital sodium (4 ml/kg) and then placed on the test bench in the prone position. The left ureter was exposed and ligated with sutures at two points near the renal hilum, followed by excision [55]. After the incision was closed, mice were given penicillin by intramuscular injection and were allowed to recover in standard rodent cages. Sham-operated control mice underwent abdominal laparotomy without excision of the right ureter. Mice were sacrificed at 3, 7, and 14 days, and renal tissue samples were collected for analysis.

Renal IR model

WT mice were anesthetized intraperitoneally with 2% pentobarbital sodium (4 ml/kg) and placed on the test bench. After abdominal laparotomy, the left renal pedicle was exposed and clamped for 45 min (ischemia); then, the clamp was removed (reperfusion). After closing the incision, mice were given penicillin by intramuscular injection and were allowed to recover in standard rodent cages. Sham-operated control mice underwent abdominal laparotomy without the clamp. Mice were sacrificed on day 42, and renal tissue samples were collected for analysis. The concentrations of blood serum urea nitrogen (BUN) and creatinine were assessed in duplicated with a commercially available assay kit (BioAssay System, Hayward, CA, USA) according to the instructions.

Enzyme-linked immunosorbent assay (ELISA)

Soluble IL-6R was quantified in plasma and tissue lysates from sham and UUO mouse kidneys using a mouse IL-6R ELISA kit (Raybiotech, Norcross, GA, USA). Plasma IL-6 was quantified using a mouse IL-6 ELISA kit (Raybiotech, Norcross, GA, USA). Renal lysate and plasma samples were diluted in reagent diluent and subjected to ELISA analysis according to the manufacturer's protocol.

Antibodies

Antibodies against Fibronectin (ab2413), Collagen I (ab34710), α -SMA (ab5694), F4/80 (ab6640), CD4 (ab183685), CD8 (ab209775), p-Smad3 (ab52903) and pan Cadherin (ab51034) were from

Abcam (Cambridge, UK). Antibodies against p-STAT3 (9145), STAT3 (9139), NF- κ B p65 (8242), p-p65 (3033), p-AKT (4060), p-Smad2 (3108), Smad2 (5339), Smad3 (9523), p-Smad1/5/8 (13820), Smad1 (6944), Vimentin (5741), Tubulin (2148), rabbit IgGs (7074) and mouse IgGs (7076) were from Cell Signaling Technology (Danvers, MA, USA). Antibodies against IL-6 (21865), IL-6R (23457), AKT (10176), TGF- β (21898), gp130(21175) and Collagen III (22734) were from Proteintech (Rosemont, IL, USA).

Histological analysis

Renal tissues were fixed in 4% formaldehyde, dehydrated, embedded, and sectioned at 4 μ m thickness. Renal sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome (Solarbio Life Sciences, Beijing, China) to evaluate the degree of fibrosis. Sections were observed and scored in a blinded manner. HE slides were scored on a scale from 0 to 4: 0, normal; 1, mild (<25% of the cortex); 2, moderate (25–50%); 3, severe (50–75%); 4, extensive damage (>75%) [56, 57]. The scoring and the positive area of collagen fibers (Masson's trichrome, blue) was calculated in 10 bright fields (\times 200) in the eyepiece of the microscope. Images were acquired using a Leica DM 1000 microscope image system with a digital camera (Wetzlar, Germany).

Immunohistochemistry

After dewaxing and rehydration, slides were incubated with 3% H₂O₂ for quenching endogenous peroxidase activity and then heated in boiling antigen unmasking solution for antigen retrieval. Slides were blocked with 5% goat serum (Vector Laboratories, UK) for 1 h, then incubated with primary antibody in a humidified chamber at 4°C overnight. Positive signal was visualized by DAB solution (ZSGB-BIO, Beijing, China). The images were visualized and acquired using the Leica DM 1000 microscope image system. Quantitative evaluation involved the use of Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) [58]. Positive signals and the number of positive cells in the kidney were quantified in 10 bright fields (\times 200) in the eyepiece of the microscope.

Cell culture and treatment

Renal fibroblast cell line NRK-49F was from American Type Culture Collection (ATCC, Manassas, USA), and cultured with Dulbecco's modified Eagle's medium (DMEM, Wisent, Nanjing, China) containing 10% (v/v) fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA) at 37°C with 5% CO₂. Fibroblasts were pretreated with serum-free medium for 12 h and then exposed to 5 ng/ml TGF- β 1, 50 ng/ml IL-6, 200 ng/ml IL-6R (R&D Systems, MN, USA), or 500 ng/ml Fc-gp130 for 3, 6, or 12 h.

Cell proliferation assay

Cell viability was measured at 0, 24, 48, 72 h using a CCK-8 assay kit (Vazyme Biotech, Nanjing, China). Cells were washed and then incubated with a working solution at 37°C for 30 min. Wst-8 in working solution can be reduced to highly soluble formazan (orange product) by various dehydrogenases in the mitochondria. The absorption value of each well, which is proportional to cell viability, was detected at 450 nm using a spectrophotometer system (TECAN, Männedorf, Switzerland).

Western blotting analysis

Renal tissues or cell samples were lysed on ice with lysis buffer containing cocktail proteinase inhibitors and protein phosphatase inhibitors (Thermo Fisher, Rockford, IL, USA). The extracted protein concentrations were quantified via BCA assay, mixed with loading buffer and boiled. Proteins were resolved on 10% SDS-PAGE in running buffer, transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, USA), blocked with blocking buffer, and blotted with the primary antibodies at 4°C overnight. Blots were washed and followed by incubation with secondary antibodies. After washing three times with PBST, signals were detected by chemiluminescence (Tanon, Shanghai, China), and visualized by ChemiScope 3300 Mini Imaging System (CLiNX, Shanghai, China). The western blot data was quantified by IMAGE J.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA samples were extracted with TRIzol reagent (Invitrogen, Carlsbad, USA). Reverse transcription were performed using the PrimeScript RT Reagent Kit (Takara, Otsu, Japan). PCR reaction systems were performed using AceQ qPCR SYBR Green Master Mix (Vazyme). Reactions were run on a StepOne Plus (Applied Biosystems, Foster City, USA). The mRNA levels were normalized to that of actin mRNA and calculated using the 2^{- $\Delta\Delta$ CT} method. The specific primers used for analysis were as follows: MCP-1 forward, 5'-TTAAAAACCTGGATCGGAACC AA-3'; MCP-1 reverse, 5'-GCATTAGCTTCAGATTT ACGGGT-3'; TNF α forward, 5'-ACCCTCACACTCA GATCATCTTC-3'; TNF α reverse, 5'-TGGTGGTTTG CTACGACGT-3'; CXCL16 forward, 5'-AGTGTCGC TGGAAGTTGTTCT-3'; CXCL16 reverse, 5'-GTTCC ACACTCTTTGCGCTC-3'; IL-6 forward, ACAAAG CCAGAGTCCTCAGAGA; IL-6 reverse, CTGTTA GGAGAGCATTGGAAATTG; TGF- β forward, CGGT GCTCGCTTTGTA; TGF- β reverse, GCCACTCAGG CGTATC; Actin forward, 5'-TAAAGACCTCTATGCC

AACACAGT-3'; Actin reverse, 5'-CACGATGGAGG GGCCGGACTCATC-3'.

Statistical analysis

All data are expressed as mean \pm SD. Statistical analysis involving two groups was performed by means of Student's t test. Multiple group comparisons were performed by one-way ANOVA statistical test. All data were processed with IBM SPSS Statistics 21 and GraphPad Prism 6. A p -value < 0.05 was considered statistically significant.

Abbreviations

UUO: unilateral ureteral occlusion; IL-6: interleukin-6; sIL-6R: soluble IL-6 receptor; gp130: glycoprotein 130; STAT3: signal transducer and activator of transcription 3; ECM: extracellular matrix; α -SMA: α -smooth muscle actin; TGF- β 1: transforming growth factor- β 1; TNF α : tumor necrosis factor- α ; MCP-1: monocyte chemoattraction protein 1; CXCL16: CXC chemokine ligand 16; AKI: acute kidney injury; CKD: chronic kidney disease; IR: ischemia reperfusion.

Supplementary Material

Supplementary figures.

<http://www.thno.org/v09p3980s1.pdf>

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Contributions

WC and XZ designed the study. HY, TW, WC and HY performed animal experiments. WC and YF performed histological analysis and IHC. WC and BJ performed WB and RT-qPCR. WC performed cell experiments. WC and XZ analyzed data. WC, HZ and XZ wrote the manuscript. XZ and HG supervised research.

Competing Interests

The authors have declared that no competing interest exists.

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