see commentary on page 231

A novel STAT3 inhibitor, S3I-201, attenuates renal interstitial fibroblast activation and interstitial fibrosis in obstructive nephropathy

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Accumulation of both interstitial myofibroblasts and excessive production of extracellular matrix proteins is a common pathway contributing to chronic kidney disease. In a number of tissues, activation of STAT3 (signal transducer and activator of transcription 3) increases expression of multiple profibrotic genes. Here, we examined the effect of a STAT3 inhibitor, S3I-201, on activation of renal interstitial fibroblasts and progression of renal fibrosis. Treatment of cultured rat renal interstitial fibroblasts with S3I-201 inhibited their activation, as evidenced by dose- and time-dependent blockade of α-smooth muscle actin and fibronectin expression. In a mouse model of renal interstitial fibrosis induced by unilateral ureteral obstruction, STAT3 was activated, and administration of S3I-201 attenuated both this activation and extracellular matrix protein deposition following injury. S3I-201 reduced infiltration of the injured kidney by inflammatory cells and suppressed the injury-induced expression of fibronectin, α-smooth muscle actin, and collagen type-1 proteins, as well as the expression of multiple cytokines. Furthermore, S3I-201 inhibited proliferation and induced apoptosis preferentially in renal interstitial fibroblasts of the obstructed kidney. Thus, our results suggest that increased STAT3 activity mediates activation of renal interstitial fibroblasts and the progression of renal fibrosis. Inhibition of STAT3 signaling with S3I-201 may hold therapeutic potential for fibrotic kidney diseases.

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KEYWORDS: chronic kidney disease; renal interstitial fibroblasts; STAT3; transforming growth factor; unilateral ureteral obstruction

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Renal tubulointerstitial fibrosis is the final common pathway in end-stage renal disease and is characterized by aberrant activation and growth of the renal fibroblasts.[1,2] The activation of interstitial fibroblasts to become α-smooth muscle actin (α-SMA)-positive myofibroblasts is the key step in the evolution of chronic kidney disease (CKD). Therefore, targeting the signaling pathway(s) that mediate the activation of myofibroblasts may be a way to attenuate the progression of renal fibrosis.

Among the signaling pathways reported to be associated with activation of renal interstitial fibroblasts,[1,3] transforming growth factor-β1 (TGF-β1) signaling has a major role in this process.[4–6] TGF-β1 induces its biological effects by interaction with TGF-β receptor type-I (TβRI), type-II (TβRII), and type-III (TβRIII).[7,8] Upregulation of TGF-β1 and TGF-β receptors has been observed in various forms of chronic renal fibrotic diseases in animal models and in humans.[4,9] In addition, accumulation of inflammatory cells and expression of some proinflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1) also contribute to the development of renal fibrosis in human and animal models of CKD.[3,10–12]

Signal transducer and activator of transcription 3 (STAT3) has been reported to induce expression of multiple genes, including TGF-β1.[13–15] STAT3 belongs to a family comprised of seven members (STAT1–4, STAT5α, STAT5β, and STAT6) and mediates various cellular functions, including cell survival and proliferation.[16,17] In response to numerous growth factors and cytokines such as TGF-β1,[18] platelet-derived growth factor,[19,20] and IL-6,[21,22] STAT3 is activated by tyrosine phosphorylation at tyrosine 705 through Janus kinases. Phosphorylated STAT3 forms dimers and then translocates to the nucleus, where it directly binds to the DNA sequence and regulates expression of target genes.[13–15] Increased STAT3 activation by phosphorylation at tyrosine 705 has been observed in interstitial fibroblasts of the fibrotic kidney induced by unilateral ureteral obstruction (UUO).[23]

However, the functional role of STAT3 in activation of renal fibroblasts and development of renal interstitial fibrosis has not been examined *in vivo*.

Recently, S3I-201, a novel selective inhibitor of STAT3, has been synthesized and used to treat tumors. S3I-201 preferentially inhibits STAT3 DNA-binding activity and diminishes STAT3 tyrosine phosphorylation.[24] In this study, we evaluated the therapeutic effect of S3I-201 on the activation of renal interstitial fibroblasts *in vitro* using rat NRK-49F cells, a rat renal interstitial fibroblast line, and the progression of renal fibrosis in a murine model of the fibrotic kidney induced by UUO injury. Furthermore, we investigated the effect of S3I-201 on the expression of some cytokines associated with the development of progressive renal fibrosis.

RESULTS

S3I-201 decreases expression of α-SMA and fibronectin in cultured renal interstitial fibroblasts

Myofibroblasts are active fibroblasts characterized by expression of α-SMA and fibronectin.[3] To understand whether STAT3 activation is required for activation of renal interstitial fibroblasts, we examined the effect of S3I-201 on expression of α-SMA and fibronectin in NRK-49F cells. As shown in Figure 1a–d, both α-SMA and fibronectin were highly expressed in NRK-49F cells, indicating that the cultured renal interstitial fibroblasts are phenotypically myofibroblasts. Incubation of cells with S3I-201 dramatically decreased the expression of α-SMA and fibronectin in a dose- and time-dependent manner, suggesting that STAT3 mediates activation of renal interstitial fibroblasts.

The STATs are activated by tyrosine phosphorylation.[25,26] p-STAT3 and p-STAT5, but not p-STAT1, were constitutively expressed in cultured NRK-49F cells. Treatment with S3I-201 dose-dependently inhibited the expression of p-STAT3 with a complete inhibition at 100 μM (Figure 1e and f). S3I-201 treatment did not affect STAT5 phosphorylation, but increased expression of p-STAT1 in a dose-dependent manner (Supplementary Figure S1). These data suggest that S3I-201 is a highly selective inhibitor of STAT3 and is able to block expression of the constitutively activated STAT3 in

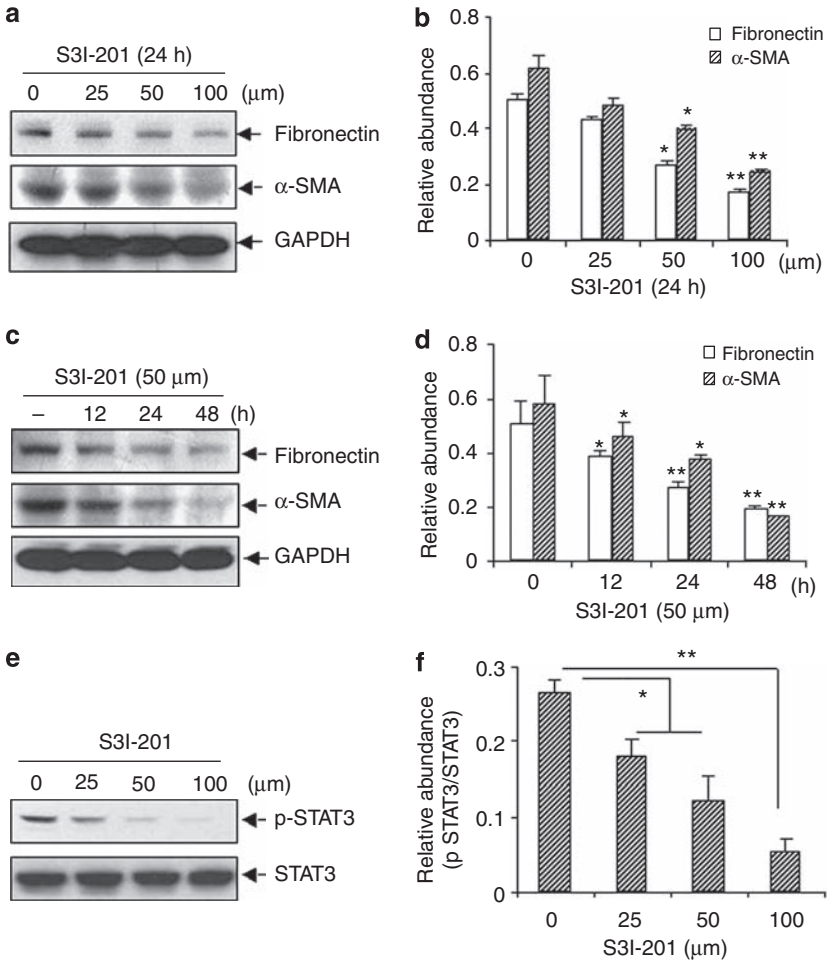

Figure 1 | Effect of S3I-201 on the expression of α-smooth muscle actin (α-SMA) and fibronectin. Cultured NRK-49F cells were treated with S3I-201 for the indicated concentrations for 24 h (**a**) or 3 h (**e**), or exposed to 50 μM S3I-201 for the indicated time period (**c**). Cell lysates were subjected to immunoblot analysis using antibodies to α-SMA, fibronectin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Representative immunoblots from three or more experiments are shown. Expression levels of the indicated proteins were quantified by densitometry and normalized with GAPDH (**b**, **d**). Activated signal transducer and activator of transcription 3 (STAT3) was depicted with p-STAT3/STAT3 ratio (**f**). Data are means ± s.e.m. Significant *P*-values reflecting differences are indicated over the bars (*$P<0.05$; **$P<0.01$).

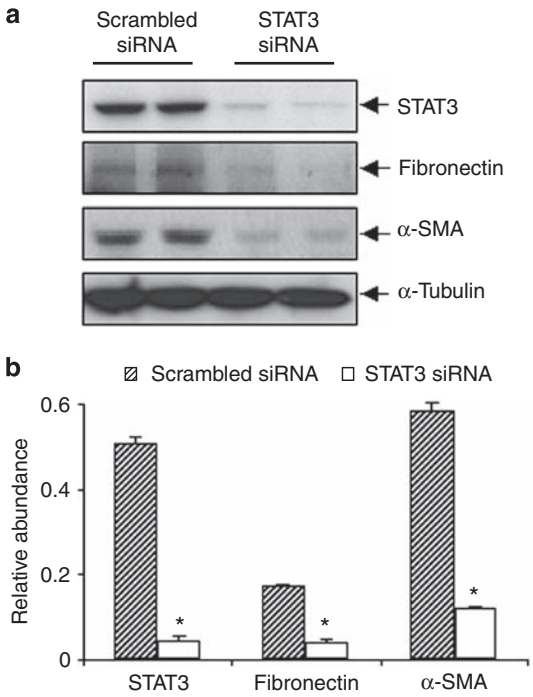

Figure 2 | Effect of signal transducer and activator of transcription 3 (STAT3) small interfering RNA (siRNA) on the expression of α-smooth muscle actin (α-SMA) and fibronectin. Cultured NRK-49F cells were transfected with siRNA targeting STAT3 or scrambled siRNA. At 48 h after transfection, cell lysates were subjected to immunoblot analysis using antibodies to STAT3, α-SMA, fibronectin, and α-tubulin. Representative immunoblots from three or more experiments are shown (**a**). Expression levels of the indicated proteins were quantified by densitometry and normalized with α-tubulin (**b**). Data are means ± s.e.m. Significant *P*-values reflecting differences in the expression of individual proteins as indicated between cells treated with STAT3 siRNA and those treated with scrambled siRNA (*$P < 0.05$).

renal interstitial fibroblasts. Furthermore, STAT3 may negatively regulate STAT1 activation. As recent studies have documented that both STAT1 and STAT5 have a role in suppression of tissue fibrosis, in contrast to STAT3,[27,28] increased expression of p-STAT1 and preservation of p-STAT5 may be beneficial to the alleviation of renal fibrosis after injury.

Downregulation of STAT3 by siRNA inhibits expression of α-SMA and fibronectin in cultured renal interstitial fibroblasts

To confirm the role of STAT3 in mediating α-SMA and fibronectin expression in NRK-49F cells, small interfering RNA (siRNA) targeting STAT3 was transfected to NRK-49F cells by electroporation to downregulate STAT3, and expression of α-SMA and fibronectin was examined by immunoblot analysis. Figure 2 shows that knockdown of STAT3 decreased expression of α-SMA and fibronectin in NRK-49F cells (Figure 2). These data support our conclusions that STAT3 is critically involved in activation of cultured renal interstitial fibroblasts.

STAT3 phosphorylation and expression in the obstructive kidney

On the basis of the above observation, we hypothesized that STAT3 has an essential role in mediating the development of renal interstitial fibrosis *in vivo*. To test this hypothesis, we first examined the activation and expression of STAT3 in obstructive nephropathy, a model of predominantly tubulointerstitial lesions that are characterized by accumulation and activation of myofibroblasts.[29] As shown in Figure 3a and b, the basal level of STAT3, but not p-STAT3, was observed in sham-operated kidneys. UUO injury caused STAT3 tyrosine phosphorylation, which evolves in two waves: the first on day 1 and the second on day 7. STAT3 tyrosine phosphorylation then remained elevated until at least 14 days after UUO injury. Whereas p-STAT3 expression declined on day 3 after UUO injury, total STAT3 expression was still upregulated at this point. On day 7, expression of STAT3 reached its maximum and kept this level until at least 14 days after UUO injury.

Previous work indicates that p-STAT3 is preferentially expressed in myofibroblasts in rat kidneys subjected to UUO injury.[23] To determine whether this is also the case in the mouse model of UUO, we performed double immunostaining for α-SMA and p-STAT3 in kidney tissue after UUO injury. Most of the cells localized to the interstitium stained for both α-SMA and p-STAT3, and a small number of cells stained only for α-SMA. Cells stained only for p-STAT3 were observed only occasionally (Figure 3d and e). Therefore, these data illustrate that STAT3 is preferentially activated in renal interstitial fibroblasts after UUO injury.

S3I-201 inhibits STAT3 phosphorylation in the obstructive kidney

As an initial step toward understanding the role of STAT3 in renal fibrosis, we examined the effect of S3I-201 on the expression of p-STAT3 in the kidney following obstructive injury. Contralateral kidneys were used as controls. As shown in Figure 4, daily administration of S3I-201 at 10 mg/kg largely suppressed STAT3 phosphorylation on day 7 and a small inhibition on day 14 after UUO (Supplementary Figure S3). Total STAT3 expression was not affected by this treatment at both time points. These data indicate that UUO injury induces both activation of STAT3 and upregulation of STAT3 protein, and that S3I-201 is an effective inhibitor of STAT3 activation without changing STAT3 protein levels. Furthermore, S3I-201 is more effective in inhibiting STAT3 activation in the early phase of obstructive nephropathy. The reason for the different effect of this inhibitor at the early and later time points is currently unknown, but may be associated with change in the signal responsible for STAT3 activation due to increased injury over time. Further studies are needed to address the mechanism that regulates STAT3 activation at different phases.

S3I-201 attenuates deposition of ECM components

An increase in extracellular matrix (ECM) is the major feature of renal fibrosis.[4,30] To determine whether STAT3 activation is involved in the regulation of renal interstitial

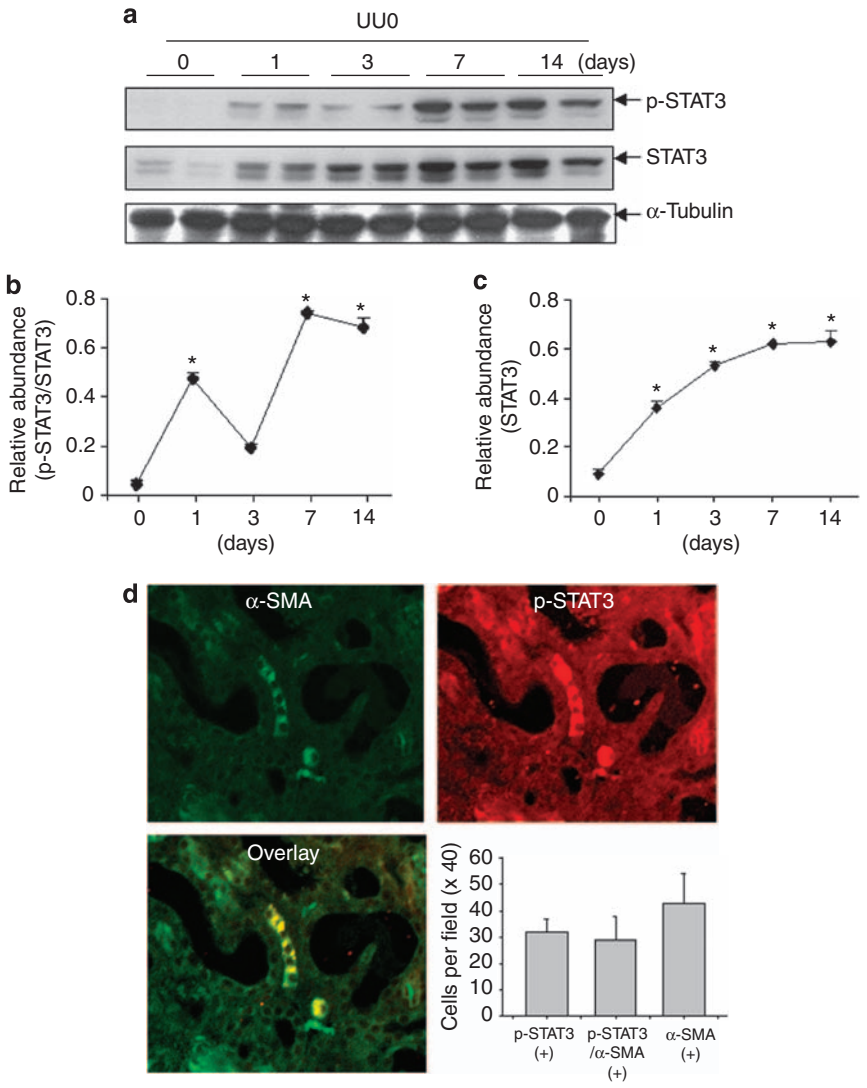

Figure 3 | Expression of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) and STAT3 following unilateral ureteral obstruction (UUO) injury. The left ureter was ligated. (**a**–**c**) At days 1, 3, 7, and 14, the kidneys were taken for analysis of expression of p-STAT3, STAT3, and tubulin. Representative immunoblots from three or more experiments are shown. After urethra ligation, kidneys were taken at 1, 3, 7, and 14 days. Expression levels of p-STAT3 and STAT3 (*$P<0.05$) (**b** and **c**) were quantified by densitometry and normalized with tubulin. Kidney tissue collected at day 7 was used for costaining with antibodies to α-smooth muscle actin (α-SMA) and p-STAT3. Interstitial cells with positive staining for both α-SMA and p-STAT3, or α-SMA, or p-STAT3 alone were counted in 25 high-power fields and expressed as means ± s.e.m. (**d**).

ECM deposition after obstructive injury, we evaluated the effect of S3I-201 on the expression of interstitial collagen fibrils by Sirius red staining. An increase in Sirius red-positive areas was observed within the tubulointerstitium after UUO injury. S3I-201 treatment significantly reduced such areas (Figure 5c and d). A semiquantitative analysis of Sirius red-positive areas reveals a 9.5-fold increase in the obstructive kidney compared with sham-operated kidneys. Treatment with S3I-201 decreased the expression of ECM components by ~60% (Figure 5e).

S3I-201 inhibits expression of collagen type-1 and α-SMA, but not β-catenin and snail after obstructive injury

To further elucidate the role of STAT3 in fibroblast activation and production of a particular ECM protein, we examined the effect of S3I-201 on the expression of collagen type-1 and α-SMA, the hallmark of fibroblast activation, after UUO injury.[4] As indicated in Figure 6a–c, basal levels of α-SMA and abundant collagen type-1 were detected in the kidney tissue of sham-operated mice. UUO injury resulted in a marked increase in their expression. S3I-201 treatment inhibited α-SMA expression and also decreased collagen type-1 to the basal level in obstructive kidneys. Interestingly, S3I-201 did not affect the basal levels of collagen type-1 and α-SMA expression in control kidneys. In addition, we also examined the effect of S3I-201 on the expression of collagen type-1 at mRNA level and found that UUO injury also resulted in increased expression of collagen type-1 (Figure 6d), whereas S3I-201 treatment attenuated this response. In contrast to those observations, administration

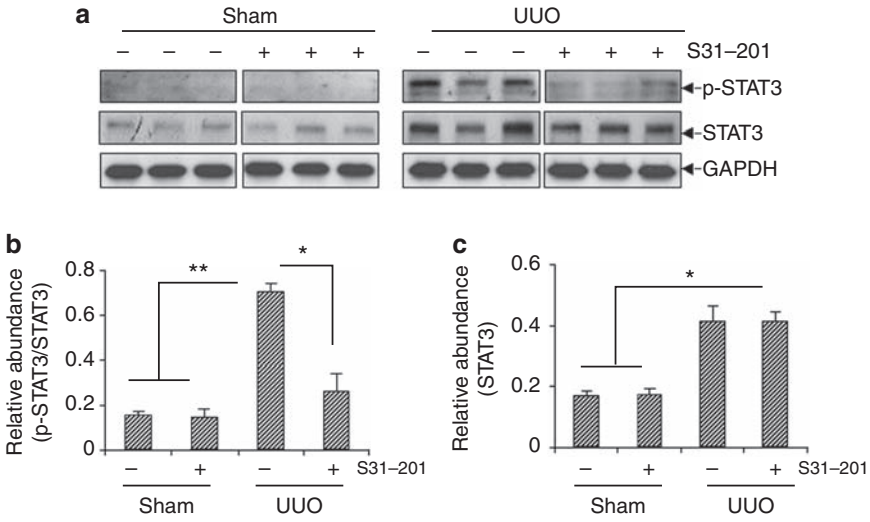

Figure 4 | Effect of S3I-201 on UUO-induced signal transducer and activator of transcription 3 (STAT3) activation. Kidney tissue lysates were subjected to immunoblot analysis with specific antibodies against p-STAT3 (Tyr705), STAT3, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (**a**). Activated STAT3 was depicted with p-STAT3/STAT3 ratio (**b**). Protein expression levels of STAT3 were quantified by densitometry and normalized with GAPDH (**c**). Data are means ± s.e.m. ($n = 6$). Significant *P*-values reflecting differences are indicated over the bars (*$P < 0.05$; **$P < 0.01$).

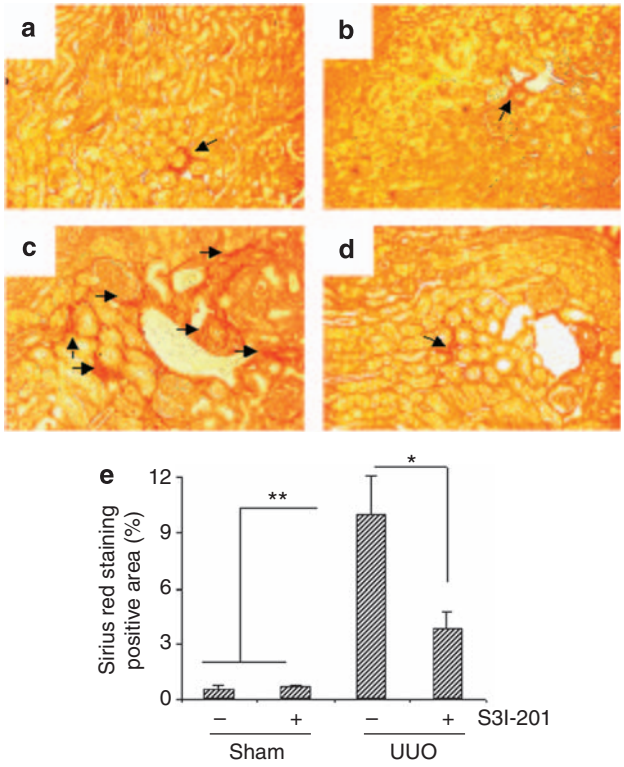

Figure 5 | Effect of S3I-201 on the deposition of extracellular matrix (ECM) in obstructive kidneys. Photomicrographs illustrating Sirius red staining of kidney tissue after various treatments: (**a**) sham with vehicle; (**b**) sham with S3I-201; (**c**) UUO with vehicle; and (**d**) UUO with S3I-201. The graph shows the percentage of Sirius red-positive tubulointerstitial area (red) relative to the whole area from 10 random cortical fields (× 200) (means ± s.e.m.) (**e**). Significant *P*-values reflecting differences are indicated over the bars (*$P < 0.05$; **$P < 0.01$). Note the ECM deposition areas (arrows).

of S3I-201 did not affect expression of β-catenin and snail (Supplementary Figure S4). Therefore, increased collagen type-1 and α-SMA after UUO injury, rather than their basal levels, are regulated by STAT3. Furthermore, STAT3 does not have a role in regulation of β-catenin and snail, two transcriptional regulators involved in the epithelial–mesenchymal transition.[31,32]

We further examined the effect of S3I-201 on activation of renal interstitial fibroblasts on day 14 after UUO injury. S3I-201 at 10 mg/kg also decreased α-SMA and collagen type-1 expression at that time, but not to significant levels (Supplementary Figure S3). Reduced effects of S3I-201 on activation of renal interstitial fibroblasts at the later time point is currently unclear and needs further investigations.

S3I-201 inhibits expression of fibronectin after obstructive injury

To determine whether S3I-201 administration inhibits other ECM proteins, we assessed the effect of S3I-201 on fibronectin expression by immunostaining and immunoblot analysis. Fibronectin staining showed increased expression of fibronectin after UUO injury. S3I-201 administration decreased its expression (Figure 7a–d). Similar results were also obtained by immunoblot analysis of kidney tissue lysates (Figure 7e). Densitometry analysis of immunoblot results showed a significant increase in the expression of fibronectin in the kidneys of mice following UUO, compared with control sham-operated mice. In contrast, fibronectin expression was blocked in mice treated with S3I-201 (Figure 7f). These data, taken together with the inhibitory effect of S3I-201 on collagen type-1 expression, suggest that STAT3 is critically involved in the production of ECM proteins after UUO injury.

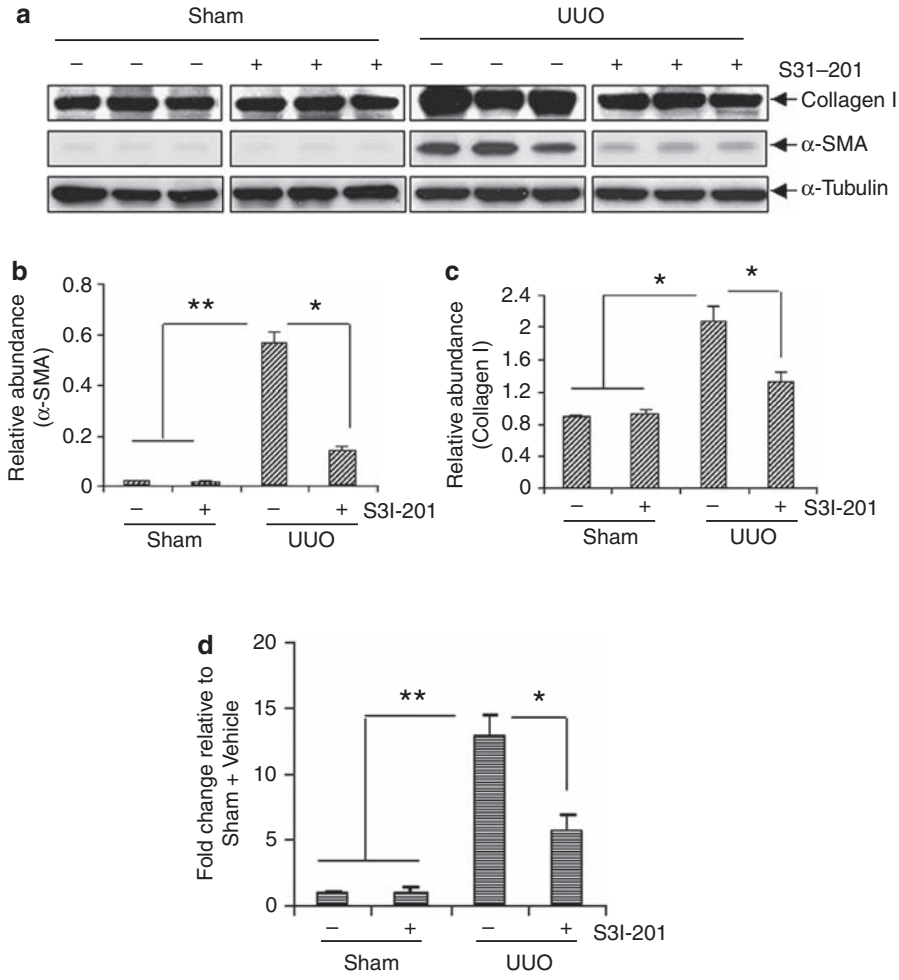

Figure 6 | Effect of S3I-201 on UUO-induced α-smooth muscle actin (α-SMA) and collagen type-I (collagen I) expression. (**a**) Kidney tissue lysates were subjected to immunoblot analysis with specific antibodies against α-SMA, collagen I, or α-tubulin. Expression levels of α-SMA (**b**) and collagen I (**c**) were quantified by densitometry and normalized with α-tubulin. Data are means ± s.e.m. ($n = 6$). (**d**) mRNA was extracted from kidney tissues of sham-operated or obstructed kidneys with/without S3I-201 administration and subjected to quantitative real-time reverse transcriptase PCR, as described in 'Materials and Methods'. mRNA expression levels of collagen I were indicated as fold induction over control (sham-operated mice treated with vehicle). Significant *P*-values reflecting differences are indicated over the bars (*$P < 0.05$; **$P < 0.01$).

S3I-201 decreases TGF-β1 and TβRII upregulation induced by obstructive injury

As increased expression of TGF-β1 and/or TGF-β receptors is found in almost all forms of kidney diseases with interstitial fibrosis,[4] we examined the effects of S3I-201 on the expression of TGF-β1 and three TGF-β receptors (type-I, type-II, and type-III) in obstructive kidneys using real-time PCR. Expression of TGF-β1 and TβRII was significantly increased in mice after obstructive injury, and administration of S3I-201 reduced their levels (Figure 8a and c). In contrast, expression levels of TβRI and TβRIII were not changed after UUO injury compared with control groups (Figure 8b and d). Interestingly, S3I-201 still reduced the basal level of TβRIII, whereas TβRI expression at the basal level was not affected by this treatment. Similarly, S3I-201 significantly decreased expression of TGF-β1, TβRII, and TβRIII, but not of TβRI in cultured renal interstitial fibroblasts (Supplementary Figure S2). In addition, we examined expression levels of TGF-β1 protein using the enzyme-linked immunosorbent assay (ELISA) assay and showed that S3I-201 also decreased expression of TGF-β1 protein in obstructive kidneys (Figure 8e). These data indicate that STAT3 mediates expression of both TGF-β1 and its receptor, TβRII, in the obstructive kidney.

Effect of S3I-201 on leukocyte infiltration and expression of proinflammatory cytokines after obstructive injury

We also examined the effect of S3I-201 on leukocyte infiltration and expression of multiple proinflammatory cytokines, including TNF-α, IL-1β, MCP-1, and ICAM-1. Staining of kidney sections with naphthol-AS-D-chloroacetate esterase showed prominent interstitial infiltration of neutrophils and monocytes after obstructive injury. S3I-201 treatment reduced leukocyte infiltration to basal levels

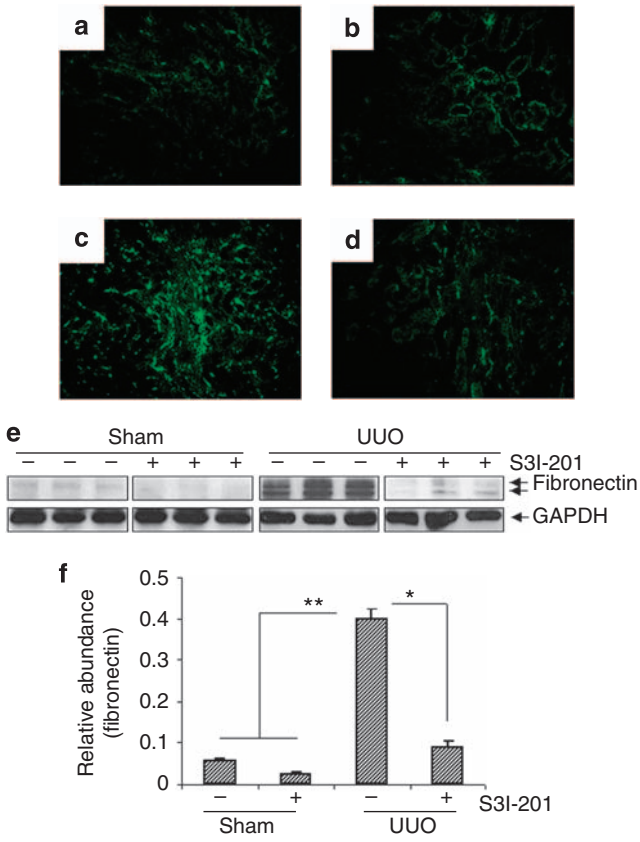

Figure 7 | Effect of S3I-201 on UUO-induced fibronectin expression. Photomicrographs illustrate fibronectin (green color) with immunofluorescent staining after various treatments: (**a**) sham with vehicle; (**b**) sham with S3I-201; (**c**) UUO with vehicle; and (**d**) UUO with S3I-201. Kidney tissue lysates were subjected to immunoblot analysis with specific antibodies against fibronectin (**e**). Expression levels of fibronectin in different groups were quantified by densitometry and normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (**f**). Data are means ± s.e.m. ($n = 6$). Significant P-values reflecting differences are indicated over the bars (*$P < 0.05$; **$P < 0.01$).

(Figure 9). The expression levels of TNF-α, IL-1β, ICAM-1, and MCP-1 were also increased in obstructive kidneys. S3I-201 treatment reduced expression of TNF-α, IL-1β, and ICAM-1 mRNA, but did not affect UUO injury-induced MCP-1 mRNA expression (Figure 10a–d). To verify these observations, we examined the effect of S3I-201 on the expression of TNF-α and IL-1β proteins using the ELISA assay. S3I-201 also significantly suppressed their expression at the protein levels (Figure 10e and f). These data suggest that STAT3 activity contributes to leukocyte infiltration and is selectively involved in the production of proinflammatory cytokines in UUO-induced renal fibrosis.

Effect of S3I-201 on proliferation and apoptosis of renal interstitial fibroblasts after obstructive injury

We further examined the effect of S3I-201 on apoptosis and proliferation of renal interstitial fibroblasts after UUO injury using the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay and by evaluating cells for phosphohistone (Ser-10) positivity, respectively. Phosphorylation of histone H3 at this site specifically mark cells undergoing mitosis.[33,34] As shown in Supplementary Figures S5 and S6, animals with UUO injury displayed a greater number of TUNEL-positive and p-histone H3-positive cells, predominantly in the renal interstitium. S3I-201 treatment resulted in increased apoptosis of interstitial cells and decreased their proliferation. S3I-201 administration did not induce apoptosis of renal tubular cells (Supplementary Figure S5b). These data illustrate that UUO injury-induced STAT3 activation contributes to survival and proliferation of interstitial cells, most probably renal interstitial fibroblasts.

DISCUSSION

Using S3I-201, a selective inhibitor of STAT3,[24] we provided evidence that STAT3 activity is necessary for the activation and proliferation of renal interstitial fibroblasts and the destruction of the kidney. We have shown that S3I-201 treatment inhibits the expression of α-SMA and interstitial ECM proteins, decreases proliferation of renal interstitial fibroblasts, attenuates progression of renal fibrosis, and suppresses infiltration of leukocytes and expression of multiple inflammatory cytokines in obstructive nephropathy.

In this study, we observed that UUO injury induces STAT3 phosphorylation at tyrosine 705 in the fibrotic mouse kidney, as early as day 1, reaching a maximum level at day 7, and remaining elevated for 14 days after UUO injury. Further immunohistochemical analysis localized p-STAT3 in most tubulointerstitial cells expressing α-SMA (Figure 3). These observations correlate with the findings of Kuratsune *et al.*,[23] indicating that p-STAT3 preferentially colocalizes with α-SMA in the tubulointerstitium in the rat kidney. The marked and persistent increase in activated STAT3 in the interstitial cells of fibrotic kidney suggests the importance of STAT3 in mediating activation of renal interstitial fibroblasts and progression of renal fibrosis following UUO injury. Indeed, inactivation of STAT3 by S3I-201 significantly attenuated the pathogenesis of renal fibrosis as described above. Furthermore, inhibition of STAT3 with either S3I-201 or its specific siRNA blocked the expression of α-SMA and fibronectin in cultured renal interstitial fibroblasts (Figures 1a–d and 2a–b).

The STAT3 activation can be induced by multiple growth factors and cytokines such as TGF-β1,[18] platelet-derived growth factor,[19,20] and IL-6,[21,22] that contribute to the development of renal fibrosis,[35,36] and increased expression of activated STAT3 was detected in several kidney diseases associated with progressive fibrosis, including glomerulonephritis and diabetic nephropathy.[37,38] Thus, we suggest that STAT3 may act as a common mediator in chronic renal damage. Consistent with our observations on the profibrotic role of STAT3 in the kidney, hyperactivated STAT3 has also been reported to be associated with chemically induced liver fibrosis[15] and scar formation after spinal cord injury.[39]

Inhibition of STAT3 activation may elicit an antifibrotic effect by multiple mechanisms. As TGF-β1 signaling has a central role in a variety of fibrogenic processes such as

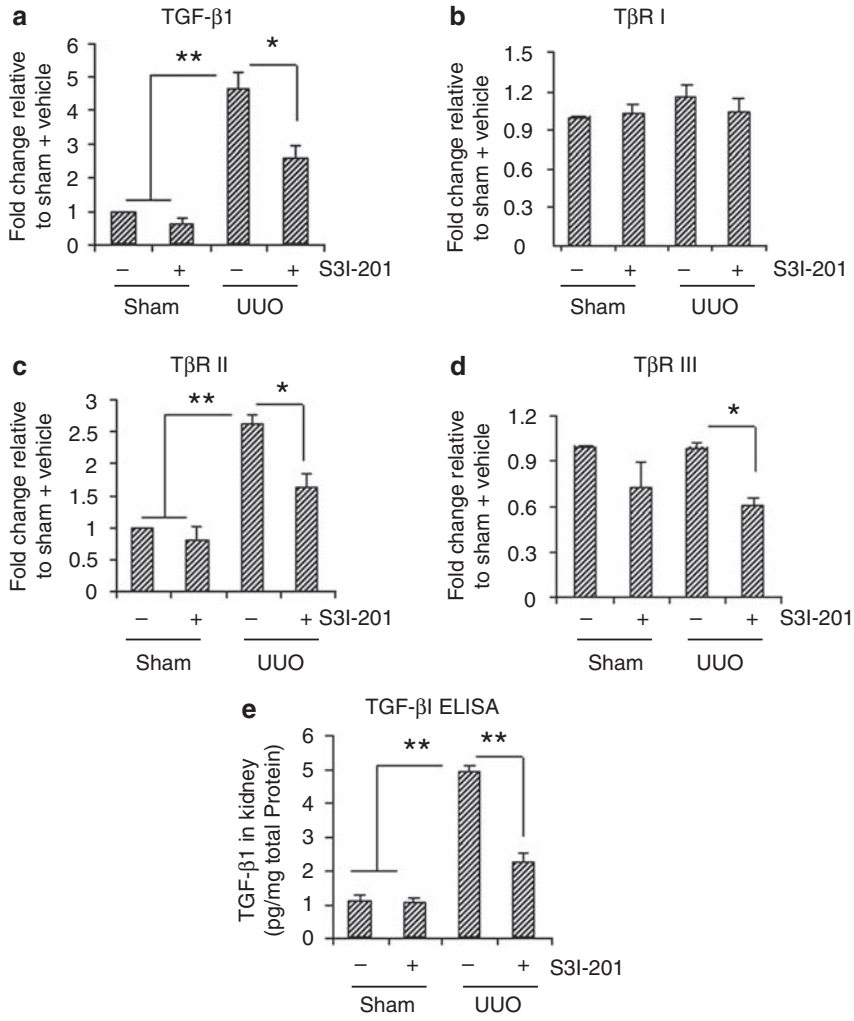

Figure 8 | Effect of S3I-201 on mRNA and protein expression of transforming growth factor-β1 (TGF-β1) and mRNA expression of TGF-β receptors. mRNA was extracted from kidney tissues of sham-operated or obstructed kidneys with/without S3I-201 administration and subjected to quantitative real-time reverse transcriptase PCR, as described in 'Materials and Methods'. mRNA expression levels of TGF-β1 (**a**), TGF-β receptor type-I (TβR I) (**b**), type-II (TβR II) (**c**) and type-III (TβR III) (**d**) were indicated as fold induction over control (sham-operated mice treated with vehicle). Protein expression levels of TGF-β1 in each group were measured by the enzyme-linked immunosorbent assay (ELISA) (**e**). Data are mean ± s.e.m. ($n = 6$). Significant *P*-values reflecting differences are indicated over the bars (*$P < 0.05$; **$P < 0.01$).

fibroblast activation, and STAT3 is a known transcription factor,[4–6,16] we examined the effect of S3I-201 on the expression of TGF-β1 and TGF-β receptors in the kidney after UUO. Our results clearly indicated that expression levels of TGF-β1 and TβRII mRNA were upregulated in the obstructed kidney, and S3I-201 treatment suppressed their expression (Figure 8a and c). Furthermore, treatment with S3I-201 also suppressed the expression of TGF-β1 protein in the obstructed kidney (Figure 8e). Thus, we suggest that S3I-201-elicited inhibition of myofibroblast activation is likely mediated by antagonizing TGF-β1 signaling through suppression of TGF-β1 and TβRII expression. Two potential STAT3-binding sites have been reported to exist in the promoter region of TGF-β1 and STAT3 activates promoter activity *in vitro*, suggesting that STAT3 may directly regulate TGF-β1 gene expression.[15]

Progression of renal fibrosis is closely associated with the inflammatory response. We observed that administration of S3I-201 inhibited infiltration of neutrophils and monocytes and suppressed the expression of TNF-α, IL-1β, and ICAM-1, but not MCP-1, in the obstructed kidney. These results suggest that inhibition of inflammatory responses may be another mechanism by which S3I-201 attenuates renal fibrosis. As MCP-1 is a chemokine for monocytes, we also suggest that STAT3 may induce monocyte infiltration into sites of injury in the kidney through a mechanism that is not involved in MCP-1 expression. In addition to MCP-1, other chemokines such as RANTES, monocyte inflammatory protein-1α, and macrophage inflammatory protein are also associated with the progression of renal fibrosis.[40–42] It will be interesting to examine whether STAT3 diminishes monocyte infiltration by reducing expression of those chemokines.

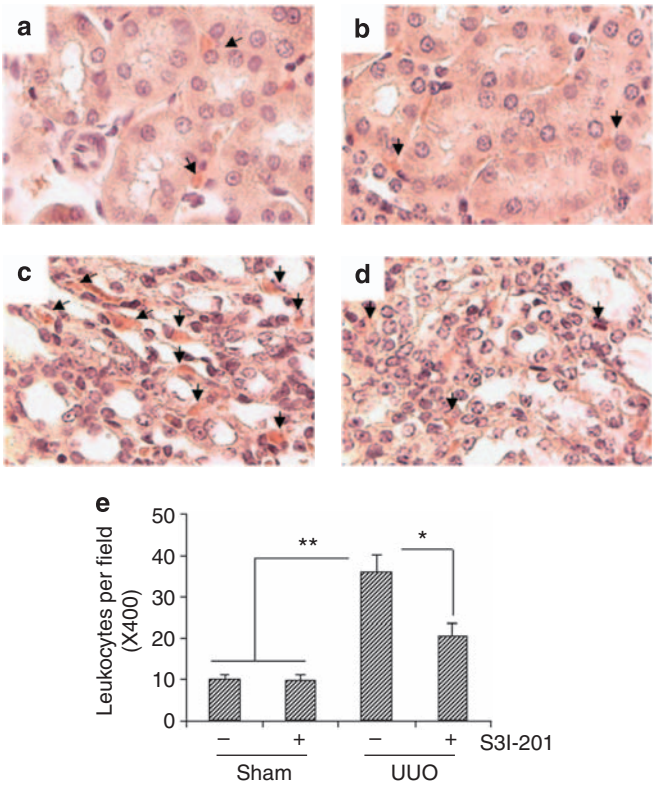

Figure 9 | Effect of S3I-201 on infiltration of leukocytes. Kidney sections were evaluated for infiltration of neutrophils and monocytes with naphthol-AS-D-chloroacetate esterase staining. Photomicrographs illustrate infiltration of neutrophils and monocytes (red color directed by arrows) in different groups: (**a**) sham with vehicle; (**b**) sham with S3I-201; (**c**) UUO with vehicle; and (**d**) UUO with S3I-201. (**e**) Infiltrated neutrophils and monocytes were counted in three random fields of each sample, and 18 fields (1 × 200) were analyzed for each condition. Data are mean ± s.e.m. ($n = 6$). Significant P-values reflecting differences are indicated over the bars (*$P < 0.05$; **$P < 0.01$).

S3I-201 is a selective and potent inhibitor of constitutive activation of STAT3 DNA-binding and STAT3-mediated gene expression.[24] Unlike other commonly used STAT3 inhibitors such as AG490, which act on Janus kinases, the upstream activators of STAT3, and other STAT isoforms,[43–45] S3I-201 inhibits STAT3–STAT3 complex formation and STAT3 DNA-binding and transcriptional activities in cells that contain constitutively activated STAT3.[24] By binding to STAT3, S3I-201 may also prevent STAT3 protein from binding to the pTyr motifs of the receptor tyrosine kinases and subsequently block *de novo* phosphorylation by tyrosine kinases.[24] These properties are very important for the treatment of renal fibrosis, as recent studies using knockout mice showed that in contrast to STAT3, STAT1 and STAT5 have a role in abrogating liver and lung fibrosis after injury.[27,28] Our results showed that S3I-201, at the concentration that blocked STAT3 phosphorylation, increased expression of p-STAT1 but did not affect that of p-STAT5 in NRK-49F cells (Supplementary Figure S1), suggesting that the resultant increased expression of p-STAT1 and preservation of p-STAT5 in renal interstitial fibroblasts after STAT3 inhibition may also be important for attenuating the progression of tissue fibrosis.

The expanded population of fibroblasts in the diseased kidney could result from an increase in proliferation and/or a decrease in cell death.[35,46] Our results showed that administration of S3I-201 inhibited proliferation and promoted death in renal interstitial cells at day 7 after UUO injury (Supplementary Figures S5 and S6). In view of the fact that most of those cells are expressed with both α-SMA and STAT3, as shown in Figure 3d, we suggest that STAT3 may also mediate the progression of renal fibrosis through increasing survival and proliferation of renal interstitial fibroblasts. Consequently, inhibition of STAT3-mediated renal interstitial fibroblast proliferation and survival would be additional mechanisms for alleviating the development of renal interstitial fibrosis.

In summary, our results clearly show that inhibition of STAT3 with S3I-201 can suppress activation and proliferation, and induce death of renal interstitial fibroblasts. Furthermore, S3I-301 treatment inhibits excessive deposition of ECM and transcriptional expression of TGF-β1, TβRII, and several proinflammatory cytokines that are associated with progressive kidney diseases. Therefore, inhibition of the STAT3 signaling pathway by S3I-201 may provide a novel approach to prevent the development of nephropathy and attenuate the progression of renal fibrosis.

MATERIALS AND METHODS

Chemicals and antibodies

S3I-201 was purchased from Calbiochem (La Jolla, CA, USA). Antibodies to STAT3, p-STAT1, p-STAT3, p-STAT5, and β-catenin were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-fibronectin was purchased from BD transduction Laboratory (Lexington, KY, USA). Primers were synthesized from Invitrogen (Carlsbad, CA, USA). STAT3 siRNA and silencer negative control siRNA were purchased from Invitrogen. Antibodies to α-SMA and α-tubulin, the naphthol-AS-D-chloroacetate esterase kit, and all other chemicals and blocks were obtained from Sigma (St Louis, MO, USA).

Cell culture and treatments

NRK-49F cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 10% fetal bovine serum, 0.5% penicillin and streptomycin in an atmosphere of 5% CO_2 and 95% air at 37 °C.

Transfection of siRNA into cells

The siRNA oligonucleotides targeted specifically to STAT3 (200 pmol) were transfected into NRK-49F cells (1×10^6) using the Amaxa Cell Line Nucleofector Kit T (Lonza Cologne AG, Cologne, Germany) and the Amaxa Nucleofector device according to the manufacturer's instructions (Amaxa Biosystem, Gaithersburg, MD, USA).

Unilateral ureteral obstruction (UUO) model and S3I-201 treatment

The UUO model was established in male C57 black mice that weighed 20–25 g (Jackson Laboratory, Bar Harbor, ME, USA), as

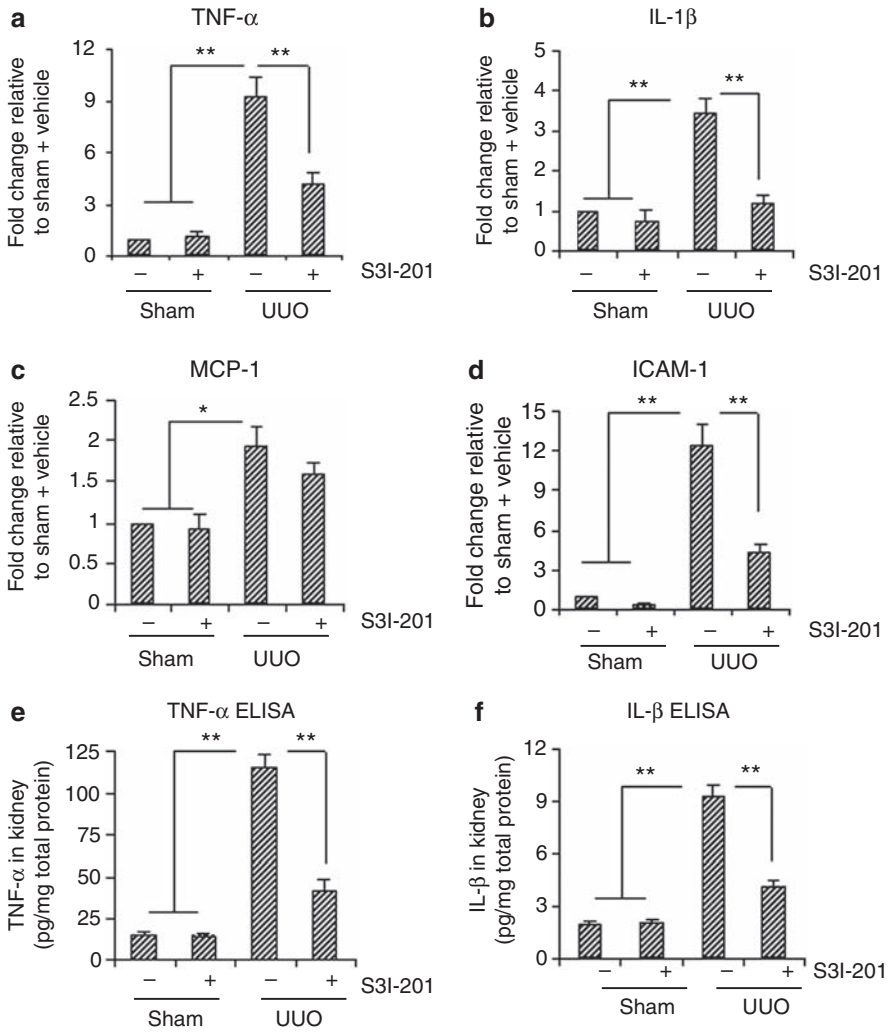

Figure 10 | Effect of S3I-201 on the expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), monocyte chemotactic protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1). mRNA extracted from kidney tissues were subjected to quantitative real-time reverse transcriptase PCR as described in 'Materials and Methods'. mRNA expression levels of TNF-α (**a**), IL-1β (**b**), MCP-1 (**c**), and ICAM-1 (**d**) were indicated as fold induction over control (sham with vehicle). Protein expression levels of TNF-α (**e**) and IL-1β (**f**) in each group were measured by the enzyme-linked immunosorbent assay (ELISA). Data are means ± s.e.m. ($n = 6$). Significant *P*-values reflecting differences are indicated over the bars (*$P < 0.05$; **$P < 0.01$).

described previously.[47] The contralateral kidneys were used as controls. To examine the efficacy of S3I-201 in renal fibrosis after UUO injury, S3I-201 (10 mg/kg) in 50 µl of dimethyl sulfoxide was intraperitoneally administered to mice immediately after ureter ligation and then given daily for 6 or 13 days. Mice were randomized into four groups with 6–7 mice in each group as follows: (1) sham injury with dimethyl sulfoxide as vehicle; (2) sham injury with S3I-201; (3) UUO injury with dimethyl sulfoxide; and (4) UUO injury with S3I-201.

Western blot analysis

Immunoblot analysis for NRK-49F cells and tissue samples was carried out according to our previous protocols.[47]

Sirius red and immunofluorescent staining

Sirius red and immunofluorescent staining was based on the procedure described in our previous studies.[47]

Quantitative real-time PCR

Real-time quantitative reverse transcriptase PCR amplifications were performed in 20 µl reactions (Table 1). The amplification protocol was as follows: denaturation for 10 min and enzyme activation at 95 °C, 40 cycles of 95 °C × 10 s, 59 °C × 30 s, and 72 °C × 20 s, followed by one-step annealing at 72 °C for 5 min. Relative mRNA abundance was determined from the ratios of specific mRNA to 18S ribosomal RNA measured in the same samples, and fold change was calculated relative to the group of sham-operated mice with vehicle treatment.

ELISA

Protein levels of TGF-β1, TNF-α, and IL-1β in kidney homogenates were determined using specific sandwich enzyme immunometric assay kits, ELISA Ready-SET-Go (eBioscience San Diego, CA, USA), as described previously.[48] The results were normalized by total protein content in kidney homogenates.

Table 1 | Forward and reverse primers used for quantitative real-time reverse transcriptase PCR

Gene	Type	Sequences (5′→3′)
18S rRNA	Forward	ACCGCGGTTCTATTTTGTTG
	Reverse	CCCTCTTAATCATGGCCTCA
TGF-β1	Forward	TGAGTGGCTGTCTTTTGACG
	Reverse	AGCCCTGTATTCCGTCTCCT
TGF-β R I	Forward	CTGGGCAAAGATTAGGGTGA
	Reverse	GCTGGCCACTACTTCTGAGG
TGF-β R II	Forward	ACAGGGGTCACACAGGACTC
	Reverse	GCTCATTCCCTGCTCTCATC
TGF-β R III	Forward	ATGGTCCCCTGTGTAGCTTG
	Reverse	GCGGAGTATCAGGAGTCAGC
TNF-α	Forward	TAGCCAGGAGGGAGAACAGA
	Reverse	TTTTCTGGAGGGAGATGTGG
IL-1β	Forward	CCCAAGCAATACCCAAAGAA
	Reverse	GCTTGTGCTCTGCTTGTGAG
MCP-1	Forward	AGCACCAGCCAACTCTCACT
	Reverse	CGTTAACTGCATCTGGCTGA
ICAM-1	Forward	CTTCCAGCTACCATCCCAAA
	Reverse	CTTCAGAGGCAGGAAACAGG
Collagen I	Forward	GAGCGGAGAGTACTGGATCG
	Reverse	GTTCGGGCTGAGTACCAGT

Abbreviations: Collagen I, collagen type-I; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; MCP-1, monocyte chemotactic protein-1; TGF-β1, transforming growth factor-β1; TGF-β R, transforming growth factor receptor-β; TNF, tumor necrosis factor.

In situ TUNEL assays

The TUNEL staining was used to detect DNA strand breaks using a detection kit, according to the instructions provided by the manufacturer. The number of TUNEL-positive nuclei per field was evaluated in five fields per section and five sections per kidney.

Statistical analysis

All the experiments were conducted at least three times. Data depicted in graphs represent the mean ± s.e.m. for each group. Multiple means were compared using Tukey's test. The differences between two groups were determined by Student's *t*-test. Statistical significant difference between mean values was marked in each graph. $P<0.05$ is considered to be significant.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Effect of STAT3 inhibition on the expression and phosphorylation of STAT1 and STAT5.
Figure S2. Effect of S3I-201 on the mRNA expression of TGF-β1, TβR1, TβR II and TβR III in cultured renal interstitial fibroblasts.
Figure S3. Effect of S3I-201 on the phosphorylation of STAT3 and expression of collagen type I and α-SMA on day 14 after UUO injury.
Figure S4. Effect of S3I-201 on the expression of β-catenin and Snail on day 7 after UUO injury.
Figure S5. Effect of S3I-201 on apoptosis after UUO injury.
Figure S6. Effect of S3I-201 on proliferation of interstitial cells after UUO injury.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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