Conditional Knockout of Src Homology 2 Domain-containing Protein Tyrosine Phosphatase-2 in Myeloid Cells Attenuates Renal Fibrosis after Unilateral Ureter Obstruction

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Abstract

Background: Src homology 2 domain-containing protein tyrosine phosphatase-2 (SHP-2) is a kind of intracellular protein tyrosine phosphatase. Studies have revealed its roles in various disease, however, whether SHP-2 involves in renal fibrosis remains unclear. The aim of this study was to explore the roles of myeloid cells SHP-2 in renal interstitial fibrosis.

Methods: Myeloid cells *SHP-2* gene was conditionally knocked-out (CKO) in mice using loxP-Cre system, and renal interstitial fibrosis was induced by unilateral ureter obstruction (UUO). The total collagen deposition in the renal interstitium was assessed using picrosirius red stain. F4/80 immunostaing was used to evaluate macrophage infiltration in renal tubular interstitium. Quantitative real-time polymerase chain reaction and enzyme linked immunosorbent assay were used to analyze the production of cytokines in the kidney. Transferase-mediated dUTP nick-end labeling stain was used to assess the apoptotic renal tubular epithelial cells.

Results: Src homology 2 domain-containing protein tyrosine phosphatase-2 gene CKO in myeloid cells significantly reduced collagen deposition in the renal interstitium after UUO. Macrophage infiltration was evidently decreased in renal tubular interstitium of *SHP-2* CKO mice. Meanwhile, the production of pro-inflammatory cytokines was significantly suppressed in *SHP-2* CKO mice. However, no significant difference was observed in the number of apoptotic renal tubular epithelial cells between wild-type and SHP-2 CKO mice.

Conclusions: Our observations suggested that SHP-2 in myeloid cells plays a pivotal role in the pathogenesis of renal fibrosis, and that silencing of *SHP-2* gene in myeloid cells may protect renal from inflammatory damage and prevent renal fibrosis after renal injury.

Key words: Fibrosis; Inflammation; Myeloid Cells; Obstructive Nephropathy; Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase-2

Introduction

It is estimated that about 14% of American adults are suffering from chronic kidney disease, and many of them eventually progress to renal failure.[1] In this process, renal fibrosis is usually the final common pathway leading to progressive loss of renal function. As the predominant infiltrating cell type in response to kidney injuries, myeloid cells play a pivotal role in the development of renal fibrosis.[2] Activated macrophages secrete several pro-inflammatory cytokines and growth factors, which induce renal tubular epithelial cell apoptosis, activate fibroblast, stimulate extracellular matrix (ECM) production, and finally result in renal fibrosis.[3,4] Nevertheless, the mechanism how myeloid cells regulate renal fibrosis remains unclear.

The Src homology 2 domain-containing protein tyrosine phosphatase-2 (SHP-2), characterized by two N-terminal Src homology 2 domains, a central catalytic domain and a C-terminal protein tyrosine phosphatase (PTP) domain, is an evolutionarily conserved intracellular PTP.[5] It has been found that SHP-2 acts as a key regulator in various signal transduction pathways[6] and is involved in many diseases, such as Noonan syndrome,[7] leukemia,[8] prostate cancer,[9] and so on. Recent studies also revealed that the SHP-2 regulates the production of pro-inflammatory cytokines and plays important roles in several inflammatory diseases. However, the role of SHP-2 in the pathogenesis of renal fibrosis has not been studied so far.

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In this work, we introduced a *SHP-2* conditional knockout (CKO) mouse, and studied the function of myeloid cells SHP-2 in the pathogenesis of renal fibrosis under unilateral ureter obstruction (UUO).

Methods

Animal preparation

C57/BL6 mice with floxed exon 4 of *SHP-2* (SHP-$2^{flox/+}Cre^{-/-}$) were mated with mice expressing Cre recombinase from the endogenous lyzs locus in myeloid cells (SHP-$2^{+/+}Cre^{+/-}$) (Jackson laboratory). SHP-$2^{flox/+}Cre^{+/-}$ mice were selected from the offspring and crossed with SHP-$2^{flox/+}Cre^{-/-}$ mice to generate *SHP-2* CKO mice (SHP-$2^{flox/flox}Cre^{+/-}$). For genotyping, DNA was isolated from tails using Mouse Tail DNA Mini Kit (Foregene, Chengdu, China) following the manufacturer's instructions. DNAs were amplified by polymerase chain reaction (PCR) using Takara PCR Amplification Kit (Takara, Dalian, China). Primers for the Cre gene were: 5'-ATGCCCAAGAAGAAGAGGAAGGT-3' (forward), 5'-GAAATCAGTGCGTTCGAACGCTAGA-3' (reverse), Primers for lox gene were 5'-ACGTCATGATCCGCTG TCAG-3' (forward), 5'-ATGGGAGGGACA GTGCAGTG-3' (reverse). DNA agarose gel electrophoresis was performed to identify the target genes. In this study, SHP-$2^{flox/flox}Cre^{-/-}$ mice were used as controls. Our animal experiment protocols were approved by the Ethics Committee of Second Military Medical University.

Surgical procedure

Unilateral ureter obstruction or shame operation was performed under general anesthesia using pentobarbital (60 mg/kg, i.p.). An abdominal median incision was made, and the left ureter was visualized. In UUO group, the ureter was permanently ligated using 9–0 silk sutures at the pyeloureteric junction. While in sham-operation group, the ureter was exposed without ligation. The abdomen was then closed in 2 layers using 6–0 silk sutures. After surgery, the mouse was housed under controlled environmental conditions with sufficient food and water. On day 7, the kidney was harvested, and the mouse was killed by cervical dislocation.

Histological analysis

Formalin-fixed paraffin-embedded kidneys were cut into 4-μm sections and stained with picrosirius red to evaluate interstitial fibrosis. The sections were scanned under a light microscope and photographed at a magnification of ×200. For each section, 10 randomly chosen fields in the cortex of the kidney were captured. The integrated optical density of the collagen area was determined using Image-Pro Plus software version 5.1 (Media Cybernetics, USA) to assess the degree of fibrosis.

Immunohistochemistry

The 4-μm-thick slide-mounted sections were deparaffinized in xylene and rehydrated with graded ethanols. The slides were incubated in 1:50 dilution of rat anti-mouse F4/80 monoclonal antibody (Abcam, Cambridge, MA, USA) overnight at 4°C. Then, the slides were incubated with 1:200 diluted biotinylated goat anti-rat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min, and subsequently reacted with streptavidin-peroxidase conjugate and 3', 3'-diaminobenzidine. The slides were examined under light microscope at a magnification of ×200 and macrophages positive for F4/80 were counted in 10 microscopic fields of the cortex. Results were expressed as the number of macrophages per high power field (HPF).

Terminal transferase-mediated dUTP nick-end labeling assay

Apoptotic cells were examined by transferase-mediated dUTP nick-end labeling (TUNEL) assay using an *in situ* cell death detection kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. All slides were counterstained with hematoxylin. The slides were observed under a light microscope at a magnification of ×200 and the number of apoptotic cells was counted in 10 fields of the cortex. Results were reported as TUNEL-positive cells per HPF.

Quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using ReverTra Ace-α–(Toyobo, Shanghai, China) following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed with Stepone™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (Takara, Dalian, China) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The following primers were used: GAPDH: 5'-TGACCACAGTCCATGCCATC-3' (forward), 5'-GACGGACACATTGGGGGTAG-3' (reverse); Exon 4: 5'-CTGAACTGTGCAG ACCCTACCTC-3' (forward), 5'-TTTGGACTTGCCGTCGTTG-3' (reverse); Exon 8, 9: 5'-GCTCGTGACCAAGCAACCG-3' (forward), 5'-ACAGCAGTCTCCACATTC CCAC-3' (reverse); tumor necrosis factor-α (TNF-α): 5'-AAGCCTGTAGCCCACGTCGTA-3' (forward), 5'-GGCACCACTAGTTGGTTGTCTTTG-3' (reverse); transforming growth factor-β (TGF-β): 5'-GGCCAGATCC TGTCCAAGC-3' (forward), 5'-GTGGGTTTCCACCATT AGCAC-3' (reverse); interleukin-1β (IL-1β): 5'-GGT GTGTGACGTTCCCATTAGAC-3' (forward), 5'-CATGGAGAATA TCACTTGTTGGTTGA-3 (reverse); IL-6: 5'-ACAACCACGGCCTTCCCTACTT-3' (forward), 5'-CACGATTTCCCAGAGAACATGTG-3'(reverse). The RNA expression levels of TNF-α, TGF-β, IL-1β and IL-6 were normalized to that of GAPDH.

Enzyme linked immunosorbent assay

Kidney tissues were homogenized in M-PER tissue protein extraction reagent (Thermo Scientific, Waltham, MA, USA) supplemented with protease inhibitor mixture (Calbiochem, Darmstadt, Germany) at 4°C for 30 min, and then centrifuged at 4000 g for 15 min, the supernatants were collected. The protein concentrations were measured

using BCA assay (Thermo Scientific, Waltham, MA, USA) and equalized to 4 μg/μl using extraction reagent. The TNF-α, TGF-β, IL-1β and IL-6 levels of the kidney were determined by commercial enzyme linked immunosorbent assay (ELISA) kits (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer's instructions.

Statistical analysis

Data were shown as mean ± standard deviation (SD); comparisons between groups were performed with Student's *t*-test or one-way analysis of variance (ANOVA) followed by SNK-*q* test for multiple comparisons using SPSS version 19.0 (IBM SPSS Statistics, Armonk, NY, USA). $P < 0.05$ was considered as statistically significant.

Results

Identification of Src homology 2 domain-containing protein tyrosine phosphatase-2 gene conditionally knocked-out in myeloid cells

To generate *SPH-2* CKO mice, mice with loxP flanked *SPH-2* allele were crossed with transgenic mice expressing Cre recombinase under the control of lyzs promoter. PCR and DNA agarose gel electrophoresis were performed to identify SHP-2$^{flox/flox}$Cre$^{+/-}$ (CKO) and SHP-2$^{flox/flox}$Cre$^{-/-}$ (control) mice [Figure 1a]. Abdominal macrophages from *SPH-2* CKO and control mice were collected and total RNA was extracted. qRT-PCR showed that SHP-2 mRNA expression was reduced by 68% in *SPH-2* CKO mice compared to control mice [Figure 1b].

Src homology 2 domain-containing protein tyrosine phosphatase-2 gene conditionally knocked-out attenuates renal fibrosis

To determine the role of SHP-2 in renal fibrosis, SHP-2$^{flox/flox}$Cre$^{+/-}$ and SHP-2$^{flox/flox}$Cre$^{-/-}$ mice were subjected to UUO surgery and sham-operation, respectively. The degrees of collagen deposition in the tubulointerstitium were studied between both groups. Seven days after operation, kidneys were harvested and picrosirius red stain was performed to evaluate collagen deposition. Both genotypes exhibited enhanced collagen deposition in UUO group compared sham-operation group. In UUO group, control mice displayed significantly more collagen deposition than *SHP-2* CKO mice, suggesting that *SHP-2* gene knockout in myeloid cells may attenuate renal fibrosis under mechanical damage [Figure 2].

Src homology 2 domain-containing protein tyrosine phosphatase-2 gene conditionally knocked-out results in reduced macrophage infiltration after unilateral ureter obstruction

Myeloid cell infiltration, including macrophage, monocyte and dendritic cells, is the key event in the pathogenesis of renal fibrosis. F4/80 immunohistochemistry stain was performed to identify interstitial macrophages. UUO increased the number of infiltrated macrophages in both genotypes compared to sham-operation. When compared to control mice, the number of macrophages on day 7 after UUO was significantly smaller in *SPH-2* CKO mice [Figure 3].

Src homology 2 domain-containing protein tyrosine phosphatase-2 gene conditionally knocked-out prevents pro-inflammatory cytokines production after unilateral ureter obstruction

Several pro-inflammatory cytokines were up-regulated and played important roles in the process of renal fibrosis. To test whether *SPH-2* CKO may affect pro-inflammatory cytokine production, qRT-PCR and ELISA were performed. qRT-PCR revealed significantly decreased production of TNF-α, TGF-β, IL-1β and IL-6 mRNA in *SPH-2* CKO mice at day 7 after UUO compared to sham-operation mice [Figure 4a]. At protein level, ELISA revealed the similar results [Figure 4b].

Src homology 2 domain-containing protein tyrosine phosphatase-2 gene conditionally knocked-out does not affect renal tubular epithelial cells apoptosis after unilateral ureter obstruction

Transferase-mediated dUTP nick-end labeling analysis was performed to test renal tubular epithelial cells apoptosis. Despite increased apoptotic cells after UUO in both genotypes

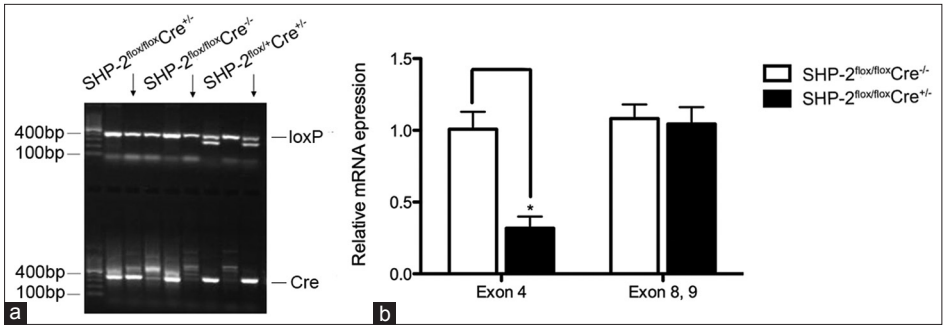

Figure 1: Myeloid cells specific deletion of the Src homology 2 domain-containing protein tyrosine phosphatase-2 (*SHP-2*) gene. (a) DNA agarose gel electrophoresis. SHP-2$^{flox/flox}$ represents a bright band at about 400 bp, SHP-2$^{flox/-}$ represents a band at about 400 bp and another band at about 300 bp, Cre$^{+/-}$ represents a bright band at about 400 bp; (b) SHP-2 is down-regulated in conditionally knocked-out (CKO) macrophages. Exon 4 of *SHP-2* gene was floxed, thus in SPH-2 CKO mice, exon 4 was deleted. The expression of SHP-2 in abdominal macrophages was measured by quantitative real-time polymerase chain reaction. ($n = 5$, $^*P < 0.01$, vs. control mice).

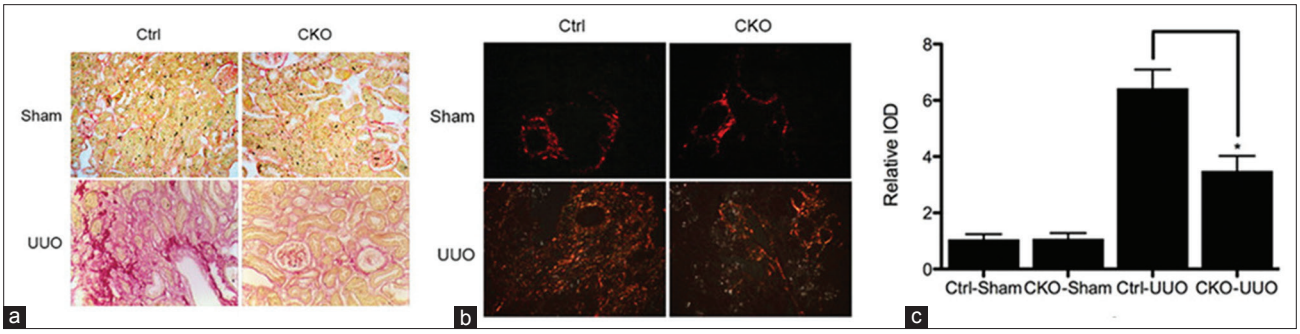

Figure 2: Histologic evaluations of kidneys from mice subjected to unilateral ureter obstruction or sham-operation; (a) Picrosirius red stain, light microscopic images (original magnification, ×200); (b) Picrosirius red stain, polarization microscopic images (original magnification, ×200); (c) Integrated optical density of collagen areas were calculated by Image-Pro Plus software under light microscopion ($n = 7$, $^*P < 0.01$). Ctrl: control mice; CKO: SHP-2 conditional Knockout mice; Sham: sham-operation; UUO: unilateral ureter obstruction; Ctrl-Sham: control mice with sham-operation; CKO-Sham: SHP-2 conditional Knockout mice with sham-operation; Ctrl-UUO: control mice with unilateral ureter obstruction; CKO-UUO: SHP-2 conditional Knockout mice with unilateral ureter obstruction; IOD: integrated optical density.

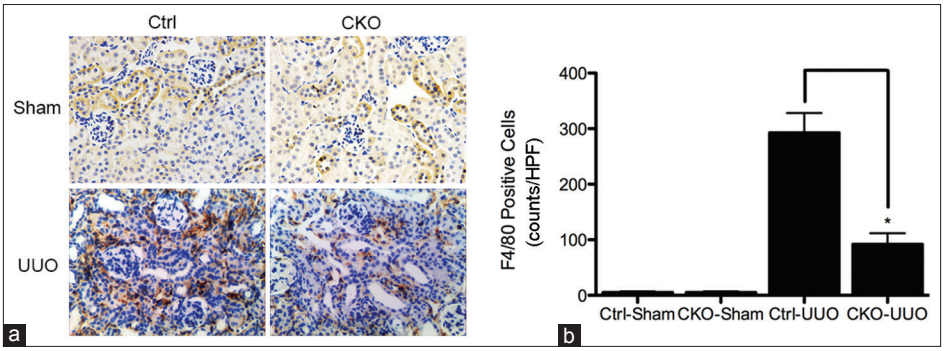

Figure 3: Renal tubular interstitium macrophage infiltration after unilateral ureter obstruction or sham-operation. (a) Immunostaining for F4/80 in the kidney (original magnification, ×200); (b) The number of F4/80-positive cells (counts/HPF) in the sections. ($n = 7$, $^*P < 0.01$). Ctrl: control mice; CKO: SHP-2 conditional Knockout mice; Sham: sham-operation; UUO: unilateral ureter obstruction; Ctrl-Sham: control mice with sham-operation; CKO-Sham: SHP-2 conditional Knockout mice with sham-operation; Ctrl-UUO: control mice with unilateral ureter obstruction; CKO-UUO: SHP-2 conditional Knockout mice with unilateral ureter obstruction.

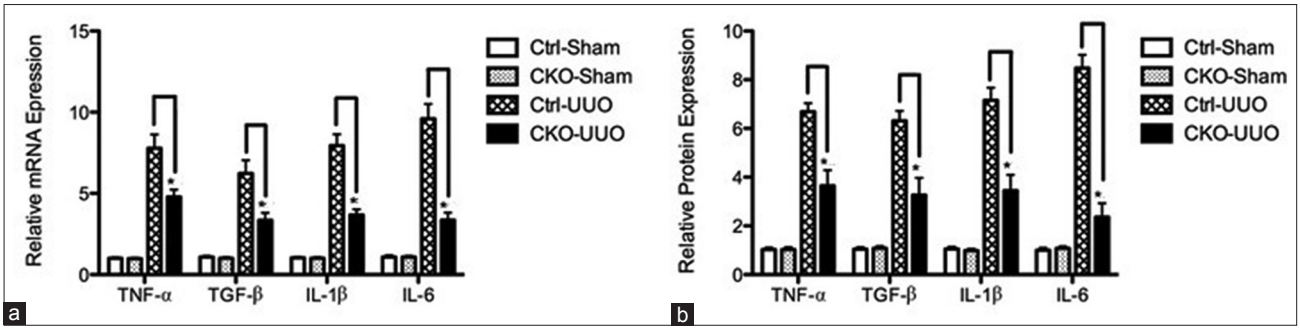

Figure 4: Src homology 2 domain-containing protein tyrosine phosphatase-2 conditionally knocked-out suppresses pro-inflammatory cytokine production after unilateral ureter obstruction (UUO). The kidneys were harvested 7 days after UUO, total RNA was extracted for quantitative real-time polymerase chain reaction (qRT-PCR), and total protein was prepared for enzyme linked immunosorbent assay (ELISA). (a) Pro-inflammatory cytokine mRNA expression, determined by qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase were used as internal control. ($n = 7$, $^*P < 0.01$, vs. control-UUO); (b) Pro-inflammatory cytokine protein expression, determined by ELISA. ($n = 7$, $^*P < 0.01$, vs. control-UUO). Ctrl: control mice; CKO: SHP-2 conditional Knockout mice; Sham: sham-operation; UUO: unilateral ureter obstruction; Ctrl-Sham: control mice with sham-operation; CKO-Sham: SHP-2 conditional Knockout mice with sham-operation; Ctrl-UUO: control mice with unilateral ureter obstruction; CKO-UUO: SHP-2 conditional Knockout mice with unilateral ureter obstruction.

compared to sham-operation group, no significant difference was observed between *SHP-2* CKO mice and control mice after UUO, suggesting *SHP-2* CKO does not affect renal tubular epithelial cells apoptosis after UUO [Figure 5].

Discussion

Renal fibrosis could be regarded as a result of imbalanced wound healing process in response to various renal injuries.[10] Studies have suggested that macrophages are critical players

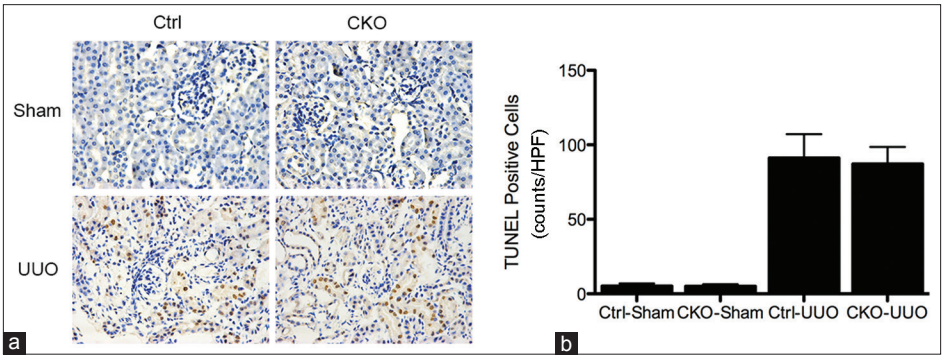

Figure 5: Src homology 2 domain-containing protein tyrosine phosphatase-2 conditionally knocked-out does not affect renal tubular epithelial cells apoptosis after unilateral ureter obstruction. (a) Transferase-mediated dUTP nick-end labeling (TUNEL) staining detecting apoptotic cells (original magnification, ×200); (b) Quantification of TUNEL-positive cells (counts/HPF) in the sections. ($n = 7$). Ctrl: control mice; CKO: SHP-2 conditional Knockout mice, Sham: sham-operation; UUO: unilateral ureter obstruction; Ctrl-Sham: control mice with sham-operation; CKO-Sham: SHP-2 conditional Knockout mice with sham-operation; Ctrl-UUO: control mice with unilateral ureter obstruction; CKO-UUO: SHP-2 conditional Knockout mice with unilateral ureter obstruction.

involving in many forms of renal injuries, repairs, and contributing to renal fibrosis.[11-14] In obstructive nephropathy, hydronephrosis causes elevated intrapelvis pressure and renal hemodynamic changes, which result in ischemic oxidative stress and activation of renin-angiotensin system.[15] With the production of reactive oxygen species and nuclear factor-κB (NF-κB), macrophages were enriched in the renal interstitium.[16,17] Activated macrophages in the injured kidney produce TNF-α and TGF-β, which are central mediators for renal fibrosis. TNF-α induces apoptosis of renal epithelial cells.[3] TGF-β is a pleiotropic cytokine, it can promote renal epithelial and nonepithelial cells undergo epithelial-mesenchymal transition, becoming myofibroblasts and contribute to ECM deposition.[18] Besides, TGF-β can also induce loss of PTEN,[19] up-regulation of Notch[20] and down-regulation of Klotho,[21] and thus promote renal fibrosis. This study also proved that reduced macrophages infiltration as well as the production of pro-inflammatory cytokines prevented renal fibrosis.

Recent studies have revealed the indispensible roles of SHP-2 in various signal transduction pathways and cellular activities. An *et al*.[22] used small interfering RNA technique targeting macrophage *SHP-2* gene, and found that SHP-2 negatively regulate MyD88-independent pro-inflammatory cytokine and type I interferon production through directly binding to the kinase domain of TANK binding kinase. You *et al*.[23] found that SHP-2 positively regulates IL-1α or TNF-α induced IL-6 production by modulating NF-κB pathway in a mitogen-activated protein kinase (MAPK)-independent manner. SHP-2 can also mediate ERK activation in several growth factor signaling, which might be necessary for IL-1 to associate with focal adhesion.[6] Besides, SHP-2 has been reported to positively regulate the signaling pathways of insulin, epidermal growth factor, platelet-derived growth factor, fibroblast growth factor and in the contrast, negatively regulate the Jak-Stat signaling pathway initiated by interferon-α/γ.[24,25] This study demonstrated that UUO strongly induced pro-inflammatory cytokine productions, including TNF-α, TGF-β, IL-1β and IL-6, which were suppressed by SHP-2 knockout, indicating that SHP-2 took an important part in the inflammatory process of UUO-mediated renal fibrosis.

Gene knockout has been the most efficient method for function study of target genes. Cre-loxP system was a site-specific recombinase system and was widely used in conditional gene knockout. Cre recombinase recognizes loxP site and mediate loxP flanked gene mutations. To achieve tissue-specific gene knockout, a Cre transgenic mouse with Cre recombinase gene inserted into downstream of a tissue-specific promoter was needed. In the present study, transgenic mice expressing Cre recombinase under the control of the endogenous Lyz2 promoter/enhancer elements were crossed with mice in which exon 4 of *SHP-2* gene were flanked by loxP sequence. qRT-PCR showed that the expression of SHP-2 in macrophages decreased about 68%.

In our study, picrosirius red stain was performed 7 days after UUO, and the result showed that the renal interstitial collagen fibrils in *SHP-2* CKO group were significantly less than those in control group, indicating that SHP-2 deficient attenuates renal fibrosis. It is reported that SHP-2 promotes cell migration and invasiveness.[26,27] We thus hypothesize that SHP-2 might affect macrophage infiltration after UUO. We then performed F4/80 stain to evaluate the macrophage infiltration in both groups and found that the number of macrophages was significantly less in SHP-2 CKO group compared to the control group. Meanwhile, another study found that the expression of SHP-2 was up-regulated in rheumatoid arthritis fibroblast-like synoviocyte (RA FLS), knockdown of SHP-2 resulted in inhibited invasiveness, migration, adhesion and spreading of RA FLS.[28]

To test whether SHP-2 influence inflammatory factors secretion after UUO, qRT-PCR and ELISA were performed. We found that TNF-α, TGF-β, IL-1β and IL-6 were inhibited in *SHP-2* CKO group, indicating that SHP-2 might positively regulate cytokine production under UUO. These results were contrary to those of An *et al*.[22] However, several studies have reported that SHP-2 was positively involved in inflammatory responses. Li *et al*.[29] found that SHP-2 positively regulates

IL-8 production in acute cigarette smoke-mediated lung inflammation through MAPK pathway, *SHP-2* CKO in lung epithelia reduced IL-8 release and pulmonary inflammation in cigarette smoke-exposed mice. Stanford *et al*.[28] found that SHP-2 was positively involved in RA. In the contrast, Coulombe *et al*.[30] found that epithelial SHP-2 protects against intestinal inflammation. The detailed mechanism for the up-regulated expression of inflammatory factors in the kidney was unclear, possibly SHP-2 regulates inflammatory factors expression after UUO through another signaling pathway, which was different from that reported by An *et al*.[22]

In conclusion, the present study found that myeloid cells SHP-2 knockout results in decreased macrophage infiltration and inflammatory factors production after UUO, thus attenuates renal fibrosis; however, it does not affect renal tubular epithelial cells apoptosis. These results suggested myeloid cells SHP-2 is positively involved in renal fibrosis. To the best of our knowledge and belief, this is the first study discussing the function of myeloid cells SHP-2 in renal fibrosis. Our results shed light on the management of renal fibrosis caused by obstructive nephropathy.

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