Original Paper

Lentivirus-Mediated Silencing of Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase 2 Inhibits Release of Inflammatory Cytokines and Apoptosis in Renal Tubular Epithelial Cells Via Inhibition of the TLR4/NF- κ B Pathway in Renal Ischemia-Reperfusion Injury

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Key Words

Shp-2 • TLR4/NF- κ B signaling pathway • Renal ischemia-reperfusion injury • Renal tubular epithelial cell • Inflammatory cytokine • Apoptosis

Abstract

Background/Aims: Renal ischemia-reperfusion injury occurs after the blood flow to the ischemic kidney is reestablished under various clinical conditions, such as organ transplantation, renal artery stenosis, end-stage disease, and the repair of descending aortic. The current study aims to explore the effects of src homology 2 domain-containing protein tyrosine phosphatase 2 (Shp-2) on the release of inflammatory cytokines and the apoptosis of renal tubular epithelial cells by regulating the TLR4/NF- κ B signaling pathway in rats with renal ischemia-reperfusion (IR) injury. **Methods:** A total of 60 normal clean Sprague Dawley (SD) (WT) rats were used in this study. The levels of creatinine (Cr) and blood urea nitrogen (BUN) were determined using an automatic biochemical analyzer. The apoptosis in renal tissue was detected by TUNEL assay. Renal tubular epithelial cells of rats were cultured, infected and treated with different lentivirus vectors. The serum levels of interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), IL-1 β and SHP27 were measured. Reverse transcription quantitative polymerases chain reaction and Western blot analysis were performed to measure the expression of relevant genes and

proteins. Furthermore, the effect of SHP-2 on the proliferation, cell cycle and apoptosis of renal tubular epithelial cells was also investigated. **Results:** In the serum of rats with renal I/R injury and prolonged reperfusion time, the contents of Cr and BUN were increased, the positive expression of SHP-2 was higher, the level of apoptosis was promoted, IL-6, TNF- α , IL-1 β and SHP27 expression in the serum was increased, the expression of SHP2, TLR4, NF- κ B, IL-6, TNF- α and Bax was up-regulated, and the expression of Bcl-2 was down-regulated. Lentivirus-mediated silencing of SHP-2 promoted the proliferation of renal tubular epithelial cells, inhibited their apoptosis, and reduced the expression of inflammatory factors in the cells by functionally suppressing the TLR4/NF- κ B signaling pathway. **Conclusion:** The results indicated that lentivirus-mediated silencing of SHP-2 inhibited the release of inflammatory cytokines and the apoptosis of renal tubular epithelial cells, and promoted the proliferation of these cells by inhibiting the TLR4/NF- κ B signaling pathway in rats with renal I/R injury.

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Introduction

Renal ischemia-reperfusion (I/R) injury is a common cause of acute kidney injury (AKI) in patients suffering from sepsis, renal artery stenosis, renal transplantation and shock, and can also lead to high morbidity and mortality [1, 2]. In addition, renal I/R injury is closely related to acute tubular-epithelial injury, vascular dysfunction, inflammation and hemodynamic alterations [3, 4]. It is estimated that over 1 million deaths are caused by AKI every year globally, and the mortality of patients with severe AKI remains high at about 40 ~ 60% [5, 6]. I/R injury, a metabolic disorder of cells, is caused by I/R and usually leads to renal dysfunction and structural damage [7]. Currently, major therapeutic approaches used for treating AKI include renal transplantation, dialysis and stem cell transplantation [8]. Additionally, pharmacological agents with therapeutic effects, such as anti-inflammation, anti-oxidative and anti-regulation agents, can be used to potentially prevent the development and progression of AKI [9]. The tubular epithelial cells injured by I/R persistently release inflammatory factors and induce tubulointerstitial inflammation, thus triggering the loss of renal functions and renal fibrosis [10]. Despite significant progresses in the pathophysiology and knowledge of I/R-induced renal injury, the detection, prevention and treatment of I/R-induced renal injury require to be further improved in order to increase the life quality of AKI patients [11].

Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 2, acts as a tumor suppressor in cancers and can be activated by binding directly to the scaffolds of tyrosine kinases [12]. In addition, it has been previously reported that SHP-2 can protect neurons from neurodegeneration during I/R injury, and its immunomodulatory effects can attenuate H₂O₂-mediated oxidative stress, whose reduction is conducive to ameliorate renal I/R injury [13, 14]. As a key member of transmembrane proteins [15], toll-like receptors (TLRs) play vital roles in adaptive immune responses by promoting the secretion of inflammatory cytokines via interactions with nuclear factor-kappa B (NF- κ B) [16]. TLR4 might be expressed in kidney tubular epithelial cells upon injuries; TLR4 signal as a vital factor in intrinsic kidney cells mediating kidney damages [17]. Additionally, NF- κ B, a transcription factor, activates the transcription of cytokines and the cascade of pro-inflammatory chemokines [18]. A previous study by Li et al. suggested that the pre-treatment with tea polyphenol could inhibit the TLR4/NF- κ B signaling pathway, and consequently protect renal tubular epithelial cells against I/R-induced apoptosis [19]. Importantly, a recent report demonstrated that I/R-induced AKI could be suppressed when cell apoptosis is inhibited [20]. Therefore, the current study aims to investigate the effects of lentivirus-mediated silencing of SHP-2, which regulates the TLR4/NF- κ B signaling pathway, on the release of inflammatory cytokines and the apoptosis of renal tubular epithelial cells in rats with renal I/R injury.

Materials and Methods

Ethics statement

All animal experiments were approved by the Animal Ethics Committee of PLA Army General Hospital (201705004), and great efforts were made to minimize the suffering of the animals used in this study.

Experimental animals

Sixty male Sprague Dawley (SD) rats (WT rats) with a calculated mean body weight of 200 ± 10 g and an age of 6 weeks old were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The rats were raised in a controlled environment with constant humidity (50%–60%) and temperature ($22^{\circ}\text{C} \sim 24^{\circ}\text{C}$) under 12 h light/dark cycles. The rats had free access to food and water. The rats were randomly assigned into the following five groups: the sham group, the Model-0 h group, the Model-24 h group, the Model-48 h group and the Model-72 h group, with each group containing 12 rats.

Establishment of the I/R injury rat model

Prior to operation, the rats in the model groups were fasted for 24 h without water deprivation, and subsequently anaesthetized with intraperitoneal injections of 2% pentobarbital sodium (40 mg/kg). Fixed in the supine position, the rats underwent a midline incision of $3 \sim 4 \text{ cm}$ in the abdominal wall, and the right renal pedicle of the right kidney was exposed and clamped for occlusion with a non-invasive microvascular clamp. The color of right kidney changed from a bright red color to a dark purple color, and finally to a gray color within 3–5 min after the clamp was installed, indicating that the occlusion was effective. The aforementioned steps were carried out to treat the left kidney as well. The artery clamp was completely loosened after the blood flow was occluded for 45 min. After the restoration of blood flow, reperfusion was successfully achieved when the kidney gradually turned its color from dark purple to bright red. Bilateral renal tissues from the rats in the Model-0 h group were collected, whereas the abdominal incisions of rats in the Model-24 h and Model-48 h groups were sutured. The rats in the Model-24 h group, Model-48 h group and Model-72 h group were given intraperitoneal injections of normal saline (1 mL) after operation before they were placed back into the cages. At 24 h, 48 h and 72 h, respectively, to collect bilateral renal tissues. The rats in the sham group underwent the same anesthesia and laparotomy as those in the model groups. However, after bilateral renal artery occlusion, the abdominal incisions of rats in the sham group were sutured without using the non-invasive artery clamp to clamp the bilateral renal pedicles.

Test of renal functions

After the successful establishment of the models, venous blood samples were collected from the inferior vena cava. Subsequently, the rats were sacrificed and the contents of Cr and BUN in each rat were measured using an automatic biochemical analyzer (Au2700, Olympus Corporation, Tokyo, Japan).

Hematoxylin-eosin (HE) staining

The renal tissues of rats were rinsed with normal saline and a portion of the renal tissues was stored in a refrigerator at -80°C for further experiments. The remaining portion of renal tissues was fixed in a 10% neutral formalin solution, paraffin-embedded and then sliced into $3 \mu\text{m}$ -thick sections. The sections were deparaffinized twice (each for 10 min) with xylene and then dehydrated with 100% ethanol for 5 min, 90% ethanol for 2 min and 70% ethanol for 2 min, respectively, followed by rinsing with distilled water for 2 min. In the next step, the sections were stained with hematoxylin for 7 min before being washed with running water for 10 min. Excessive staining solution on the sections was rinsed off by distilled water. Subsequently, the sections were immersed in 95% ethanol for 5 s, and then stained with eosin for 1 min. In the next step, 95% ethanol was used to carry out two rounds of conventional dehydration twice (2 min each round), followed by two round of cleaning using xylene (5 min each round). After being dried in air and mounted in resin, the sections were observed and photographed under a microscope (wi102502, Beijing Ruoshuihe Science and Technology Co., Ltd., Beijing, China).

Immunohistochemistry

The extracted tissue sections were dried in an incubator at 60°C for 1 h. Subsequently, the sections were conventionally de-paraffinized using xylene and dehydrated using gradient ethanol. In the next step, the sections were immersed in 3% H₂O₂ for 10 min, and washed with distilled water three times (3 min each time). Antigen retrieval was performed using a pressure cooker for 1 ~ 3 min as follows: 1500 mL ~ 3000 mL sodium citrate buffer solution (pH 6.0) or 0.001 M ethylene diaminetetraacetic acid (EDTA, pH 8.0) was heated to boil. In the next step, the sections were cooled down to room temperature using an ice water bath, and rinsed twice (3 min each time) with 0.01 M phosphate buffered saline (PBS, pH 7.4). Subsequently, the sections were incubated for 20 min at room temperature with 10% normal goat serum (Beijing ComWin Biotech Co., Ltd., Beijing, China) and the excessive serum was discarded. In the next step, rabbit anti-rat SHP-2 antibodies (diluted to a ratio of 1:500, ab131541, Abcam, Cambridge, MA, USA) were added onto the sections, followed by overnight incubation in a wet box at 4°C. Biotin-labeled goat anti-rabbit immunoglobulin G (IgG, diluted to a ratio of 1:2000, ab80948, Abcam, Cambridge, MA, USA) secondary antibodies were then added onto the sections, followed by incubation at 37°C for 1 h. A solution of 1:100 streptavidin-peroxidase (SP) complex (horseradish peroxidase labeled streptavidin, SP30100, Beijing Hongyuechuangxin Technology Co., Ltd., Beijing, China) was then added onto the slides, followed by incubation at 37°C for 1 h. A diaminobenzidine (DAB) kit (ab64221, Abcam, Cambridge, MA, USA) was used for color development for 10 min. The cells with negative expression showed no color changes, while the cells with weak positive expression were in a light yellow color, while the cells with moderately positive expression were in a brown yellow color, while the cells with strongly positive expression were brownish [21]. A total of 5 view fields were randomly selected from each section for image acquisition under an inverted microscope (XDS-800D, Shanghai Caikon Optical Instrument Co., Ltd., Shanghai, China). The percentage of SHP-2 positive cells in each view field was calculated as follows: SHP-2 positive expression rate = number of positive cells/total number of cells.

TdT-mediated biotin-16-dUTP nick-end labeling (TUNEL) assay

TUNEL assay was employed to analyze the apoptosis profiles in different samples. In brief, several paraffin sections were cut at 4 µm intervals, and the middle of the long axis along the left rat kidneys. A total of 5 sections from different parts of the rat kidneys were selected for staining. In the next step, the sections were dewaxed, rinsed twice (5 min each time) with double distilled water and rinsed two more times (5 min each time) with PBS. Subsequently, the sections were immersed in 3% hydrogen peroxide (10 mL of 30% hydrogen peroxide solution + 90 mL methanol) at 37°C for 40 min to block endogenous peroxidase, and then rinsed three times (5 min each time) with the PBS solution. After drying, the sections were incubated with a proteinase K solution (1 mg/mL) at 37°C for 10 min, and rinsed three times (5 min each time) with the PBS solution. After rinsing, the sections were incubated with a mixed TUNEL reaction solution (50 µL) (enzyme concentration and labeled solution = 1 : 9) at 37°C for 1 h, and rinsed three times (5 min each time) with the PBS solution. In the next step, the sections were incubated with a POD-conversion solution at 37°C for 30 min and then rinsed three times (5 min each time) with the PBS solution. DAB was used for full color development at room temperature for 3-10 min and the sections were then rinsed thoroughly under running water. Subsequently, the sections were re-stained with hematoxylin, rinsed under running water until they became colorless, subsequently dehydrated with alcohol, cleaned with xylene, and then mounted with balsam. A labeling solution instead of the mixed TUNEL reaction solution was used as the negative control (NC). Condensed and brownish nuclei of renal tubular epithelial cells indicated positive results. Ten non-overlapping visual fields were randomly selected on each section and observed under a light microscope (magnification of X200) (Noesis S.A. French). The mean optical density (MOD) and the positive staining rate (percentage of positive nuclei to the total number of nuclei in the field of vision) were measured and recorded. The apoptotic index (AI) = MOD × positive staining rate × 100 %.

In vitro cell culture

The rats in the Model-48 h group and sham group were starved for 4 h prior to the experiment, and were subsequently sacrificed by cervical dislocation. The bilateral kidneys of the rats were removed under sterile conditions. The renal capsule and pedicle tissues were separated, and the renal cortex was extracted and sliced into 1 mm³ tissue blocks, which were then transferred onto an 80-mesh stainless steel sieve and

ground thoroughly. The liquid below the sieve was then collected and transferred onto a 100-mesh sieve. A Hank's balanced salt solution (HBSS) containing 0.5 mM Ethylene Glycol Tetraacetic Acid (EGTA) (without Ca^{2+}) was used to wash the tissue sections. In the next step, the tissues filtered by the 100-mesh sieve were collected and centrifuged at 1500 rpm for 6 min, and the supernatant was abandoned. A total of 3 mL of 0.25% trypsin and 6 mL of Dulbecco's Modified Eagle's Medium (DMEM)-F12 containing 15% fetal calf serum were added into the test tube to resuspend and precipitate the cells, which were then mixed evenly using straw. In the next step, the cells were inoculated in a 6-well culture plate and cultured at 37°C in a humidified incubator containing 5% CO_2 . After 72-h incubation, the culture solution was replaced for the first time, and was later replaced once every 48 h. During the 48 h time interval, the renal tubular epithelial cells started to grow around the renal tubular segment, and gradually attained a cobblestone-like and disordered structure surrounding the renal tubules. Renal tubular epithelial cells and 293T cells were cultured at 37°C and 5% CO_2 in a DMEM (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (HyClone, Logan, Ut, USA). In the next step, the cells were detached by 0.25% trypsin (Gibco, Gaithersburg, MD, USA) and were into a single cell suspension using DMEM containing 10% FBS, followed by conventional cell culture. Cells in the logarithmic phase of growth were harvested for subsequent experiments.

Sterile slides were placed into a 24-well plate and inoculated with renal tubular segment cells. The cells were allowed to reach 60% - 70% confluence, followed by harvest for identification. Cell slides were rinsed with PBS, fixed with paraformaldehyde at -10°C for 5 min, rinsed thrice with PBS, and incubated in 3% H_2O_2 at room temperature for 5 min to deactivate the endogenous peroxidase. Subsequently, the cell slides were rinsed thrice with PBS, blocked with a 10% rabbit serum blocking buffer at room temperature for 30 min, and incubated for 1 h after adding the cytokeratin 18 (CK18) antibody (ab181597, diluted at a ratio of 1:100, Abcam, Cambridge, MA, USA). Subsequently, the cell slides were rinsed thrice with PBS, incubated at 37°C for 15 min with diluted biotinylated secondary antibodies, and rinsed thrice with PBS. The S-A/horse radish peroxidase (HRP) solution was added to the cells, which were then incubated at 37°C for 10-15 min and then incubated with a fresh DAB solution for 1 - 2 min to develop colors. Running water was used to stop development and the slides were counted for microscopic observation.

Lentiviral vector transfection of renal tubular epithelial cells

Renal tubular epithelial cells under good growth conditions were infected using lentiviral vectors. A day prior to transfection, the cells were inoculated in a 24-well plate at a density of 5×10^4 cells/well. Subsequently, the cells were randomly divided into the sham-blank group (renal tubular epithelial cells in the sham group without treatment), the model-blank group (renal tubular epithelial cells in the model group without any treatment), the model-NC group (renal tubular epithelial cells in the model group transfected with lentiviral vectors LV3-NC carrying nonsense gene sequences), the model-si-SHP-2 group (renal tubular epithelial cells in the model group transfected with lentiviral vector LV3-TSG-6 carrying SHP-2-siRNA according to the instructions for vector of Shanghai GenePharma Co., Ltd., Shanghai, China), the model-LPS group [renal tubular epithelial cells in the model group transfected with TLR4/NF- κB activator LPS (100 ng/mL, Sigma-Aldrich Chemical Company, St Louis, MO, USA)], and the model-E5564 group [renal tubular epithelial cells in the model group transfected with TLR4/NF- κB activator E5564 (10 ng/mL, Sigma-Aldrich Chemical Company, St Louis, MO, USA)]. After 24 h of incubation, the cells in the NC and si-SHP-2 groups were co-transfected according to the instructions of Lipofectamine 2000 (11068019, Invitrogen, New York, California, USA). In the next step, the cells were collected after 72 h of infection. A day prior to transfection, the cells were sub-cultured and inoculated into a 6-well plate with 1×10^5 cells in each well, and were subsequently cultured to reach 70%-80% confluence. The pcDNATM6 (100 pmol) was diluted by 250 μL of an Opti-MEM serum free medium (51985042, Gibco, Gaithersburg, MD, USA). Subsequently, 2-GM/EmGFP-siRNA and SHP-2-siRNA sequences (final concentration: 50 nM) were added onto the cells, which were then uniformly mixed and incubated at room temperature for 5 min. Lipofectamine 2000 (5 μL) was diluted with 250 μL of Opti-MEM serum-free medium, and uniformly mixed and incubated at room temperature for 5 min. Both parts of the aforementioned solutions were mixed and incubated at room temperature for 20 min before the mixture was added into the wells. The transfected cells were further incubated at 37°C in a humidified incubator containing 5% CO_2 . A complete medium was used after 6 - 8 h of cell culture, and subsequent experiments were conducted after 24 - 48 h of cell culture.

Enzyme-linked immunosorbent assay (ELISA)

Venous blood samples (3 mL) were collected from the rats by puncturing the abdominal artery prior to euthanasia. The blood samples were centrifuged at 1500 rpm for 15 min, and the supernatant was obtained. ELISA kits (ml038325, Shanghai Meilian Biotechnology Co., Ltd., Shanghai, China) were employed to detect the expression of interleukin-6 (IL-6) (ab100713, Abcam, Cambridge, MA, USA), tumor necrosis factor- α (TNF- α) (ab34674, Abcam, Cambridge, MA, USA), IL-1 β (ab100768, Abcam, Cambridge, MA, USA), and SHP47 (ab202407, Abcam, Cambridge, MA, USA). The antibodies were initially diluted to 1 ~ 10 mL using a coating buffer and the diluted antibody (0.1 mL) was added into each well to incubate with the cells overnight at 4°C. Excessive solution was discarded the following day before 5% of serum was used to block the cells for 40 min at 37°C. In the next step, the wells were rinsed three times (3 min each time) with a washing solution. The test samples (diluted to a ratio of 1: 50 - 1: 400) were then added into enzyme-labeled reaction wells in triplicate with 100 μ L in each well, and incubated at 37°C for 40 - 60 min. Subsequently, the wells were washed 3 times (3 min each time) using a washing solution. At the same time, blank (containing no sample or enzyme labeled reagent), negative (containing no sample but with enzyme labeled reagent) and positive (containing both a positive control sample and enzyme-labeled reagent) wells were set for later comparisons. Freshly diluted enzyme-labeled second antibodies (0.1 mL, Abcam, Cambridge, MA, USA) were then added into the wells and incubated at 37°C for 25 ~ 60 min before the wells were rinsed with double distilled H₂O (ddH₂O, PER 018-1, Beijing Damao Chemical Reagents Co., Ltd., Beijing, China). Tetramethylbenzidine (TMB, 0.1 mL, EL0001, Huzhou Huiyuan Chemical Reagents Co., Ltd., Huzhou, Zhejiang, China) was used as a substrate and was added to each well for 10 ~ 30 min of incubation at 37°C. A total of 50 μ L stop buffer was added for termination of color development, and the optical density (OD) value of each well was determined at a wavelength of 450 nm within 20 min.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The renal tissues in all groups were frozen in liquid nitrogen and then ground to a fine powder. Total RNA content was extracted from renal tissue using RNeasy RNeasy spin column (15596-018, Invitrogen, New York, California, USA), and the concentration and purity of RNA were determined through the detection of the optical density value. According to the instructions of the PrimeScript RT reagent Kit (RR037A, Takara Biotechnology, Dalian, Liaoning, China), total RNA was reverse transcribed into cDNA. Reaction conditions, with a total reaction volume of 25 μ L, were as follows: reverse transcription at 37°C for 3 cycles (15 min each cycle), and reverse transcriptase activation at 85°C for 5 s. In the next step, cDNA was diluted by 65 μ L diethyl pyrocarbonate (DEPC) and uniformly mixed. Subsequently, SYBR Premix Ex Taq II (RR037A, Takara Biotechnology, Dalian, Liaoning, China) was used for RT-qPCR according to the instructions of a SYBR Premix Ex Taq II (RR037A, Takara Biotechnology, Dalian, Liaoning, China). The reaction system (20 μ L) included the following: 1 μ L of SYBR Premix Ex Taq II (2 μ L of PCR buffer, 1 μ L of ROX Reference Dye (10⁴), 1 μ L of DNA template and 16 μ L of ddH₂O. RT-qPCR was carried out using an ABI PRISM 7300 system (Applied Biosystems Inc., Foster City, CA, USA). The reaction conditions were as follows: pre-denaturation at 95°C for 4 min, and 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, followed by a final extension cycle at 72°C for 7 min. β -actin was used as the internal reference, and all primers (Table 1) were designed and synthesized by Wuhan Bojie Biological Engineering Company (Wuhan,

Table 1. Primer sequence for RT-qPCR. Notes: RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; TLR4, Toll-like receptor 4; NF- κ B p65, nuclear factor kappa B; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α ; Bcl-2, B cell lymphoma/leukemia-2; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2

Gene	Primer sequence
SHP-2	Forward: 5'-CAGTTGCAACTTCTTACCTC-3' Reverse: 5'-GCAGGAGACTGCAGC TCAGTGATG-3'
TLR4	Forward: 5'-CCTGAGACCAGGAAGCTTGA-3' Reverse: 5'-TCCAGCCACTGAAGTTGTGA-3'
NF- κ B p65	Forward: 5'-TCCAGCCACTGAAGTTGTGA-3' Reverse: 5'-CGTAACCGCTAGTCGAAG-3'
IL-6	Forward: 5'-ATGGATGCTACCAACTGGAT-3' Reverse: 5'-TGAAGGACTCTGGCTTTGTCT-3'
TNF- α	Forward: 5'-ACTGAATTCGGGGTGATCG-3' Reverse: 5'-GGCTACAGCCTTGTCACTCG-3'
Bax	Forward: 5'-GACCAGGGTGGCTGGGAAGG-3' Reverse: 5'-GATGGTGAGCGAGGCGGTGA-3'
Bcl-2	Forward: 5'-CTTCCAGCTGAGAGCAACC-3' Reverse: 5'-CATCCCAGCCTCCGTTATCC-3'
β -actin	Forward: 5'-GTCTTTTACATCCTGGCTCACA-3' Reverse: 5'-GACGGTTTGGGCTCCTTCA-3'

Hubei, China). The ratio between the target gene in the experimental and control groups was calculated using the $2^{-\Delta\Delta Ct}$ method as follows: $\Delta\Delta Ct = \Delta Ct_{\text{experimental group}} - \Delta Ct_{\text{control group}}$ and $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$. Ct represented the number of amplification cycles when the real time fluorescence intensity reached the set threshold value while the gene amplification was still in the logarithmic growth phase. The experiment was repeated three times. The method stated above was also suitable for cell experiments.

Western blot analysis

Renal tissues were frozen using liquid nitrogen and then ground to a fine powder. A lysis buffer (C04 Sigma Aldrich, St. Louis, MO, USA) was added to the renal tissues and centrifuged at 13000 rpm and 4°C for 15 min. The supernatant was collected for future use. A bicinchoninic acid (BCA) assay was conducted for protein quantification. In the next step, 10% sodium dodecyl sulfate (SDS) running gel and transferring buffer were prepared. Total protein (40 µg per group) and 10 × SDS sample buffer were mixed and denatured by boiling at 100°C for 5 min. After ice-bath and centrifugation, the mixture was added to each lane for electrophoretic separation. The resolved proteins on the gel were then transferred onto a nitrocellulose membrane using a wet transfer method. The nitrocellulose membrane was then blocked with 5% milk at 4°C overnight. The rabbit anti-rat primary antibodies against SHP-2 (dilution ratio of 1:500, ab131541), TLR4 (dilution ratio of 1:500, ab13556), NF-κB p65 (dilution ratio of 1:2000, ab16585), Bcl-2 (dilution ratio of 1:2000, ab100772), TNF-α (dilution ratio of 1:1000, ab6671), Bcl-2 (dilution ratio of 1:2000, ab32124), and Bax (dilution ratio of 1:2000, ab3250) were added to the membrane and incubated overnight at 4°C, followed by three times (5 min each time) with PBS washing at room temperature. Subsequently, goat anti-rabbit IgG secondary antibodies with a dilution ratio of 1:2000, ab6721, Abcam, Cambridge, MA, USA) were added onto the membrane and incubated at 37°C for 1 h, and the membrane was rinsed three more times (5 min each time) with PBS. In the next step, the membrane was completely immersed in an enhanced chemiluminescence (ECL, KLS, Pierce, Rockford, IL, USA) solution for luminescence development. The gel color was developed in a dark room and photographed. GAPDH was used as the internal reference. The ratio between the target band and the internal reference band was used as the relative protein expression. The experiment was repeated three times. The aforementioned method was also suitable for cell experiments.

MTT assay

After being infected with virus of the determined concentration, the cells were cultured for 48 h in an RPMI 1640 medium at 37°C in a humidified incubator containing 10% FBS and 5% CO₂. Subsequently, the cells in the logarithmic phase of growth were collected for subsequent experiments. After trypsinization at room temperature, the cell suspension was transferred to centrifuge tubes and counted after uniform mixing. In the next step, cells were inoculated (at a density of $3 \times 10^3 \sim 6 \times 10^3$ per well) for incubation in a 96-well plate with each well containing 0.1 mL cell suspension, and 6 duplicated wells were set. A total of 3 time points, i.e., 24 h, 48 h and 72 h, were set, to conduct the following experiments. Each well was supplemented with 10 µL MTT solution (5 mg/mL), and the cells were further incubated at 37°C for 2 h. After terminating the incubation, the supernatant was discarded. Dimethyl sulfoxide (DMSO, 150 µL for each well) was added to the wells, and OD value of each well was determined using an ELISA plate reader (Model 680, Beijing Nuoyawei Instrument Co., Ltd., Beijing, China) excited at a wavelength of 570 nm. The experiment was repeated three times and the mean values were recorded. Cell viability curves were drawn with time points as the abscissa and OD values as the ordinate.

Hoechst 33258 staining

After the cells in each group were treated, the culture medium was discarded and the cells were rinsed twice with sterile PBS. Subsequently, paraformaldehyde (4%) was added into each culture dish to fix the cells for 10 min. After polyformaldehyde was removed carefully, the culture dishes were rinsed twice with sterile PBS, and incubated with 5 mg/L⁻¹ Hoechst 33258 reagent and vibrated gently for 30 min on a shaker at room temperature. The fluorescent microscope was turned on at 20 min before the measurement. After the Hoechst33258 reagent in the culture dishes was discarded, the cells were observed under the microscope, which showed that chromatin was evenly distributed. The cells stained with a uniform blue color were

regarded as normal cells, and the cells showing concentrated and bright blue nuclei were considered as apoptotic cells.

Flow cytometry

After the cells were transfected for 48 h, the culture medium was discarded and the cells were rinsed with a PBS balanced salt solution. In the next step, the cells were trypsinized with a 0.25% trypsin solution until the cells started to shrink and detach from the bottom. A serum-containing medium was used to terminate the trypsinization. Subsequently, the cells were gently shaken until they were completely detached and become a suspension, which was then centrifuged at 1000 rpm for 5 min before the supernatant was discarded. In the next step, the cells were rinsed twice with PBS, fixed for 30 min with precooled ethanol, and then collected after centrifugation. Subsequently, the cells were rinsed with PBS containing with RNase-containing 1% propidium iodide (PI) for 30 min. After the cells were rinsed twice with PBS to wash away excessive PI, the volume of the cells was adjusted to 1 mL using a PBS balanced salt solution. Subsequently, flow cytometry (FACS Calibur, Beckman Coulter, Inc., Brea, CA, USA) was employed to detect the cell cycle distribution in each group. A total of 3 samples were set in each group, and the experiment was repeated three times.

After infection for 48 h, the cells were detached with EDTA-free trypsin and collected in a flow tube. After the cells were centrifuged, the supernatant was discarded. In the next step, the cells were rinsed thrice with cold PBS and the supernatant was discarded after centrifugation. According to the instructions of an Annexin-V-fluorescein isothiocyanate (FITC) cell apoptosis assay kit (Beyotime Biotechnology Co., Ltd., Shanghai, China), a total of 1×10^6 cells were resuspended in 100 μ L of Annexin-V-FITC/PI staining solution containing Annexin-V-FITC, PI and N-2-Hydroxyethylpiperazine-N'-Ethanesulfonic Acid (HEPES) (mixed at a ratio 1:2:50). Subsequently, the cells were incubated at room temperature for 15 min. In the next step, a HEPES buffer (1 mL) solution was added to the cells and gently mixed. Flow cytometry was employed to record cell apoptosis at a wavelength of 488 nm. Blue fluorescence at wavelengths of 525 nm and 620 nm were used to detect the signals of FITC-fluorescence and PI fluorescence, respectively. Apoptosis rate = $[(An + PI) + (An + PI)] / [(An + PI) + (An + PI) + (An - PI)]$ [22].

Statistical analysis

Statistical analyses were performed using SPSS 22.0 software (IBM Corp. Armonk, NY, USA). Data were tested for normality of distribution and homogeneity of variance. Measurement data were expressed as mean $\pm$ standard deviations. Comparisons between two groups were performed using the *t* test, and the comparisons among multiple groups were analyzed by one-way analysis of variance (ANOVA). A *p* value of < 0.05 was considered to be statistically significant.

Results

Increased levels of BUN and Cr in serum of rats at 24 h, 48 h and 72 h time points

The contents of Cr and BUN in each group were determined in order to test renal function in all groups. Compared with the sham group, the BUN and Cr levels in the Model-0 h group were not statistically significant (*p* > 0.05). Compared with the sham group and Model-0 h group, the Model-24 h, Model-48 h and Model-72 h groups were found to show increased levels of BUN and Cr (all *p* > 0.05) (Table 2).

Table 2. Levels of BUN and Cr in serum of rats are increased at 24 h, 48 h and 72 h. Notes: Measurement data were expressed as mean $\pm$ standard deviation. Comparisons among multiple groups were analyzed by one-way analysis of variance. The experiment was conducted for three times. N = 12 per group; *, *p* < 0.05 vs. the sham group; #, *p* < 0.05 vs. in the Model-24 h group

Factors	Sham	model-0 h	model-24 h	model-48 h	model-72 h
BUN	7.83 $\pm$ 0.76	9.36 $\pm$ 1.43	22.39 $\pm$ 1.75#	29.36 $\pm$ 1.64#	33.45 $\pm$ 2.16#
Cr	86.32 $\pm$ 5.31	97.26 $\pm$ 6.28	323.92 $\pm$ 19.47#	394.17 $\pm$ 114.35#	423.27 $\pm$ 20.13#

Rats with renal I/R injury showed most serious histopathological changes of renal tissues at the 72 h time point

HE staining was conducted to observe the histopathological changes of renal tissues in all groups. The results (Fig. 1) demonstrated that the rats in the Model-0 h and sham groups showed no evident abnormalities, whereas the rats in the Model-24 h, Model-48 h and Model-72 h groups exhibited visible pallid renal cortex and severe medullary congestion. For the Model-24 h, Model-48 h and Model-72 h groups, in addition to intraluminal inflammatory exudation, some renal tubular epithelial cells showed signs of necrosis and abscission under the microscope. In addition, cell casts were found in some distal tubules. Blood vessel dilatation and inflammatory cell infiltration were observed in renal interstitium with apparent glomerulopathy. The renal tissues of rats in the Model-72 h group exhibited more severe damages compared to the rats in the Model-24 h and Model-48 h groups. Therefore, it could be suggested that the extent of damages in renal tissues was positively related to the reperfusion time.

Higher positive expression of SHP-2 in rats with renal I/R injury

Immunohistochemistry was employed to detect the location and positive expression of SHP-2 in all groups (Fig. 2). The results showed that the positive expression of SHP-2 was primarily localized to the cytoplasm as evidenced by brown-yellow granules. The positive expression rate of SHP-2 protein was $(18.83 \pm 2.32)\%$ in rats in the sham group, which was considerably lower than $(31.56 \pm 3.06)\%$ achieved in the Model-0 h group, $(49.56 \pm 4.21)\%$ in the Model-24 h group, $(73.24 \pm 6.83)\%$ in the Model-48 h group, and $(83.18 \pm 5.21)\%$ in the Model-72 h group ($p < 0.05$), respectively. Compared with the Model-24 h and Model-48 h groups exhibited lower SHP-2 protein expression compared with the Model-72 h group ($p < 0.05$). The aforementioned data indicated that the positive expression of SHP-2 was higher in rats with renal I/R injury.

Fig. 1. The morphology of renal tissues was observed using HE staining under a microscope (magnified at $\times 400$), and rats with renal I/R injury at 72 h exhibited most serious histopathological changes of renal tissues; $\blacktriangledown$, renal tubular epithelial cells; I/R, ischemia-reperfusion; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; HE, hematoxylin and eosin.

Fig. 2. Positive expression of SHP-2 was higher in rats with renal I/R injury (magnification $\times 400$). Panel A and B, SHP-2 protein expression in the sham, Model-0 h, Model-24 h, Model-48 h and Model-72 h groups was determined using immunochemical methods, and the Model-72 h group showed the highest protein expression; *, $p < 0.05$ vs. the sham group; #, $p < 0.05$ vs. the Model-24 h group. Stained particles with positive expression; in immunohistochemistry, the brown staining was positive cells. Measurement data were analyzed by one-way analysis of variance; the experiment was repeated three times ($n = 12$ per group); I/R, ischemia-reperfusion; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2.

Apoptosis was more serious in rats with renal I/R injury and prolonged reperfusion time

Apoptosis in cells from all groups was detected by the TUNEL assay. The results (Fig. 3A) showed that there was a significant increase in the number of apoptotic cells in each model group after renal I/R. Compared to that in the cortex of proximal convoluted tubules, the number of apoptotic cells was considerably higher in the distal convoluted tubules of the outer medullary area. In addition, there were more apoptotic renal tubular epithelial cells when the reperfusion time became longer. The above results of the TUNEL assay were consistent with the increased renal tubular injury in I-stained sections over longer reperfusion time. The results of TUNEL assay are shown in Fig. 3B. The number of apoptotic cells in the Model-0 h, Model-24 h, Model-48 h and Model-72 h groups was significantly higher than that in the sham group ($p < 0.05$). The number of apoptotic cells in the model-48 h and Model-72 h groups was significantly higher than that in the model-24 h group ($p < 0.05$). The number of apoptotic cells in the Model-72 h group was significantly higher than that in the Model-0 h group ($p < 0.05$). Therefore, the above results suggested that the severity of apoptosis in each group increased with prolonged perfusion time.

Cultured cells were identified as renal tubular epithelial cells

In order to identify whether the cultured cells were renal tubular epithelial cells, immunohistochemistry was used to stain the specific marker protein CK18. After cell culture, immunohistochemical staining was conducted and revealed that CK18 was distributed uniformly around the nucleus in the cytoplasm of the cells, and the intensity of staining was different. However, no specific staining was detected in the NC. Therefore, the cultured cells were confirmed to be proximal renal tubular epithelial cells (Fig. 4).

Rats with renal I/R injury exhibited higher expression of IL-6, TNF- α , IL-1 β and SHP27 in serum with prolonged reperfusion time

ELISA was performed for all groups in order to measure the expression of serum inflammatory cytokines. The results in Table 3 indicated that, compared with the rats in the sham group, the rats in the Model-0 h, Model-24 h, Model-48 h and Model-72 h groups exhibited increased serum expression of IL-6, TNF- α , IL-1 β and SHP27 (all $p < 0.05$). In addition, the IL-6, TNF- α , IL-1 β and SHP27 serum expression in the Model-24 h group was higher than that in the Model-0 h group, whereas the IL-6, TNF- α , IL-1 β and SHP27 serum expression in the Model-48 h and Model-72 h groups was higher than that in the Model-0 h and Model-24 h

groups (all $p < 0.05$). The aforementioned results indicated the association between the length of reperfusion time and the severity of inflammatory reactions in each group.

Fig. 3. More severe apoptosis in rats with renal I/R injury with a prolonged reperfusion time. Panel A, TUNEL staining showed that the positive cells were apoptotic cells; Panel B, number of TUNEL positive cells in the sham, Model-0 h, Model-24 h, Model-48 h and Model-72 h groups were detected by TUNEL staining, and the Model-72 h group displayed the highest number of TUNEL positive cells; The apoptotic cells were brown yellow; $p < 0.05$ vs. the sham group; #, $p < 0.05$ vs. the Model-24 h group; the measurement data were analyzed by one-way analysis of variance; the experiment was repeated three times ($n = 12$ per group); I/R, ischemia-reperfusion; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2.

Fig. 4. Cultured cells were identified as renal tubular epithelial cells (magnified at $\times 200$) in vitro by immunohistochemical staining. Panel A, negative results of immunohistochemical staining showed no brown staining; Panel B, positive results of immunohistochemical staining showed that CK18 was distributed unevenly in the cytoplasm of the cells around the nucleus, and the intensity was different.

Table 3. Lentivirus-mediated silencing SHP2 reduces expression of inflammatory factors in renal tubular epithelial cells. Notes: TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; *, $p < 0.05$ vs. the sham group; #, $p < 0.05$ vs. in the Model-24 h group. The measurement data were presented as mean $\pm$ standard deviation and analyzed by one-way analysis of variance; the experiment was repeated three times ($n = 12$ per group)

Groups	IL-6 (pg/mL)	TNF- α (pg/mL)	IL-1 β (pg/mL)	SHP27 (pg/mL)
Sham group	36.98 $\pm$ 5.02	11.75 $\pm$ 3.58	32.84 $\pm$ 5.32	35.08 $\pm$ 5.27
Model-0 h group	53.21 $\pm$ 7.45*#	18.29 $\pm$ 3.90*#	48.74 $\pm$ 7.12*#	51.09 $\pm$ 7.63*#
Model-24 h group	78.24 $\pm$ 8.60*	31.40 $\pm$ 4.31*	73.18 $\pm$ 8.22*	83.09 $\pm$ 8.51*
Model-48 h group	144.97 $\pm$ 13.22*#	72.33 $\pm$ 8.42*#	132.72 $\pm$ 11.93*#	152.85 $\pm$ 14.07*#
Model-72 h group	164.74 $\pm$ 10.15*#	92.28 $\pm$ 7.68*#	152.72 $\pm$ 8.32*#	167.61 $\pm$ 13.25*#

SHP2, TLR4, NF- κ B, IL-6, TNF- α and Bax expression was upregulated while Bcl-2 expression was downregulated in the serum of rats with renal I/R injury

RT-qPCR and Western blot analysis were performed to measure the mRNA and protein expression of SHP2, TLR4, NF- κ B, IL-6, TNF- α , Bax and Bcl-2 in all groups. The results showed that, compared with the rats in the sham group, the rats in the Model-0 h, Model-24 h, Model-48 h and Model-72 h groups exhibited elevated mRNA and protein expression of SHP2, TLR4, NF- κ Bp65, IL-6, TNF- α and Bax, in addition to reduced expression of Bcl-2 (all $p < 0.05$). In addition, the rats in the Model-24 h group exhibited higher mRNA and protein expression of SHP2, TLR4, NF- κ Bp65, IL-6, TNF- α and Bax, but lower Bcl-2 mRNA and protein expression compared to the rats in the Model-0 h group (all $p < 0.05$). Compared to the rats in the Model-24 h group, the rats in the Model-48 h and Model-72 h groups exhibited higher mRNA and protein expression of SHP2, TLR4, NF- κ B, IL-6, TNF- α and Bax, but reduced mRNA and expression of Bcl-2 (all $p < 0.05$) (Fig. 5). The above results revealed that the rats with renal I/R injury were associated with elevated mRNA and protein expression of SHP2, TLR4, NF- κ B, IL-6, TNF- α and Bax but decreased mRNA and protein expression of Bcl-2.

Lentivirus-mediated silencing of SHP-2 functionally suppressed the TLR4/NF- κ B pathway

QPCR and Western blot analysis were conducted for SHP-2, TLR4, NF- κ Bp56, IL-6, TNF- α , Bcl-2, Bax, and the results are shown in Fig. 6. Compared with the sham-blank group, all other groups exhibited significantly higher mRNA and protein expression of NF- κ Bp56, IL-6, TNF- α and Bax, in addition to significantly increased mRNA and protein expression of Bcl-2 (all $p < 0.05$). Compared with the sham-blank group, the Model-si-SHP2 group demonstrated significantly decreased SHP-2 expression, while the model-blank, model-NC, model-E5564 and model-LPS groups exhibited significantly higher SHP-2 expression (all $p < 0.05$). Compared with the sham-blank group, the model-E5564 group showed significantly decreased TLR4 expression ($p < 0.05$), while the model-blank, model-NC, model-si-SHP2 and model-LPS groups showed significantly higher TLR4 expression (all $p < 0.05$). Between the model-blank and the model-LPS groups, there were no significant differences in terms

Fig. 5. SHP2, TLR4, NF- κ B, IL-6, TNF- α and Bax expression was up-regulated and Bcl-2 expression was down-regulated in serum of rats with renal I/R. Panel A, RT-qPCR was used to determine the mRNA levels of SHP2, TLR4, NF- κ B, IL-6, TNF- α , Bax and Bcl-2. The Model 72 h group showed the most significantly increased mRNA expression of SHP2, TLR4, NF- κ B, IL-6, TNF- α and Bax, and most significantly decreased Bcl-2 expression; Panel B, Western blot analysis was used to determine the protein expression of SHP2, TLR4, NF- κ B, IL-6, TNF- α , Bax and Bcl-2, showing that the Model 72 h had the most significantly increased protein expression of SHP2, TLR4, NF- κ B, IL-6, TNF- α and Bax, and most significantly decreased Bcl-2 expression. *, $p < 0.05$ vs. the sham group; #, $p < 0.05$ vs. the Model-24 h group; the measurement date were analyzed by one-way analysis of variance; the experiment was repeated three times ($n = 12$ per group); SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; I/R, ischemia-reperfusion; NF- κ B, nuclear factor-kappa B; TLRs, Toll-like receptors; HE, hematoxylin-eosin; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α ; RT-qPCR, reverse transcription quantitative polymerase chain reaction; Bcl-2, B cell lymphoma/leukemia-2.

Fig. 6. Lentivirus-mediated silencing of SHP-2 functionally suppressed the TLR4/NF-κB pathway. Panel A, RT-qPCR was used to determine the mRNA levels of SHP2, TLR4, NF-κB, IL-6, TNF-α, Bax and Bcl-2, and the model-si-SHP-2 and model-E5564 groups showed suppressed mRNA expression of TLR4, NF-κB, IL-6, TNF-α and Bax, and significantly increased Bcl-2 mRNA expression; Panel B, Western blot analysis was used to determine the protein expression of SHP2, TLR4, NF-κB, IL-6, TNF-α, Bax and Bcl-2. The model-si-SHP-2 and model-E5564 groups showed suppressed protein expression of TLR4, NF-κB, IL-6, TNF-α and Bax, and significantly increased Bcl-2 protein expression. *, $p < 0.05$ vs. the sham group; #, $p < 0.05$ vs. the Model-24 h group; the measurement data were analyzed by one-way analysis of variance; the experiment was repeated three times; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; I/R, ischemia-reperfusion; NF-κB, nuclear factor-kappa B; TLRs, Toll-like receptors; HE, hematoxylin-eosin; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; RT-qPCR, reverse transcription quantitative polymerase chain reaction; Bcl-2, B cell lymphoma/leukemia-2.

of the mRNA and protein expression of TLR4, NF-κB p56, IL-6, TNF-α, Bcl-2, and Bax (all $p > 0.05$). Compared with the model-blank and model-NC groups, the model-si-SHP2 group exhibited significantly decreased SHP-2 expression (all $p < 0.05$). In addition, there was no significant change in the model-E5564 group regarding SHP-2 expression ($p > 0.05$). In contrary, the mRNA and protein expression of NF-κB p56, IL-6, TNF-α and Bax was suppressed in the model-si-SHP2 and model-E5564 groups, while the mRNA and protein expression of Bcl-2 was significantly increased in these two groups (all $p < 0.05$). The mRNA and protein expression of TLR4, NF-κB p56, IL-6, TNF-α and Bax was increased significantly in the model-LPS group (all $p < 0.05$), while the mRNA and protein expression of Bcl-2 was decreased significantly in this group. Based on these results, it could be concluded that lentivirus-mediated silencing of SHP-2 functionally suppressed the TLR4/NF-κB pathway.

Lentivirus-mediated silencing of SHP2 promoted the proliferation of renal tubular epithelial cells

A MTT assay was carried out in order to detect the cell viability in all groups. As depicted in Fig. 7, the results revealed that, compared with the sham-blank group, all other groups showed decreased proliferation at the 48th h and 72nd h time points after transfection (all $p < 0.05$). No significant differences were observed in the proliferation rates between the model-blank and model-NC groups (all $p > 0.05$). Compared with the model-blank and model-NC groups, the model-LPS group exhibited significantly decreased proliferation, while the model-E5564 and model-si-SHP2 groups demonstrated enhanced renal tubular epithelial cell proliferation (both $p < 0.05$). The aforementioned results indicated that the lentivirus-mediated silencing of SHP2 promoted the proliferation of renal tubular epithelial cells.

Lentivirus-mediated silencing of SHP2 improved the morphology of apoptotic cells

Hoechst33258 stained cells were placed in Petri dishes and the morphology of apoptotic cells was observed under a fluorescence microscope. Fig. 8 showed that a minimum of 400 cells were evaluated in 12 visual fields selected from each group. In the sham-blank group, the chromatin of the cells was homogeneous and diffused with low density fluorescence, and the fluorescence of large and deep staining of nucleus was rare. All model groups showed dense pyknosis or granular fluorescence. Compared with the model-blank and model-NC groups, the dense pyknotic morphology or granular fluorescence in the model-si-SHP2 and

model-E5564 groups was decreased, while that in the model-LPS group was increased. The aforementioned results suggested that the lentivirus-mediated silencing of SHP2 improved the morphology of apoptotic cells.

Lentivirus-mediated silencing of SHP2 affected cell cycle distribution in renal tubular epithelial cells

Flow cytometry was conducted to study the effect of SHP2 on the cell cycle distribution of renal tubular epithelial cells. The results of PI single staining (Fig. 9) showed that the proportions of cells in the G_0/G_1 phase were $(47.22 \pm 1.97)\%$, $(64.13 \pm 2.35)\%$, $(62.89 \pm 2.05)\%$, $(79.13 \pm 3.41)\%$, $(55.16 \pm 2.01)\%$ and $(76.82 \pm 3.06)\%$ respectively, and those in the S phase were $(40.24 \pm 4.10)\%$, $(22.29 \pm 2.16)\%$, $(23.17 \pm 2.04)\%$, $(8.14 \pm 1.04)\%$, $(33.46 \pm 2.12)\%$, and $(8.69 \pm 1.08)\%$, respectively, in the sham-blank, model-blank, model-NC, model-si-SHP2, model-LPS and model-E5564 groups. Compared with the sham-blank group

Fig. 7. Renal tubular epithelial cells treated by si-SHP2 and TLR4/NF- κ B inhibitor E5564 showed the highest OD values at the 72 h time point after treatment. *, $p < 0.05$ vs. the sham-blank group; #, $p < 0.05$ vs. the model-blank group; the measurement data were analyzed by one-way analysis of variance; the experiment was repeated three times. SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; NF- κ B, nuclear factor-kappa B; TLR4, toll-like receptors; OD, optical density.

Fig. 8. Lentivirus-mediated silencing of SHP2 improved the morphology of apoptotic cells. Hoechst33258 staining was used to observe the morphology of apoptotic cells in each group, and the number of dense pyknotic morphology or granular fluorescence in the model-si-SHP2 and model-E5564 groups was decreased; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2.

Fig. 9. Lentivirus-mediated silencing of SHP2 affected cell cycle distribution in renal tubular epithelial cells. Panel A and B, cell cycle distribution was detected by PI staining, and the results showed that the model-si-SHP-2 and model-E5564 groups were associated with an extended G₀/G₁ phase and a shortened S phase; *, $p < 0.05$ vs. the sham-blank group; #, $p < 0.05$ vs. the model-blank and model-NC groups; the measurement data were analyzed by one-way analysis of variance; the experiment was repeated three times; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; PI, propidium iodide; NC, negative control.

model-blank, model-NC, model-si-SHP-2, model-LPS and model-E5564 groups were showed extended G₀/G₁ phases and shortened S phases (both $p < 0.05$). Compared with the model-blank and model-NC groups, the model-si-SHP-2 and model-E5564 groups showed extended G₀/G₁ phases and shortened S phases (both $p < 0.05$), whereas the model-LPS group exhibited a prolonged G₀/G₁ phase and a shortened S phase (both $p < 0.05$). No significant differences in the length of G₂ phase were observed among the groups ($p > 0.05$). The above results indicated that the silencing of SHP-2 might be responsible for altered cell cycle distribution of renal tubular epithelial cells.

Lentivirus-mediated silencing of SHP2 inhibited the apoptosis of renal tubular epithelial cells

The effect of SHP2 on the apoptosis of renal tubular epithelial cells was detected using flow cytometry and Annexin V-FITC/PI double staining. The results (Fig. 10) revealed that, as compared with the sham-blank group, all other groups exhibited gradually increased rates of apoptosis (all $p < 0.05$). There were no significant differences in apoptotic rates between the model-blank and model-NC groups ($p > 0.05$). Compared with the model-blank and model-NC groups, the model-si-SHP-2 and model-E5564 groups exhibited decreased apoptosis rates ($p < 0.05$), while the model-LPS group exhibited a significantly increased apoptosis rate ($p < 0.05$). Therefore, the obtained results suggested that the silencing of SHP-2 could inhibit the apoptosis of renal tubular epithelial cells.

Lentivirus-mediated silencing of SHP2 reduced the expression of inflammatory factors in renal tubular epithelial cells

Finally, ELISA was employed to confirm whether SHP2 could influence the expression of inflammatory factors. The results of ELISA using the supernatant of cell culture are shown in Table 4. The levels of IL-6, TNF- α , IL-1 β and SHP27 in the sham-blank group were significantly lower than those in other groups (all $p < 0.05$). There were no significant differences in the expression of IL-6, TNF- α , IL-1 β and SHP27 between the model-blank and model-NC groups (all $p > 0.05$). Compared with the model-blank and model-NC groups, the model-si-SHP-2

Fig. 10. Lentivirus-mediated silencing of SHP2 inhibited the apoptosis of renal tubular epithelial cells. Panel A and B, the apoptosis rate in all groups was detected by Annexin V-FITC and propidium iodide staining, and the results showed that the model-si-SHP-2 and model-E5564 groups had decreased apoptosis rates; Panel B, cell apoptosis in all groups. *, $p < 0.05$ vs. the sham-blank group; #, $p < 0.05$ vs. the model-blank and model-NC groups; the measurement data were analyzed by one-way analysis of variance; the experiment was repeated three times; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; PI, propidium iodide; NC, negative control; FITC, fluorescein isothiocyanate.

Table 4. Rats with renal I/R injury have higher IL-6, TNF- α , IL-1 β and SHP27 expression in serum with reperfusion time prolonging. Notes: TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; LPS, lipopolysaccharide; NC, negative control; *, $p < 0.05$ vs. the sham group; #, $p < 0.05$ vs. the model-blank group

Groups	IL-6 (pg/mL)	TNF- α (pg/mL)	IL-1 β (pg/mL)	SHP27 (pg/mL)
Sham-blank group	25.11 $\pm$ 3.22	15.94 $\pm$ 3.51	24.03 $\pm$ 3.22	26.93 $\pm$ 3.47
Model-blank group	42.1 $\pm$ 7.1*	48.29 $\pm$ 3.90*	61.09 $\pm$ 6.43*	64.85 $\pm$ 7.41*
model-NC group	3.08 $\pm$ 7.03	49.73 $\pm$ 5.29*	60.28 $\pm$ 7.13*	65.29 $\pm$ 7.16*
model-si-SHP2 group	3.87 $\pm$ 5.13*#	32.56 $\pm$ 5.29*#	42.19 $\pm$ 4.92*#	44.37 $\pm$ 5.12*#
model-LPS group	80.27 $\pm$ 7.51*#	70.29 $\pm$ 7.84*#	80.27 $\pm$ 7.51*#	86.65 $\pm$ 7.96*#
Model-E5564 group	3.12 $\pm$ 5.13*#	31.38 $\pm$ 5.09*#	43.82 $\pm$ 5.26*#	45.06 $\pm$ 4.83*#

and model-E5564 groups exhibited decreased expression of IL-6, TNF- α , IL-1 β and SHP27 (all $p < 0.05$), whereas the model-LPS group exhibited increased expression of IL-6, TNF- α , IL-1 β and SHP27 (all $p < 0.05$). The aforementioned results indicated that the silencing of SHP-2 and the inhibition of the TLR4 signaling pathway could reduce the expression of inflammatory factors (IL-6, TNF- α , IL-1 β and SHP27) in renal tubular epithelial cells.

Discussion

Renal I/R injury, a leading cause of acute kidney injury (AKI) with no effective and accessible therapies, is known to lead to unfavorable prognoses and significantly high mortality rates [1, 20]. The findings of the current study suggested that the lentivirus-mediated SHP-2 silencing decreased the release of inflammatory cytokines, promoted the proliferation and inhibited the apoptosis of renal tubular epithelial cells by inhibiting the TLR4/NF- κ B signaling pathway in rats with renal I/R injury, thus providing a new therapeutic target for the treatment of renal I/R injury.

As a tyrosine phosphatase, SHP-2 is highly correlated with multiple cell signaling processes, such as growth, survival, proliferation, differentiation and apoptosis, and is particularly important in chronic inflammation [23]. The current study demonstrated that the silencing of SHP-2 inhibited the release of inflammatory cytokines. Inflammatory response is considered as one of the major mechanisms underlying renal I/R injury [24]. Previous studies have indicated the protective role played by SHP-2 against neurodegeneration during I/R injury [25]. Moreover, it has been previously demonstrated that SHP-2 can inhibit the activation of the TRIF pathway by competitively inhibiting the TBK-1 protein in TRIF pathway. Therefore, the reduction in the expression of pro-inflammatory factors downstream of the TRIF pathway may exert protective effects against I/R injury [26]. In addition, inflammatory cytokines such as ILs and TNF have been noted to play a role in the pathogenesis of renal I/R injury [28, 29]. Interestingly, a previous study revealed that SHP-2 suppressed the expression of TLR3-activated pro-inflammatory cytokines IL-6 and TNF- α , and the silencing of SHP-2 increased the expression of TLR3-activated kinase-activated IFN- β and TNF- α [30]. The absence or inhibition of TLRs suppresses cardiac hypertrophy induced by I/R, whereas TLR2 and TLR4 have been shown to mediate systemic inflammatory responses and NF- κ B activation [31]. Moreover, inflammation is a key factor in the occurrence and development of ischemic damage, and secondary to an intense inflammatory response [32]. Therefore, the inhibition of apoptosis and inflammation might serve as an effective strategy to attenuate renal I/R injury [2]. Additionally, I/R injury was demonstrated to activate NF- κ B signaling by increasing the NF- κ B p65-p50 dimer and by degrading I κ B [33, 34]. A previous study confirmed that the inhibition of NF- κ B and TLR4 by deactivating the anti-inflammatory and anti-apoptosis TLR4/NF- κ B signaling pathway could promote the neuroprotective effect of isoquercetin, thereby attenuating I/R injury [35].

Remarkably, various downstream target genes activated by NF- κ B have been reported to regulate the immune system, cell survival, differentiation and cell proliferation [33]. The findings of the current study demonstrated that the inhibition of the TLR4/NF- κ B signaling pathway repressed the release of inflammatory cytokines, and promoted the proliferation and inhibited the apoptosis of tubular epithelial cells in renal I/R injury. A previous study reported that TLR could activate the NF- κ B signaling pathway, which plays a key role in promoting the production of pro-inflammatory cytokines. In addition, the aberrant TLR4 expression might induce the excessive expression of pro-inflammatory cytokines and result in detrimental inflammation [36]. LPS was found to activate the TLR4/NF- κ B signaling pathway, and suppress proliferation and induce the apoptosis of MAC-T epithelial cells, whereas the inhibition of NF- κ B also inhibited the apoptosis of MAC-T cells [37]. Previously, NF- κ B was demonstrated to regulate the genes, such as TNF- α , IL-1 and IL-6, responsible for mediating inflammation. In addition, the inhibition of NF- κ B could protect against ischemic brain damage and the acute stage of ischemia [19, 38]. Furthermore, it was shown that the functional inhibition of pro-inflammatory cytokines (including TNF- α and IL-6) could prevent the progression of renal I/R injury, while TLR4-deficient mice were less susceptible to ischemic renal injury [39]. In line with the current findings, a previous study also demonstrated that picoside II exerts a protective effect on renal I/R injury due to its inhibitory effect on the TLR4/NF- κ B signaling pathway, thus leading to the attenuation of oxidative stress [15].

This study further highlighted that the lentivirus-mediated silencing of SHP2 could promote the proliferation and inhibit the apoptosis of renal tubular epithelial cells. In addition, accumulating evidence suggests that tubular epithelial cells act as the largest cell population participating in renal inflammation, and NF- κ B also plays a crucial role in activating the inflammatory cascade [40]. Moreover, one study has reported that SHP-2 could regulate the NF- κ B signaling pathway by initiating the expression of pro-inflammatory cytokines [41]. Additionally, IL-6 expression is modulated by SHP-1 through the activation of NF- κ B and the protein tyrosine kinases of TLR4 [42]. Another important study indicated that

the inhibition of the TLR4/NF- κ B signaling pathway could attenuate the oxidative stress and immune responses in rat models with renal I/R injury [15].

Conclusion

The findings of the current study suggested that the lentivirus-mediated SHP-2 silencing inhibited the release of inflammatory cytokines, promoted the proliferation and inhibited apoptosis of renal tubular epithelial cells by inhibiting the TLR4/NF- κ B signaling pathway in renal I/R injury. Thereby, the aforementioned findings might provide a new therapeutic target for the treatment of renal I/R injury. However, further studies based on large sample sizes are still required in order to enhance the prognostic significance of SHP-2 silencing.

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Disclosure Statement

The authors have declared that no competing interests exist.

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