

# miR-27a suppresses TLR4-induced renal ischemia-reperfusion injury

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**Abstract.** Ischemia reperfusion injury (IRI) is one of the primary causes of acute renal injury and even acute renal failure. An increasing body of evidence has indicated that the aberrant expression of microRNAs (miRNA/miR) is closely associated with the progression of IRI. In the process of renal IRI, inflammatory reactions, cell adhesion and the death of renal tubular epithelial cells serve important roles. The present study investigated the expression of renal miRNAs following renal IRI in an attempt to identify the miRNAs that exert pivotal functions in renal IRI. The present study revealed that miR-27a, which was downregulated in IRI, and the 3'-untranslated region (UTR) of Toll-like receptor 4 (TLR4) have associated binding sites. The levels of TLR4, miR-27a and specific associated proteins in the renal tissues of gestational rats were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, immunohistochemistry and western blotting. The associations between miR-27a and TLR4 were analyzed, and the regulatory effect of miR-27a on TLR4 was detected via a luciferase reporter gene assay, western blotting and RT-qPCR *in vivo* and *in vitro*. In addition, the present study demonstrated that miR-27a suppressed the expression of TLR4 by binding to the 3'UTR of TLR4. The overexpression of miR-27a downregulated the expression of TLR4, which in turn inhibited the inflammation, cell adhesion and cell death in IRI. Therefore, miR-27a inhibited inflammation in IRI by decreasing the expression of TLR4.

## Introduction

Ischemia reperfusion injury (IRI) is one of the primary causes of acute renal injury, and even acute renal failure (1). It would

be of great clinical benefit to examine the physiological and pathological process of IRI and to explore the molecular biological mechanisms governing these processes in order to identify effective protective or intervention measures for the improved treatment of acute renal injury.

The present study demonstrated that when renal IRI occurred, the increased expression of Toll-like receptor (TLR)-2 and TLR4 subsequently activated the downstream NF- $\kappa$ B signaling pathway, which then produced a large number of inflammatory factors such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 and IL-6. Previous research has demonstrated that the apoptosis of renal tubule cells is mediated by inflammatory injury (2,3). At present, the expression of Bcl-2-like 1 (Bcl-x) and baculoviral IAP repeat containing 8 is typically reported to be downregulated during IRI, and the expression of Bcl-x, which serves an important role in cell apoptosis, is usually upregulated (4,5). Following IR, there is also an upregulation of adhesion factors in the ischemic area, which leads to an inflammatory cascade reaction, which is induced by the adhesion factors that mediate the adhesion of endothelial cells to neutrophils (6). The aim of the present study was to investigate how to inhibit the inflammatory reactions induced by IRI, reduce the apoptosis of glomerular cells, promote the regeneration and repair of renal tubular cells, and inhibit adhesion factors, which are key issues in the intervention of IRI.

At present, numerous miRNAs have been reported to exert pivotal functions in renal IRI (7,8). An experimental study has demonstrated that miR-21 is upregulated in renal IRI (7). In a rat model of renal IRI, researchers observed that miR-21 was involved in regulating renal IRI by affecting cell adhesion and the structure of the cytoskeleton (9,10). It has been reported that miR-494 is highly expressed in a rat model of renal IRI, which can activate transcription activator 3, thereby promoting apoptosis and further aggravating renal impairment (11). However, despite the aforementioned studies, the role of miR-27a in renal IRI has not been completely elucidated.

In order to further identify the miRNAs regulated during IRI, the present study investigated miRNA expression in renal IRI, and demonstrated that the expression of miR-27 is downregulated in renal IRI. miR-27a can inhibit renal IRI by binding through the 3'UTR of TLR4, and miR-27 exhibits potential as a novel therapeutic for the treatment of renal IRI.

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**Key words:** ischemia-reperfusion injury, Toll-like receptor 4, microRNA-27a

## Materials and methods

**Ethics statement.** All animal studies were conducted in accordance with, and with the approval of the China Medical University Ethics Committee for Animal Experimentation (Liaoning, China).

**Renal IRI model.** A total of 40 Sprague-Dawley female rats, 8 weeks of age and weighing 200-250 g were acquired from Department of Animal Department of China Medical University (Shenyang, China). The rats were acclimated to standard experimental conditions: 12 h light/dark cycle, 22±2°C room temperature, with humidity levels ranging between 50-65%, and with food and water available *ad libitum*. The animals were randomized into 4 groups comprised of 10 rats each, including a Sham group, IRI group, IRI+control group and an IRI+miR-27a agomir group.

The sham group was anesthetized with 2% isoflurane and received an abdominal incision. The IRI group, IRI+control group and an IRI+miR-27a agomir group rats were also anesthetized following the inhalation of 2% isoflurane. Subsequently, an abdominal incision was created, and the renal pedicle was clamped for 45 min with a microaneurysm clamp as previously described (12). Following the removal of the clamp, the kidney was inspected for the restoration of blood flow. Animal wellbeing was monitored every 8 h. The rats were sacrificed 7 days following the first procedure, through complete exsanguination via cardiac puncture under anesthesia with 2% isoflurane delivered via inhalation. The kidneys were harvested, cut longitudinally and fixed in 10% buffered formalin (4°C, overnight).

miR-27a control or agomir (Guangzhou RiboBio, Co., Ltd., Guangzhou, China) for *in vivo* use were delivered by the protocol outlined in a previous study (13). Briefly, miR-27a agomir or control in 0.1 ml of saline buffer were injected into the tail vein of animals in the IRI+miR-27a agomir group or the IRI+control group for 48 h prior to the aforementioned ischemic surgery via the tail vein at 20-40  $\mu$ l/sec.

**Cell lines.** Normal rat kidney epithelial cells (NRK52E cells) were purchased from the Beijing Concorde Cell Resource Center (Beijing, China), and were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), at 37°C in 5% CO<sub>2</sub>.

**Reverse transcription-quantitative PCR (RT-qPCR) analysis.** Total RNA was extracted from the NRK52E cells and kidney tissues using the RNA Isolation kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol, and samples were stored at -80°C until subsequent use. The RNA Reverse Transcription kit (Beyotime Institute of Biotechnology, Shanghai, China) was used for the RT of RNA into cDNA using the following conditions: 37°C for 15 min, and followed by 85°C for 5 sec. RT-qPCR was subsequently performed using a SYBR<sup>®</sup> Green Real-time PCR Master Mix (Toyobo Co. Ltd., Osaka, Japan). RT-qPCR was performed using MX3000P Real-time PCR instrument according to the manufacturer's protocol (Beyotime Institute of Biotechnology), using the following conditions: 94°C for

5 min, and followed by 30 cycles of 94°C for 30 sec, 58-61°C for 30 sec. GAPDH and U6 were used as the internal controls. The expression of miRNAs was detected using a miRcute miRNA qPCR Detection kit (SYBR Green, Tiangen Biotech Co., Ltd.) (14,15). The primer sequences used are listed in Table I. The relative gene expression levels were calculated using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (16). All experiments were performed in triplicate.

**miRNA microarray analysis.** A total of 500 ng RNA, which was extracted as above, was subjected to Agilent miRNA microarray analysis (Guangzhou RiboBio, Co., Ltd.). The differences in miRNAs between the experimental groups were examined for statistical significance using an unpaired Student's t-test.

**Western blot analyses.** Tissues and 1x10<sup>6</sup> cells were lysed with ice-cold lysis buffer containing NP40 buffer and a proteinase inhibitor cocktail (Invitrogen; Thermo Fisher Scientific, Inc.). The lysates were sonicated with an oscillation frequency of 15-25 kHz at 4°C for 10 sec, followed by centrifugation at 12,000 x g for 10 min at 4°C and the supernatants were retained. Total protein concentration was quantified using a bicinchoninic acid protein assay kit (Applygen Technologies, Inc., Beijing, China), and western blot analysis was performed. A total of 30  $\mu$ g protein/lane were resolved by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Thermo Fisher Scientific, Inc.). Following blocking with 3% BSA (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) in TBS containing 0.1% Tween-20 for 3 h at room temperature, the membranes were incubated overnight at 4°C with primary antibody. After several washes, the membranes were incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The proteins bands were visualized using enhanced chemiluminescence kits (GE Healthcare, Chicago, IL, USA), and the optical density of the protein bands was quantified using the ImageJ v1.8.0 software (National Institutes of Health, Bethesda, MD, USA), using GAPDH as an internal control. The primary antibodies used included: TLR4 (1:1,000; cat. no. sc-13593), MyD88 (1:1,000; cat. no. sc-74532), NF- $\kappa$ B (1:1,000; cat. no. sc-166588), TNF- $\alpha$  (1:1,000; cat. no. sc-52746), IL-1 $\beta$  (1:1,000; cat. no. sc-52012), Bcl-2 (1:1,000; cat. no. sc-56015), Bax (1:1,000; cat. no. sc-20067), E-cadherin (E-cad, 1:1,000; cat. no. sc-71009), Vimentin (vim, 1:1,000; cat. no. sc-80975) and GAPDH (1:1,000; cat. no. sc-51907), all purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The secondary antibodies used were HRP-conjugated anti-rabbit (1:1,000; cat. no. sc-2370) and anti-mouse (1:1,000; cat. no. sc-516102, Santa Cruz Biotechnology, Inc.) were used.

**miRDB analysis.** In order to analyze the target binding sites of microRNA-27a and TLR4, miRDB software (<http://mirdb.org/>) was used.

**Dual luciferase reporter assay.** The TLR4 wild-type and TLR4 deletion (TLR DEL, where the binding sites with miR-27a were removed) 3'-untranslated region (3'-UTR) were PCR amplified and cloned into the pMIR-REPORT<sup>™</sup> vector (Ambion; Thermo Fisher Scientific, Inc.). A total of 100,000 NRK52E

Table I. Primer sequences.

| Name       | Forward primer (5'-3') | Reverse primer (5'-3') |
|------------|------------------------|------------------------|
| miR-448    | ACACTCAUUGCAUAUGUAGG   | AUGGGACAUCGAGAGTACA    |
| miR-651-3p | ACACTCAAAAGGAAAGUGU    | CUUUUAGGAUACGAGTACAT   |
| miR-27a    | ACACTCAUUCACAGUGGC     | GCGGAACUUAGCAGTACAT    |
| miR-21     | ACACTCCAACACCAGUCG     | CAGCCCAUTTGGAGAGTACAT  |
| miR-24-3p  | ACACTCUGGCUCAGUUCAG    | CUGUCCUGTTGAGAGTACA    |
| miR-494-3p | ACACTCUGAAACAUCACAG    | GAGGUUUCAGAGTACAT      |
| U6         | CTCGCTTCGGCAGCAC       | ACGCTTCACGAATTTGCGT    |
| TLR4       | TCCTGGCTAGGACTCTGATCAT | CATGGCATGGCTTACACCACC  |
| MyD88      | GAAACTCCACAGGCGAGCG    | GTAAAGCGCGACCAAGGG     |
| NF-κB      | GTGTGGAGGCTGCCTTGCG    | GGCTTTCAAGACTGGAACGGTC |
| TNF-α      | TCTCATTCCTGCTTGTGGC    | GCTGGCCCACTAGTTGGTT    |
| IL-1β      | TGAGCACAGAAAGCATGATC   | CATCTGCTGGTACCACCAGTT  |
| Bcl-2      | TTCTTTGAGTTCGGTGGGGTC  | TGCATATTTGTTTGGGGCAGG  |
| Bax        | TCCACCAAGAAGCTGAGCGAG  | GTCCAGCCCATGATGGTTCT   |
| E-cad      | GGAGGCTCTCCCGTCTTTTG   | CTTTGTGACCGGTGCAAT     |
| Vimentin   | GGACCAGCTAACCAACGAC    | GGTCAAGACGTGCCAGAG     |
| GAPDH      | CATCCCTTCTCCCACACAC    | AGTCCCAGGGCTTTGATTTG   |

miR, microRNA; E-cad, E-cadherin; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

cells were seeded in 24-well plates, and the experiment was performed when cells reached 80% confluency. The plasmids were transfected using 2.5 μl of HiGene (Beyotime Institute of Biotechnology). Control, miR-27a and miR-27a antisense (AS; Guangzhou RiboBio, Co., Ltd.) were simultaneously transfected. After 24 h, passive lysis buffer (1x) was used to lyse the transfected cells, and 20 μl supernatant was used to measure for luciferase activity using a Dual-Luciferase Reporter Assay System (BioTek Instruments, Inc., Winooski, VT, USA). *Renilla* luciferase activity was used to normalize reporter activity. Experiments were performed in triplicate.

**MTT assay.** MTT assay (Beijing Solarbio Science and Technology Co., Ltd.) was used to measure cell viability. Cells were seeded at a density of 1x10<sup>3</sup> cells per well in 96-well plates, and transfected with 30 nM miR-27a mimic/control (CGCCUU GAAUCGGUGACACUU/UUCUCCGAACGUGUCACGU) and miR-27a inhibitor/control (GCGGAACUUAGCCAC UGUGUGAA/UUCUCCGAACGUGUCACGU) (Guangzhou RiboBio, Co., Ltd.) using HiGene transfection reagent (Beyotime Institute of Biotechnology). Each transfection condition was carried out in triplicate. At the end of the each time point, 50 μl of 5 mg/ml MTT was added to each well, and incubated at 37°C for 4 h. A total of 500 μl of DMSO was added in to each well and swirled gently to stop the reaction. The plate was left in the dark for another 4 h at room temperature. Optical density at 490 nm was measured by a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Apoptosis assay.** After 24 h from transfection with miR-27a mimic/control and miR-27a inhibitor/control, cells were subsequently stained with Annexin V-fluorescein isothiocyanate/propidium iodide (Shanghai Shenggong Biology

Table II. Differential miRNA expression in IRI.

| miRNA      | P-values | Fold change (sham/IRI) | Regulation trend |
|------------|----------|------------------------|------------------|
| miR-494-3p | 0.8470   | -                      | -                |
| miR-448    | 0.0002   | 19.875                 | Up               |
| miR-24-3p  | 0.0012   | 20.221                 | Down             |
| miR-21     | 0.0010   | 9.283                  | Up               |
| miR-27a    | 0.0001   | 35.298                 | Down             |
| miR-34a    | 0.0010   | 7.192                  | Down             |
| miR-651-3p | 0.0001   | 18.28                  | Down             |

miR, microRNA; IRI, ischemia reperfusion injury.

Engineering Technology Service, Ltd., Shanghai, China) for 20 min at room temperature in the dark. Cell apoptosis was then detected using a flow cytometry (BD FACSCalibur; BD Biosciences, San Jose, CA, USA) at 488 nm. Data were analyzed using FlowJo version 10 (FlowJo LLC, Ashland, OR, USA). Experiments were carried out in triplicate.

**Immunohistochemical staining.** Kidney samples were fixed in 10% neutral buffered formalin (4°C, overnight), embedded in paraffin, sliced into sections 5 μm thick. The paraffin sections were subsequently de-waxed in water, and incubated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature. The sections were incubated with 3% goat serum (cat. no. 16210064; Thermo Fisher Scientific, Inc.) at room temperature for 10 min, and subsequently incubated with a primary antibody against TLR4 (1:100, cat. no. sc-13593, Santa Cruz Biotechnology, Inc.)

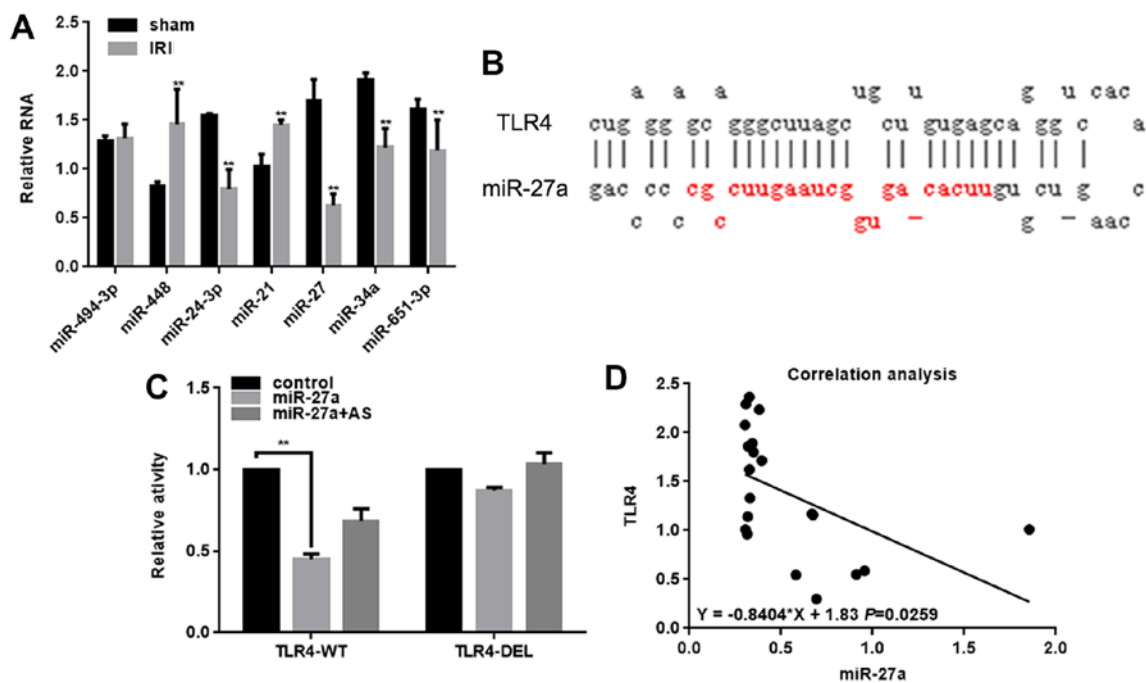


Figure 1. Changes in miRNA expression levels in renal IRI. (A) The expression of miRNAs in the sham and IRI groups were detected by reverse transcription-quantitative PCR. \*\* $P < 0.01$  vs. sham group. (B) miRDB predicted that miR-27a could be combined specifically with TLR4. (C) Interactions between miR-27a and the TLR4 3'-UTR were examined via a luciferase reporter assay. \*\* $P < 0.01$ , as indicated. (D) Correlation between the expression levels of miR-27a and TLR4 in IRI tissues. Data are presented as the mean  $\pm$  standard error of the mean. miRNA/miR, microRNA; IRI, ischemia reperfusion injury; TLR4, Toll-like receptor 4; WT, wild-type; DEL, deletion.

4°C overnight. An avidin-biotin-HRP complex immunodetection kit (Shanghai Shenggong Biology Engineering Technology Service, Ltd.) was used to detect the reaction according to the manufacturer's instructions, and the samples were examined by light microscopy at x400 magnification (Leica DM2500M; Leica Microsystems GmbH, Wetzlar, Germany).

**ELISA.** Experiments were performed according to the protocol outlined in the TNF- $\alpha$  (cat. no. ab236712; Abcam, Cambridge, UK) and IL-1 $\beta$  ELISA kits (cat. no. ab100768; Abcam). Tissues and cells were incubated at 37°C for 30 min. According to the manufacturer's protocol: A total of 100  $\mu$ l plasma or supernatant was added onto the TNF- $\alpha$ /IL-1 $\beta$  antibody-coated plate, and incubated at 25°C for 2 h. After adding the biotin-conjugated detecting TNF- $\alpha$ /IL-1 antibody and incubating for 2 h, streptavidin-HRP was added, and 3,3',5,5' tetramethylbenzidin was used for development. The optical density value was measured at 450 nm by Multiskan spectrum (Thermo Fisher Scientific, Inc.). Experiments were performed in triplicate.

**Detection of blood urea nitrogen (BUN) and serum creatinine (Scr).** The blood of rats in each group was extracted via the abdominal aorta and centrifuged 37°C for 5 min at 3,000  $\times$  g. The BUN and Scr levels of mice in each group were measured using an automatic biochemical analyzer.

**Statistical analysis.** All data were analyzed with SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean  $\pm$  standard error of the mean.  $P < 0.05$  was considered to indicate a statistically significant difference. Statistical analyses were performed one-way analysis of variance when there

were  $>2$  groups, followed by Tukey's post hoc test for multiple comparisons. A Student's t-test was used to compare differences between 2 groups. Actuarial survival rates were calculated using the Kaplan-Meier method, and differences in survival in univariate comparisons were compared using the log-rank test.

## Results

**Changes in miRNA levels in renal IRI.** The miRNA microarray revealed that various miRNA changes occurred following renal IRI when compared with the sham group (Table II).

The expressions of these miRNAs in the IRI and sham groups were detected by RT-qPCR (Fig. 1A). The results demonstrated that the expression of miR-27a was significantly decreased in the IRI group. In addition, the miRDB software indicated that miR-27a could bind to the 3'UTR of TLR4 (Fig. 1B), which serves an important role in renal IRI (17). The luciferase reporter gene assay also confirmed that miR-27a could inhibit the activity of TLR4 (Fig. 1C). However, when the miR-27a and TLR4 binding sites were mutated, the inhibitory effect of miR-27a on TLR4 was attenuated (Fig. 1C). In addition, the co-transfection of miR-27a and miR-27a AS did not affect the activity of TLR4 (Fig. 1C). miR-27a also inhibits the activity of TLR4, by binding to the 3'UTR region of TLR4, as indicated by the negative correlation between the expression of TLR4 and miR-27a in renal IRI (Fig. 1D). Taken together, the results of the present study suggest that miR-27a serves an important role in renal IRI by regulating TLR4.

**Expression of TLR4 and its downstream pathway in renal IRI.** Immunohistochemical staining revealed that the expression of TLR4 was higher in renal tissue following IRI (Fig. 2A). The

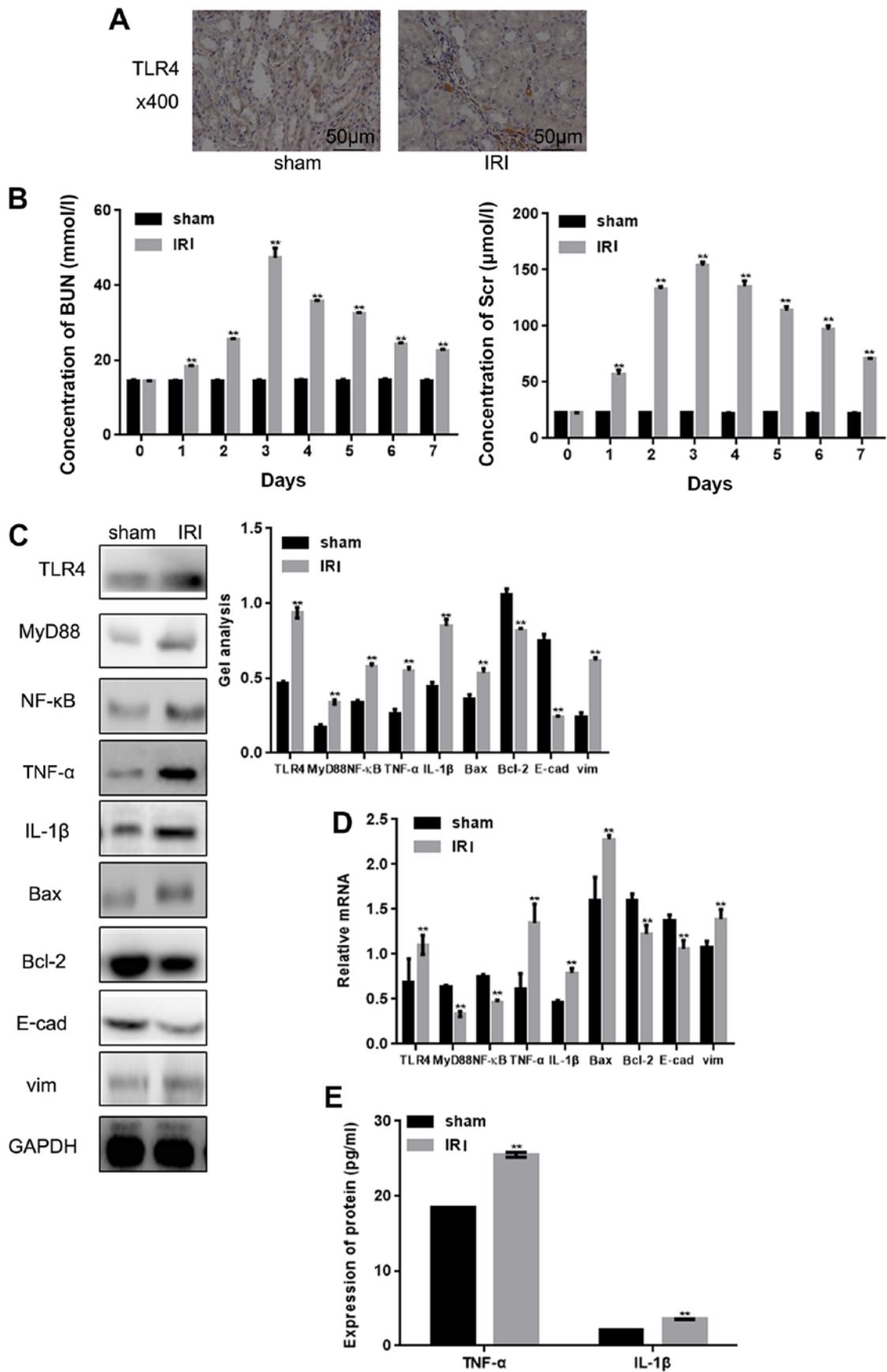


Figure 2. Expression of TLR4 and its downstream pathway in renal IRI. (A) TLR4 expression in renal tissues was detected by immunohistochemical staining (magnification, x400; scale bars, 50 µm) (B) The expression of BUN and Scr were detected by automatic biochemical analyzer. (C and D) Levels of TLR4, MyD88, NF-κB, TNF-α, IL-1β, Bax, vim, Bcl-2 and E-cad in tissues were detected with (C) western blotting and (D) reverse transcription-quantitative PCR. (E) Levels of TNF-α and IL-1β in tissues were detected by ELISA. Data are presented as the mean ± standard error of the mean. \*\*P<0.01 vs. sham group. IRI, ischemia reperfusion injury; TLR4, Toll-like receptor 4; E-cad, E-cadherin; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; BUN, blood urea nitrogen; Scr, serum creatinine.

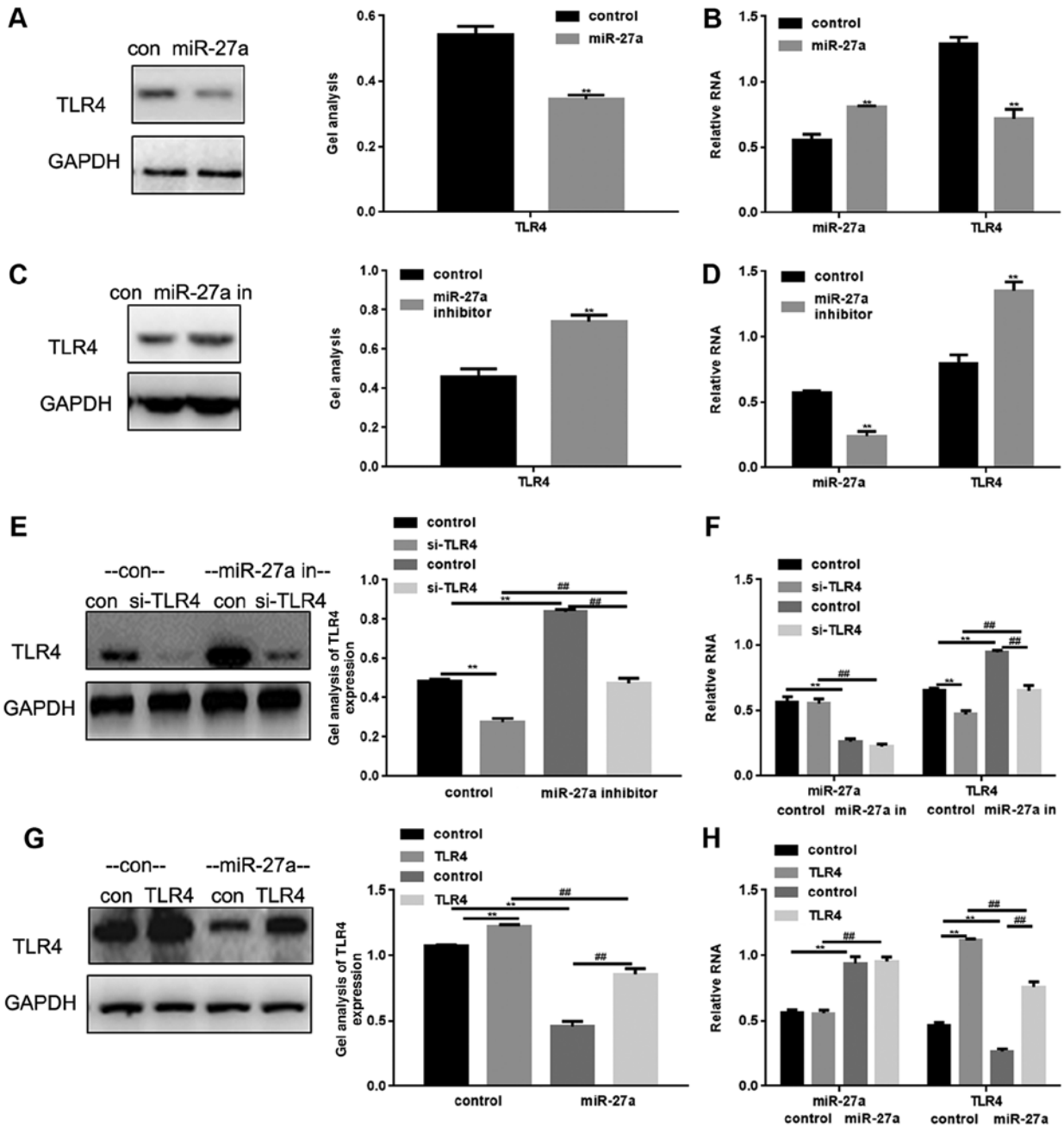


Figure 3. Association between miR-27a and TLR4 in NRK52E cells. (A) Western blotting and (B) RT-qPCR revealed that when the miR-27a was overexpressed, TLR4 was downregulated in cells. \*\*P<0.01 vs. control group. (C) Western blotting and (D) RT-qPCR demonstrated that when the expression of miR-27a was decreased, TLR4 was upregulated in cells. \*\*P<0.01 vs. control group. (E and F) Effects of the miR-27a inhibitor and TLR4 siRNA on TLR4 were detected by (E) western blotting and (F) RT-qPCR. \*\*P<0.01 vs. the indicated control group; ##P<0.01 vs. the indicated si-TLR4+miR-27a inhibitor group. (G and H) The effects of miR-27a and TLR4 on TLR4 were detected by (G) western blotting and (H) RT-qPCR. Data are presented as the mean ± standard error of the mean. \*\*P<0.01 vs. the indicated control group, ##P<0.01 vs. the indicated TLR4+miR-27a group. miR, microRNA; TLR4, Toll-like receptor 4; RT-qPCR, reverse transcription-quantitative PCR; siRNA/si-, small interfering RNA; in, inhibitor.

subsequent detection of BUN and Scr levels demonstrated that their expressions were upregulated following IRI (Fig. 2B). Western blot analysis and RT-qPCR also demonstrated that TLR4 and its downstream signaling pathways were altered in renal IRI (Fig. 2C and D). Among them, the expressions of TLR4, MyD88, NF-κB, TNF-α, IL-1β, Bax and vim were upregulated, and the expressions of Bcl-2 and E-cad were downregulated. This phenomenon suggests that inflammation, apoptosis and the enhancement of cell adhesion occurred in the renal IRI tissues. An ELISA also demonstrated that there was

an upregulation of TNF-α and IL-1β in the IRI group, which demonstrated that IRI can lead to inflammation (Fig. 2E).

*Association between miR-27a and TLR4 in NRK52E cells.* Following transfection with an miR-27a mimic or miR-27a inhibitor into NRK52E cells, western blotting and RT-qPCR were performed in order to detect the effect of miR-27a on TLR4 (Fig. 3). Transfection efficiency was confirmed by RT-qPCR and western blotting (Fig. 3B and D-H). The results demonstrated that the expression of TLR4 was markedly

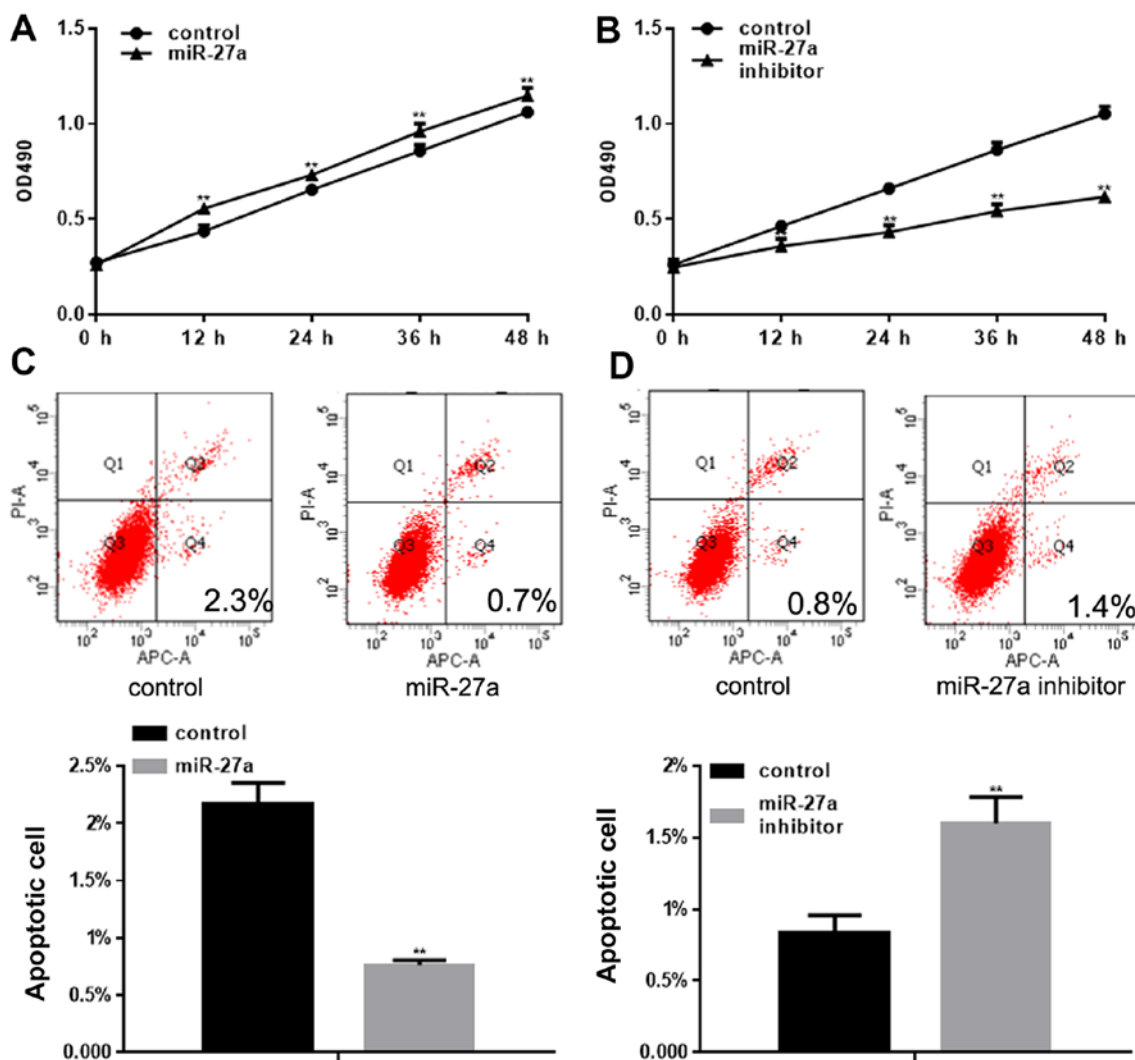


Figure 4. miR-27a promotes the growth of NRK52E cells. (A) The MTT assay demonstrated that the proliferation of cells was promoted following transfection with miR-27a. (B) The MTT assay also demonstrated that the proliferation of cells was inhibited by transfection with an miR-27a inhibitor. (C) Following transfection with miR-27a, cell apoptosis was significantly inhibited. (D) Following transfection with the miR-27a inhibitor, cells apoptosis was significantly promoted. Data are presented as the mean  $\pm$  standard error of the mean. \*\* $P < 0.01$  vs. control group. miR, microRNA; OD, optical density; PI, propidium iodide; APC, allophycocyanin.

downregulated by miR-27a (Fig. 3A and B) and upregulated with miR-27a inhibitor (Fig. 3C and D). The present study also observed that treatment with the miR-27a inhibitor maintained the downregulated expression of TLR4 induced by si-TLR4 (Fig. 3E and F). miR-27a was also observed to reduce the degree of the TLR4 expression upregulation induced by TLR4 transfection (Fig. 3G and H).

*miR-27a promotes the growth of NRK52E cells.* An MTT assay was used to detect the proliferation of NRK52E cells following transfection with miR-27a or the miR-27a inhibitor. miR-27a was observed to promote the proliferation of cells; however, the miR-27a inhibitor significantly inhibited the proliferation of cells (Fig. 4A and B). The present study also demonstrated that miR-27a could inhibit cell apoptosis, and the miR-27a inhibitor significantly promoted cell apoptosis (Fig. 4C and D).

*miR-27a inhibits renal IRI by inhibiting TLR4.* The present study increased the expression of miR-27a in rats with renal

IRI, and following this, the expression of TLR4 was partially decreased (Fig. 5A). The subsequent detection of BUN and Scr demonstrated that their expressions were downregulated following the overexpression of miR-27a (Fig. 5B). Western blotting and RT-qPCR also demonstrated that the overexpression of miR-27a could downregulate the expressions of TLR4, MyD88, NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , Bax and vim, and upregulate the expressions of Bcl-2 and E-cad (Fig. 5C and D). Furthermore, the ELISA also observed that the expressions of TNF- $\alpha$  and IL-1 $\beta$  were significantly decreased by miR-27a (Fig. 5E).

## Discussion

IRI is commonly observed in a clinical setting and ailments such as shock, myocardial infarction and vascular occlusion during surgery may lead to IRI (18).

A recent study has demonstrated that specific Dicer enzymes in the epithelial cells of the proximal renal tubular epithelial cells can mitigate, and to an extent cause, renal IRI by regulating changes in the expression of various miRNAs (19).

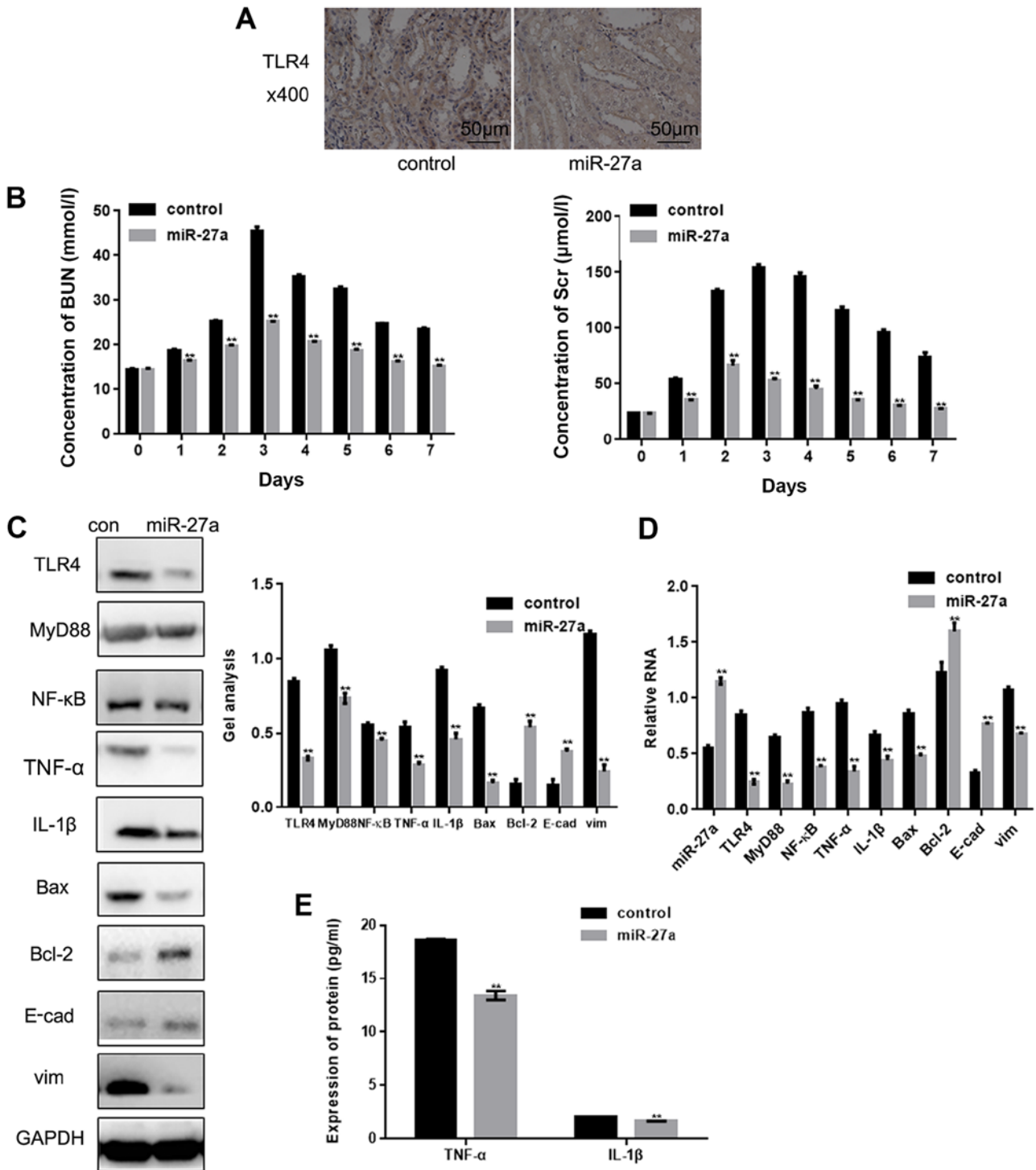


Figure 5. miR-27a inhibits renal IRI by inhibiting TLR4. (A) Expression of TLR4 in tissues was detected by immunohistochemical staining (magnification, x400; scale bars, 50 µm). (B) The expressions of BUN and Scr were detected by an automatic biochemical analyzer. \*\*P<0.01 vs. control group at each time point. (C and D) The levels of miR-27a, TLR4, MyD88, NF-κB, TNF-α, IL-1β, Bax, vim, Bcl-2 and E-cad in tissues were detected by western blotting and reverse transcription-quantitative PCR. (E) The levels of TNF-α and IL-1β in tissues were detected by ELISA. Data are presented as the mean ± standard error of the mean. \*\*P<0.01 vs. control group. miR, microRNA; IRI, ischemia reperfusion injury; TLR4, Toll-like receptor 4; E-cad, E-cadherin; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; BUN, blood urea nitrogen; Scr, serum creatinine.

miR-126 and miR-24 are also considered to be important miRNAs in regulating renal IRI (7,19-21). The results of the present study indicated that miR-27a could improve the pathological damage of the renal tubular epithelium by reducing apoptosis, and also improved the repair of renal function by alleviating inflammatory reactions. A previous

study demonstrated that the overexpression of miR-27 in a rat subarachnoid IR model reduced the expression of Toll-like receptor adaptor molecule-2 (22). It has also been reported that the sustained intrathecal injection of an miR-27a agomir prior to ischemia can significantly alleviate ischemic symptoms (22). This suggests that miR-27a may be particularly



important for the pathophysiology of IRI models. In the present study, a significant downregulation of miR-27a was observed following renal IRI using microarray technology.

The present study also observed a negative correlation between miR-27a and the expression of TLR4 in a renal IRI model, and that miR-27a and the 3'UTR of TLR4 have target binding sites. Taken together, these results demonstrate that miR-27a can inhibit the activity of TLR4.

In the early stage of IR, TLR4 recognizes the activation of endogenous ligands released by injured kidney cells, and subsequently activates downstream signaling pathways through the binding protein MyD88 (21). NF- $\kappa$ B is an important signal transduction pathway downstream of TLR4, which controls the transcription of the majority of apoptotic and immunoinflammatory genes (23). In addition, apoptosis can be regulated by affecting the expression of Bax and Bcl-2 (24), and TLR4 also exerts specific regulatory effects on E-cad and vim, which in turn affects cell adhesion (25). The present study constructed an IRI rat model, and observed that the expression of TLR4 was significantly upregulated. The downstream signaling molecules MyD88 and NF- $\kappa$ B of TLR4 were activated, the secretion of pro-inflammatory cytokines and chemokines increased, and apoptosis levels also increased, suggesting that the TLR4 signaling pathway was overactivated in the IRI model.

A previous study confirmed that when IR occurs, cell proliferation ability decreases and apoptosis increases (26). The present study demonstrated that miR-27a can promote cell proliferation and inhibit cell apoptosis, which may provide an alternative explanation for the protective effect of miRNA-27a in the kidneys.

In the rat model of renal IRI, the present study observed that the expressions of TLR4, MyD88, NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , Bax and vim were upregulated, whereas the expressions of Bcl-2 and E-cad were downregulated, and the changes in the expressions of these proteins were partially restored following the overexpression of miR-27a. This suggests that miR-27a can inhibit the inflammatory response, cell adhesion enhancement and apoptosis by regulating the expression of TLR4 *in vivo*.

In conclusion, the present study demonstrated that miR-27a is downregulated in renal IRI, and that miR-27a may partially influence the process of IRI by targeting TLR4. Furthermore, miR-27a may serve as a potential therapeutic target for the treatment of IRI in the future.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

YW performed the majority of the experiments, and was one of the contributors in writing the manuscript. DW conducted the miRNA analysis, and ZJ designed the experiment and wrote the manuscript. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

The current study followed internationally recognized guidelines on animal welfare, and obtained approval of China Medical University Ethics Committee for Animal Experimentation (Liaoning, China; no. CMU201603). The care and use of laboratory animals was performed in accordance to the National Institutes of Health guide. All animal studies complied with the ARRIVE guidelines and the AVMA euthanasia guidelines of 2013.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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