

miR-30a-5p mitigates autophagy by regulating the Beclin-1/ATG16 pathway in renal ischemia/reperfusion injury

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Abstract. Renal ischemia/reperfusion (I/R) injury often occurs during multiple organ failure and sepsis, and autophagy may serve a role in I/R injury. The aim of the present study was to explore the effect of microRNA (miR)-30a-5p on autophagy in renal I/R injury. miR-30a-5p and autophagy-related protein expression levels in renal I/R injury mouse models and in hypoxia/re-oxygenation HK-2 cell models were determined using reverse transcription-quantitative PCR or western blotting; apoptosis was analyzed using flow cytometry. The effects of miR-30a-5p, Beclin-1 and autophagy-related gene 16 (ATG16) on the proliferation and autophagy of HK-2 cells were analyzed through gain- and loss-of-function studies. miR-30a-5p expression was significantly decreased after renal I/R injury in the *in vivo* and *in vitro* experiments. Renal I/R injury led to upregulated expression of autophagy-related proteins microtubule-associated protein light chain 3 (LC3)-II and Beclin-1, and downregulated expression of p62. miR-30a-5p overexpression decreased the number of LC3 punctae, decreased HK-2 cell apoptosis, increased p62 expression and decreased LC3-II and Beclin-1 expression. Inhibition of miR-30a-5p exhibited the opposite effects. A luciferase reporter assay demonstrated that miR-30a-5p targeted Beclin-1. Beclin-1 overexpression led to a significant increase in LC3-II expression and a decrease in p62 expression, as well as a significant increase in apoptosis. Beclin-1 overexpression also increased the protein expression level of ATG16. Downregulation of Beclin-1 decreased the expression of LC3-II, elevated the p62 level and decreased apoptosis. ATG16 knockdown showed similar effects as those of Beclin-1 downregulation. In conclusion, miR-30a-5p was increased in renal I/R injury and might mitigate autophagy by regulating the Beclin-1/ATG16 pathway.

Introduction

Acute kidney injury (AKI) is one of the most common clinical syndromes following surgical operations and sepsis; it is accompanied by a disorder in kidney function (1). AKI occurs in 10-15% of patients admitted to hospital, whereas its incidence in intensive care has been reported in >50% of patients. AKI is evaluated by examining the levels of serum creatinine (SCr) and blood urea nitrogen (BUN) (2). Among the etiology of AKI, prerenal etiologies account for 25-60% and renal etiologies account for 35-70% of AKI cases (3). In the kidney, proximal tubules are more susceptible to ischemia than are the inner medulla and deep papillae (4). Ischemia/reperfusion (I/R) injury of the kidney is the leading cause of AKI (5). It was reported that ischemic injury or nephrotoxins contribute to 80-90% of the renal etiologies (3).

Autophagy is a cellular stress response that serves important roles in cellular homeostasis (6,7). Autophagy can be caused by stress, including cell starvation, oxidant injury and endoplasmic reticulum stress, as well as hypoxia (6,7). Most of these stresses occur in the pathogenesis of AKI (8). Accumulating evidence indicates that autophagy may serve a protective role in cell survival during renal ischemia/reperfusion injury (9,10).

MicroRNAs (miRNAs) are a family of small (19-22 nucleotides), non-coding RNAs (11). Previous studies have implicated dysregulated miRNAs in the pathogenesis of kidney injury (5,12,13). miRNA (miR)-30a-5p is a tumor suppressor in several cancer types (14-16). Previous studies have linked miR-30a-5p to ischemic heart disease (17) and cerebral ischemic stroke (18). miR-30a-5p is also reported to regulate renal cell carcinoma aggressiveness (15). However, the function and underlying mechanisms of miR-30a-5p in ischemic kidney injury remain unclear. The present study aimed to explore whether miR-30a-5p affected ischemic kidney injury and the autophagy of tubular epithelial cells, as well as to investigate the underlying mechanism.

Materials and methods

Animal models. Male C57BL/6 mice (age, 6-8 weeks; weight, 18-22 g) were from Chengdu Dossy Experimental Animals Co., Ltd. The mice were maintained in a temperature (22±2°C) and humidity-controlled (55±5%) vivarium with a 12-h light/dark cycle and allowed free access to food and water. Mice were

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anesthetized with an intraperitoneal injection of pentobarbital (70 mg/kg) and kept on a warm blanket to maintain the body temperature at 37°C during surgery. To induce renal I/R injury, the bilateral renal pedicles were clamped for 60 min with a microaneurysm clip followed by 24 h reperfusion. For miR-30a-5p detection, mice with 6 h reperfusion after 60 min clamping were also used. Mice were sacrificed by cervical dislocation, and their kidneys and blood were collected for examination. Mice in the sham group underwent the same surgical procedures without clamping. The Ethics Committee of the General Hospital of Central Theater Command (Wuhan, China) approved this study, which followed the regulatory standards of the National Institutes of Health guide for the care and use of laboratory animals (19).

For the *in vivo* study of the function of miR-30a-5p, adenovirus miR-30a-5p mimic (Ad-mimic) and miR-30a-5p inhibitor (Ad-inhibitor) were purchased from Shanghai GenePharma Co. Ltd. Mice were intraperitoneally injected with 1×10^9 pfu Ad-mimic or Ad-inhibitor 24 h before renal I/R injury.

Histopathological evaluation. Freshly harvested kidney tissues from mice were fixed in 4% paraformaldehyde for 16 h at room temperature. The tissues were then dehydrated through an ethanol gradient. Tissues were paraffin embedded and cut into 5 μ m sections. The sections were then deparaffinized with xylene and rehydrated in an ethanol gradient and distilled water. The sections were stained with hematoxylin for 5 min, washed with water, differentiated for 30 sec and stained with eosin for 30 sec at room temperature. Histological damage was quantified in a blinded manner using a light microscope. Tubular necrosis scores were determined by assessing the percentage of tubules that displayed epithelial necrosis or had luminal necrotic debris, tubular dilation and hemorrhage: 0, none; 1, mild (<25%); 2, moderate (25-50%); 3, severe (51-75%); and 4, extensive (>75%) damage (20).

Detection of SCr, BUN and kidney injury molecule 1 (Kim-1). Serum levels of SCr and BUN were analyzed using commercially available Creatinine Assay kit (cat. no. C011-1-1) and Urea Assay kit (cat. no. C013-2-1) from Nanjing Jiancheng Bioengineering Institute, respectively, according to the manufacturer's procedure. Urine concentrations of Kim-1 were analyzed using commercially Kidney Injury Molecule 1 Assay kit (cat. no. H436-1; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's procedure.

ELISA analysis. Serum levels of TNF- α , IL-1 β and IL-6 were determined by commercially available mouse TNF- α ELISA kit (cat. no. PT512), mouse IL-1 β ELISA kit (cat. no. PI301) and mouse IL-6 ELISA kit (cat. no. PI326) from Beyotime Institute of Biotechnology, respectively. Absorbance was measured at 450 nM using a microplate reader (Bio-Rad Laboratories, Inc.). All samples were analyzed in triplicate.

Cell lines. HK-2 human tubular epithelial and 293T cell lines were obtained from the American Type Culture Collection (ATCC) and were cultured according to ATCC protocols. Both cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), streptomycin (100 μ g/ml), penicillin (100 U/ml) and

2 mM of L-glutamate. All cells were cultured at 37°C in a 5% CO₂ incubator (Thermo Fisher Scientific, Inc.). To induce hypoxia/re-oxygenation (H/R) injury, HK-2 cells were incubated in a hypoxic chamber with 0.3% O₂, 5% CO₂, and 95% N₂ for 16 h, and then were re-oxygenated in a normoxic chamber with 5% CO₂ and 95% N₂ for 4 h.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from renal cortex tissues and cells using a RNeasy mini kit (Qiagen Sciences, Inc.) according to the manufacturer's protocols. cDNA was synthesized using the SuperScript III First-Strand Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. qPCR was performed using TaqMan Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and an ABI 7300 Real Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 10 sec, annealing and extension at 60°C for 1 min. The following primers were used: miR-30a-5p, forward 5'-TGTAACAT CCTCGACTGGAAG-3' and reverse 5'-TGCGTGTCTGAGTC-3'; U6, forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'; Beclin-1, forward 5'-AGGTTGAGAAAGGCGAGACA-3' and reverse 5'-TTTTGATGGAATAGGAGCCG-3'; and β -actin, forward 5'-GTGGGGCCGCTAGGCACCA-3' and reverse 5'-CGG TTGGCCTTAGGGTTTCTAG-3'. β -actin or U6 were used as the internal controls. Relative expression levels were analyzed using $2^{-\Delta\Delta C_q}$ method (21).

Western blotting. Proteins were obtained from mouse renal cortex and HK-2 cells using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). Protein concentration was measured with a BCA Protein Assay kit (cat. no. P0011; Beyotime Institute of Biotechnology). Equal amounts (30 μ g) of protein from each sample were separated by 10% SDS-PAGE. The separated bands were then electrotransferred onto a polyvinylidene difluoride membrane (Roche Diagnostics). After being blocked with 5% skim milk on a rotary shaker at room temperature for 2 h, the blots were incubated with the following primary antibodies: Rabbit anti-human microtubule-associated protein light chain 3 (LC3)-I/II monoclonal antibody (1:2,000; cat. no. 12741; Cell Signaling Technology, Inc.), rabbit anti-human Beclin-1 monoclonal antibody (1:1,500; cat. no. 3495; Cell Signaling Technology, Inc.), rabbit anti-human p62 monoclonal antibody (1:2,000; cat. no. ab207305; Abcam), rabbit anti-human autophagy-related gene16 (ATG16) monoclonal antibody (1:2,000; cat. no. 8089; Cell Signaling Technology, Inc.), and mouse anti-human β -actin (1:2,500; cat. no. ab8226; Abcam) overnight at 4°C, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:10,000; cat. no. ab6789; Abcam) or HRP-conjugated goat anti-rabbit IgG (1:10,000; cat. no. ab6721; Abcam) for 30 min at 37°C. Protein bands were visualized using ECL Western Blotting Substrate kit (cat. no. 32209; Pierce; Thermo Fisher Scientific, Inc.) and semi-quantified using the ImageJ software (version 1.47; National Institutes of Health); β -actin was used for normalization.

Cell transfection. miR-30a-5p mimic (sense, 5'-UGUAAA CAUCCUCGACUGGAAG-3'; anti-sense, 5'-CUUCCAGUC GAGGAUGUUUACA-3'), miR-30a-5p inhibitor (5'-CUU CCAGUCGAGGAUGUUUACA-3'), miRNA mimic-negative control (nc) (sense, 5'-UCACAACCUCCUAGAAAGAGU AGA-3'; anti-sense, 5'-UACUCUUUCUAGGAGGUUGUG AUU-3') and inhibitor-nc (5'-CACAUUGTGCUCUCUGCA CUGCTC-3'), Beclin-1 small interfering (si)RNA (sense, 5'-GGAGCCAUUUUAUUGAAACUTT-3'; anti-sense, 5'-AGU UUCAAUAAAUGGCUCCTT-3'), siRNA-scramble control (sense, 5'-GUACGCCAAAAGUUAACCT-3'; anti-sense, 5'-GGUUUAACUUUUGGCGUAC-3') and ATG16 siRNA (sense, 5'-AGAGCUUGACUCAGACCAAGU-3'; anti-sense, 5'-UUGGUCUGAGUCAAGCUCUGA-3') were purchased from Shanghai GenePharma Co. Ltd. Beclin-1 cDNA was amplified from the HK-2 cells and cloned into the pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). For miR-30a-5p function analysis, 3×10^5 HK-2 cells were transfected with miR-30a-5p mimic (50 nM), miR-30a-5p inhibitor (100 nM), miRNA-nc (50 nM) or inhibitor-nc (100 nM) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h. To downregulate the expression of Beclin-1 or ATG16, 3×10^5 HK-2 cells were transfected with siRNA-scramble control (50 nM), Beclin-1 siRNA (50 nM) or ATG16 siRNA (50 nM) using Lipofectamine[®] 2000 for 48 h. To overexpress Beclin-1, 3×10^5 HK-2 cells were transfected with pcDNA3.1 empty vector (0.5 μ g) or pcDNA3.1-Beclin-1 (0.5 μ g) using Lipofectamine[®] 2000 for 48 h.

Flow cytometry. Apoptosis was detected using the Annexin V-FITC Apoptosis Detection kit (cat. no. C1062L; Beyotime Institute of Biotechnology) according to previously reported protocols (22). Briefly, cells (1×10^6) were re-suspended in 100 μ l binding buffer. Annexin V-FITC (5 μ l) and propidium iodide staining solution (10 μ l) were added to the cell suspension and the cells were cultured in the dark for 15 min at room temperature. The percentage of early + late stage apoptotic was detected using a BD FACSCalibur flow cytometer (BD Biosciences), and data were analyzed using FlowJo 7.6.1 (FlowJo LLC).

Immunofluorescence staining. Immunofluorescence staining of LC3 was performed according to previously described methods (23). Briefly, 3×10^5 cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.02% Triton X-100 for 5 min at room temperature, blocked with 5% bovine serum albumin (Beyotime Institute of Biotechnology) in PBS for 20 min at 4°C and incubated with rabbit anti-human LC3 monoclonal antibody (1:1,000; cat. no. 3868; Cell Signaling Technology, Inc.) for 12 h at 4°C. After washing three times with PBS, cells were incubated with the Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:200; cat. no. A-11034; Thermo Fisher Scientific, Inc.) for 2 h. Images were captured using fluorescence microscopy (Zeiss AG). The number of LC3 punctae in each cell were counted. A minimum of 50 cells were included for each group.

In silico analysis. The predicted targets of miR-30a-5p were analyzed by the EIMMo miRNA target prediction server (24),

microRNA.org database (25), and TargetScan (Release 7.2; March 2018; <http://www.targetscan.org>) database.

Luciferase reporter assay. The pGLO-Beclin-1-wild-type (WT) plasmids were constructed by cloning the 3'-untranslated region (3'-UTR) of Beclin-1 containing the predicted miR-30a-5p binding site downstream of the firefly luciferase structural gene of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation). Similarly, the mutant (Mut) 3'-UTR of Beclin-1 was generated using a QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Inc.), according to the manufacturer's protocol, and used to make the pGLO-Beclin-1-Mut plasmids. The 293T cells were plated into 24-well plates (1.5×10^5 cells/well) and incubated at 37°C for 24 h. Subsequently, the cells were transfected at 37°C with 100 nM miR-30a-5p or a mimic-nc combined with 100 ng pGLO-Beclin-1-WT or pGLO-Beclin-1-Mut plasmid using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 48 h after transfection, luciferase activities were measured using a Dual-Luciferase Reporter Assay kit (Promega Corporation) according to the manufacturer's instructions using a GloMax 20/20 luminometer (Promega Corporation). The relative luciferase activity was normalized by *Renilla* luciferase activity.

RNA immunoprecipitation (RIP). RIP was performed using an EZ-Magna RIP RNA-Binding Protein Immunoprecipitation kit (cat. no. 17-701; MilliporeSigma) in HK-2 cells transfected with miR-30a-5p mimic or mimic-nc. 5×10^6 cells were lysed with complete RIP lysis buffer. Subsequently, 100 μ l of cell lysate was incubated with RIP buffer containing magnetic beads conjugated with mouse anti-human ago2 antibody (1:10; cat. no. MA5-23515; Thermo Fisher Scientific, Inc.) or negative control normal mouse IgG (1:10; cat. no. ab188776; Abcam). Samples were incubated with Proteinase K buffer, washed with RIP Wash Buffer, and then the bound RNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1, v/v) for RT-qPCR assay, aforementioned.

Statistical analysis. All statistical analyses were conducted using SPSS 20.0 software (IBM Corp.). Data are expressed as the mean \pm standard deviation. Differences between two groups were assessed using an independent samples t-test. For multiple-group comparisons, a significant one-way analysis of variance F-test was used, followed by the Tukey-Kramer test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-30a-5p expression is decreased in mouse models of renal I/R injury and in H/R-exposed HK-2 cells. To induce renal I/R injury, the bilateral renal pedicles were clamped for 60 min with a microaneurysm clip followed by 24 h reperfusion (model group). Mice were subsequently euthanized, and their kidneys and blood were collected for examination. Mice in the sham group underwent the same surgical procedures without clamping. Compared with the sham surgery group, the mouse models of renal I/R injury exhibited significantly increased serum levels of SCr, BUN and urine concentrations

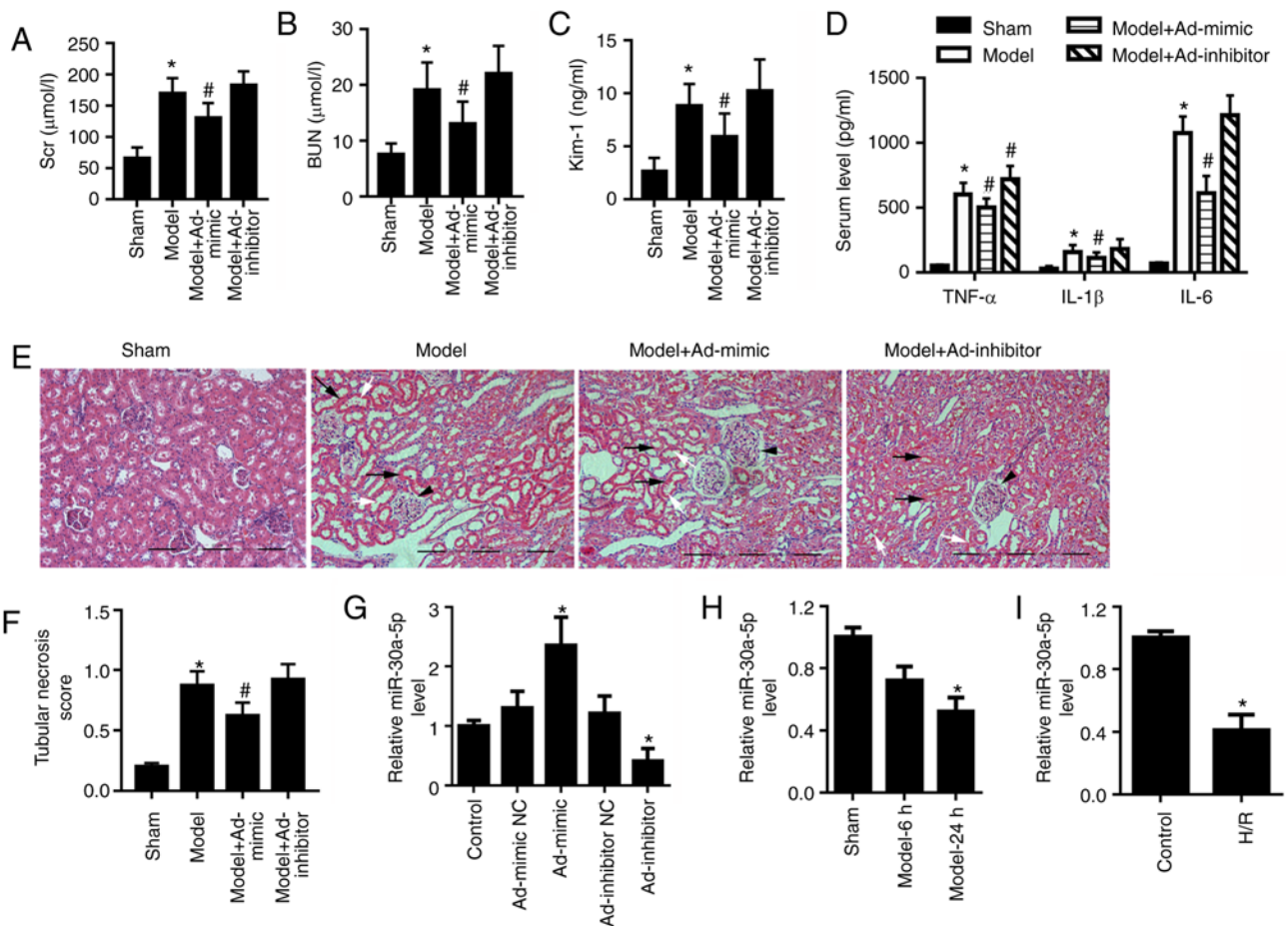


Figure 1. miR-30a-5p expression is decreased in *in vivo* and *in vitro* models of renal I/R injury. Mice were intraperitoneally injected with Ad-mimic or Ad-inhibitor 24 h before renal ischemia/reperfusion injury. ELISA analysis for serum levels of (A) SCr and (B) BUN, and (C) urine concentration of Kim-1 in mice (n=6). (D) ELISA analysis for serum levels of TNF- α , IL-1 β and IL-6. (E) Renal histological changes in mice were observed by H&E staining; scale bar, 40 μ m. Black arrows indicate the loss of epithelial cell nuclei; white arrows point to abscission of tubular epithelial cells; and black arrowheads indicate glomerular hypertrophy. (F) The histological tubular necrosis scores were evaluated. (G) miR-30a-5p expression levels in the cortex region of mouse kidneys following intraperitoneal injection with Ad-mimic or Ad-inhibitor were detected by RT-qPCR. (H) miR-30a-5p expression levels in the renal cortex region of mice was detected using RT-qPCR at 6 and 24 h post-reperfusion (n=6). (I) miR-30a-5p expression in H/R-exposed HK-2 cells was assessed by RT-qPCR (n=3). *P<0.05 vs. sham or control; #P<0.05 vs. model. Ad, adenovirus; H/R, hypoxia/reoxygenation; Kim-1, kidney injury molecule 1; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; BUN, blood urea nitrogen; SCr, serum creatinine.

of Kim-1 (Fig. 1A-C, respectively). To assess the levels of inflammation during injury, the concentrations of TNF- α , IL-1 β and IL-6 were also examined using ELISA kit. The result showed that serum levels of TNF- α , IL-1 β and IL-6 were significantly elevated after renal I/R compared with the sham group (Fig. 1D). Histopathological examination revealed tubular epithelial cell necrosis in renal I/R injury model group, including abscission of tubular epithelial cells, glomerular hypertrophy and loss of epithelial cell nuclei (Fig. 1E and F). These results confirmed the successful establishment of the mouse models of kidney injury.

For the *in vivo* study of the function of miR-30a-5p, model mice were intraperitoneally injected with Ad-mimic or Ad-inhibitor 24 h before renal I/R injury. miR-30a-5p expression levels in the cortex region of mice were detected by RT-qPCR, which demonstrated that miR-30a-5p was significantly upregulated by Ad-mimic injection and was significantly downregulated by Ad-inhibitor injection compared with control group (Fig. 1G). The result showed that miR-30a-5p overexpression partially relieved renal I/R injury *in vivo*, whereas miR-30a-5p knockdown exhibited less effect on

serum levels of SCr, BUN, TNF- α , IL-1 β and IL-6, urine concentrations of Kim-1 and tubular necrosis score in the renal I/R injury mouse models compared with the untreated model group (Fig. 1A-F). In the cortex region of mice with renal I/R injury, miR-30a-5p expression decreased compared with the sham group (Fig. 1H). Consistent with the results of the *in vivo* experiments, miR-30a-5p expression was also significantly decreased in the H/R-exposed HK-2 cells as compared with the control cells (Fig. 1I).

miR-30a-5p suppresses autophagy and apoptosis in renal I/R injury. Compared with those of the sham group, the cortex region of mice with renal I/R injury exhibited an upregulation in the protein expression levels of autophagy-related proteins LC3-II and Beclin-1, and downregulated expression of p62 (Fig. 2A). Ad-mimic infection reduced the expression of LC3-II and Beclin-1 but increased the expression of p62 compared with the model group (Fig. 2A). Ad-inhibitor infection increased the expression of LC3-II and decreased the expression of p62 compared with the model group (Fig. 2A); however, no significant difference was detected for the

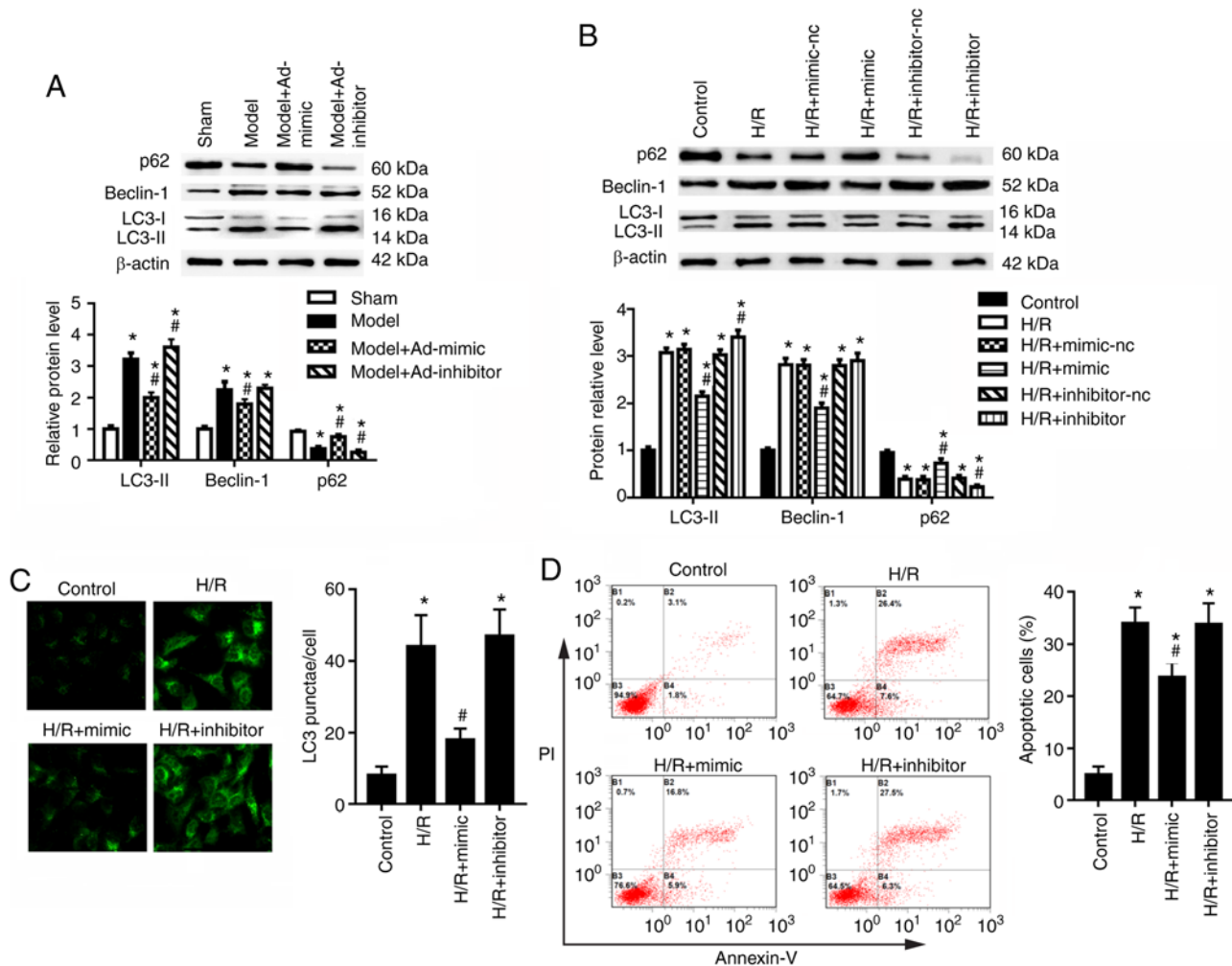


Figure 2. miR-30a-5p regulates autophagy in HK-2 cells under H/R injury. (A) I/R model mice were intraperitoneally injected with Ad-mimic or Ad-inhibitor 24 h before renal I/R injury. Western blot analysis was used to determine the protein expression levels of LC3-I-II, Beclin-1 and p62 in the renal cortex (n=6). *P<0.05 vs. sham; #P<0.05 vs. model. (B) HK-2 cells were transfected with miR-30a-5p mimic or miR-30a-5p inhibitor, and the protein expression levels of LC3, Beclin-1 and p62 were measured in HK-2 cells (n=3). *P<0.05 vs. control; #P<0.05 vs. H/R. (C) Immunofluorescence staining was performed to detect LC3 punctae in HK-2 cells under H/R injury with the use of miR-30a-5p mimic or miR-30a-5p inhibitor (n=3; magnification, x40). *P<0.05 vs. control; #P<0.05 vs. H/R. (D) Apoptosis was analyzed by flow cytometry following staining for Annexin V-FITC/PI (n=3). *P<0.05 vs. control; #P<0.05 vs. H/R. Ad, adenovirus; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; LC3, microtubule-associated protein light chain 3; miR, microRNA; nc, negative control.

expression levels of Beclin-1. In the HK-2 cells, miR-30a-5p mimic transfection significantly increased and miR-30a-5p inhibitor transfection significantly decreased the expression levels of miR-30a-5p (Fig. S1). Moreover, HK-2 cells in the H/R condition showed significantly increased LC3-II and Beclin-1 levels, and decreased p62 level, compared with the control group (Fig. 2B). To confirm the effects of miR-30a-5p on the pathogenesis of renal I/R injury, HK-2 cells were transfected with miR-30a-5p mimic or miR-30a-5p inhibitor and were then exposed to H/R. Compared with H/R-injured cells, miR-30a-5p overexpression decreased LC3-II and Beclin-1 protein expression levels but increased p62 expression in HK-2 cells (Fig. 2B). miR-30a-5p knockdown increased LC3-II and decreased p62 expression but had no significant effect on Beclin-1 expression compared with the H/R group. LC3 punctae analysis was performed using immunofluorescence staining; the data showed that the number of LC3 punctae was significantly elevated in H/R-exposed HK-2 cells compared with the control cells (Fig. 2C). Transfection of miR-30a-5p mimic significantly decreased the number of

punctae compared with the H/R group, whereas miR-30a-5p inhibitor exhibited no significant effect of number of LC3 punctae under H/R condition (Fig. 2C). Following H/R exposure, apoptotic rates significantly increased compared with the control group (Fig. 2D); miR-30a-5p mimic transfection significantly decreased cell apoptosis compared with the H/R group, whereas the miR-30a-5p inhibitor had little effect on cell apoptosis. These results suggested that miR-30a-5p may suppress autophagy and apoptosis under H/R injury.

miR-30a-5p directly interacts with Beclin-1. Beclin-1 was reported to be closely related to cell autophagy (26). Bioinformatics analysis demonstrated Beclin-1 to be a potential target of miR-30a-5p (Fig. 3A). The dual-luciferase reporter assay demonstrated that, compared with the miR-30a-5p mimic-nc, the miR-30a-5p mimic resulted in an ~60% reduction in the luciferase activity of 293T cells transfected with the pGLO-Beclin-1-WT plasmid (Fig. 3B); however, the miR-30a-5p mimic did not affect the luciferase activity of 293T cells that were transfected with the pGLO-Beclin-1-Mut

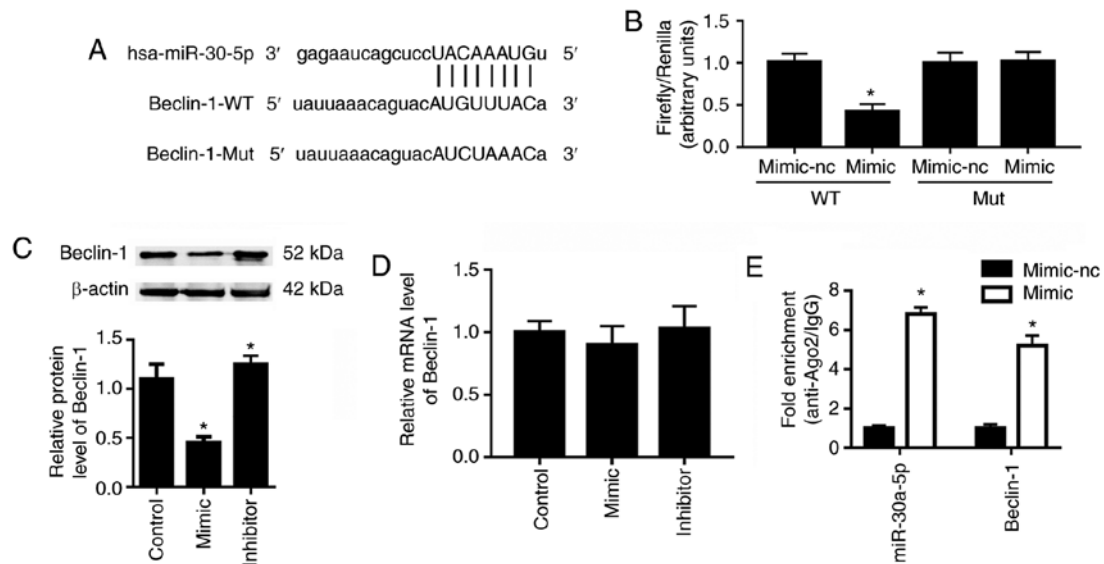


Figure 3. miR-30a-5p directly interacts with the 3'UTR of Beclin-1. (A) The potential binding sites of miR-30a-5p and the 3'UTR of Beclin-1. (B) 293T cells were co-transfected with the pGLO-Beclin-1-WT or pGLO-Beclin-1-Mut plasmid and miR-30a-5p mimic or miR-30a-5p mimic-nc, and luciferase activity was detected (n=3). (C) HK-2 cells were transfected with a miR-30a-5p mimic or miR-30a-5p inhibitor, and the protein levels of Beclin-1 were determined by western blot and semi-quantified. The untransfected cells were used in control group (n=3). (D) mRNA expression levels of Beclin-1 were detected by RT-qPCR. (E) RNA immunoprecipitation assay was performed using anti-Ago2 in HK-2 cells transfected with miR-30a-5p mimic or mimic-nc. miR-30a-5p and 3'UTR of Beclin-1 mRNA expression levels in the anti-Ago2 immunoprecipitated products were measured by RT-qPCR. *P<0.05 vs. miR-30a-5p mimic-nc or control group. miR, microRNA; Mut, mutant; nc, negative control; RT-qPCR, reverse transcription-quantitative PCR; UTR, untranslated region; WT, wild-type.

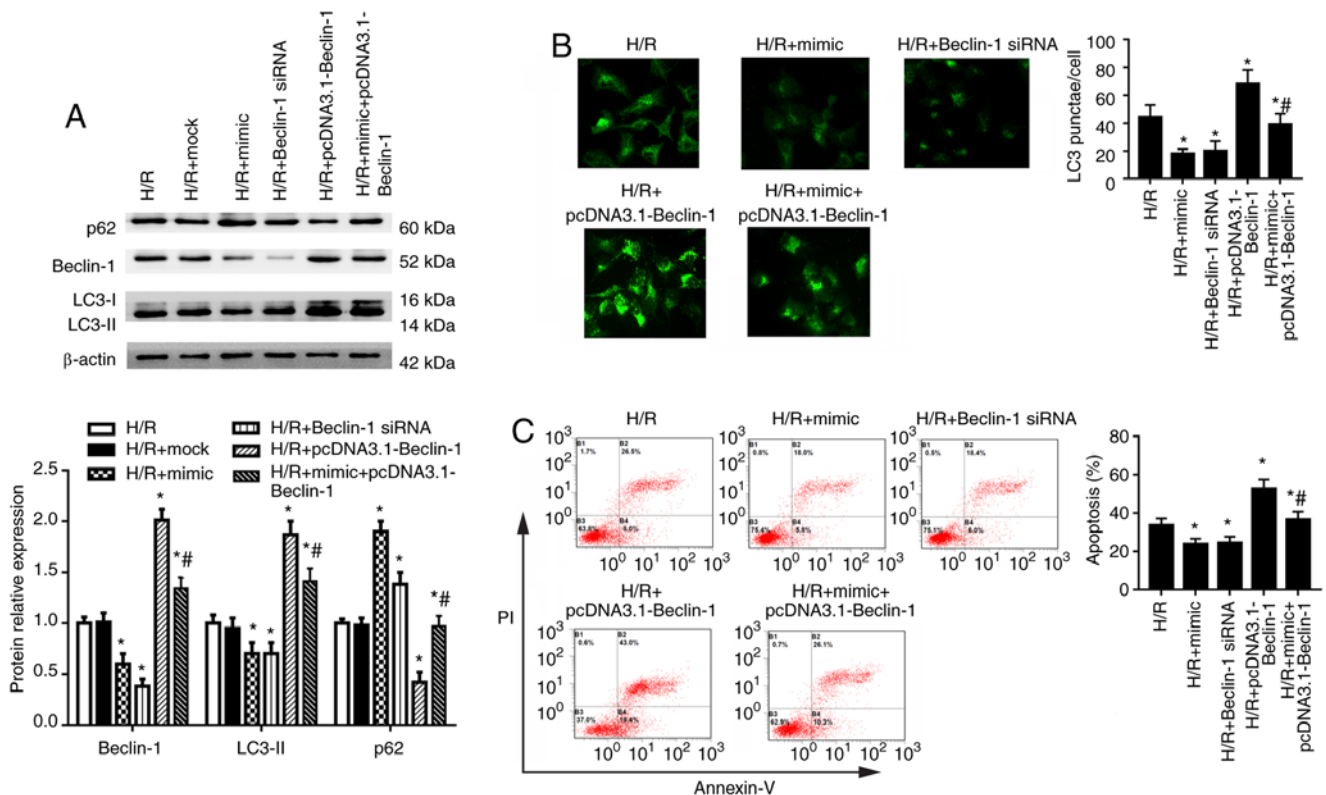


Figure 4. Beclin-1 serves a crucial role in autophagy in HK-2 cells. (A) HK-2 cells were transfected with miR-30a-5p mimic, Beclin-1 siRNA, pcDNA3.1-Beclin-1 or a combination of the miR-30a-5p mimic with pcDNA3.1-Beclin-1 for 48 h and then exposed to H/R. The protein expression levels of Beclin-1, LC3-II and p62 were measured by western blot and semi-quantified (n=3). (B) Immunofluorescence staining was performed to detect LC3 punctae in HK-2 cells (n=3; magnification, x40). (C) Cell apoptosis was analyzed by flow cytometry following staining for Annexin V-FITC/PI. *P<0.05 vs. H/R; #P<0.05 vs. H/R + pcDNA3.1-Beclin-1. H/R, hypoxia/reoxygenation; LC3, microtubule-associated protein light chain 3; miR, microRNA; siRNA, small interfering RNA.

plasmid. Western blot analysis showed that the miR-30a-5p mimic transfection significantly reduced the protein expression

level of Beclin-1, whereas miR-30a-5p inhibitor transfection significantly upregulated Beclin-1 protein levels compared

with the control group (Fig. 3C). However, neither miR-30a-5p mimic nor inhibitor transfection affected the mRNA expression levels of Beclin-1 significantly compared with the control group (Fig. 3D). To confirm the direct interaction between miR-30a-5p and Beclin-1, RIP assays were conducted. As shown in Fig. 3E, expression levels of miR-30a-5p and 3'UTR of Beclin-1 mRNA in the Ago2-RIP products of miR-30a-5p mimic transfected HK-2 cells were both significantly increased compared to the mimic-nc group.

Beclin-1 serves a crucial role in HK-2 cell autophagy. To investigate the role of Beclin-1 in autophagy of HK-2 cells under H/R condition, cells were transfected with miR-30a-5p mimic, Beclin-1 siRNA, pcDNA3.1-Beclin-1 or a combination of the miR-30a-5p mimic with pcDNA3.1-Beclin-1 for 48 h and then exposed to H/R. Beclin-1 siRNA transfection significantly decreased and pcDNA3.1-Beclin-1 transfection significantly increased the mRNA and protein expression levels of Beclin-1 compared with the control group (Fig. S2A and B). In HK-2 cells under H/R condition, Beclin-1 siRNA significantly decreased and pcDNA3.1-Beclin-1 significantly increased Beclin-1 expression compared with the H/R group (Fig. 4A). Compared with cells in the H/R group, Beclin-1 siRNA significantly inhibited expression of LC3-II and increased p62 expression. Conversely, pcDNA3.1-Beclin-1 transfection led to increased LC3-II and decreased p62 expression (Fig. 4A). miR-30a-5p mimic transfection partly reversed the effects of pcDNA3.1-Beclin-1 on the expression of LC3-II, Beclin-1 and p62. LC3 puncta analysis using immunofluorescence staining showed that the number of LC3 punctae was significantly decreased by Beclin-1 knockdown and increased by Beclin-1 overexpression compared with the H/R group (Fig. 4B). Co-transfection of miR-30a-5p mimic combined with Beclin-1 overexpression decreased the number of LC3 puncta compared with the Beclin-1 overexpression group (Fig. 4B). Beclin-1 siRNA transfection also significantly decreased cell apoptosis, whereas Beclin-1 overexpression significantly increased cell apoptosis, compared with cells in the H/R group (Fig. 4C). Co-transfection of miR-30a-5p mimic and pcDNA3.1-Beclin-1 significantly reduced cell apoptosis compared with the Beclin-1 overexpression group. These data indicate that Beclin-1 may serve a crucial role in autophagy of HK-2 cells.

Beclin-1 contributes to HK-2 cell autophagy by modulating ATG16 expression. A previous study demonstrated that ATG16 serves a crucial role in ischemic kidney injury (27). We hypothesized that miR-30a-5p may regulate HK-2 cell autophagy and proliferation by suppressing Beclin-1 expression and ATG16 expression. As expected, in the ischemic kidney injury mouse models and in the H/R-exposed HK-2 cells, ATG16 expression was significantly upregulated (Fig. 5A and B). In the H/R-exposed HK-2 cells, it was revealed that Beclin-1 siRNA decreased and pcDNA3.1-Beclin-1 increased the expression levels of ATG16, compared with the H/R group (Fig. 5C). In addition, miR-30a-5p mimic transfection significantly down-regulated, whereas miR-30a-5p inhibitor increased ATG16 expression in H/R-injured HK-2 cells.

The effects of ATG16 on autophagy and apoptosis of HK-2 cells were examined following transfection with ATG16 siRNA, which was used to downregulate ATG16 expression (Fig. S3).

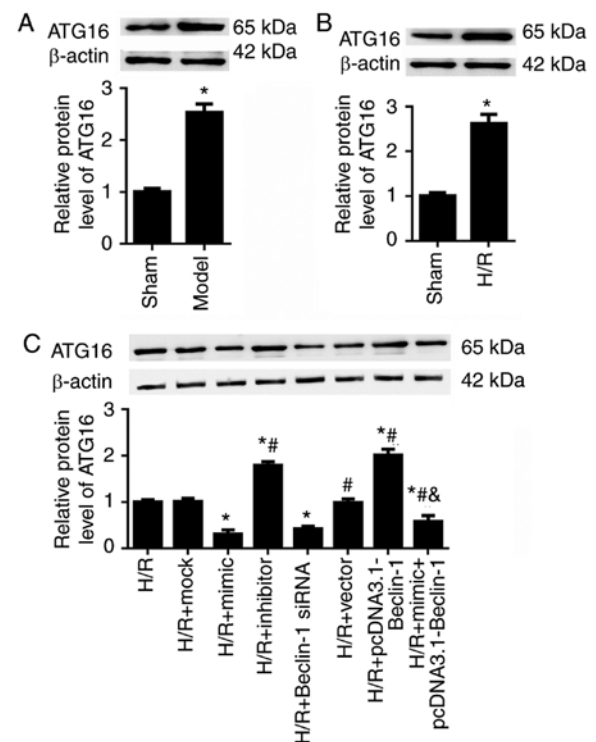


Figure 5. ATG16 expression is modulated by Beclin-1. ATG16 protein expression levels in the (A) renal cortex of IR model mice (n=6) and (B) H/R-exposed HK-2 cells (n=3) were detected by western blot. *P<0.05 vs. sham or control. (C) ATG16 protein expression was analyzed by western blot in HK-2 cells transfected with miR-30a-5p mimic, miR-30a-5p inhibitor, Beclin-1 siRNA, pcDNA3.1-Beclin-1 or a combination of the miR-30a-5p mimic with pcDNA3.1-Beclin-1 for 48 h, followed by exposure to H/R (n=3). *P<0.05 vs. H/R; #P<0.05 vs. H/R + mimic; &P<0.05 vs. H/R + inhibitor. ATG16, autophagy-related gene16; H/R, hypoxia/reoxygenation; miR, microRNA; siRNA, small interfering RNA.

In HK-2 cells under H/R condition, ATG16 knockdown significantly decreased ATG16 and LC3-II protein expression levels, and increased p62 expression in HK-2 cells compared with the H/R group (Fig. 6A). miR-30a-5p mimic transfection enhanced, whereas miR-30a-5p inhibitor transfection partly reversed, the effect of ATG16 knockdown on the expression of LC3-II (Fig. 6A). Compared with cells in the H/R group, ATG16 knockdown significantly decreased the number of LC3 punctae and decreased cell apoptosis (Fig. 6B and C). miR-30a-5p mimic enhanced, but miR-30a-5p inhibitor reversed, the effect of ATG16 knockdown on the number of LC3 punctae and apoptosis compared with the H/R + ATG16 siRNA group (Fig. 6B and C). Taken together, these results indicate that miR-30a-5p may mediate HK-2 cell autophagy in renal I/R injury by directly targeting Beclin-1 to modulate ATG16 expression.

Discussion

AKI is one of the most common complications in hospitalized patients. It is defined as a sudden decline in renal function, including cell death and inflammation in the proximal tubule (1). The underlying mechanism of AKI has not been fully elucidated (28). Kidney I/R injury is a major cause of AKI (1,29). Data from the present study demonstrated a significant decrease of miR-30a-5p expression, along

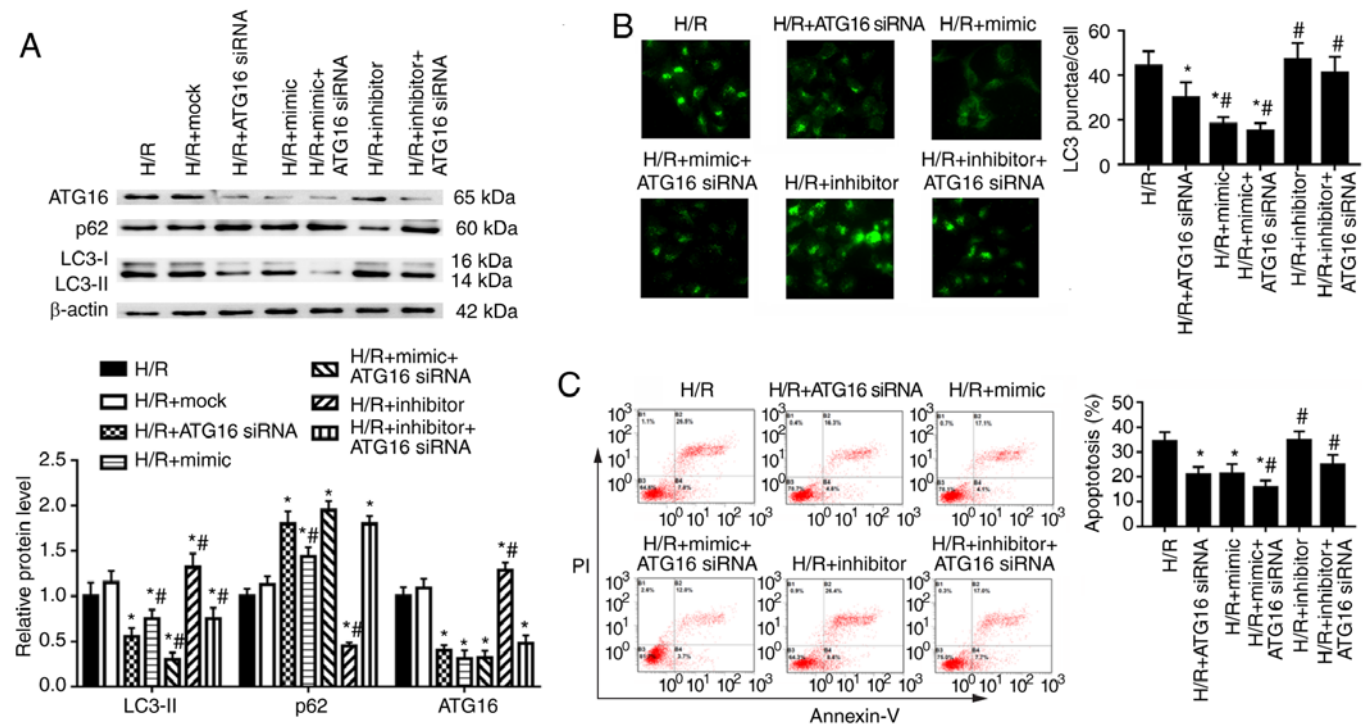


Figure 6. Beclin-1 contributes to HK-2 cell autophagy by modulating ATG16 expression. (A) HK-2 cells were transfected with ATG16 siRNA, miR-30a-5p mimic, miR-30a-5p inhibitor, or a combination of ATG16 siRNA with miR-30a-5p mimic or miR-30a-5p inhibitor, and then exposed to H/R injury. Protein expression levels were determined by western blot analysis (n=3). (B) Immunofluorescence staining was performed to detect LC3 punctae in HK-2 cells (n=3; magnification, x40). (C) Cell apoptosis was analyzed by flow cytometry following staining for Annexin V-FITC/PI. (n=3). *P<0.05 vs. H/R. #P<0.05 vs. H/R + ATG16 siRNA. ATG16, autophagy-related gene16; H/R, hypoxia/reoxygenation; miR, microRNA; siRNA, small interfering RNA.

with upregulated expression of autophagy-related proteins LC3-II and Beclin-1 and decreased p62 expression, in mice with renal I/R injury and in HK-2 cells exposed to hypoxic conditions *in vitro*. It was also revealed that miR-30a-5p directly interacted with the 3'-UTR of Beclin-1, and that Beclin-1 effectively promoted cell autophagy by enhancing ATG16 levels. These data suggested that Beclin-1/ATG16 signaling may be a novel and promising downstream target of miR-30a-5p for therapeutic intervention in ischemic kidney injury. Autophagy may serve as a cellular homeostatic mechanism under stress conditions, including H/R injury (27). Thus, in the current study, autophagy was examined by detecting the expression of LC3-II, Beclin-1 and p62, as well as by the LC3 punctae analysis. The I/R-injured mice showed increased LC3-II and Beclin-1, and decreased p62 protein expression levels. Similar results were observed in H/R-exposed HK-2 cells *in vitro*.

miRNAs are important in the maintenance of normal cellular functions, and dysregulation of miRNAs can result in a number of pathological states, including cellular differentiation, proliferation and apoptosis (30). Several studies have reported that miRNAs regulate tubular epithelial cell growth, apoptosis and renal injury (31,32). Studies have also demonstrated that miR-30a-5p may regulate mitochondrial dynamics and autophagy (33), and it may also promote replication of porcine circovirus type 2 by enhancing autophagy (34). The present study data demonstrated that administration of miR-30a-5p mimic markedly decreased HK-2 cell apoptosis, downregulated expression of LC3-II and Beclin-1, and enhanced p62 expression in H/R-exposed

HK2 cells. Conversely, transfection with a miR-30a-5p inhibitor enhanced autophagy of H/R exposed HK-2 cells; the miR-30a-5p inhibitor had little effect on cell apoptosis. Several studies have associated dysregulated miRNAs not only to the pathogenesis of kidney injury but also to renal fibrosis (35-37). Li *et al* (38) reported that miR-30 expression was significantly inhibited by TGF-β1 in the kidney fibroblast, and miR-30 was downregulated in renal fibrosis. Adenovirus-mediated upregulation of miR-30 in the kidney fibroblast significantly reduced unilateral ureteral obstruction-induced renal fibrosis by targeting SRY-box transcription factor 9 in mouse models (38). Unfortunately, the role of miR-30a-5p in renal fibrosis was not examined in the present study; we hypothesized that miR-30a-5p may affect renal fibrosis in the I/R model.

miRNAs conduct their biological function by binding to their target molecules (39). The mechanism of miR-30a-5p in ischemic kidney injury is unclear, and bioinformatic tools [microRNA.org (25), EIMMo miRNA target prediction (24) and TargetScan] were used in the present study to search for potential targets of miR-30a-5p. The results from many tools identified Beclin-1 to be an important element of autophagy that may be associated with the development of ischemic kidney injury (40). In a previous study, Beclin-1, which was identified as a target gene of miR-30a-5p, was shown to serve a crucial role in the drug-resistance of small cell lung cancer (16). In the present study, miR-30a-5p was shown to regulate autophagy of I/R injury renal by targeting Beclin-1.

A previous study revealed that Beclin-1 and the ATG5-ATG12-ATG16 complex serve important roles in

autophagy of thyroid papillary carcinoma cells (41) and are involved in initial autophagosome formation (42). To clarify the mechanism underlying the positive effects of Beclin-1 on cell apoptosis and autophagy, the expression of ATG16 was examined in the present study. In accordance with our hypothesis, ATG16 expression was significantly upregulated in the renal I/R injury mouse models and in the H/R-exposed HK-2 cells. Beclin-1 positively regulated and miR-30a-5p negatively modulated ATG16 expression. Further analysis of the mechanism revealed that ATG16 siRNA significantly decreased HK-2 cell apoptosis, downregulated LC3-II expression and the number of LC3 punctae, and promoted p62 expression. These results demonstrated that Beclin-1 may regulate autophagy in H/R injured HK2 cells by regulating ATG16 expression.

In conclusion, the present data indicated that miR-30a-5p may moderate renal I/R injury by inhibiting autophagy and apoptosis. Furthermore, mechanistic analysis demonstrated that miR-30a-5p directly interacted with Beclin-1, and ATG16 was involved in this process. Collectively, our data suggested that miR-30a-5p may regulate autophagy after renal I/R injury via the Beclin-1/ATG16 pathway. These results may provide a novel therapeutic approach to combat ischemic kidney injury.

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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

YF conceived and designed the study, performed the cell experiments and was a major contributor to the writing of the manuscript. LZ performed the animal experiments and helped write the manuscript. WH designed the study, performed the cell experiments and reviewed the manuscript. YF, LZ and WH confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of the General Hospital of Central Theater Command (Wuhan, China) approved this study, which followed the regulatory standards of the National Institutes of Health guide for the care and use of laboratory animals.

Patient consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

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