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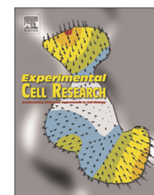

Research Article

# MiR-21 inhibits autophagy by targeting Rab11a in renal ischemia/reperfusion

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ABSTRACT

Renal ischemia–reperfusion (I/R) is one of the main causes of the acute kidney injury (AKI) that usually occurs during clinical surgery. Autophagy plays an important role in recovery from acute ischemic kidney injury. MicroRNA-21 (miR-21) was reported to inhibit autophagy in several diseases. However, the molecular mechanism of miR-21 on autophagy during renal I/R is still unclear. For the *in vitro* study, NRK-52E cells were transfected with miR-21 mimics and subjected to I/R. Results showed that miR-21 mimics inhibited cell viability and induced cell apoptosis in NRK-52E cells. Expression of beclin-1 and LC3-II was induced, and p62 was decreased by I/R. miR-21 mimics inhibited this induction. RT-PCR and western blotting assay showed that miR-21 mimics inhibited the protein level of Rab11a, but not the mRNA level. A luciferase activity assay showed that miR-21 directly targeted Rab11a 3′-UTR. Rab11a overexpression attenuated the effect of miR-21 mimics and I/R on cell viability and cell apoptosis. The expression of beclin-1 and LC3-II was increased, and p62 was decreased by Rab11a overexpression. For the *in vivo* assay in a rat I/R model, the miR-21 level was increased during renal I/R injury. Pre-treatment with miR-21 inhibitor injection attenuated the renal injury, and enhanced expression of LC3-II and beclin-1. The results showed that miR-21 inhibited autophagy by targeting Rab11a in renal I/R, indicating that Rab11a might be a new medical target for the treatment of renal I/R.

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## 1. Introduction

Renal ischemia–reperfusion (I/R) injury is one of the main causes of the acute kidney injury (AKI) that frequently occurs during cardiac surgery, renal vascular obstruction, and kidney transplantation [1]. Renal I/R usually results in cellular injury in the renal tubular epithelial cells [2]. Autophagy is a basic catabolic process to maintain cellular homeostasis [3,4]. The autophagic process specifically identifies and digests the surplus, unnecessary, or dysfunctional proteins and organelles to sustain cell function, while also recycling the reusable cytoplasmic constituents [3]. Autophagy-deficient cells accumulate destroyed mitochondria and cytoplasmic inclusions, leading to cellular injury. Emerging evidence indicates that autophagy plays an important role in maintaining proximal tubule function and recovery from acute ischemic kidney injury [5,6].

The formation of double-membrane vesicles, which is called autophagosome (AP), and its fusion with endolysosome are key steps in the autophagic process [7]. Rab proteins, a superfamily of RAS GTPases, are the main regulators of membrane trafficking and fusion [8,9]. There are numerous studies that have focused on the role of Rab proteins on the regulation of the autophagic process [9,10]. For instance, Rab1, Rab11, Rab32 and Rab33 participate in the formation of the phagophore assembly site (PAS) [9]. Rab4, Rab7, Rab8, Rab32 and Rab33 are involved in the formation and maturation of AP [9]. Numerous studies have primarily focused on the roles of Rab proteins in autophagy, although the upstream regulators in this process need further investigation.

MicroRNAs are a group of single-stranded non-coding RNAs that play key roles in the regulation of gene expression [11,12]. It has been shown that several microRNAs are associated with renal I/R, such as miR-20a, -21, -132, -362, and -379 [13]. MiR-21 is a well-known onco-microRNA and has been reported to inhibit autophagy in some diseases [14–16]. The molecular mechanism of miR-21 on autophagy in renal I/R is still unknown.

In the present study, we found that the miR-21 level was increased during renal I/R injury. MiR-21 inhibited autophagy activity and aggravated I/R injury by targeting Rab11a. Rab11a overexpression attenuated the effect of miR-21 mimics and I/R in NRK-52E cells. The results indicated that miR-21 inhibited autophagy by

*Abbreviations:* I/R, ischemia–reperfusion; AKI, acute kidney injury; AP, autophagosome; PAS, phagophore assembly site; COPD, chronic obstructive pulmonary disease

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targeting Rab11a in renal I/R.

## 2. Materials and methods

### 2.1. Cell culture and transfection

NRK-52E cells (ATCC, USA), rat renal proximal tubular cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 4 mM L-glutamine, 0.15% sodium bicarbonate, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified incubator (37 °C, 5% $CO_2$).

The pcDNA3.1-Rab11a plasmid was constructed by inserting the cDNA fragment retro-transcribed from the full-length of Rab11a mRNA. MiR-21 mimics (5′-UAGCUUAUCAGCUGAUGUUGA-3′), control miRNA (5′-UUCUCCGAACGUGUCACGUTT-3′), Rab11a siRNA (5′-AATGTCAGACAGACGCGAAAA-3′), control siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′ Genepharma, Shanghai, China), pcDNA3.1-Rab11a, or empty vector were transfected into NRK-52E cells using the Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA).

### 2.2. Ischemia–reperfusion model in NRK-52E cells

Twenty-four hours after transfection, cells were seeded into 96-well plates or 6-well plates. Hypoxia/reoxygenation treatment of the cells were performed to mimic *in vitro* I/R. NRK-52E cells were cultured in serum-free media with 1% oxygen in a Galaxy® 48R incubator (Eppendorf/Galaxy Corporation, Connecticut, USA) at 37 °C for 24 h before then being exposed to normoxic conditions (20% oxygen) for another 24 h.

### 2.3. Cell viability assay

Cell vitality of the NRK-52E cells was measured using the MTT assay. A total of 20 μl of the MTT (5 mg/ml, Sigma, USA) solution was added per well, and the cells were incubated for 4 h at 37 °C. The supernatants were removed and the formazan crystals were dissolved in 150 μl of dimethylsulfoxide (Sigma, USA). Finally, optical density was determined at 570 nm. In the assay, five parallel wells were made.

### 2.4. Cell apoptosis assay

Cell apoptosis of the NRK-52E cells was detected with the flow cytometry method (FCM) using an Annexin V-PI Apoptosis Detection Kit (Abcam, Cambridge, UK). Briefly, the cells were collected, washed with PBS, and suspended in 500 μl of binding buffer. The cells were incubated with Annexin V at room temperature for 10 min and were then stained by Propidium Iodide (PI). The relative quantitative apoptosis was analyzed using FCM.

### 2.5. Quantitative RT-PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA) and was then reverse transcribed. Quantitative RT-PCR was carried out on an ABI Prism 7500 System (Applied Biosystems Inc. USA). SYBR Green Gene Expression Assay (Qiagen, Valencia, CA) was used for Rab11a and β-actin expression. The primers used for Rab11a were 5′-CCTGGTCCCACAGATACCAC-3′ and 5′-CTCAGACCTGGGAAATGGAC-3′. The primers used for β-actin were 5′-ATTGCCGACAGGATGCAGAA-3′ and 5′-CAAGATCATTGCTCCTCCTGAGCGCA-3′. A TaqMan microRNA assay kit (reference number: 4427012, Applied Biosystems, Foster, CA, USA) was used for the miR-21 analysis. U6 ribosomal RNA was used as the internal control. The reverse transcription primer sequence for miR-21 was 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAACATC-3′. The sequences of quantitative RT-PCR primers for miR-21 were 5′-ACACTCCAGCTGGGTAGCTTATCAGACTGA-3′ and 5′-GTGTCGTGGAGTCGGCAATTC-3′. Fold changes were calculated using the formula: $R=2^{-\Delta\Delta CT}$.

### 2.6. Western blot analysis

The proteins were separated using SDS-PAGE before being transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in 5% (w/v) powdered low fat skim milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 2 h. The membrane was then incubated with rabbit polyclonal antibodies of beclin-1 (catalogue number: ab55878), LC-3 II (catalogue number: ab63817), p62 (catalogue number: ab91526), Rab11a (catalogue number: ab128913) and β-actin (catalogue number: ab129348, Abcam, Cambridge, UK) in TBST at 4 °C for 8 h, followed by a goat anti-rabbit (1:3000, catalogue number: ab6721) peroxidase secondary antibody (Abcam, Cambridge, UK) for 1 h at 37 °C. Chemiluminescent detection was performed using the Bio-Rad ChemiDoc™ MP Imaging System. The gray value of the bands was analyzed using the Image J2x software.

### 2.7. Luciferase activity assay

A 67 bp segment of Rab11a's 3′-UTR was amplified from the NRK-52E cells with specific primers (sense: 5′-CCAAGCTTACCTCCTATTGGTCCTGATTA-3′ and anti-sense: 5′-GGACTAGTTGCTCTGAGGCAAAACAATAG-3′), and was then cloned into pMiR-Report (Ambion Inc., Austin, Texas, USA). A mutated 3′-UTR of Rab11a was introduced in the potential miR-21 binding site by using a two-step PCR approach. NRK-52E cells were co-transfected with the reporter vectors containing the wild type or mutant of Rab11a 3′-UTR and miR-21 mimic. After 48 h, luciferase activity was measured using a dual-luciferase reporter assay system (Promega, WI, USA).

### 2.8. Animal treatment

A total of 20 male Sprague-Dawley rats (Vital River Laboratory Animal, Beijing, China), weighing 150–180 g, were used in the study. They were kept at a constant temperature (23 ± 1 °C) with a 12 h light-dark cycling and were allowed free access to water and food. All animal procedures were performed in accordance with the university guidelines for the care and use of laboratory animals. The study was approved by the Ethics Committee of Nanchang University.

For the renal I/R surgery, the rats were anesthetized with pentobarbital (50 mg/kg, i.p.). Bilateral renal pedicles were clamped to induce renal ischemia. After 30 min, the clamps were removed for reperfusion. Animals in the sham group were subjected to incisions without I/R. To detect the effect of miR-21, the mice were injected with miR-21 inhibitors (5′-UCAACAUCAGUCUGAUAAGCUA-3′ Genepharma, Shanghai, China) or control inhibitors (5′-CAGUACUUUUGUGUAGUAGUACAA-3′ Genepharma, 60 mg/kg, i.p.) 24 h before renal ischemia. After 24 h, the rats were anesthetized with pentobarbital, and the unilateral kidneys were collected for RT-PCR and western blotting.

### 2.9. Histopathology examination

The unilateral kidneys were fixed in paraformaldehyde fixative at room temperature. The fixed kidneys were embedded in paraffin wax before being cut into sections (5 μm). The paraffin sections were stained with hematoxylin–eosin (H&E). Histological features were observed using a light microscope.

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