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# Microvesicle-containing miRNA-153-3p induces the apoptosis of proximal tubular epithelial cells and participates in renal interstitial fibrosis

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**Abstract. – OBJECTIVE: To uncover the role of microvesicle-containing (MV-containing) miRNA-153-3p in inducing the apoptosis of proximal tubular epithelial cells and RIF (renal interstitial fibrosis), and its potential mechanism.**

**MATERIALS AND METHODS: Mice were subjected to unilateral ureteral obstruction (UUO) to establish the in vivo RIF model. MVs were extracted from the obstructed kidney tissues of mice, to further isolate the RNAs. MiRNA-153-3p levels in RIF mice and MVs were determined. In vitro RIF model was constructed by TGF-β1 induction in NRK-52E and NRK-49F cells. The regulatory effect of miRNA-153-3p on the apoptosis of tubular epithelial cells was examined. Subsequently, potential target gene of miRNA-153-3p was predicted and identified. Rescue experiments were finally carried out to uncover the role of miRNA-153-3p/Bcl-2 in influencing RIF.**

**RESULTS: MiRNA-153-3p was upregulated in mice undergoing UUO, MVs extracted from obstructed kidney tissues of mice and TGF-β1-induced NRK-52E and NRK-49F cells. The overexpression of miRNA-153-3p remarkably induced apoptosis in tubular epithelial cells. Bcl-2 was verified to be the target gene of miRNA-153-3p, and the Bcl-2 level was negatively regulated by miRNA-153-3p. Overexpression of Bcl-2 reversed the effect of miRNA-153-3p on inducing cell apoptosis.**

**CONCLUSIONS: MV-containing miRNA-153-3p released by tubulointerstitial fibroblasts transmits to the proximal tubular epithelial cells via the damaged tubule basement membrane. It induces the apoptosis of proximal tubular epithelial cells by inhibiting Bcl-2 level and further aggravates RIF.**

*Key Words:*
MiRNA-153-3p, MVs, Tubulointerstitial fibroblasts, Apoptosis, RIF, Proximal tubular epithelial cells.

## Introduction

Renal interstitial fibrosis (RIF), also known as tubulointerstitial fibrosis (TIF), is the main pathological feature of the progressive renal damage caused by various pathogenic causes. RIF is one of the fundamental pathological changes leading to end-stage renal disease (ESRD)[1]. RIF exists in most progressive kidney diseases and chronic kidney diseases (CKD), and its occurrence indicates a poor prognosis[2,3]. Apoptosis is a key event during organ fibrosis, indicating that cell apoptosis triggers the occurrence and progression of fibrosis[4,5].

MiRNAs are a class of endogenous non-coding RNAs found in eukaryotes with 20-25 nucleotides in length[6]. Mature miRNAs recognize the target mRNAs through complementary base pairing. The pairing degree makes miRNA degrade the target mRNAs or inhibit their translation[7]. Functionally, miRNAs are extensively involved in various biological processes[8]. In tumor diseases, miRNAs exert crucial functions in influencing tumorigenesis, tumor progression, and metastasis[9,10]. Meanwhile, miRNAs are also of significance in kidney development and kidney diseases[11].

MVs are small vesicles (30-150 nm) containing complex RNAs and proteins[12]. A variety of cells can secrete MVs under normal and pathological conditions, which are mainly derived from polyvesicles formed by microparticles invagination of the intracellular lysosome. After fusion of extracellular membrane and extracellular membrane of polyvesicles, MVs are finally released into extracellular matrix[13,14]. All types of cultured cells are capable of secreting MVs, and they are naturally

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present in body fluids, including blood, saliva, urine, cerebrospinal fluid, and milk[15,16].

In this paper, we established *in vivo* and *in vitro* RIF models by performing UUO and TGF-β1 induction, respectively, to investigate the specific molecular mechanism of MV-containing miRNA-153-3p derived from tubulointerstitial fibroblasts in regulating the apoptosis of renal tubular epithelial cells, so as to provide a new theory for explaining the pathogenesis of RIF.

## Materials and Methods

### Experimental Animals

Male CD-1 mice (18-20 g) were obtained from Model Animal Research Center, housed in a standard SPF environment, and fed with normal diet. Mice were treated with the sham operation, or UUO for 1, 3, and 7 days, respectively. They were sacrificed at the 1st, 3rd, and 7th day postoperatively, respectively for collecting the kidney tissues. This study was approved by the Animal Ethics Committee of the Medical School of Huanghuai University Animal Center.

### Cell Culture

Rat proximal tubular epithelial cell line (NRK-52E) and renal interstitial fibroblast cell line (NRK-49F) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin, and maintained in an incubator with 5% $CO_2$ at 37°C. When 80% of cells were fused, they were cultured in the serum-free medium and treated with different doses of TGF-β1. The cells and supernatant were collected for the following experiments.

### Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The tissues were lysed for harvesting the total RNA and subjected to reverse transcription using PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). 1 μL of complementary deoxyribose nucleic acid (cDNA) was collected for PCR using SYBRGreen method. CT value was recorded through ABI7300 system and the relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems, Foster City, CA, USA). PCR conditions were as follows: 5-min denaturation at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C and 90 s at 72°C. The primer sequences used in this study were as follows: Bcl-2, F: 5'-CGAGGAGCCCGCTGTAGTCG-3', R: 5'-GCTAGGGTGTCCGAAGCC-3'; miR-153-3p, F: 5'-GCTGTAAGACGATCCTCGGACTG-3', R: 5'-AGGTTGATGTACGATGGAAGTCAG-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

### Western Blot

The total protein was extracted for determining protein concentration. The protein sample was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and blocked in 5% skim milk for 1 hour. The specific primary antibody was used to incubate with the membrane overnight at 4°C, followed by incubation with secondary antibody for 2 h at room temperature. After washing with 1×Tris buffered saline-Tween (TBST) for 1 min, the chemiluminescent substrate kit was used for the exposure of the protein band.

### MVs Isolation

MVs were isolated from cell supernatant by ultracentrifugation. Briefly, cell culture was collected and centrifuged at 4°C, 300 g/min for 5 min, 1,200 g/min for 20 min, and 10,000 g/min for 30 min sequentially. Suspension was harvested for centrifugation again at 110,000 g/min for 60 min and the precipitate was MVs. MVs were resuspended in Phosphate-Buffered Saline (PBS) and observed using the transmission electron microscope.

### TUNEL

The tissues were dehydrated and embedded, and the sections were routinely dewaxed, washed, hydrated, and fixed strictly in accordance with the TUNEL Apoptosis Kit (Sigma-Aldrich, St. Louis, MO, USA). DAPI-labeled and TUNEL-labeled cells were captured (magnification 20×).

### Dual-Luciferase Reporter Gene Assay

The binding sequences in the promoter regions of Bcl-2 and miRNA-153-3p were cloned into the psi-CHECK2 vector (Promega, Madison, WI, USA), that was Bcl-2 WT. Meanwhile, mutant-type Bcl-2 vector was constructed as well. The cells were co-transfected with Bcl-2 WT/Bcl-2 MT and miRNA-153-3p mimics/negative

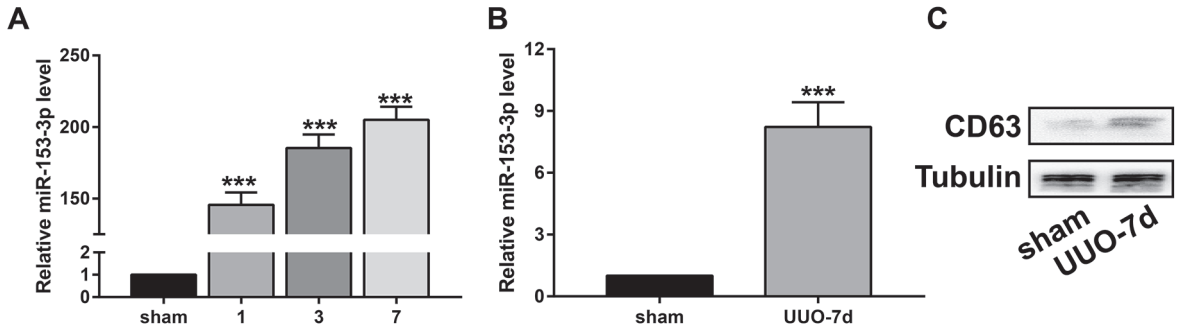


**Figure 1.** MiR-153-3p upregulation in RIF. **A,** MiR-153-3p level in mouse kidney tissues of the sham group, and those undergoing UUO for 1, 3, and 7 days. **B,** MiR-153-3p level in MVs extracted from mice in the sham group and those undergoing 7-day UUO. **C,** Protein level of CD63 in MVs extracted from mice in the sham group and those undergoing 7-day UUO.

control. After 48 h of transfection, the cells were harvested for the determination of the Luciferase activity.

### Plasmid Construction and Transfection

The cDNA of Bcl-2 was cloned into the mammalian expression vector pcDNA3.0, that was, pcDNA3.0-Bcl-2. The cells were subjected transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, cells were collected for transfection efficacy verification and the following experiments.

### Statistical Analysis

The Statistical Product and Service Solution (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. GraphPad Prism 6.0 (La Jolla, CA, USA) was used for depicting figures. The data were expressed by x±s. The *t*-test was used to compare the differences between two different groups. Comparison between groups was also done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

## Results

### MiRNA-153-3p Upregulation in RIF

Mice were subjected to UUO for establishing the *in vivo* RIF model. At postoperative day 1, 3, and 7, miRNA-153-3p level in obstructed kidney tissues of mice gradually increased (Figure 1A). MVs were extracted in obstructed kidney tissues of mice undergoing UUO for 7 days and those in the sham group. Upregulated miRNA-153-3p was observed in MVs extracted from RIF mice (Figure 1B). Subsequently, MVs marker protein CD63 was determined by Western blot. Compared with kidney tissues harvested from mice of the sham group, protein level of CD63 was markedly upregulated in mice undergoing 7-day UUO, indicating much more MVs secreted at post-UUO (Figure 1C). The above data illustrated that RIP triggered MVs secretion from the obstructed kidney tissues, and miRNA-153-3p level in MVs was upregulated as well.

### TGF-β1 Induction Upregulated MiRNA-153-3p Level and Stimulated Apoptosis

TGF-β1 induction was performed in NRK-52E and NRK-49F cells with different doses (0, 1, 2, and 5 ng/mL) for different time (0, 6, 12, 24, and 48 h). MiRNA-153-3p level was dose-dependently and time-dependently upregulated (Figures 2A, 2B). Subsequently, NRK-49 cells were treated with 5 ng/mL TGF-β1 for 24 h. The protein levels of fibrosis markers FN and α-SMA were remarkably upregulated (Figure 2C). To further explore the role of miRNA-153-3p in RIF, we constructed miRNA-153-3p mimics and inhibitor (Figure 2D). The number of TUNEL-positive cells was elevated by transfection of miRNA-153-3p mimics in NRK-49F cells, suggesting the induced apoptosis (Figures 2E, 2F).

### MiRNA-153-3p Targeted Bcl-2

Through bioinformatics prediction, the binding sites in the promoter regions of Bcl-2 and miRNA-153-3p were discovered (Figure 3A). Luciferase activity was reduced after the co-transfection of Bcl-2 WT and miRNA-153-3p mimics, verifying that Bcl-2 was the downstream target of miRNA-153-3p (Figure 3B). Both mRNA and the protein levels of Bcl-2 were downregulated in NRK-49F cells overexpressing miRNA-153-3p. Conversely,

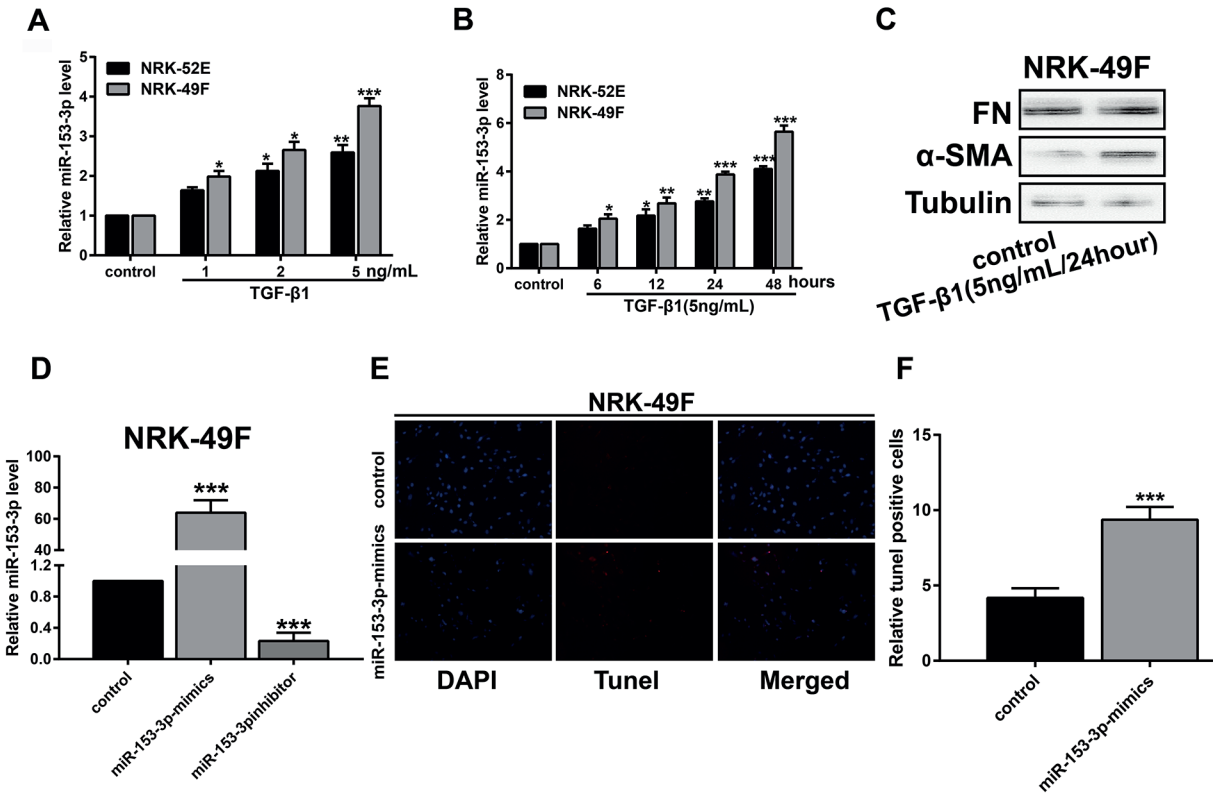


**Figure 2.** TGF-β1 induction upregulated miR-153-3p level and stimulated apoptosis. **A,** MiR-153-3p level in NRK-52E and NRK-49F cells treated with 0, 1, 2, and 5 ng/mL TGF-β1 for 24 h. **B,** MiR-153-3p level in NRK-52E and NRK-49F cells treated with 5 ng/mL TGF-β1 for 0, 6, 12, 24, and 48 h. **C,** Protein levels of FN and α-SMA in NRK-49F cells treated with 5 ng/mL TGF-β1 for 24 h. **D,** Transfection efficacy of miR-153-3p mimics and inhibitor in NRK-49F cells. **E,** TUNEL-labeled, DAPI-labeled, and merged images in NRK-49F cells transfected with negative control or miR-153-3p mimics (magnification 20×). **F,** TUNEL-positive cell number in NRK-49F cells transfected with negative control or miR-153-3p mimics.

transfection of miRNA-153-3p inhibitor markedly upregulated the Bcl-2 level (Figures 3C, 3D).

### *Overexpression of Bcl-2 Partially Reversed the Role of MiRNA-153-3p in Inducing Apoptosis*

We further investigated the role of Bcl-2 in the apoptosis of tubular epithelial cells. In kidney tissues extracted from mice undergoing 7-day UUO, both mRNA and the protein levels of Bcl-2 were downregulated (Figures 4A, 4B). Transfection of pcDNA-Bcl-2 greatly upregulated Bcl-2 level in NRK-49F cells (Figures 4C, 4D). Notably, the overexpression of Bcl-2 reversed the increased TUNEL-positive cell ratio in NRK-49F cells overexpressing miRNA-153-3p (Figure 4E).

## Discussion

RIF is characterized by tubular atrophy and extracellular matrix deposition[17,18]. Apoptosis is a common form of cell death, and apoptosis of proximal tubular epithelial cells is one of the major causes of tubular atrophy and interstitial fibrosis[19,20]. In this paper, *in vivo* and *in vitro* RIF models were constructed by performing UUO and TGF-β1 induction, respectively. We aim to uncover the role of MV-containing miRNA-153-3p in inducing the apoptosis of renal tubular epithelial cells and further aggravation into RIF.

MiRNA-153-3p is involved in the regulation of tumor cell apoptosis and acts as a tumor suppressor in many tumors[21,22]. For example, miRNA-153-3p inhibits melanoma to proliferate and invade by targeting SNAIL1[23]. In glioma, miRNA-153-3p enhances radiotherapy sensitivity by targeting Bcl-2[24]. Our findings illustrated that MV-containing miRNA-153-3p was upregulated in the obstructed kidney tissues. In addition, TGF-β1 induction elevated miRNA-153-3p level in dose-dependent and time-dependent ways.

Bcl-2 is an oncogene with an inhibitory effect on apoptosis[25]. Bcl-2 protein is the encoded product of the proto-oncogene Bcl-2, and it is a promotor of cell survival[26]. The targeted inhibition and degradation of Bcl-2 can promote cancer cell apoptosis, thus contributing to tumor treatment[27,28]. Some miRNAs may have binding sites with Bcl-2, and they are involved in cell apoptosis by regulating Bcl-2 level[29]. It is reported that the overexpression of miR-34a-5p suppresses proliferative ability and induces apoptosis of cervical cancer cells by downregulating Bcl-2[30]. MiRNA-184 weakens the proliferation and induces the apoptosis of SW480 and HCT116 cells by downregulating Bcl-2 and C-Myc[31]. In breast cancer, miR-148a induces apoptosis by targeting B-cell lymphoma 2[32]. In this study, Bcl-2 was verified to be the target gene of miRNA-153-3p, and Bcl-2 level was negatively regulated by miRNA-153-3p. Notably, the overexpression of Bcl-2 reversed the effect of miRNA-153-3p on inducing cell apoptosis.

MVs are specifically secreted membranes vesicles involved in cell communication[33]. MiRNAs not only regulate the gene expressions by themselves, but also regulate the target genes of other cells through MVs delivery[34]. MiRNAs in tumor-derived MVs participate in tumor metastasis and apoptosis[35]. For example, the exosome-derived miR-25-3p and miR-92a-3p stimulate the progression of liposarcoma[36].

Our findings demonstrate that during the progression of RIF, tubulointerstitial fibroblasts secrete a large amount of MVs containing miRNA-153-3p. These MVs are transmitted to the proximal tubular epithelial cells, where Bcl-2 level is inhibited and apoptosis is induced. As an important mediator that contributes to cell-to-cell communication, MVs are of significance in the pathological progression of RIF.

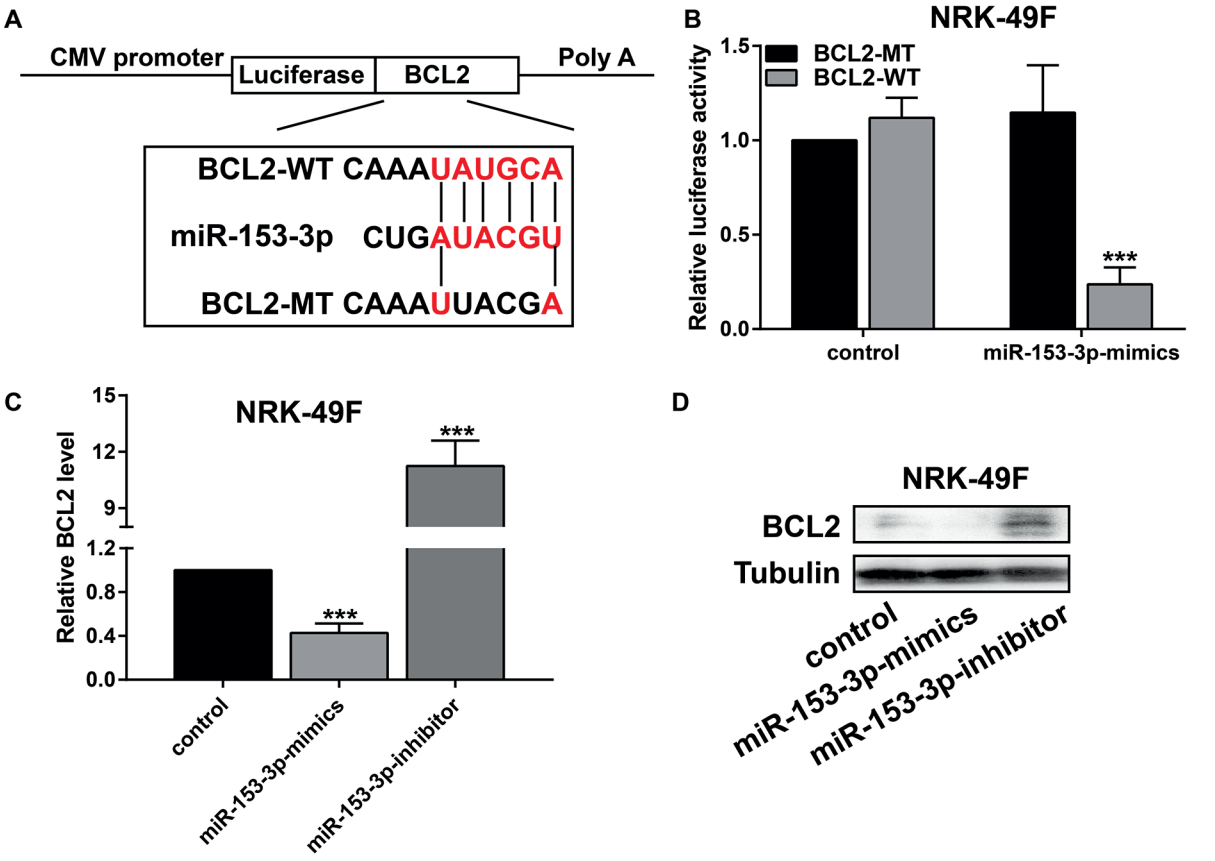


**Figure 3.** MiR-153-3p targeted Bcl-2. **A,** Binding sites in the promoter regions of Bcl-2 and miR-153-3p. **B,** Luciferase activity in NRK-49F cells co-transfected with Bcl-2 WT/Bcl-2 MT and miR-153-3p mimics/negative control. **C,** The mRNA level of Bcl-2 in NRK-49F cells transfected with control, miR-153-3p mimics or miR-153-3p inhibitor. **D,** The protein level of Bcl-2 in NRK-49F cells transfected with the control, miR-153-3p mimics or miR-153-3p inhibitor.

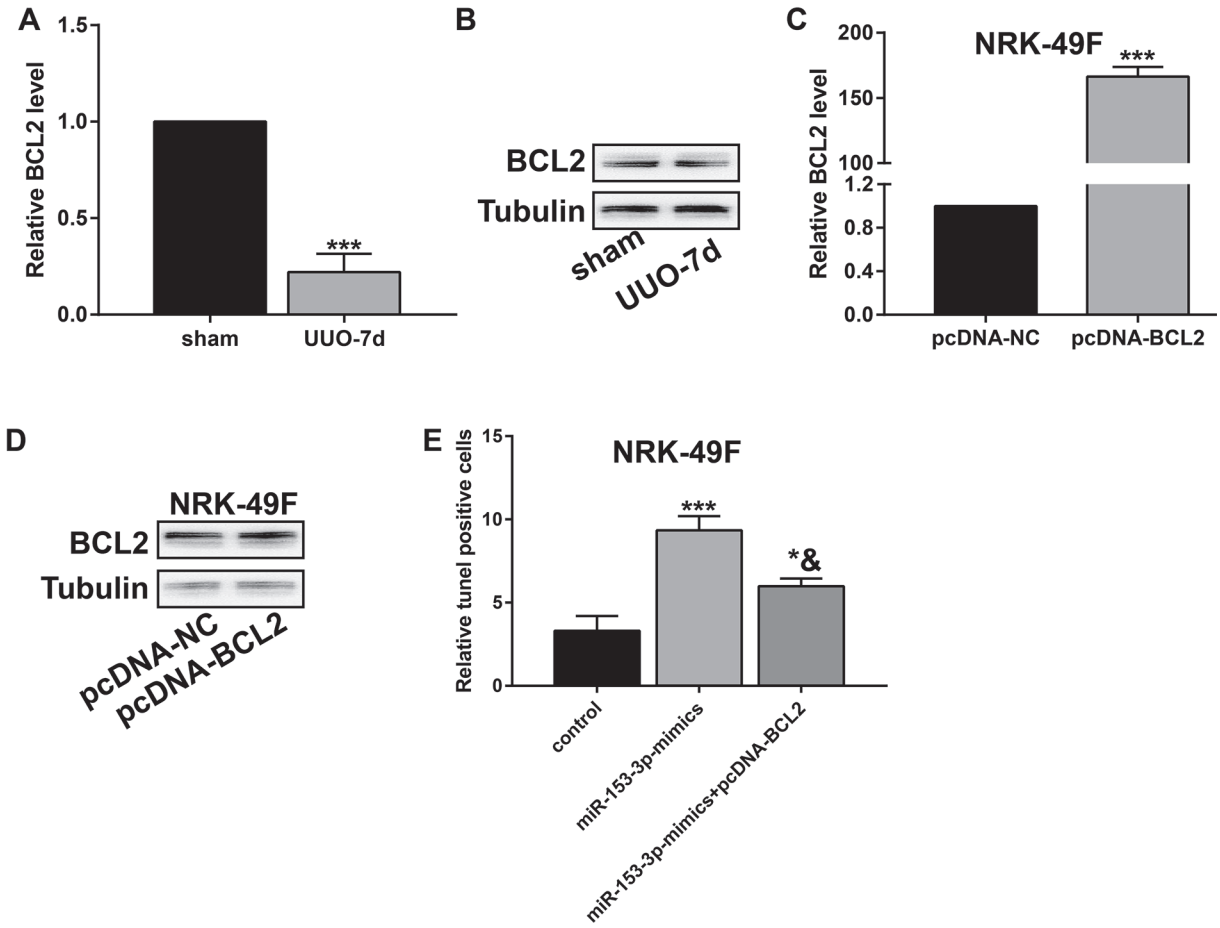


**Figure 4.** Overexpression of Bcl-2 partially reversed the role of miR-153-3p in inducing apoptosis. **A,** The mRNA level of Bcl-2 in mouse kidney tissues of the sham group, and those undergoing 7-day UUO. **B,** The protein level of Bcl-2 in mouse kidney tissues of the sham group, and those undergoing 7-day UUO. **C, D,** Transfection efficacy of pcDNA-Bcl-2 in NRK-49F cells. **E,** TUNEL-positive cell number in NRK-49F cells transfected with the negative control, miR-153-3p mimics, or miR-153-3p+pcDNA-Bcl-2.

## Conclusions

MV-containing miRNA-153-3p released by tubulointerstitial fibroblasts transmits to the proximal tubular epithelial cells, where they induce the apoptosis of proximal tubular epithelial cells by inhibiting Bcl-2 level and further aggravating RIF.

### Conflict of Interests

The Authors declare that they have no conflict of interests.

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