

# MicroRNA-24 protects against myocardial ischemia-reperfusion injury via the NF- $\kappa$ B/TNF- $\alpha$ pathway

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**Abstract.** Acute myocardial infarction (AMI) is a form of cardiomyopathy in which a blocked coronary artery leads to an irreversible loss of cardiomyocytes due to inadequate blood and oxygen supply to the distal myocardium tissues, eventually leading to heart failure. Recently, studies have revealed that microRNA (miRNA/miR)-24 has diagnostic value in the pathogenesis of AMI by affecting multiple cell processes such as cell proliferation, differentiation and apoptosis. However, the specific mechanism of miR-24 in ischemia-reperfusion injury (IRI) after AMI remains to be fully elucidated. The present study aimed to investigate the effects and mechanisms of miR-24 in IRI. *In vitro*, the current study detected cellular apoptosis and apoptotic-related protein expression levels in the cardiomyocyte H9C2 cell line (negative control group, model group and miRNA group) via flow cytometry and western blot analysis. In the *in vivo* study, rats were randomly divided into sham, model and miRNA groups. The infarct area was observed using nitro blue tetrazolium staining, pathological changes of the myocardium were detected via hematoxylin and eosin staining and TUNEL staining was used to detect cardiomyocyte apoptosis. The expression levels of related proteins were evaluated via immunohistochemistry and western blot analysis. The *in vitro* and *in vivo* results demonstrated that miR-24 significantly inhibited cardiomyocyte apoptosis compared with the model group. Concurrently, the expression levels of proteins associated with the NF- $\kappa$ B/TNF- $\alpha$  pathway (NF- $\kappa$ B, caspase-3, Bax, Bcl-2, TNF- $\alpha$ , vascular cell adhesion molecule 1, intercellular adhesion molecule 1 and monocyte chemoattractant protein-1) in the miRNA group were significantly different from the model group ( $P < 0.001$ ). Compared

with the model group, miR-24 significantly improved pathological damage and infarct size of rat myocardium. Overall, the present results suggested that miR-24 improves myocardial injury in rats by inhibiting the NF- $\kappa$ B/TNF- $\alpha$  pathway.

## Introduction

Acute myocardial infarction (AMI) is one of the leading causes of mortality in patients with cardiovascular disease worldwide (1), accounting for 80% of cardiogenic shock (2). AMI is caused by coronary artery blockage and myocardial ischemic necrosis, which eventually leads to heart failure (3). Therefore, early reperfusion therapy can save the dying myocardium, which is essential for blood supply and nutritional support of ischemic myocardium, as well as improving the prognosis of patients with AMI (1,3). However, there is evidence that traditional reperfusion therapy can cause additional damage to myocardial structure and function, namely myocardial ischemia-reperfusion injury (IRI) (3,4). IRI is a pathological phenomenon in which the reintroduction of oxygen and other nutrients into the myocardium further aggravates pathological damage to the myocardium, resulting in decreased cardiac function, arrhythmias and irreversible death of cardiomyocytes (1,3,4). Therefore, elucidating the mechanism of IRI and improving myocardial damage caused by IRI is important for the clinical treatment of patients with AMI to restore coronary blood flow.

MicroRNAs (miRNAs/miRs) are RNA regulators consisting of 18-22 nucleotides, which can bind to mRNAs and decrease protein translation (5). In recent years, it was reported that miRNAs, as a small regulator, are closely associated with numerous diseases, including myocardial infarction (MI), heart failure and acute coronary syndrome (5). Several miRNAs have been identified as potential biomarkers for cardiovascular disease, and their expression levels are closely associated with the occurrence and prognosis of cardiovascular disease (5). Previous studies have revealed that miR-24 is essential in myocardial infarction. For example, bone marrow mesenchymal stem cell exosomes inhibited myocardial cell apoptosis in AMI rats via the upregulation of miR-24 under hypoxic conditions (6). miR-24 inhibited the proliferation of vascular smooth muscle cells and regulated vascular remodeling in the diabetic rat model by inhibiting the platelet-derived growth factor/BB pathway (7). Furthermore, miR-24 is abnormally expressed in myocardial fibrosis after

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infarction, which is regulated by TGF- $\beta$ 1 signaling (8,9). Compared with wild-type mice, miR-24 transgenic mice with MI showed decreased myocardial cell apoptosis and improved cardiac function after MI (10). Following induction of MI, inhibiting the expression of miR-24 has significant biological effects on cardiomyocytes and fibroblasts, which can reduce the infarct size and markedly improve cardiac function (11).

It has been previously reported that the occurrence of IRI involves a variety of factors, and the damage caused by inflammatory response to cells is an important pathological mechanism (12). NF- $\kappa$ B, as an inflammation marker, is involved in the occurrence of various diseases, such as chronic inflammatory diseases, heart disease and cancer (13,14). NF- $\kappa$ B signaling regulates the immune response, hypoxia response, apoptosis and embryonic development, as well as tumorigenesis and development (12-14). TNF- $\alpha$  serves an important role as a proinflammatory cytokine in NF- $\kappa$ B-mediated inflammation (15). However, whether miR-24 can alleviate IRI-induced MI via the NF- $\kappa$ B/TNF- $\alpha$  pathway remains to be elucidated. The present study aimed to investigate the role and mechanism of miR-24 in IRI-induced myocardial damage via *in vitro* and *in vivo* studies.

## Materials and methods

**Cell culture.** Cardiomyocytes (H9C2 cell line; American Type Culture Collection) were cultured in high-glucose DMEM containing 10% FBS and 1% penicillin-streptomycin (all from Thermo Fisher Scientific, Inc.). Cells were cultured in a 5% CO<sub>2</sub> incubator with 95% saturation humidity at 37°C. Subsequent experiments were performed with cells in the logarithmic growth phase.

**IRI model.** Cardiomyocytes in the logarithmic phase were randomly divided into three groups: i) Cells in the negative control (NC) group were cultured in normal DMEM; ii) cells in the model group were cultured in glucose-free DMEM (Thermo Fisher Scientific, Inc.) with a mixture of 5% CO<sub>2</sub> and 95% N<sub>2</sub> for 10 h at 37°C under ischemia, followed by addition of high-glucose (4,500 mg/l) DMEM for 2 h of routine culture and iii) cells in the miRNA group were incubated in glucose-free DMEM with a mixture of 5% CO<sub>2</sub> and 95% N<sub>2</sub> gas for 10 h at 37°C under ischemia, followed by addition of normal DMEM for 2 h of routine culture after transfection of miR-24 (1). Briefly, H9C2 cells were seeded in a 6-well plate at a density of 1×10<sup>5</sup> cells/well. Once cell fusion reached 70%, Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect cells in the NC and miR-24 (Sangon Biotech Co., Ltd.) groups according to the kit instructions. miRNA NC sequence, 5'-CCTTGGATGGCCTAGGAGATAG-3'; and miR-24 mimic sequence, 5'-TGGCTCAGTTCAGCAGGAACAG-3'. The cells were incubated in an incubator at 37°C and 5% CO<sub>2</sub> for 48 h before subsequent experiments.

**Cell apoptosis via flow cytometry.** The apoptosis of H9C2 was analyzed via flow cytometry (Beckman Coulter, Inc.). Cells were collected and washed twice with PBS. Cells were resuspended in 300  $\mu$ l binding buffer and incubated in 5  $\mu$ l Annexin V-FITC and 5  $\mu$ l PI at room temperature in the dark for 15 min. Finally, 200  $\mu$ l binding buffer was added to resuspend the cells, which were then filtered. Flow cytometry was performed within 1 h.

**Western blotting.** Total protein in H9C2 cells and tissues were extracted with RIPA buffer (Thermo Fisher Scientific, Inc.), and proteins were quantified using a BCA assay protein kit. Loading buffer (Thermo Fisher Scientific, Inc.) was added to the lysate solution, boiled at 100°C for 10 min and then stored at -20°C. Equal amounts of protein samples (30  $\mu$ g) were separated via 10% SDS-PAGE. Separated proteins were transferred to nitrocellulose filter membranes. Membranes were blocked with 5% skimmed milk powder [prepared with TBS-0.1% Tween-20 (TBS-T)] at room temperature for 1 h. Subsequently, membranes were incubated with the following primary antibodies: NF- $\kappa$ B (cat. no. 8242S), caspase-3 (cat. no. 9662S), Bax (cat. no. 2772S), Bcl-2 (cat. no. 3498S), TNF- $\alpha$  (cat. no. 3707S), vascular cell adhesion molecule 1 (VCAM-1; cat. nos. 13662S and 39036S), intercellular adhesion molecule 1 (ICAM-1; cat. no. 4915S) and monocyte chemoattractant protein-1 (MCP-1; cat. nos. 81559S and 41987S) (all from Cell Signaling Technology, Inc.) and incubated overnight at 4°C. Membranes were washed three times with TBS-T (5 min each time) and then incubated at room temperature for 1 h with the corresponding secondary antibody (anti-rabbit IgG HRP-linked antibody; 1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.) under gentle rotation. An ECL kit (Thermo Fisher Scientific, Inc.) was used to detect protein bands. GAPDH was used as an internal reference.

**Animal experiments.** Healthy male Sprague-Dawley rats (weight, 200±20 g; age, 8 weeks) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Under normal conditions, rats were fed under a normal circadian rhythm of 12/12 h light/dark cycles. Establishment of an IRI model was as follows: Rats were anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. Rats were placed in the right decubitus position, and microscopic scissors were used to open the chest cavity between the three and four intercostals of the left forelimb to fully expose the heart. Then, microscopic straight forceps were used to gently pick up a small amount of pericardium and slightly tear the pericardium under the left atrial appendage to fully expose the left anterior descending coronary artery (LAD) or the area. A 7-0 needle suture with a needle holder was utilized, and the needle was inserted 2-mm from the lower edge of the left atrial appendage. The suture was passed through the LAD to completely block the blood flow of LAD. Changes in the electrocardiogram and myocardial color were observed, and the ligation was removed after 30 min and reperfusion was conducted for 120 min. The experimental rats were randomly divided into three groups (15 rats in total; five rats in each group): i) Sham group, threaded but with no ligated artery; ii) Model group, where animals underwent IRI modeling after ischemia for 30 min and reperfusion was performed for 120 min; and iii) miRNA group, where animals underwent IRI modeling, ischemia was first performed for 30 min and then rats underwent reperfusion for 120 min. After 7 days, miR-24 was injected into the myocardium. The animal experiments involved in the present study were approved by the Ethics Committee of Zhoupu Hospital affiliated to Shanghai Medical University (approval no. 2017-C-040-E01).

**MI area determination.** After reperfusion in each group, the animals were euthanized by cervical dislocation. The rat

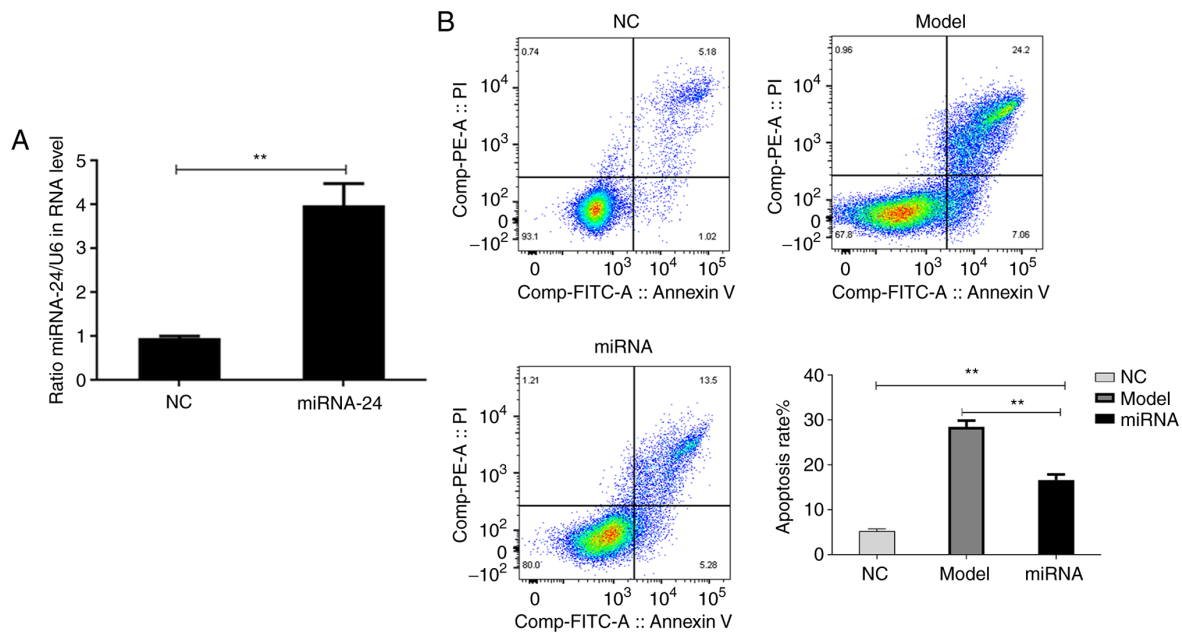


Figure 1. miR-24 inhibits cardiomyocyte apoptosis. (A) Reverse transcription-quantitative PCR results showing that miR-24 expression is increased following miR-24 transfection compared with the negative control group. (B) The apoptotic rate of cardiomyocytes in each group were detected via flow cytometry. \*\* $P < 0.01$ . NC, negative control; miR, microRNA; miRNA, cells transfected with miR-24.

hearts were removed, and 1 ml 3% Evans blue (Sigma-Aldrich; Merck KGaA) was injected into the left ventricle. Perpendicular to the long axis direction of the heart, the heart tissue was cut into thin slices with a thickness of 5  $\mu$ m and 1% 2,3,5-triphenyltetrazolium chloride solution was incubated with the heart tissue section at 37°C for 15 min. Image Pro Plus 6.0 analysis software (Media Cybernetics, Inc.) was used to determine the infarct size.

**Hematoxylin and eosin (H&E) staining.** Heart tissues were fixed with 4% paraformaldehyde for 30 min at room temperature, and paraffin sections (5  $\mu$ m) were sliced, followed by H&E staining. The heart tissue sections were immersed in hematoxylin solution for 5-20 min at room temperature and differentiated with 1% hydrochloric acid alcohol for 1-5 sec, and then stained with 1% eosin solution for 1 min at room temperature. Myocardial injury was observed under an optical microscope (magnification, x200).

**TUNEL assay.** Cardiomyocyte apoptosis in heart tissues were detected using the TUNEL method (cat. no. 11684817910; Roche Diagnostics). Tissue sections were washed twice with xylene for dewaxing, washed once with gradient ethanol (100, 95, 90, 80 and 70%) for rehydration and then treated with cell permeate for 8 min. Subsequently, 50  $\mu$ l TUNEL reaction mixture was added to the specimens and incubated for 1 h at 37°C in the dark in a wet box. A total of 50  $\mu$ l converter-POD was added and sections were incubated for 30 min. Finally, 50-100  $\mu$ l diaminobenzidine (DAB) substrate was added to the tissues and incubated at 15-25°C for 10 min. Sections were observed and imaged using an optical microscope (magnification, x200) and Image-Pro Plus software (Media Cybernetics, Inc.).

**Immunohistochemical staining.** Slices were placed in an oven for 30 min for dewaxing and were then hydrated with gradient

alcohol. A total of 1 ml 30%  $H_2O_2$  and 9 ml methanol were used to remove endogenous catalase. Subsequently, 5% BSA blocking solution (cat. no. A1933-25g; Sigma-Aldrich; Merck KGaA) was added and slides were incubated in a 37°C incubator for 30 min. Heart tissues were incubated with primary antibodies (NF- $\kappa$ B and TNF- $\alpha$ ) (as aforementioned; dilution, 1:500) at 4°C overnight, and then incubated with corresponding secondary antibody [SignalStain® Boost IHC Detection Reagent (HRP, Rabbit); cat. no. 8114; Cell Signaling Technology, Inc.] at room temperature for 1 h. DAB was added immediately after the liquid around the tissue dried, slides were incubated at room temperature for 5 min and the reaction was terminated with water washing. The slices were dehydrated and made transparent after counter-staining with hematoxylin for 5 min at room temperature. The results were observed using an inverted microscope (Nikon Corporation).

**Statistical analysis.** Statistical analysis was performed with GraphPad Prism 5 software (GraphPad Software, Inc.). Data are presented as the mean  $\pm$  SD. Comparison between two groups was analyzed using Student's t-test, and one-way ANOVA followed by Tukey's post hoc test was used for comparison between  $\geq 3$  groups. All experiments were repeated  $\geq 3$  times.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**miR-24 inhibits cardiomyocyte apoptosis.** After the cells were transfected with miR-24 mimic, transfection efficiency analysis by RT-qPCR indicated that the expression of miR-24 was significantly higher in the transfection group compared with the NC group (Fig. 1A). The apoptotic rate of H9C2 cells in each group was detected via flow cytometry (Fig. 1B). The results demonstrated that cardiomyocyte apoptosis

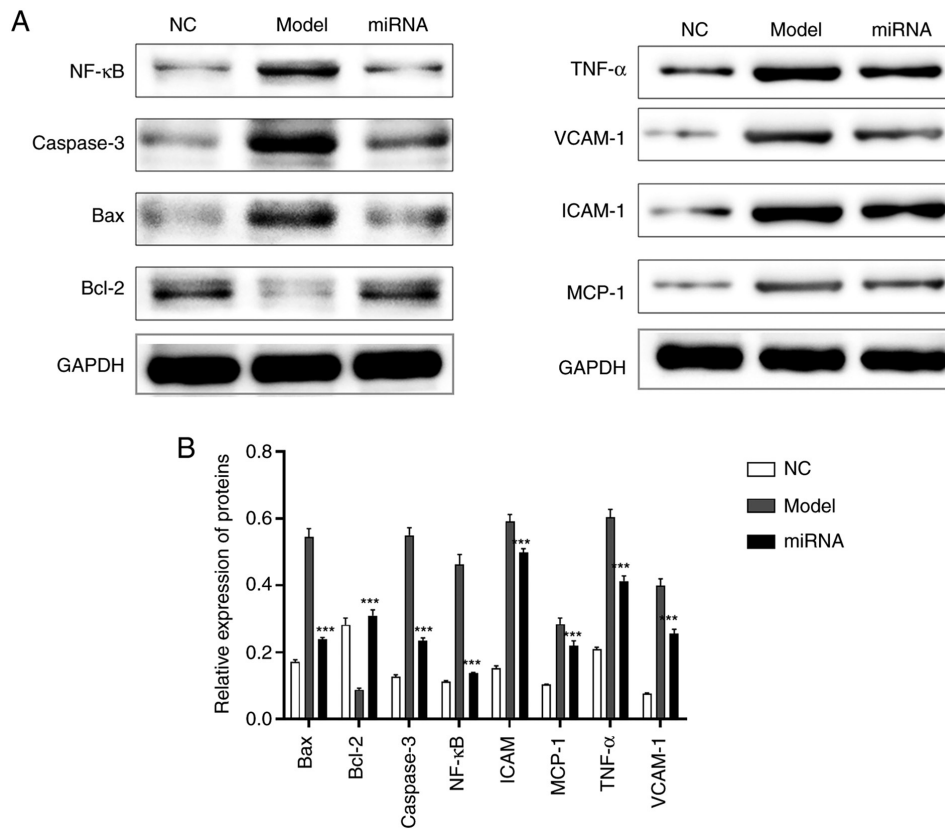


Figure 2. miR-24 decreases H9C2 cell apoptosis via NF- $\kappa$ B/TNF- $\alpha$  signaling *in vitro*. (A) Relative protein expression levels associated with the NF- $\kappa$ B/TNF- $\alpha$  pathway were measured via western blotting. (B) Results of gray-scale analysis of protein expression. \*\*\*P<0.001 vs. model group. NC, negative control; miR, microRNA; miRNA, cells transfected with miR-24; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; MCP-1, monocyte chemoattractant protein-1.

was significantly induced following IRI in the model group compared with the miRNA group (Fig. 1B). Compared with the model group, the apoptotic rate of H9C2 cells in the miRNA group was significantly inhibited (Fig. 1B).

**miR-24 decreases H9C2 cell apoptosis via NF- $\kappa$ B/TNF- $\alpha$  signaling *in vitro*.** To determine whether miR-24-induced apoptosis in H9C2 cells was associated with NF- $\kappa$ B/TNF- $\alpha$  signaling, the expression of proteins associated with the NF- $\kappa$ B/TNF- $\alpha$  pathway were detected using western blotting (Fig. 2A and B). The results identified that compared with the NC group, the protein expression levels of NF- $\kappa$ B, TNF- $\alpha$ , caspase-3, Bax, Bcl-2, VCAM-1, ICAM-1 and MCP-1 were significantly increased in the model group (Fig. 2A and B). However, in the miRNA group significantly inhibited protein expression levels of NF- $\kappa$ B, TNF- $\alpha$ , caspase-3, Bax, Bcl-2, VCAM-1, ICAM-1 and MCP-1 were recorded compared with the model group (Fig. 2A and B).

**miR-24 pretreatment improves myocardial function in IRI.** To evaluate the role of miR-24 in rat heart function, an IRI animal model was established and an electrocardiogram was performed before and after animal experiments (Fig. 3A). MI in rats was detected using Evans blue staining (Fig. 3B). The area of MI in the model group was significantly larger compared with the sham group, but the area of MI in the miRNA group was significantly reduced compared with the model group (Fig. 3B). The pathological results of H&E staining indicated that the

myocardial fibers in the sham group were neatly arranged and were regular in structure (Fig. 3C). However, myocardial cell disorder, inflammatory cell infiltration and myocardial fibrosis were observed in the model group (Fig. 3C). The pathological changes in cardiomyocytes in the miRNA group were markedly improved compared with the model group (Fig. 3C).

Myocardial tissue apoptosis was analyzed using the TUNEL method (Fig. 3D). The number of TUNEL-positive cells in the model group was significantly higher compared with the sham group (Fig. 3D). Compared with the model group, following miR-24 overexpression, the apoptosis rate in myocardial tissue was significantly decreased (Fig. 3D).

**miR-24 protects MIRI via the NF- $\kappa$ B/TNF- $\alpha$  pathway.** In order to examine the mechanism underlying miR-24 influencing myocardial function in rats, the expression levels of related proteins were measured *in vivo* using immunohistochemistry and western blotting. The staining showed that myocardial cells in the model group were disordered and pink protein mucous exudated, while myocardial lesions in the miRNA group were significantly alleviated. Immunohistochemistry results suggested that the proportion of NF- $\kappa$ B (Fig. 4A) and TNF- $\alpha$  (Fig. 4B) positive cells in the miRNA group was significantly lower compared with that in the model group (Fig. 4A and B). Subsequently, proteins were extracted from rat cardiac tissue in each group. The results were consistent with those of the *in vitro* studies, as the western blotting results suggested that, compared with the sham group, the



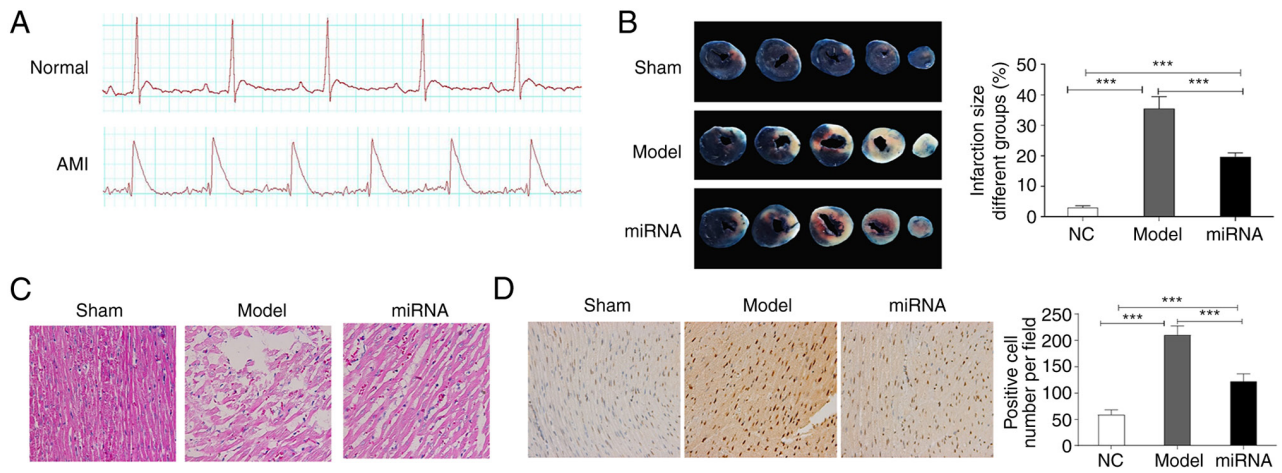


Figure 3. miR-24 can prevent myocardial ischemia-reperfusion injury. (A) Changes in the electrocardiogram of rats before and after modeling. (B) Comparison of infarct size between groups. Blue-stained areas represent healthy tissue and unstained pale areas indicate infarcted tissue (magnification, x40). (C) Pathology of different groups (magnification, x200). (D) Cellular apoptosis of different groups was assessed using a TUNEL assay (magnification, x200). \*\*\* $P < 0.001$ . NC, negative control; miR, microRNA; miRNA, animals injected with miR-24; AMI, acute myocardial infarction.

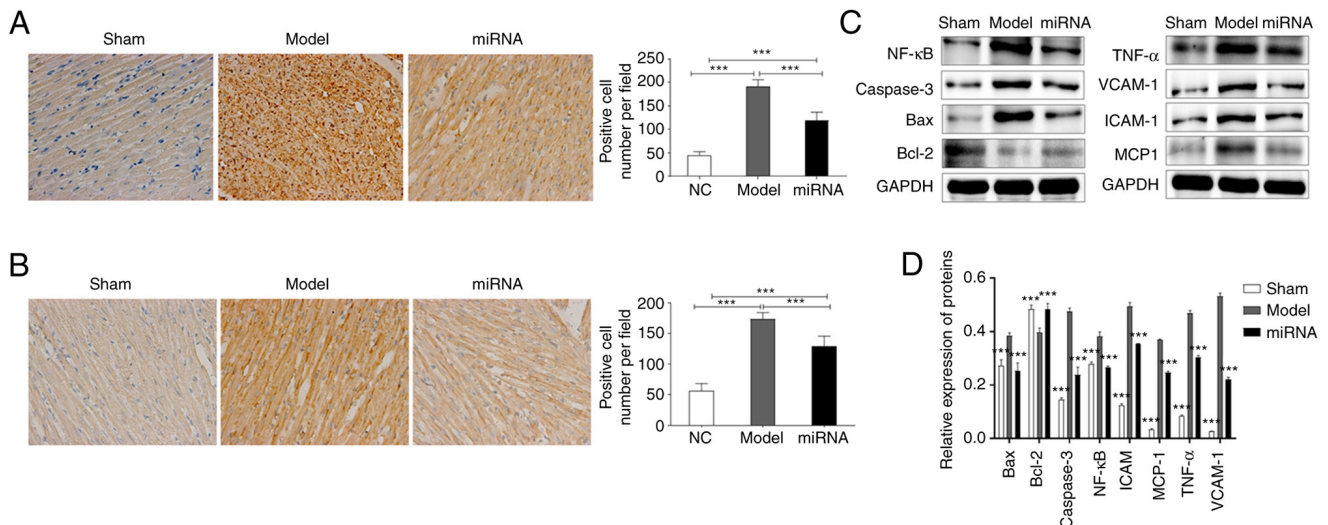


Figure 4. Effects of miR-24 on the expression of NF- $\kappa$ B/TNF- $\alpha$  signaling-related proteins in tissues. (A) Immunohistochemical staining of NF- $\kappa$ B expression in each group (magnification, x200). (B) Immunohistochemical staining of TNF- $\alpha$  expression in each group (magnification, x200). (C and D) Relative protein expression levels of proteins in the NF- $\kappa$ B/TNF- $\alpha$  pathway were detected via western blotting. \*\*\* $P < 0.001$  vs. model group. NC, negative control; miR, microRNA; miRNA, animals injected with miR-24; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; MCP-1, monocyte chemoattractant protein-1.

protein expression levels of NF- $\kappa$ B, TNF- $\alpha$ , caspase-3, Bax, VCAM-1, ICAM-1 and MCP-1 were significantly upregulated in the model group (Fig. 4C and D). By contrast, Bcl-2 expression was downregulated in the model group compared with the sham group, but upregulated in the miRNA group compared to the model group. However, the miRNA group had significantly downregulated NF- $\kappa$ B, TNF- $\alpha$ , caspase-3, Bax, VCAM-1, ICAM-1 and MCP-1 protein expression levels compared with the model group (Fig. 4C and D).

## Discussion

IRI is caused by decreased or blocked blood flow after coronary artery reperfusion. IRI can lead to further damage of ischemic tissues, cell death and even irreversible pathological changes, such as organ dysfunction (16,17). Moreover, IRI

leads to a high mortality rate in patients with MI, which is a serious clinical complication and affects the prognosis of these patients. Oxidative stress, cardiomyocyte apoptosis and cardiac dysfunction are all associated with IRI (18,19). It has been previously reported that intervention in cardiomyocyte apoptosis can restore IRI to a certain extent (19). The present research also suggested that miR-24 can decrease cardiomyocyte apoptosis caused by IRI.

miRNAs are a type of non-coding RNA that regulate post-transcriptional gene expression. In most multicellular organisms, miRNAs perform a variety of functions, other than protein translation, and are involved in physical processes and disease development. Previous studies have revealed that miRNAs can regulate inflammation, autophagy, apoptosis and oxidative stress, and also serve an important role in the repair of myocardial damage after IRI (20). For instance, miR-703

protected against mouse cardiomyocyte injury by inhibiting NOD-, LRR- and pyrin domain-containing protein 3/caspase-1 mediated pyroptosis (21). It was also reported that miR-346 reduced the infarct size and inhibited cardiomyocyte apoptosis by targeting Bax after myocardial IRI in rats (22). miR-760 protected against myocardial IRI by inhibiting sodium hydrosulfide (23), while miR-141-3p interacts with chromodomain-helicase-DNA-binding protein 8 and reduces the expression of p21, which serves an important role in hypoxia/reoxygenation-induced cardiomyocyte apoptosis (24). In the present study, miR-24 decreased the infarct size and cardiomyocyte apoptosis, as well as improved myocardial function in rats following myocardial IRI.

NF- $\kappa$ B is an important inflammatory transcription factor widely found in eukaryotic cells and is involved in the transcriptional regulation of numerous apoptosis-related genes. Previous studies have reported that NF- $\kappa$ B is essential in cardiovascular diseases and is activated in the early pathogenesis of ischemia (25). TNF- $\alpha$ , as an inflammatory cytokine in the NF- $\kappa$ B pathway, is also involved in the regulation of IRI (26). When vascular endothelial cells undergo inflammatory activation, IL-8 and MCP-1 are induced to attract leukocytes, VCAM-1 and ICAM-1, along with other cell adhesion molecules, in order to promote the adhesion of inflammatory cells and lead to the occurrence of vascular diseases, such as atherosclerosis (27). The ratio of Bax/Bcl-2 protein acts as the molecular switch of apoptosis, and caspase-3 protein is the executor of apoptosis. Consistently, the present study found that the expression levels of NF- $\kappa$ B/TNF- $\alpha$  pathway-related proteins in the miRNA group (NF- $\kappa$ B, caspase-3, Bax, Bcl-2, TNF- $\alpha$ , VCAM-1, ICAM-1 and MCP-1) were significantly different from those in the model group.

In conclusion, the present *in vitro* and *in vivo* studies demonstrated that miR-24 improved myocardial injury in rats by inhibiting the NF- $\kappa$ B/TNF- $\alpha$  pathway. However, due to the intricate signaling pathways involved in myocardial IRI, the role of miR-24 in additional signaling pathways associated with myocardial IRI requires to be further elucidated.

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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

XL conceived and designed the experiments. CL and MF performed the experiments and wrote the manuscript. CL, MF,

ZL and WW analyzed the data. XL made substantial contributions to proofreading the manuscript and gave final approval of the version to be published. XL and CL confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The animal experiments involved in this study were approved by the Ethics Committee of Zhoupu Hospital affiliated to Shanghai Medical University (2017-C-040-E01).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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