MiR-24 Protects Cardiomyocytes Against Hypoxia/Reoxygenation-Induced Injury Through Regulating Mitogen-Activated Protein Kinase 14

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Summary

This study aimed to explore the function of miR-24 in hypoxia/reoxygenation (H/R)-induced cardiomyocyte injury. We constructed a cardiomyocyte model of H/R using the primary cardiomyocytes isolated from Sprague-Dawley rats. To explore the role of miR-24, cells were transfected with a miR-24 mimic or miR-24 inhibitor. The RNA expression levels of miR-24 and Mapk14 were determined using qRT-PCR. The proliferation and apoptosis of cells were determined using a CCK8 assay and a flow cytometer. The TargetScan website was used to predict the targets of miR-24. A dual-luciferase reporter gene assay was conducted to verify whether Mapk14 is indeed a target of miR-24. A Western blot was applied for protein detection.

H/R exposure decreased the expression of miR-24 in rat cardiomyocytes. Transfection of the miR-24 mimic into cardiomyocytes reduced H/R-induced injury as evidenced by an increase in proliferation and a decrease in the apoptotic rate. By contrast, transfection of the miR-24 inhibitor aggravated H/R-induced injury. The expression of Bcl-2 was increased while the levels of Bax and Active-caspase 3 were reduced in the H/R+ miR-24 mimic group compared to those in the H/R group. H/R+miR-24 inhibitor group showed the opposite results. Mapk14 was identified as a target of miR-24. The mRNA level of Mapk14 and its protein (p38 MAPK) level were negatively affected by miR-24. Furthermore, we discovered that depletion of Mapk14 reduced the promoting effect of the miR-24 inhibitor on cell apoptosis.

Overall, our results illustrated that miR-24 could attenuate H/R-induced injury partly by regulating Mapk14.

Key words: Myocardial ischemia, Cell apoptosis, Target, Dual-luciferase reporter gene assay

Although great progress has been made in its treatment, ischemic heart disease is still one of the major causes of death around the world. Currently, reperfusion treatment is the normal therapy for myocardial ischemia that decreases myocardial infarction, reduces cell apoptosis, and restores contractile dysfunction. However, accompanying reperfusion is the myocardial ischemia/reperfusion (MI/R) injury, which is still a challenging side effect. Moreover, previous experiments have suggested that reperfusion precipitated cell injury leading to necrosis and apoptosis. Hence, it is prerequisite for one to look into the mechanism of MI/R injury and develop novel therapeutics that could exhibit protective effects on MI/R injury.

In recent years, microRNAs (miRNAs) have attracted much attention and have been illustrated to have many physiological and pathological functions. Several miRNAs have been identified to be involved in the regulation of MI/R injury. miR-21 is found to act as a tumor promoter in several types of tumors, such as tongue squamous cell carcinoma, acute myeloid leukemia, breast carcinoma, and glioma. In myocardial infarction, miR-24 exhibited an anti-apoptotic effect that was partially mediated by Bim. However, the function of miR-
24 in M/R and the detailed mechanism remains largely unclear.

In this research, we constructed a cardiomyocyte model of hypoxia/reoxygenation (H/R) in vitro to investigate the function of miR-24 and its targets. We discovered that miR-24 that was down-regulated in cardiomyocytes suffered from H/R. Moreover, we preliminarily revealed that miR-24 possibly exhibited its protective function on H/R-induced cardiomyocyte injury by regulating Mapk14.

Methods

Ethical statement: This work was conducted with formal approval of the local animal care committees.

Primary cardiomyocyte isolation and cultivation: The isolation of primary cardiomyocytes was performed as previously described. In brief, Sprague-Dawley (SD) rats were killed by dislocation after 24 hours of birth. The hearts were isolated, ground, and digested in 0.07% trypsin and 0.05% collagenase II at 37°C for 10 minutes. After the first digestion, the released cells were discarded. Then, the digestion was repeated four times, and the cell supernatants generated in these 4 times were added to a centrifuge tube that contained Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). After filtration and centrifugation (700 × g; 5 minutes), cells were re-suspended in a DMEM medium supplemented with 10% FBS and 1% v/v penicillin/streptomycin (Gibco, Oklahoma, USA). The differential wall adhesion method was used to purify cardiomyocytes. Cell suspensions were cultured in a T-75 culture flask (BD Biosciences, Franklin Lakes, NJ, USA) at 37°C for 90 minutes. Cells in the supernatants were seeded into 96 well plates coated with gelatin at the density of 1 × 10⁶ cells/mL and cultured at 37°C with 5% CO₂. After 96 hours’ cultivation, the cells were used for the following tests. The purity of the cultured cardiac myocytes was higher than 95%.

Construction of the cardiomyocyte model of H/R in vitro: Before hypoxia treatment, the primary cardiomyocytes were transferred into a DMEM culture medium without FBS. Then, the cells were cultivated in an anaerobic incubator with 95% N₂ and 5% CO₂ at 37°C for 6 hours. Afterwards, the cells were moved to the incubator containing 95% O₂ and 5% CO₂ to re-oxygenation at 37°C for 24 hours.

Transfection: The miR-24 mimic (5’-UGGCUAGUUCCAGGAACAG-3’), an inhibitor (5’-ACCCAGUCAAGUCGUCCUUAGC-3’), a miR-24 negative control (miR-24 mimic NC: 5’-AUGUGCGAAGGAUAGGAUAGGUGU-3’), si-Mapk14 (5’-GACAAAATCTGCAGGGAATGAAAC-3’), and si-NC (5’-ATTCCAGGAACACATGAGCAAGATG-3’) were all synthesized by GenePharma Co. (Shanghai, China). Cell transfection was performed using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s description. The concentrations of the miR-24 mimic, the miR-24 inhibitor, the miR-24 mimic NC and the miR-24 inhibitor NC we used for transfection were 50 nM each.

Cell counting kit-8 (CCK8) assay: Cell suspensions were prepared and seeded into 96-well plates with 1,000 cells in each well. The cells in the plates included four groups: control, H/R, H/R + miR-24 mimic and H/R + miR-24 inhibitor. All the cells in these four groups were subjected to H/R besides the control group. Cells in the H/R + miR-24 mimic group were transfected with the miR-24 mimic. Cells in the H/R + miR-24 inhibitor group were transfected with the miR-24 inhibitor. At 24-hour and 48-hour time points, CCK-8 reagents (10 µl/well) were added in the indicated wells and incubated at 37°C for 1.5 hours. Then, the absorbance at 450 nm was detected using the ELx800 microplate reader (BioTek Instruments Inc., USA).

MTT assay: Cell suspensions were prepared and seeded into 96-well plates with 1,000 cells in each well. Cells were cultured under standard conditions. To determine cell viability, we added 20 µL MTT (5 mg/mL) in each well. After incubation for 4 hours at 37°C, the culture supernatant was discarded, and 150 µL DMSO was added into the well to dissolve the purple-colored precipitates. Then, the absorbance at 490 nm was detected using the EL x800 microplate reader (BioTek Instruments Inc., USA). Cell viability was detected at 24-hour and 48-hour time points.

qRT-PCR: Total RNA was extracted from the cells using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The cDNA was formed using a PrimeScript RT Reagent Kit (Takara bio.) and used as a template to detect the mRNA expression of MAPK14. qPCR was performed using a SYBR Premix Ex Taq II (Takara bio.) kit on the ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The sequences of the primers were as follows: Mapk14 F: 5’-A CTGGCGGCGTCCTTCCAGAGGAGA-3’, Mapk14 R: 5’-CAGG CAGATAAGTGCCTGC-3’, Gapdh F: 5’-GGCTGCTCTTTTGTCAGCAAG-3’, and Gapdh R: 5’-AGTATGATGCTGGACTGTGGGTTTTGTCCAG-3’. Gapdh was utilized as an internal reference. For the detection of miR-24, the Mir-X™ miRNA First Strand Synthesis Kit was used for reverse transcription, and the SYBR PrimeScript™ miRNA RT-PCT Kit was used to conduct qPCR on a 7500 real-time PCR system. The sequence of the miR-24 forward primer was 5’-GGTGGCTCAGTTCAGCAGCAG-3’, and the sequence of the U6 forward primer was 5’-AGATTAGCATTGAGCAGGCCTCGTACAG-3’. The reverse primers of miR-24 and U6 were provided in the kit. The mRNA expression level of U6 was used as an internal reference. The relative expression value was calculated using the 2^(-ΔΔCt) method.

Western blot: Total proteins were isolated using RIPA lysis (with a protease inhibitor). The proteins were separated by 10% SDS-PAGE and electro-transferred to polyvinylidene fluoride (PVDF) membranes. Then, the membranes were incubated with Bcl-2 (Abcam, Cambridge, UK; cat. no. ab196495; 1:1000), Bax (Abcam; cat. no. ab 53154; 1:1000), Cleaved Caspase-3 (Abcam; cat. no. ab 49822; 1:1000), p38 MAPK (Cell Signaling Technology (CST), Inc.; no. 9212; 1:1000), phospho-p38 MAPK (CST; no. 9211; 1:1000), stress-activated protein kinase (JNK) (ProteinTech Group, Inc. USA; cat. no. 51151-1-AP; 1:1000), p-JNK (Abcam; ab47337; 1:1000) and GAPDH (Abcam; cat. no. ab9485; 1:2500) primary anti-
bodies (all were polyclonal antibodies). On the next day, the membranes were incubated with the horseradish peroxidase (HRP)-linked anti-rabbit secondary antibody (CST, no. 7074). An enhanced chemiluminescence detection kit (ECL, Pierce Biotechnology Inc) was utilized to detect the luminescent signals. The protein expression of GAPDH was used as an internal reference. Image J software was used to quantify the density of the bands.

**Cell apoptosis analysis:** After treatments, we harvested the cells by concentration and washed them using pre-cooled PBS. Next, cell suspensions (1-5 × 10^6 cells/mL) were prepared using the binding buffer. Then, 100 μL of cell suspension was added into a 5 mL flow tube followed by Annexin V-FITC/PI double staining (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s description. At last, the apoptotic rates of the cells were detected by a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The results were analyzed by utilizing Flowjo 10 software.

**Dual-luciferase reporter gene assay:** The potential target sites of miR-24 in Mapk14 were predicted on the TargetScan website (http://www.targetscan.org). We synthesized the fragment of wide-type 3'UTR of Mapk14 gene (WT) and the fragment of a Mapk14 3’ UTR with mutations in predicted binding sites (Mut). Next, the WT and Mut were inserted into the pmiR-RB-Report TM vector. The constructed vectors were named as pmiR-RB-WT and pmiR-RB-Mut, respectively. Afterwards, the primary cardiomyocytes were transfected with the pmiR-RB-WT + miR-24 mimic NC, pmiR-RB-WT + miR-24 mimic, pmiR-RB-Mut + miR-24 mimic NC or pmiR-RB-Mut + miR-24 mimic, accordingly. The luciferase activity was determined using a Dual-Luciferase Reporter Assay Kit (Promega Corporation, Madison, WI, USA) after 48 hours of transfection following the manufacturer’s descriptions. The luciferase activity was normalized to the activity of Renilla luciferase.

**Statistical analysis:** All the data were presented as mean ± standard deviation (SD). Statistical analysis was carried out using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Student’s t test was performed to evaluate the statistical significance between two groups. One-way analysis of variance (ANOVA) followed by post hoc a Bonferroni test was used to evaluate the statistical significance among three or more groups. Two-way ANOVA followed by a post hoc Bonferroni test was used to analyze the results of the dual-luciferase reporter gene assay. A result was considered as statistically significant when P value was less than 0.05.

**Results**

The expression level of miR-24 was down-regulated in the models of H/R: In order to explore the effects of miR-24 on myocardial ischemia, the model of H/R was constructed using the primary cardiomyocytes isolated from the hearts of SD rats. The expression of miR-24 in this model was determined by qRT-PCR. The results indicated that the expression level of miR-24 was lower in the H/R group than that in the control group (Figure 1A, P < 0.01). Then, we examined the effect of miR-24 mimic and inhibitor on miR-24 expression. The results revealed that the expression level of miR-24 was increased in the miR-24 mimic group compared to that in the miR-24 mimic NC group. By contrast, the level of miR-24 was decreased in the miR-24 inhibitor group compared to that in the miR-24 inhibitor NC group (Figure 1B, P < 0.01). There is no significant difference between the miR-24 mimic NC group or miR-24 inhibitor NC group and the control group. Similarly, in the H/R models, the miR-24 expression was increased by the miR-24 mimic and decreased by the miR-24 inhibitor compared to that in the H/R + miR-24 mimic NC group and the H/R+miR-24 inhibitor NC group, respectively (Figure 1C, P < 0.01).

**miR-24 extenuates H/R-induced cardiomyocyte injury:** To investigate the biological functions of miR-24 in H/R injury, we explored the effect of miR-24 alteration on the proliferation and apoptosis of H/R-injured cardiomyocytes. In CCK8 assays, we found that the OD_{450} value was higher at 48 hours than that at 24 hours, suggesting the proliferation of cardiomycocytes during cultivation. More importantly, we found that the proliferative ability was inhibited in the H/R group compared with the control group at 24-hour and 48-hour time points (P < 0.01, Figure 2A). The proliferative ability was increased in the H/R + miR-24 mimic group compared to that in the H/R group (P < 0.05, Figure 2A). On the contrary, the proliferation was decreased in the H/R + miR-24 inhibitor group compared to that in the H/R group (P < 0.05, Figure 2A). The following MTT assays further verified the phenomena we observed from CCK8 assays. It is apparent that the OD_{450} value in the H/R group was lower than that in the control group at 24 hours and 48 hours. Furthermore, the OD_{450} value was increased in HR+ miR-24 mimic group, whereas the OD_{450} value was reduced in H/R + miR-24 inhibitor group compared to that in the H/R group (P < 0.05, Figure 2B). By flow cytometry, we identified that the apoptotic rate of cardiomycocytes was higher in the H/R group (26.00%) than that in the control group (11.44%). After transfection of miR-24 mimic into the cardiomyocytes suffered from H/R, the apoptotic rate was decreased to 15.99%. However, the apoptotic rate of cardiomycocytes was increased to 43.4% in the H/R + miR-24 inhibitor group compared to that in the H/R group (P < 0.01, Figure 3A). Afterwards, we examined the expression changes of apoptotic-related proteins by Western blot. The results exhibited that the Bcl-2 expression level was decreased in the H/R group compared to that in the control group. Contrarily, the levels of Bax and Active-caspase3 were increased in the H/R group. In addition, the level of Bcl-2 was increased in the H/R + miR-24 mimic group and decreased in the H/R + miR-24 inhibitor group compared to that in the H/R group. The levels of Bax and Active-caspase3 were decreased in the H/R + miR-24 mimic group and enhanced in the H/R + miR-24 inhibitor group compared to that in the H/R group (P < 0.01, Figure 3B). Collectively, these data suggested that miR-24 played a promoting role in cell growth and a suppressive role in cell apoptosis in myocardial ischemia injury.

**miR-24 could directly bind to Mapk14:** We predicted the targets of miR-24 by using the TargetScan software and identified that Mapk14 is a target of miR-24. The
miR-24 ATTENUATES H/R-INDUCED CARDIOMYOCYTE INJURY

miR-24 negatively regulates the expression of MAPK14 and possibly improves myocardial ischemia by regulating the MAPK signaling pathway: The effects of miR-24 on the mRNA and protein expression of Mapk14 (protein name: p38 MAPK) were determined using qRT-PCR and the Western blot. The results revealed that the mRNA expression of Mapk14 was increased in the H/R group compared to that in the control group. Up-regulation or down-regulation of miR-24 in the H/R model decreased or increased the Mapk14 mRNA level compared with the H/R group, respectively (P < 0.01, Figure 5A). In the Western blot, we observed a similar phenomenon. The level of p38 MAPK was enhanced in the H/R group compared to that in the control group. And the level of p38 MAPK in the H/R + miR-24 mimic group was decreased compared to that in the H/R group. The level of p38 MAPK in the H/R + miR-24 inhibitor group was enhanced compared to that in the H/R + NC group (Figure 5B). These data suggested that miR-24 negatively regulated the expression of Mapk14 (p38MAPK) in cardiomyocytes.

In order to explore the influence of miR-24 on the MAPK signaling pathway, we further examined the expression of p-p38 MAPK, p-JNK, and JNK in cardiomyocytes. We identified that the p-p38 MAPK and p-JNK protein expression levels were increased in the H/R group compared with those in the NC group. What is more, the expression of these two proteins was inhibited in the H/R + miR-24 mimic group and increased in the H/R + miR-24 inhibitor group compared to those in the H/R group (P < 0.01, Figure 5B). However, no significant change was found in the protein expression of JNK in the H/R group, H/R + miR-24 mimic group and H/R + miR-24 inhibitor group compared with the sham group. These data suggested that miR-24 exhibited an inhibitory effect on the MAPK signaling pathway. To further explore whether miR-24 affected the apoptosis of cardiomyocytes by regulating Mapk14, we depleted Mapk14 in cardiomyocytes. As illustrated in Figure 6A-C, the expression of Mapk14 was decreased in cardiomyocytes transfected with si-MAPK14 compared to that in the si-NC group at both RNA and protein levels (P < 0.01). The results of cell apoptosis analysis indicated that the apoptotic rate of the H/R + miR-24 inhibitor + si-MAPK14 group was lower than that in the H/R + miR-24 inhibitor group, indicating that depletion of MAPK14 could suppress cell apoptosis.
miR-24 promoted proliferation of cardiomyocytes suffered from H/R. A: The effect of miR-24 on cell viability were detected by CCK8 assay. B: The effect of miR-24 on the proliferative ability of cardiomyocytes was determined using the MTT assay. n = 6, ** P < 0.01 versus control; * P < 0.05 versus H/R.

induced by the miR-24 inhibitor (P < 0.01, Figure 6D, E). Collectively, these results suggested that miR-24 possibly mitigated H/R injury by inhibiting Mapk14.

**Discussion**

During the past years, more and more evidence illustrated that a series of miRNAs are involved in the regulation of MI/R injury. In the present study, we demonstrated the protective function of miR-24 in H/R injury in vitro and suggested that this protection might be achieved by regulating the MAPK signaling pathway.

In the process of fibrosis, miR-24 was found to be down-regulated and demonstrated to be an inhibitor of scar formation in skeletal muscle by inhibiting the profibrotic pathways. In the heart, repeated aggravation of myocardial ischemia and hypoxia is one of the main causes of myocardial fibrosis. In the present study, we also observed that the expression level of miR-24 was down-regulated in the cardiomyocytes after being suffered from H/R, indicating the participation of miR-24 in ischemic heart disease.

Previous studies indicated that miR-24 might take a distinct part in different tissues and different diseases. In patients with atherosclerosis, miR-24 was found to play an inhibitory role in the proliferation of endothelial cells by regulating importin-α3 and modulating inflammatory responses. In bladder cancer cells and high glucose-induced vascular smooth muscle cells, the inhibitory effects on cell proliferation were observed as well. However, in non-small cell lung cancer cells, hepatocellular carcinoma cells, and human tongue squamous cell carcinoma cells, miR-24 exhibits promoting effects on the proliferation ability of these cells. In cardiomyocytes, we found that up-regulation of miR-24 recovered the proliferation ability that was damaged by H/R, suggesting that miR-24 exerted a pro-proliferative role in H/R injury. Qian, et al. reported that miR-24 exhibited an antiapoptotic effect on mouse cardiomyocytes. Consistent with their data, we identified that up-regulation of miR-24 repressed the apoptosis of cardiomyocytes that suffered from H/R. Moreover, we found that the levels of Bax and cleaved-caspase 3 were decreased while the level of Bcl-2 was increased in H/R-treated cardiomyocytes that transfected miR-24 mimic. The foregoing outcomes suggested that miR-24 has the potential to extenuate the H/R injury by promoting cardiomyocyte proliferation and inhibiting cardiomyocyte apoptosis.

Although it has been demonstrated by Qian, et al. that miR-24 functioned as an anti-apoptotic miRNA partially by repressing Bim in the ischemic zones of the left ventricle after acute MI, the mechanism of how miR-24 involved in the MI/R injury remains largely unknown. Most miRNAs, including miR-24, are implicated in the
miR-24 ATTENUATES H/R-INDUCED CARDIOMYOCYTE INJURY

Figure 3. miR-24 inhibits apoptosis of cardiomyocytes suffering from H/R. A: The effect of miR-24 on cell apoptosis was determined by a flow cytometer (left). The apoptosis percentages were shown in the right panel. B: The effect of miR-24 on the protein expression levels of apoptotic-related proteins were determined by the Western blot (left). The relative expression level of these proteins were calculated (right). n = 6, ** P < 0.01 versus control; * P < 0.05, ## P < 0.01 versus H/R.

regulation of multiple genes.24,25) Hence, to better understand the possible mechanism of how miR-24 exerts its role in MI/R, we predicted the potential targets of miR-24 using TargetScan. We focused on Mapk14, which contains the binding sites of miR-24 in its 3’-UTR. MAPKs are a family of Ser/Thr protein kinases, which modulate many cellular activities, for example, administering cellular responses to cell stress, pro-inflammatory cytokines, cell proliferation, and differentiation.26,27) Using Multifactor Dimensionality Reduction software (MDR) analysis, Xu, et al. implied that miR-24-Mapk14 (the interaction between miR-24 and Mapk14 has been predicted previously28) are candidate susceptibility factors for schizophrenia.25) However, the correlation between miR-24 and Mapk14 has not been proved by experiments and the effects of the miR-24-Mapk14 axis on cardiomyocytes has not been investigated. By dual-luciferase reporter gene assay, we verified that miR-24 could directly bind to the 3’UTR of Mapk14. Further analysis revealed that miR-24 could negatively modulate the expression of Mapk14 in cardiomyocytes. At protein level, the expression levels of p-p38 MAPK and p38 MAPK were decreased when up-regulated the expression of miR-24 using miR-24 mimic. JNK is one of the family numbers in the MAPK signaling pathway.30) We found that the protein expression p-JNK was also negatively regulated by miR-24 (Figure 5). Our Mapk14 deple-
miR-24 could directly bind to the Mapk14. A: The sequence of wild type (WT) 3'UTR of Mapk14 and the mutated (Mut) 3'UTR of Mapk14. The potential binding sites were predicted using TargetScan. The bars represent the potential binding sites (seed sequences) between miR-24 and Mapk14. The underline represents the mutant binding sites. B: Sequence alignment of the 3'-UTR of rat and human Mapk14. The red box represents the sites complementary to the seed sequences of miR-24. C: The relative luciferase activity in cardiomyocytes was determined after the WT or Mut were co-transfected with miR-24 mimic. n = 6, ** P < 0.01 versus H/R + miR-24 mimic NC. ## P < 0.01 versus WT.

miR-24 negatively regulate the expression of Mapk14 and affect the activation of the MAPK signaling pathway. A: The effect of miR-24 on Mapk14 mRNA expression was determined by qRT-PCR. B: The effect of miR-24 on the protein expression levels of p-p38 MAPK, p38 MAPK, p-JNK and JNK were determined by the Western blot (left). The relative expression ratios of p-p38 MAPK/p38 MAPK and p-JNK/JNK were calculated (right). n = 6, ** P < 0.01 versus control; ## P < 0.01 versus H/R.

In conclusion, our present results demonstrated that miR-24 might also inhibit the expression of Mapk14 in humans. Up-regulation of miR-24 would be a promising therapeutic strategy for MIR injury. However, much experimental and clinical verification is required to support this speculation.
miR-24 facilitates the proliferation and inhibits apoptosis of cardiomyocytes that suffered from H/R, at least partly, by regulating Mapk14. Moreover, these outcomes suggested that miR-24 might be a valuable biomarker and therapeutic target for patients suffering from MIR injury. In vivo assays were urgently needed to further verify our current results and point of view.

Disclosure
Conflicts of interest: There are no competing interests in this study.
Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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