MicroRNA-15b deteriorates hypoxia/reoxygenation-induced cardiomyocyte apoptosis by downregulating Bcl-2 and MAPK3

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ABSTRACT

To investigate the role of miRNA-15b in cardiomyocyte apoptosis after ischemia reperfusion injury in acute myocardial infarction (AMI), we conducted the AMI rat model by using left anterior descending ligation and performed hypoxia/reoxygenation experiments in H9c2 cells. MiRNA-15b was measured by quantitative reverse transcription PCR (qRT-PCR). Cardiomyocyte apoptosis was determined by terminal deoxynucleotide transferase dUTP nick end labeling staining. Synthesized miRNA-15b mimic and inhibitor were transfected into H9c2 cells by Lipofectamine regent. RNA expression of B cell lymphoma/leukemia-2 (Bcl-2) and mitogen-activated protein kinase 3 (MAPK3) was examined by qRT-PCR and their protein expression was determined by western blot. Ischemia reperfusion increased miRNA-15b expression in the ischemic rat heart and resulted more severe cardiomyocytes apoptosis. In H9c2 cells, hypoxia/reoxygenation induced increased miRNA-15b expression and augmented cardiomyocyte apoptosis observed at 24 hours after 24-hour hypoxia. Compared with the vehicle group, miRNA-15b mimic further raised miRNA-15b level and increased cardiomyocyte apoptosis, whereas miRNA-15b inhibitor suppressed miRNA-15b expression and protected cardiomyocytes from apoptosis. Although the mRNA expression of the target genes Bcl-2 and MAPK3 was not changed significantly, the protein expression of these two genes were markedly reduced after miRNA-15b mimic treatment and significantly increased after transfected with miRNA-15b inhibitors. In conclusion, miRNA-15b deteriorates cardiomyocyte apoptosis by post-transcriptionally downregulating the expression of Bcl-2 and MAPK3.

Significance of this study

What is already known about this subject?

- ► MiRNA-1, miRNA-133 and miRNA-208 have close relationship with acute myocardial infarction (AMI).
- ► MiRNA-15b plays an important role in ischemia reperfusion injury (IRI) and AMI.
- ► Apoptosis is one of major mechanisms of IRI after AMI.

What are the new findings?

- ► MiRNA-15b plays an important role during AMI process.
- ► B cell lymphoma/leukemia-2 (Bcl-2) and mitogen-activated protein kinase 3 (MAPK3) are new target genes of miRNA-15b verified in the present study.
- ► Bcl-2 and MAPK3 protect cardiac cells from IRI via downregulating apoptosis.

How might these results change the focus of research or clinical practice?

- ► Although percutaneous coronary intervention and thrombolytic therapy resolve the obstruction of infarct-related artery, they cannot fully save the dying myocardium.
- ► RNA interfering and epigenetic modulation are the promising methods and techniques.
- ► Small RNA interfering modulation like miRNA-15b will alleviate cardiomyocyte apoptosis.

INTRODUCTION

Acute myocardial infarction (AMI) is the most severe manifestation of coronary artery disease, which causes >2.4 million deaths in the USA, >4 million deaths in Europe and northern Asia,[1] and more than a third of deaths in developed nations annually.[2] Aging, increased rates of comorbidities of hypertension, diabetes and hyperlipidemia and larger obesity population all contribute to the increasing incidence of AMI.[3] When MI occurred, occlusion of coronary arteries prevents oxygen from supplying to the myocardium, resulting in loss of viable cardiac tissue and impairment of cardiac contractility. Both human studies and lab experiments suggest that apoptosis occurs in most affected cardiac cells during the initial 6–24 hours after acute coronary occlusion.[4 5] Thrombolysis therapy, percutaneous coronary intervention (PCI) and coronary artery bypass graft surgery are widely used for rescuing myocytes from ischemia-caused cell death.[6] However, due to the limited time window of PCI and thrombolysis therapy for ischemic heart disease, there is

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still a considerable number of patients who do not have the chance to be treated or may be treated too late and thus suffering from deteriorated cardiac function. Therefore, exploration of additional treatments for these patients is imperative. Furthermore, reperfusion itself may aggravate the myocardial injury after ischemia, which is termed as ischemia reperfusion injury (IRI). IRI can cause disruption of normal oxidative metabolism with reactive oxygen species overproduction, triggering myocyte death and eventually cardiac failure. However, the molecular regulators of the gene expression during myocardial ischemia/reperfusion (I/R) is still obscure.

MicroRNAs (miRNAs, miR) are a number of endogenous non-protein-coding RNAs comprising 20–23 nucleotides. They post-transcriptionally modulate gene expression by hybridization to messenger RNA (mRNA), leading to translational repression or degradation of the target mRNAs.[7 8] The 5′ end 2–8 nucleotides of the miRNA, named as the 'seed sequence', play an important role for targeting mRNAs. Of note, miRNAs regulate 30%–50% of the genome and thus are potent mediators of cellular signaling.[9] MiRNAs with homologous 5′ end nucleotides formed respective families, whereas they distinct from each other in the 3′ end.[7]

In recent years, many miRNAs have been suggested to be involved in regulating cardiomyocyte apoptosis.[10–12] MiRNA-15 family is composed of miRNA-15a, miRNA-15b, miRNA-16-1, miRNA-16-2, miRNA-195, and miRNA-497 and is exclusively found to be upregulated in different cardiovascular disease.[8] Some studies indicate that miRNA-15b induces apoptosis by targeting B cell lymphoma/leukemia-2 (Bcl-2) in gastric cancer cells and hepatic stellate cells.[13 14] Also, there are many genes involved in cardiomyocyte apoptosis, such as the Bcl-2 family. Cimmino *et al*[15] demonstrated that miRNA-15a and miRNA-16-1 expression was inversely correlated to Bcl-2 expression in B cell chronic lymphocytic leukemia and that both miRNAs negatively regulated Bcl-2 at a post-transcriptional level. Hullinger *et al*[16] designed a study to determine whether miRNA-15 family and miRNA-497 were dysregulated on IRI in a porcine model. Liu *et al*[17] primarily found that miRNA-15a/b was upregulated in response to cardiac I/R injury and correlated to myocardial apoptosis. The same authors further found that miRNA-15b deteriorated cardiomyocyte apoptosis by increasing the release of mitochondrial cytochrome *c* to the cytosol, resulting activation of caspase-3 and caspase-9, without affecting Bcl-2 mRNA.[18] These studies suggested that miRNA-15b closely related to cardiomyocyte apoptosis after I/R. Although the data confirmed that the miRNA-15 family targeted both Bcl-2 and Arl-2 (another target gene) in cardiomyocytes, the increased survival of cardiomyocyte suffering from hypoxia and the protective effect of miRNA-15 inhibition after I/R possibly attributed to comprehensive influence of many potential regulatory genes. In this context, searching for other genes that can be targeted to modulate the ischemia-induced cardiomyocyte apoptosis is imperative to improve the AMI patients' long-term outcome. In this study, we identified the potential target genes of miRNA-15b as Bcl-2 and mitogen-activated protein kinase 3 (MAPK3) using bioinformatics (TargetScan, PicTar, Miranda, and miRDB). Using the miRNA-15b inhibitors and mimics, we sought to elucidate the mechanisms underlying the regulation of miRNA-15b on cardiomyocyte apoptosis following acute myocardial I/R injury.

METHODS

Myocardial I/R rat model, miRNA-15b expression, and cardiomyocyte apoptosis measurement

Thirty-six healthy Sprague-Dawley rats (18 males and 18 females, purchased from Laboratory Animal Science Department, Fudan University, Shanghai, China) weighing 250–350 g were involved in the study. Use of animals was in accordance with the regulations of the Animal Experiments Ethic Committee of Shanghai Jiaotong University and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). In the I/R group, 26 rats were anesthetized with sodium pentobarbital (45 mg/kg) and a thoracotomy was performed. Rats were incubated and ventilated with a small animal ventilator with tidal volume 2 mL and respiratory rate 80/min. An anterior thoracotomy was performed to open the pericardium. The heart was then rapidly exteriorized, and a 6-0 silk suture was tightened around the proximal left anterior descending coronary artery (before the first branch of the diagonal artery). Following a 30 min coronary artery occlusion, the suture was removed to allow coronary reperfusion followed by closure of the chest wall. The surgical procedure was done under sterile condition. After 24 hours of coronary reperfusion, the individual rat was sacrificed and the heart was exteriorized and perfused. In the sham group, the same procedure was performed without tying the suture in 10 rats.

MiRNA-15b was isolated from the border zone of infarcted hearts with the mirVana miRNA isolation kit (Ambion, Texas, USA) according to the manufacturer's protocol. The concentration of RNA was determined by quantitative reverse transcription PCR (qRT-PCR) with miRNA Detection Kit (Invitrogen, California, USA).

For Terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) measurement, all hearts were soaked in formalin after removed, and tissues were handled by gradient alcohols dehydrating and paraffin wax embedding. The 3-μm-thick tissue sections were separated from paraffin-embedded heart tissue. Through immersed in xylene for 5 min and three times, all sections were deparaffinized. Then tissue sections were rehydrated and incubated in phosphate-buffered saline (PBS) with pH 7.4. Afterwards, tissues were incubated with 20 µg/mL proteinase K for 30 min, then washed with 0.1 M PBS. Tissue sections were incubated with TUNEL reaction mixture for 60 min at 37°C using an apoptosis detection kit (Roche, Indianapolis, USA). TUNEL-positive nuclei (fragmented DNA) fluoresced bright green at 450–500 nm. The average TUNEL-positive cells were calculated in three different fields from at least two slides for each group.

Cell culture

The cardiomyocytes used in this study were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Science (Shanghai, China). H9c2 (rat ventricular cell) lines were cultured in Dulbecco's modified Eagle's

medium supplemented with 10% fetal bovine serum and 1% penicillin and 1% streptomycin.

Treatment with hypoxia/reoxygenation for miRNA-15b expression

Cultured rat cardiomyocytes dishes were placed into a hypoxic incubator filled with 95% N_2 and 5% CO_2 at 37°C. After 6-hour, 18-hour and 24-hour hypoxia, respectively, the dishes were transferred to a normoxic incubator for 24-hour reoxygenation. MiRNAs were then isolated from the cultured cells using the mirVana miRNA isolation kit. MiRNA-15b expression was determined by qRT-PCR via miRNA Detection Kit.

Measurement of cardiomyocyte apoptosis induced by hypoxia/reoxygenation

Rat ventricular cell (H9c2) cultured in 0.1% FBS were treated with hypoxia for 6, 18 and 24 hours and incubated of reoxygenation for 24 hours, respectively. Afterwards, cell apoptosis was measured by TUNEL staining. Cardiomyocytes cultured on coverslips in 18-well plates were fixed in 4% paraformaldehyde. TUNEL staining was done using the in situ cell death detection kit (Roche) according to the manufacturer's protocol. The number of TUNEL-positive cells were counted under a fluorescence microscope. Results were shown from the data of five independent experiments (n=6) with 20 random microscopic fields from each sample.

Synthesis of miRNA mimic and inhibitor

miRNA-15b mimic and its inhibitor were synthesized by Shanghai GenePharma. The sequences of miRNA-15b is 5'-UAGCAGCACAUCAUGGUUUACA-3', and its inhibitor (anti-miRNA oligonucleotides (AMOs)) is the exact antisense copy of the mature miRNA.

Transfection of miRNA

Oligo transfection was performed according to the manufacturer's instructions. Briefly, 1×10^5 cells per well were transfected with 1 OD miRNA-15b or AMOs using Lipofectamine2000 Reagent (Invitrogen) for 18 hours after 24-hour serum starvation.

RNA level determination

RNAs from cardiomyocytes were isolated with a RNA Isolation Kit (Ambion). qRT-PCR for miRNA-15b was performed on cDNA generated from 50 ng of total RNA according to the manufacturer's protocol. qRT-PCR for Bcl-2 and MAPK3 was performed on cDNA generated from 200 ng of total RNA using the protocol of a mRNA detection kit (Roche), respectively. PCR conditions were denaturation (95°C, 1 min), 40 cycles of annealing (60°C, 15 s), and extending (70°C, 15 s). As an internal control, U6 was used for miRNA-15b template normalization and glyceraldehyde phosphate dehydrogenase (GAPDH) was used for Bcl-2 and MAPK3 template normalization. The main sequences of primers are shown in table 1.

Protein level determination

Proteins isolated from cultured cardiomyocytes were determined by western blot analysis. Equal amounts of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. A standard western blot analysis was conducted using Bcl-2 and MAPK3 antibody (Invitrogen). β-Actin antibody (1:5000 dilution; BioVision) was used as a loading control.

Table 1 Primers used for reverse transcription-PCR

Primer	Sequence
miR-15b forward	5′-TAGCAGCACATCATGGTTTACA-3′
miR-15b reverse	5′-TGCGTGTCGTGGAGTC-3′
Bcl-2 forward	5′-CTAGTCTAGACGGGCCTCAGGGAAC AGAA-3′
Bcl-2 reverse	5′CTAGTCTAGAAGCAATTAGCCCCCGTGACC-3′
MAPK3 forward	5′-TGGTAGACCGTTCTGGAATGG-3′
MAPK3 reverse	5′-CGTTACATGTGGCAGCTTGAG-3′

Bcl-2, B cell lymphoma/leukemia-2; MAPK3, mitogen-activated protein kinase 3.

Statistics

All values are expressed as mean±SD Comparisons of parameters among ≥3 groups were analyzed by one-way analysis of variance for single-factor, followed by Student's t-test with Bonferroni correction for multiple comparisons. Differences were considered statistically significant at a value of $p < 0.05$. Statistical analysis was performed with Prism V.5.0 software (GraphPad Software, California, USA).

RESULTS

Temporary occlusion of the left anterior descending (LAD) artery consistently produced myocardial infarction in rats

A total of 36 rats were involved in the study. In the sham group, all rats survived. Among the 26 rats were in the I/R group, 2 died of anesthesia. Ventricular fibrillation occurred in one rat. Two rats died from hemodynamic disorders. Eventually, 21 rats survived in the I/R group. The rat I/R model was successfully established by temporary occlusion of the LAD coronary artery for 30 min under ECG monitoring; the ST segment elevation was observed in lead II during ischemia. At 24 hours after reperfusion, was performed to evaluate the extent of infarction area was assessed by H&E staining. Rats in the I/R group exhibited visible infarction (I/R infarction size, 33.39%±4.81%, n=21), whereas the sham group showed no infarction (see online supplementary figure 1A,B).

LAD I/R injury induced miRNA-15b expression and cardiomyocyte apoptosis in the ischemic cardiomyocytes

To test the impact of cardiac I/R injury on the expression of miRNA-15b, we subjected 10 rats to sham and 21 rats to LAD occlusion surgery for 30 min and collected the rat heart for RNA extraction. PCR analysis showed that the miRNA-15b expression was significantly higher than those in the sham animals (figure 1A,B). Next, we also tested the cell apoptosis in the ischemic cardiomyoctes and found that the number of apoptotic cardiomyocyte in the I/R group was significantly increased compared with that in the sham group (figure 1C). We also compared TUNEL-positive nuclei between the border zone and distal zone for all rats in the sham and I/R groups, respectively. Results indicated

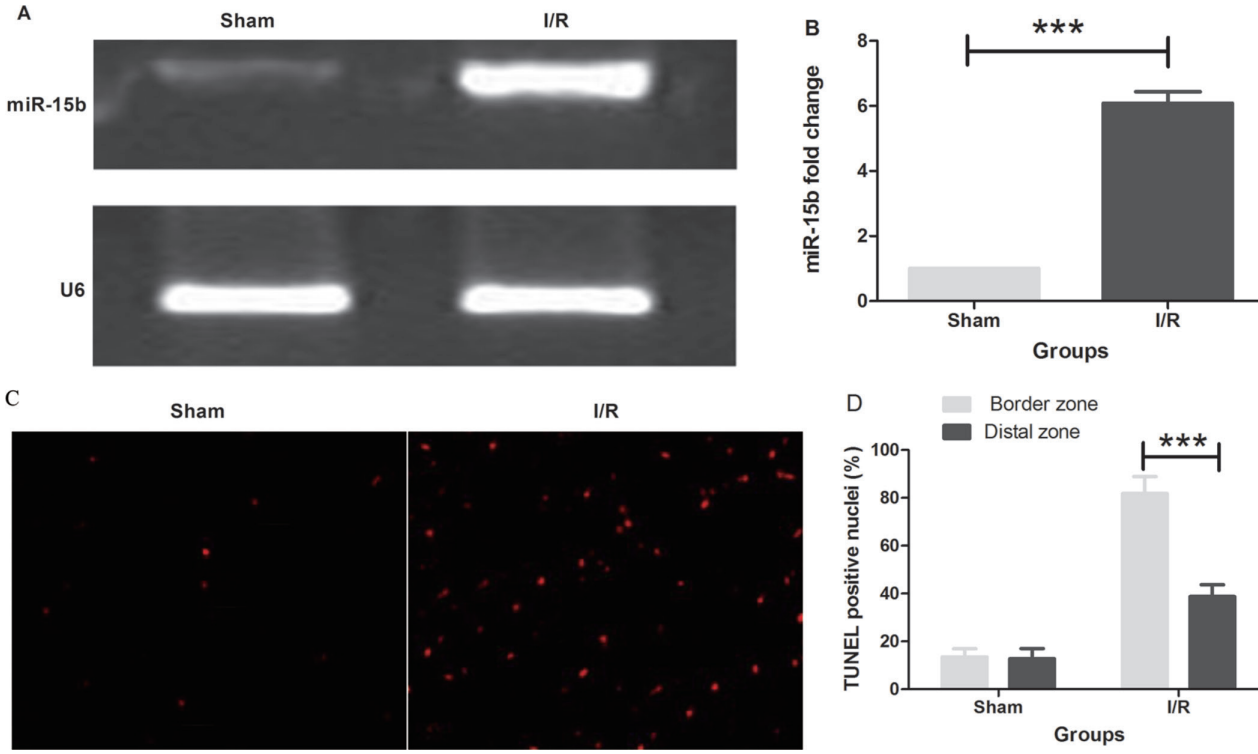

Figure 1 LAD ischemia/reperfusion (I/R) induced miRNA-15b expression and cardiomyocyte apoptosis in the ischemic cardiomyocytes. RNA or sections were prepared from the ischemic heart at 24 hours after surgery for PCR analysis or Terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) staining. (**A**) Representative images of quantitative reverse transcription PCR of rat ischemic heart tissue following LAD occlusion. (**B**) Quantification of expression level of miRNA-15b. (**C**) Representative TUNEL staining images. n=10 for the sham group and n=21 for the I/R group. (**D**) TUNEL-positive nuclei count. In the I/R group, the percentage of TUNEL-positive nuclei in border zone is higher than in distal zone. ***p<0.001.

that TUNEL-positive nuclei were higher in the border zones compared with distal zones (figure 1D).

LAD I/R injury induced Bcl-2 and MAPK3 expression in the ischemic cardiomyocytes

To investigate the effect of cardiac I/R injury on the expression of Bcl-2 and MAPK3, we made heart lysates, extracted RNA, and protein by technique mentioned above. Using qRT-PCR and western blot, we found that mRNA of Bcl-2 and MAPK3 in the I/R group did not change significantly compared with the sham group (figure 2A), whereas both protein levels in the I/R group decreased compared with the sham group (figure 2B).

Hypoxia/reoxygenation induced miRNA-15b expression in cultured H9c2 cells

To consolidate our findings in the rat cardiac I/R injury model, we further tested the impact of hypoxia/reoxygenation on the expression of miRNA-15b in cultured H9c2 cells. Cells were cultured under hypoxia conditioning for 6, 18 or 24 hours followed by reoxygenation. Cells were collected for RNA extraction at 24 hours after reoxygenation. As shown in online supplementary figure 2A,B, 6, 18 and 24 hours exposure of H9c2 cells to hypoxia followed by 24 hours reoxygenation resulted in significantly increased expression of miRNA-15b. The 18-hour hypoxia/24-hour reoxygenation treated H9c2 exhibited highest miRNA-15b expression level. Therefore, we chose 18-hour hypoxia/24-hour reoxygenation in our following experiments.

Hypoxia/reoxygenation leads to apoptosis in H9c2 cells

Next, we tested the impact of hypoxia/reoxygenation on the cell apoptosis in H9c2 cells with TUNEL staining. As shown in online supplementary figure 2C,D, hours of hypoxia resulted about 10% of cell apoptosis, while 18-hour hypoxia/24-hour reoxygenation resulted in up to 50% apoptosis.

MiRNA-15b plays an important role in the regulation of hypoxia/reoxygenation-induced cardiomyocytes apoptosis

In order to investigate the role of miRNA-15b in the regulation of hypoxia/reoxygenation-induced cardiomyocytes apoptosis, both gain-of-function and loss-of-function approaches were applied to regulate miRNA-15b in cultured H9c2 cells. First, we tested the regulation effect of miRNA-15b mimic and inhibitor on cell apoptosis in the cultured H9c2 cells. As shown in figure 3A, B, miRNA-15b mimic increased but miRNA-15b inhibitor decreased miRNA-15b relative expression after 18-hour hypoxia followed by 24-hour reoxygenation in H9c2 cells. The results suggested that chemically synthesized miRNA-15b mimic and inhibitor were successfully transfected into cardiomyocytes after co-cultured for 18 hours.

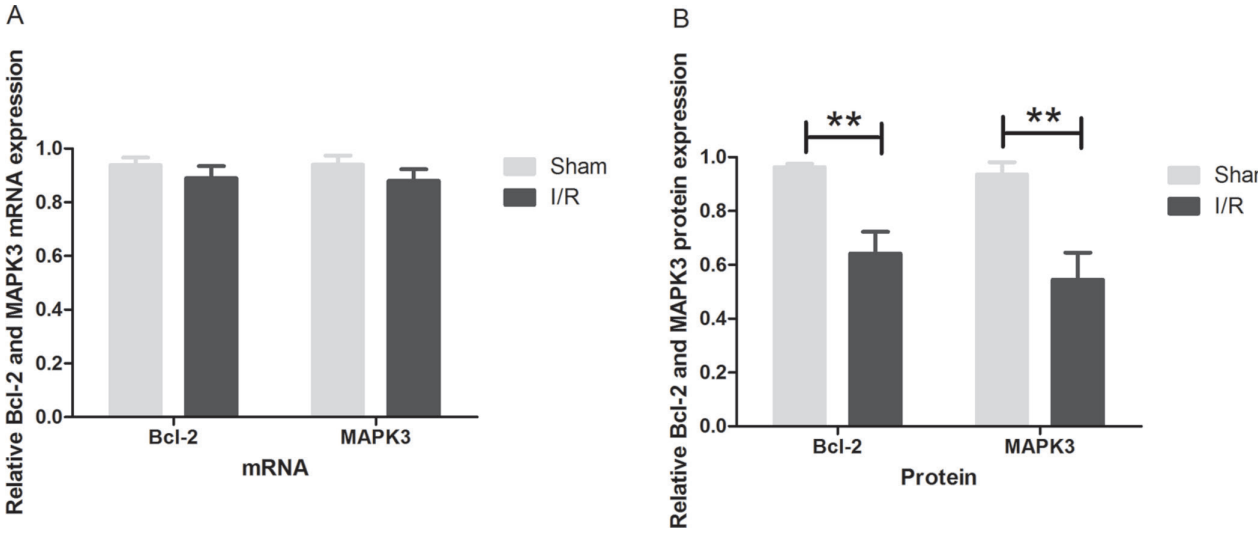

Figure 2 LAD ischemia/reperfusion (I/R) induced B cell lymphoma/leukemia-2 (Bcl-2) and mitogen-activated protein kinase 3 (MAPK3) mRNA and protein expression. (**A**) mRNA expression of Bcl-2 and MAPK3 in the I/R group did not change significantly compared with the sham group. (**B**) Protein expression of Bcl-2 and MAPK3 in the I/R group decreased in the I/R group compared with the sham group. **p<0.01.

The role of miRNA-15b on the regulation of hypoxia/reoxygenation-induced cardiomyocytes apoptosis

As shown in figure 3C, D, after cultured with miRNA-15b mimic and inhibitor under 18-hour hypoxia followed by 24-hour reoxygenation, miRNA-15b mimic increased hypoxia/reoxygenation-induced cardiomyocyte apoptosis as determined by TUNEL staining. However, cardiomyocyte apoptosis was reversed after treating with miRNA-15b inhibitor. The results suggest that miRNA-15b plays an important role in the hypoxia/reoxygenation-induced cardiomyocyte apoptosis.

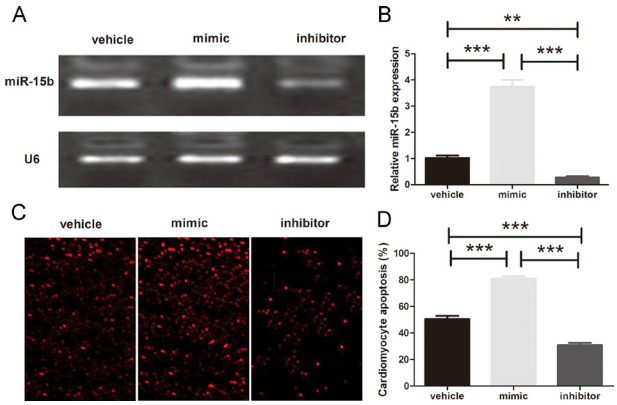

Figure 3 MiRNA-15b plays a role in in the regulation of hypoxia/reoxygenation-induced cardiomyocytes apoptosis. Inhibitors or mimics of miRNA-15b were transfected into H9c2 cells. (**A**) Representative quantitative reverse transcription PCR images of the RNA prepared from vehicle, miRNA-15b mimic or inhibitor transfected H92c cells. (**B**) Quantification expression of miRNA-15b after transfection. (**C**) Representative images of Terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) staining of H92c cells transfected with miRNA-15b mimic or inhibitor. (**D**) Quantification of TUNEL+ cells in the H92c cells. n=8/group, **p<0.01; ***p<0.001.

Post-transcriptional modulation of miRNA-15b on Bcl-2 and MAPK3

It was demonstrated that miRNA-15b had pro-apoptotic effect on cardiomyocytes.[15 16] The mechanism possibly involved the binding of miRNA-15b with the 3′ untranslated region (UTR) of the target genes, thus providing regulation of the apoptotic factors. In order to find out the post-translational target of miRNA-15b in IR-induced cardiomyocyte apoptosis, we used a computational and bioinformatics-based approach to predict the putative target related to apoptosis. According to TargetScan algorithms, we identified candidate targets of miRNA-15b as Bcl-2 and MAPK3. There are target sites for miRNA-15b at 1103–1109 of Bcl-2 3′ UTR and 503–509 of MAPK3 3 ′UTR. The specific binding sites and parameters are shown in figure 4.

To confirm this assumption, we transfected cardiomyocytes with vehicle, miRNA-15b mimic or miRNA-15b inhibitor, then incubated with 18-hour hypoxia and 24-hour reoxygenation. The mRNAs of Bcl-2 and MAPK3 were determined by qRT-PCR and the protein expression were further measured by western blot. As shown in figure 5A, B, the mRNAs of Bcl-2 and MAPK3 were not changed statistically compared with the vehicle, while the proteins were inversely regulated by synthesized miRNA-15b (figure 5C, D). These results suggest that

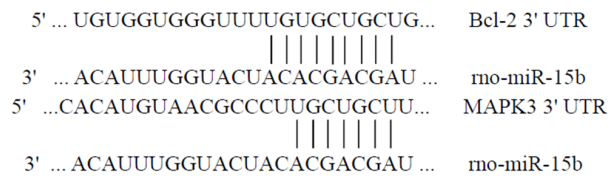

Figure 4 MiRNA-15b binding sites in B cell lymphoma/leukemia-2 (Bcl-2) and mitogen-activated protein kinase 3 (MAPK3).

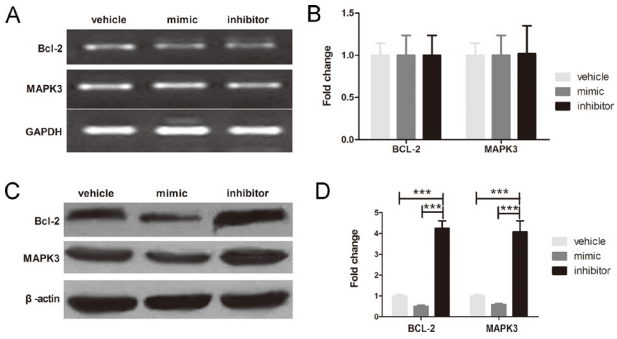

Figure 5 Post-transcriptional modulation of miRNA-15b on B cell lymphoma/leukemia-2 (Bcl-2) and mitogen-activated protein kinase 3 (MAPK3). Cultured rat cardiomyocytes pretreated with vehicle, miRNA-15b mimic and inhibitor were subjected to hypoxia for 18 hours, cells were collected at 24 hours after reoxygenation. (**A**) Representative quantitative reverse transcription PCR image of mRNA level of Bcl-2 and MAPK3. (**B**) Quantification of (A). (**C**) Representative image of western blot of Bcl-2 and MAPK3. (**D**) Quantification of (C). n=8/group. ***p<0.001.

miRNA-15b inversely modulates Bcl-2 and MAPK3 expression post-transcriptionally without affecting their mRNA expression.

DISCUSSION

The present study demonstrated that I/R injury or hypoxia/reoxygenation induced increased expression of miRNA-15b in rat myocardial cells or H9c2 cells, which was accompanied by robust apoptosis in these cells. Using mimic and inhibitor approaches, we further showed that increased miRNA-15b deteriorated IR or hypoxia/reoxygenation-induced cardiomyocyte apoptosis via downregulating the target antiapoptotic genes Bcl-2 and MAPK3. Our results suggest that miRNA-15b post-translationally downregulates target genes without modulating their mRNA levels thus exacerbates cardiomyocyte apoptosis following AMI.

Cardiomyocyte apoptosis takes place and lasts for several weeks after AMI.[19] In recent years, laboratory experiments lead to a better understanding of the important role of cell apoptosis in the regulation of cardiomyocyte death in vivo and in vitro.[19] MiRNAs, a group of small non-coding RNAs, are identified as post-transcriptional inhibitors of gene expression. They are thought to 'fine tune' the translational output of target mRNAs.[8] MiRNAs are associated with target mRNAs and act as negative regulators of gene expression by promoting mRNA degradation or inhibiting translation. Notably, several studies support that miRNAs play pivotal roles in AMI, I/R or H_2O_2-induced hypoxia followed by cardiomyocyte apoptosis.[10–12]

Among the known miRNAs, miRNA-15b is believed to be specifically expressed in adult cardiac and skeletal muscle tissues and dominantly concentrates in cardiomyocytes in rats. MiRNA-15b coexists with other family members such as miRNA-16-1, miRNA-16-2, miRNA-195, and miRNA-497. Many different heart diseases induced high expression of miRNA-15b.[8] Hullinger *et al* reported that in mice and pigs miRNA-15 expression increased in the part of infarcted heart area as a result of IRI.[16] In other studies, miRNA-15b-induced apoptosis in non-cardiac cells and the popular target gene of miRNA-15b is Bcl-2.[13 14] Consistent with a recent study,[17] we demonstrated that miRNA-15b was upregulated in response to cardiac I/R injury both in vivo and in vitro. In addition, we also observed significantly increased apoptosis in I/R-challenged cardiomyocytes or hypoxia/reoxygenation-challenged H9c2 cells. As shown in figure 2A, B, the expression of miRNA-15b was upregulated in border areas of the infarcted myocardium at 30 min after coronary ligation in adult rat hearts. Chemical mimics of miRNA-15b increased hypoxia/reoxygenation-induced cardiomyocyte apoptosis while antagomir attenuated cardiomyocytes apoptosis. These results suggest that miRNA-15b plays an important role in cardiomyocyte apoptosis following acute myocardial IRI.

To further elucidate how miRNA-15b regulates the cardiomyocyte apoptosis, we use computational algorithms to predict the target genes of miRNA-15b and identified Bcl-2 and MAPK3 as the potential target of miRNA-15b. To confirm this prediction, we conducted both miRNA mimic and inhibitor transfection experiments and found that miRNA-15b has negative correlation to its target gene Bcl-2. The putative miRNA-15b binding sequence interacted with the 3′ UTR of Bcl-2 mRNA and downregulated the protein expression of Bcl-2 without decreasing Bcl-2 mRNA level. Consistently, Guo *et al* found a similar impact of miRNA-15b on hepatic stellate cell apoptosis.[14] This evidence suggests that miRNA-15b is able to post-transcriptionally regulate Bcl-2 expression.

MAPK3 is predicted as another target gene of miRNA-15b by the TargetScan system. The MAPK family has three members: MAPK8 (JNK), MAPK14 (p-38), and MAPK3/MAPK1 (ERK1/2).[20] JNK signaling cascade activates inflammation and apoptosis, whereas ERK pathway mediates cellular survival and growth.[21 22] Several studies reported that ERK activation conferred cardioprotection.[23–25] However, there is also evidence showing that ERK activation plays a role in cardiomyocyte loss. For instance, ERK1/2 activation leads to increased apoptosis and loss of rat cardiomyocytes after hypoxia/reoxygenation or doxorubicin-induced cardiomyocyte injury,[26] while mitigation of elevated ERK cascade restores cell viability.[27 28] MAPK3 was also demonstrated to play a protective role in apoptosis.[29] In accordance, our study suggests that MAPK3 has close relationship with cardiomyocyte apoptosis in IRI.

We also reviewed other target genes of miRNA-15b. In human osteoblastic cells, cyclin E1 was verified to be one of the putative target genes.[30] Xu *et al*[31] identified the potent oncoprotein yes-associated protein as a downstream target of miRNA-15b in contractile vascular smooth muscle cells. The study conducted by Sun *et al*[32] suggested that miRNA-15b regulated cell growth and invasion by modification of target gene cripto-1 expression in glioma cells. In a very recent study, miRNA-15b was manifested to induce apoptosis of hepatoma SMCC7721 cells via downregulation of newly discovered pro-apoptotic protein XIAP.[33] Nevertheless, there was no above target genes found in cardiomyocytes.

In summary, the current study reveals that the miRNA-15b in cardiomyocytes responds promptly to cardiomyocyte I/R injury and hypoxia/reoxygenation insult. Increased miRNA-15b deteriorates cardiomyocytes apoptosis by

suppressing its target gene Bcl-2 and MAPK3 post-transcriptionally.

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Contributors YL contributed to conduct the study, write the draft and approve the final version of the manuscript. LY contributed to construct the AMI rat model. JY contributed to cell culture and hypoxia/reoxygenation treatment. DS contributed to TUNEL staining. ZP contributed to RNA isolation and qRT-PCR. PL contributed to the conception of the work, statistics analysis and western blot procedure. XW contributed to the conception of the work and agreement of all aspects of the work. All authors reviewed the manuscript.

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