MicroRNA-138 attenuates myocardial ischemia reperfusion injury through inhibiting mitochondria-mediated apoptosis by targeting HIF1-α

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Abstract. Myocardial ischemia-reperfusion (I/R) injury is considered to have a detrimental role in coronary heart disease, which is considered to be the leading cause of death worldwide. However, the molecular mechanism involved in the progression of myocardial I/R injury is still unclear. The current study aimed to investigate the expression and function of microRNA (miR)-138 in the process of myocardial I/R injury. First, miR-138 expression levels were analyzed both in myocardium with I/R injury and control myocardium using reverse transcription-quantitative polymerase chain reaction analysis. Then, the relationship between the levels of miR-138 and hypoxia-inducible factor (HIF)1-α was also investigated using a luciferase reporter assay. Assessment of myocardial infarct size, measurements of serum myocardial enzymes and electron microscopy analysis were all utilized to analyse the effect of miR-138 on myocardial I/R injury. The authors of current study also used western blotting to examine the expression levels of the mitochondrial fission-related proteins dynamin-1-like protein and mitochondrial fission 1 protein. It was found that miR-138 is downregulated and HIF1-α is upregulated after myocardial ischemia reperfusion injury. Overexpression of miR-138 reduced myocardial I/R injury-induced infarct sizes and myocardial enzyme levels, and it also inhibited the expression of proteins related to mitochondrial morphology and myocardial I/R-induced mitochondrial apoptosis by targeting HIF1-α. Taken together, these findings provide a novel insight into the molecular mechanism of miR-138 and HIF1-α in the progression of myocardial I/R injury. miR-138 has the potential to become a promising therapeutic target for treating myocardial I/R injury.

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Introduction

Myocardial ischemia-reperfusion (I/R) injury is considered to have a detrimental role in coronary heart disease (CHD), which is considered to be the leading cause of death worldwide (1-3). Myocardial infarction is caused by coronary occlusion and the subsequent insufficient supply of blood to the myocardium, which may then cause irreversible necrosis to occur (4,5). Restoring blood flow to the ischemic myocardium is the most effective treatment to rescue ischemic myocardium cells and to save the life of the patient (6). However, this treatment can cause an abrupt restoration of the oxygen supply and the reperfusion of myocardium may aggravate myocardial injury, leading to a reperfusion injury (7,8). Therefore, it is necessary to explore and to better understand the potential molecular mechanism of myocardial I/R injury.

MicroRNAs (miRNAs or miRs) are small, single-stranded, non-coding RNAs (20-22 nucleotides) that are involved in numerous biological processes (9). miRNAs participate in many biological processes, such as cell apoptosis, cell differentiation and cell development (10). More and more evidence has indicated that several miRNAs are expressed abnormally during myocardial I/R injury, suggesting the involvement of miRNAs in myocardial I/R injury development (11-13). Furthermore, the inhibition of mitochondria-mediated apoptosis to reduce cardiomyocyte apoptosis is considered to be an important mechanism (14,15). However, little evidence is available regarding the role of miRNAs and mitochondria-mediated apoptosis in myocardial I/R injury.

Hypoxia-inducible factor (HIF) 1 is known as a heterodimeric transcription factor composed of an oxygen-labile α subunit (HIF1-α) and a constitutive β subunit (HIF1-β), and HIF1 can bind to the hypoxia response element to regulate gene expression (16,17). Furthermore, HIF1-α is the regulatory subunit that senses tissue oxygen level, responds to various types of cellular stimulation and exerts a vast array of physiological functions, enabling cells to adapt to temporary hypoxia (18). Some studies found that HIF1-α activity showed some effects in preventing diabetic cardiomyopathy

and cardiac remodeling (19,20). However, the role of HIF1-α in myocardial I/R injury is incompletely understood.

The aim of the current study was to investigate the effect of miRNAs and mitochondria-mediated apoptosis in a myocardial I/R injury model. In the current study, the authors uncovered the pivotal role of miR-138 in the myocardial I/R injury model, which may be connected with the inhibition of myocardial I/R-induced mitochondrial apoptosis.

Materials and methods

Ethical statement. The experiments involving animals conform to local and national Guide for the Care and Use of Laboratory Animal guidelines. Furthermore, these experimental methods are also approved by the Clinical Ethics Committee of Affiliated Hospital of Weifang Medical University (Shandong, China).

Mouse model of myocardial I/R Injury. A total of 60 mice (30 males and 30 females; age, 8-10 weeks; weight, 18-25 g) were obtained from the Animal Center of Weifang Medical University (Weifang, China). Animals were housed at a temperature of 23-25˚C and a relative humdity of 40-60%, under a 14/10 h light/dark cycle and free access to water and food. Housing conditions also included a light intensity of ~40 lux at the position of the animal in cage. Mice were divided into five groups (each, n=12): A sham group (mice without injury), a control group (I/R injury with miR-138 negative control injection), an I/R injury group, a miR-138 mimic group (I/R injury with miR-138 mimic injection) and a miR-138 inhibitor group (I/R injury with miR-138 inhibitor injection). The mouse model of myocardial I/R injury was established as previously described (21). In short, pentobarbital (50 mg/kg) was used to anesthetize the mice. Then, a left horizontal incision was made at the third intercostal space. Subsequently, a silk suture we used to tie around the left anterior descending artery and a silicon tube (1 mm outside diameter). In the sham group, the mouse only received the identical surgical procedure without ligature. The silicon tube was then removed to achieve reperfusion for 6 h after 30 min of ischemia. After reperfusion, the heart samples were collected as quickly as possible. For some experiments, miR-138 mimic (20 nmol/l), miR-138 inhibitor (20 nmol/l), HIF1-α siRNA (100 nmol/l) or miR-138 negative control (20 nmol/l) were administered intraperitoneally for seven days before modeling. miR-138 mimic, miR-138 inhibitor, HIF1-α siRNA were all purchased from Genscript (Piscataway, NJ, USA). The miR-138 mimic sequence was 5'-AGCUGGUGUUGUGAAUCAGGCCG-3', the miR-138 inhibitor sequence was 5'-CGGCCUGAUUCACAACACCAG CU-3', the HIF1-α siRNA sequence was 5'-AUCCAGAGU CACUGGAACU-3' and the NC sequence was 5'-UGAAUC CUUGAAUAGGUGUGUU-3'. Carbon dioxide was used to euthanize the mice with a fill rate of 10-30% of the volume of the cage per minute. The authors of the current study followed the American Veterinary Medical Association guidelines' recommendations to confirm the death of the mice, including heartbeat, breathing, corneal reflex and responses to firm toe pinch, graying of the mucous membranes and rigor mortis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RNA from heart samples from each group was extracted using the TRIzol® reagent (Invitrogen). Subsequently, the RNA was reverse transcribed into cDNA using the cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and we perform RT-qPCR with SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.). GAPDH was used as the internal normalized reference. The relative levels of miR-138 and HIF1-α were calculated using the $2^{-\Delta\Delta Cq}$ method (22). The thermocycing conditions were as follows: 45˚C for 10 min and one cycle of 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 45 sec. The primers sequences were as follows: miR-138: Forward, 5'-TCCGAGCCTGACTAAGTG TTGTGGTCGA-3' and reverse, 5'-GTGCAGGGTCCGAGG T-3'; GAPDH: Forward, 5'-TGGTATCGTGGAAGGACTC-3' and reverse 5'-AGTAGAGGCAGGGATGATG-3'; HIF1-α: Forward, 5'-CTCAGCCCCAGTGCATTGTA-3' and reverse 5'-GAACCTCCTATAGCCACCGC-3'.

Western blot analysis. Myocardial tissues from each group were lysed using radioimmunoprecipitation assay buffer. Subsequently, a bicinchoninic acid protein assay to detect the concentration of protein. Protein lysates (20 μl) were then separated using 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were then blocked with 5% skim milk in TBST for 1 h at room temperature. The membranes were incubated with the following antibodies obtained from Abcam (Cambridge, UK): HIF1-α (1:2,000; cat. no. ab51608), cleaved caspase-9 (1:2,000; cat. no. ab2324), cleaved caspase-3 (1:2,000; cat. no. ab2302), Drp1 (1:2,000; cat. no. ab184247), Fis1 (1:2,000; cat. no. ab71498) and GAPDH (1:5,000; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. Then, they were washed using TBST and incubated with secondary antibodies (horseradish peroxidase conjugated Goat Anti-Rabbit IgG; 1:5,000; cat. no. ab205718, Abcam) at 4˚C for 1 h. ECL Western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.) was used to visualize and detect the results. ImageJ software 1.43v (National Institutes of Health, Bethesda, MD, USA) was used to quantify protein levels.

Assessment of myocardial infarct size. After sacrificing the mice in each group, evans blue dye was used to demarcate the ischemic area-at-risk. Subsequently, heart tissues were excised and sliced (50 μm). These samples were stained with 1% triphenyltetrazolium chloride at 37˚C for 20 min and then fixed using 4% paraformaldehyde for 8 h at 4˚C. Infarcted myocardium was separated from the non-infarcted myocardium and weighed carefully. Heart tissue from each group were dissected and weighed. The infarct size was presented as a percentage of the total ischemic area.

Measurement of serum myocardial enzymes. After 6 h of reperfusion, blood samples from each group were collected, centrifuged at 2,000 x g for 30 min at 4˚C and transferred to Eppendorf tubes. Subsequently, ELISA was performed to measure the serum levels of troponin I (cat. no. BEK-2212-1P/2P; Biosensis), cardiac muscle (cTn I; cat. no. EKC40439, R&D Systems Inc.) and creatine kinase M-type/B-type (CK-MB; cat. no. ABIN415661; R&D Systems Inc.) according to the manufacturer's protocol.

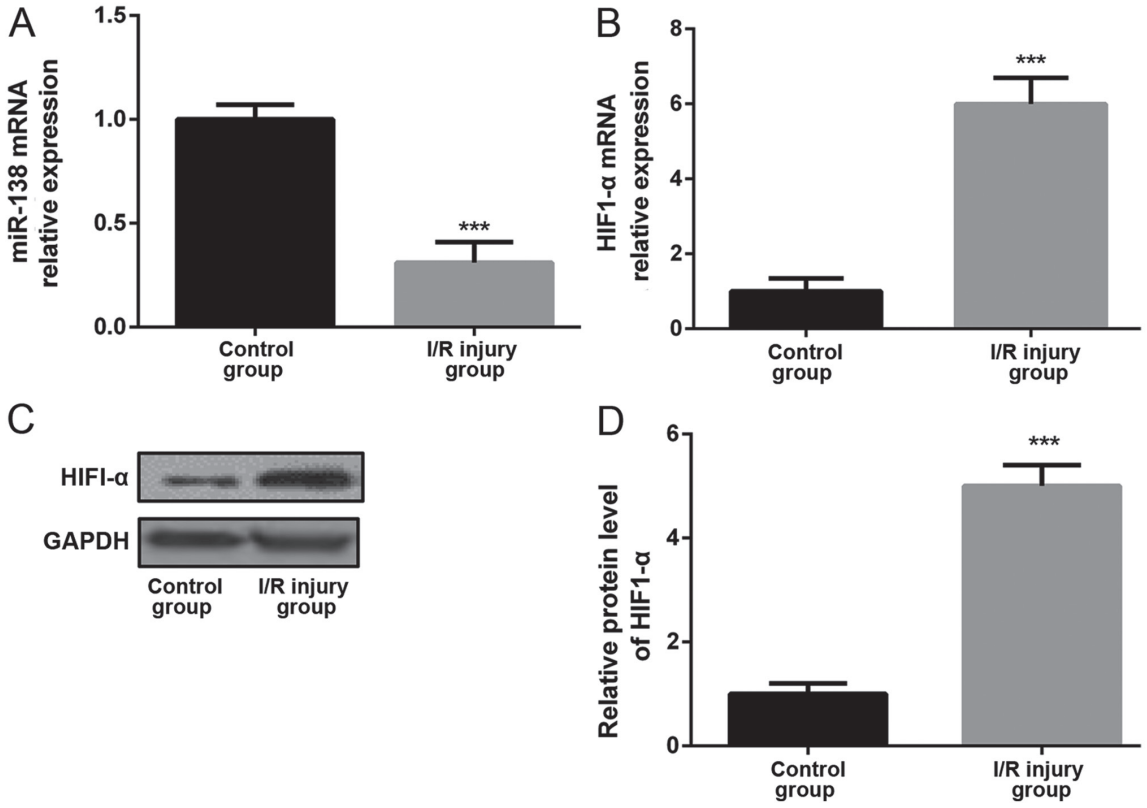

Figure 1. Expression of miR-138 and HIF1-α in the myocardium with I/R injury. (A) miR-138 mRNA expression measured by RT-qPCR. (B) HIF1-α mRNA expression measured by RT-qPCR. (C) Western blotting and (D) densitometric quantification of proteins. (***P<0.001). HIF1-α, hypoxia-inducible factor 1-α; miR, microRNA; I/R, ischemia-reperfusion; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Electron microscopy analysis. The method of electron microscopy analysis was described as previously (23). The electron microscopy images of mitochondria were analyzed using PhotoshopCS5.0 software (Adobe, Inc.). For the analysis, the number of the myocardial mitochondria in each group were measured.

Luciferase reporter assay. TargetScan (www.targetscan.org/) was used to predict the target gene of miR-138. Following, a wild-type (WT) 3'-untranslated region (UTR) fragment of HIF1-α containing the putative miR-138 binding sequence was inserted into a pmirGlO Dual-luciferase miRNA Target Expression Vector (Promega Corporation, Madison, WI, USA), while mutant (MUT) 3'-UTR was also cloned into the vector to generate a mutated binding site. Subsequently, the cells obtained from the American Type Culture Collection were co-transfected with HIF1-α-WT or HIF1-α-MUT and miR-138 mimics (20 nmol/l) using Lipofectamine™ 2000 (Thermo Fisher Scientific, Inc.). Dual Luciferase reporter assay system (DLR® Assay, Promega Corporation) was used to evaluate the luciferase activity after 48 h. Renilla Luciferase was used as a normalizing transfection control.

Statistical analysis. GraphPad Prism 4 software was used to analyze the experimental data. The data were presented as mean ± standard deviation. Statistical analyses were performed using Student's t-test and one-way analysis of variance, followed by a Tukey's post-hoc test. P<0.05 indicated that the difference between groups was statistically significant.

Results

miR-138 is downregulated and HIF1-α is upregulated after myocardial I/R injury. To identify whether miR-138 performed important functions in myocardial I/R injury, its expression level in adult mouse myocardium with and without I/R injury was examined. The results indicated that the miR-138 expression level was significantly downregulated in the myocardium of the myocardial I/R injury model compared with the control myocardium (Fig. 1A). Moreover, the expression level of HIF1-α was significantly upregulated in the myocardium of the myocardial I/R injury model compared with the control myocardium (Fig. 1B). The western blotting results also confirmed the alteration of HIF1-α expression level as HIF1-α protein expression significantly upregulated in the myocardium of the I/R injury group compared with the control group (Fig. 1C and D).

Overexpression of miR-138 reduces the myocardial I/R-induced increase in infarct size and myocardial enzymes. As mentioned previously, compared with the control group, the miR-138 expression level was significantly decreased in the myocardial I/R injury group. Subsequently, to further explore the role of miR-138 in the myocardial I/R injury model, miR-138 mimic, miR-138 inhibitor or a negative control were administered intraperitoneally before modeling. Compared with the I/R injury group, the infarct size of, and serum CTn I and CK-MB levels in the myocardium with I/R injury were significantly decreased in the miR-138 mimic group and increased in the miR-138 inhibitor group (Fig. 2).

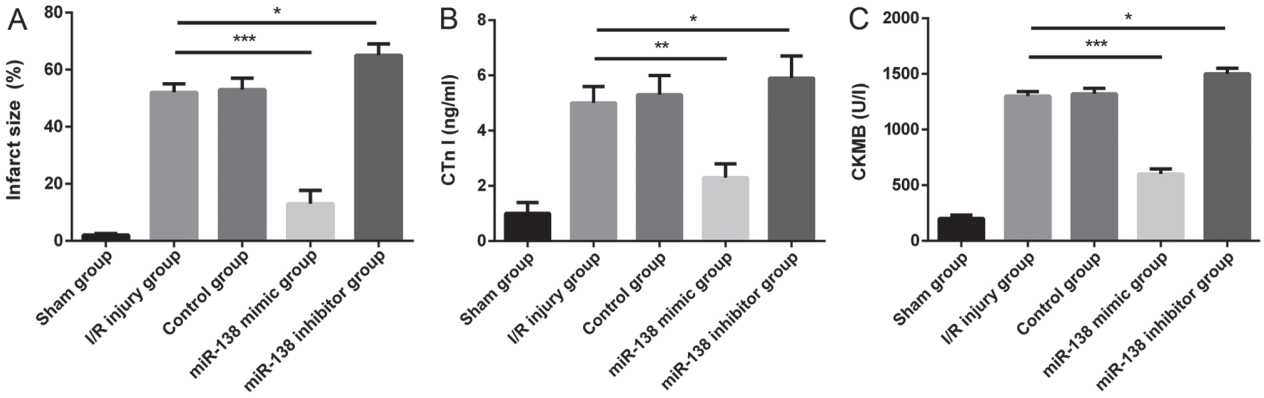

Figure 2. miR-138 influences the infarct size and myocardial enzymes in the myocardium with I/R injury. Effect of miR-138 on (A) infarct size, (B) cTn I concentration and (C) CK-MB activity. (*P<0.05, **P<0.01, ***P<0.001). miR, microRNA; I/R, ischemia-reperfusion; cTn I, Troponin I, cardiac muscle; CK-MB, creatine kinase M-type/B-type.

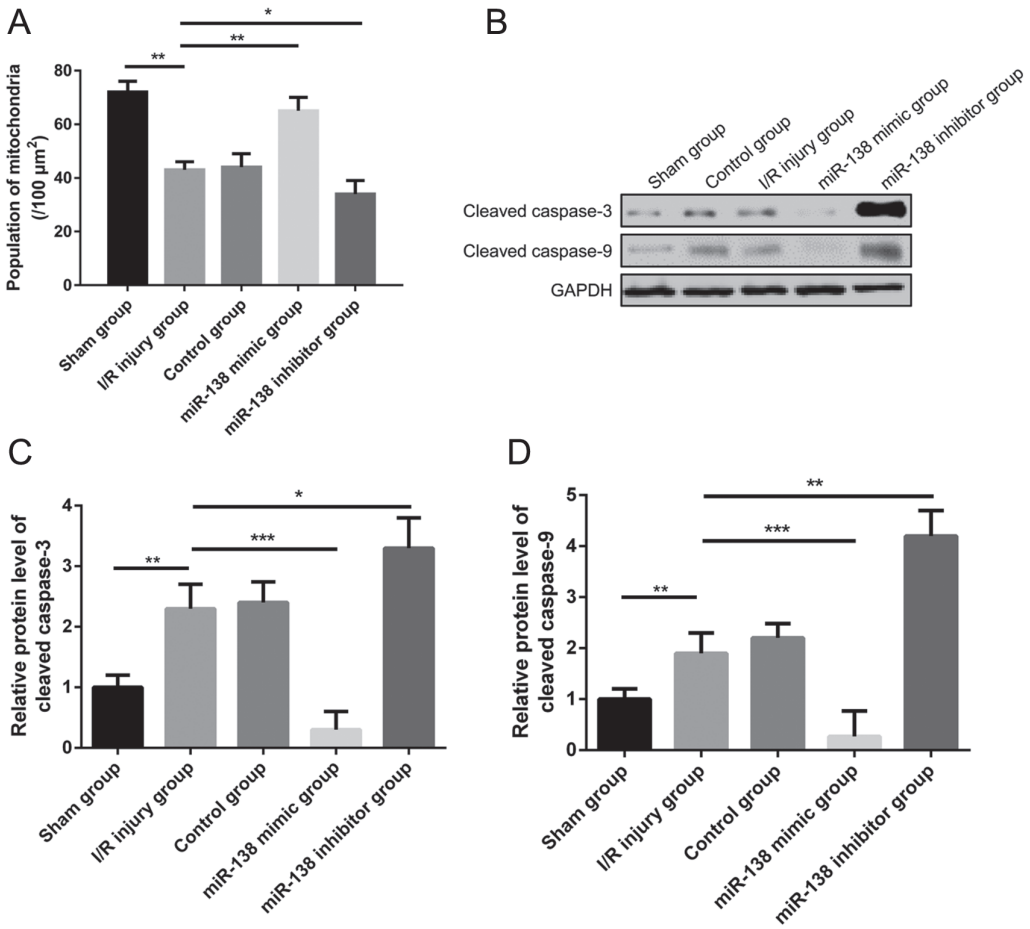

Figure 3. miR-138 inhibited the expression of cleaved caspase-9 and -3 in myocardial I/R injury. Effect of miR-138 on (A) the number of mitochondria and (B) the expression of cleaved caspase-3 and caspase-9 in the myocardium. Densitometric quantification of (C) caspase-3 and (D) caspase-9 in the myocardium. (*P<0.05, **P<0.01, ***P<0.001). miR, microRNA; I/R, ischemia-reperfusion.

miR-138 inhibits the expression of cleaved caspase-9 and -3 in myocardial I/R injury. The number of the mitochondria in the myocardial I/R injury group per area was significantly decreased compared with the sham group. However, in the miR-138 mimic group, the number of mitochondria per area were increased significantly, while in the miR-138 inhibitor group, the number of mitochondria was significantly reduced compared with the I/R injury group (Fig. 3A). Notably, the results of the western blot analysis indicated that the expression levels of cleaved caspase-9 and -3 were significantly increased in the myocardial I/R injury group compared with sham mice, and after the injection of the miR-138 mimic, expression levels were decreased compared with the I/R injury group (Fig. 3B-D). In the miR-138 inhibitor group, cleaved caspase-9

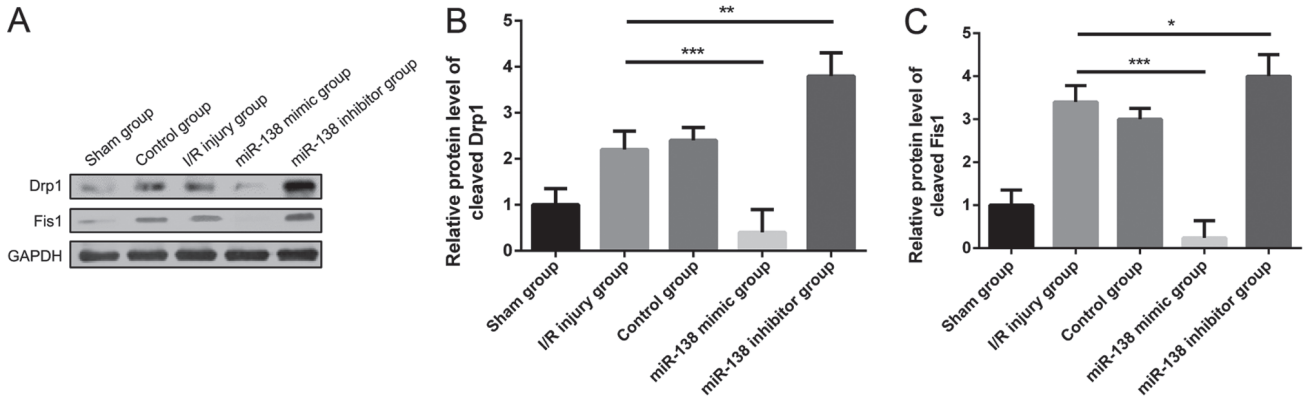

Figure 4. miR-138 inhibited the expression of proteins related to mitochondrial morphology (A) Effect of miR-138 on the expression of Drp1 and Fis1 in the myocardium. Densitometric quantification of (B) Drp1 and (C) Fis1 in the myocardium. (*P<0.05, **P<0.01, ***P<0.001). miR, microRNA; Drp1, dynamin-1-like protein; Fis1, mitochondrial fission 1 protein.

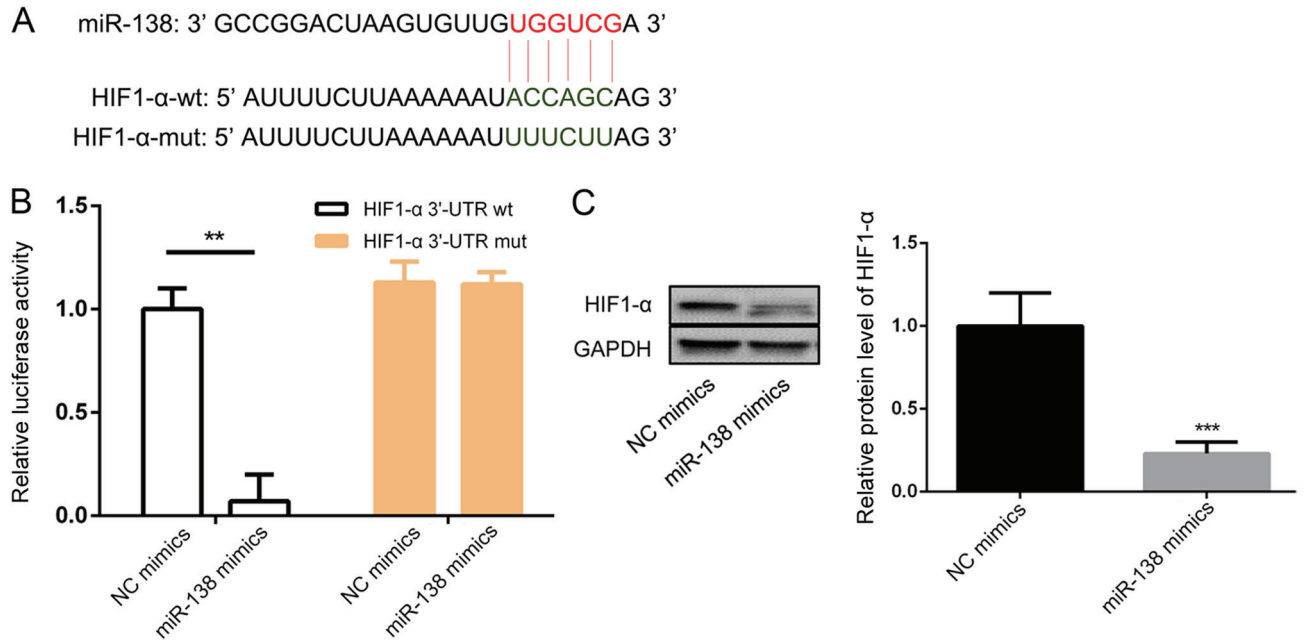

Figure 5. miR-138 directly targeted the 3'-UTR of HIF1-α mRNA. (A) Schematic representation of the mature miR-138 sequence, putative miR-138 target site in the 3'-UTR of HIF1-α mRNA. (B) The relative luciferase activity in the WT 3'-UTR of HIF1-α mRNA and MUT 3'-UTR of HIF1-α after miR-138 overexpression. (C) HIF1-α protein expression after miR-138 mimic transfection. (**P<0.01, ***P<0.001). miR, microRNA; UTR, untranslated region; HIF1-α, hypoxia-inducible factor 1-α; WT, wild-type; MUT, mutated.

and -3 expression levels were significantly increased reduced compared with the I/R injury group.

miR-138 inhibits the expression of proteins related to mitochondrial morphology. To explore the mechanism of miR-138 in relation to morphological alterations of mitochondria in myocardial I/R injury, the expression levels of the mitochondrial fission-related proteins, Drp1 and Fis1 (24,25), were examined using western blot analysis. The results indicated that the expression levels of Drp1 and Fis1 in the myocardial I/R injury group were both increased compared with the sham group (Fig. 4). When treated with miR-138 mimic, however, Drp1 and Fis1 expression levels were significantly decreased, while their expression levels were both increased in the miR-138 inhibitor group compared with the I/R injury group.

HIF1-α is the target of miR-138. The bioinformatic results were confirmed by a luciferase reporter assay. Fig. 5A shows the predicted miR-138 binding sequence in HIF1-α. The luciferase activity of the construct with the WT 3'-UTR was significantly inhibited after transfection with the miR-138 mimic (Fig. 5B). Western blotting results indicated that miR-138 mimic transfection markedly decreased the expression of HIF1-α compared with NC mimic transfection (Fig. 5C).

Downregulation of HIF1-α inhibits the expression of cleaved caspase-9 and caspase-3 in myocardial I/R injury. To explore the role of HIF1-α in the model of myocardial I/R injury, siRNA of HIF1-α were administered intraperitoneally before modeling. HIF1-α siRNA was shown to significantly decrease the expression of HIF1-α in the myocardium compared with the control group (Fig. 6A). The number of mitochondria per

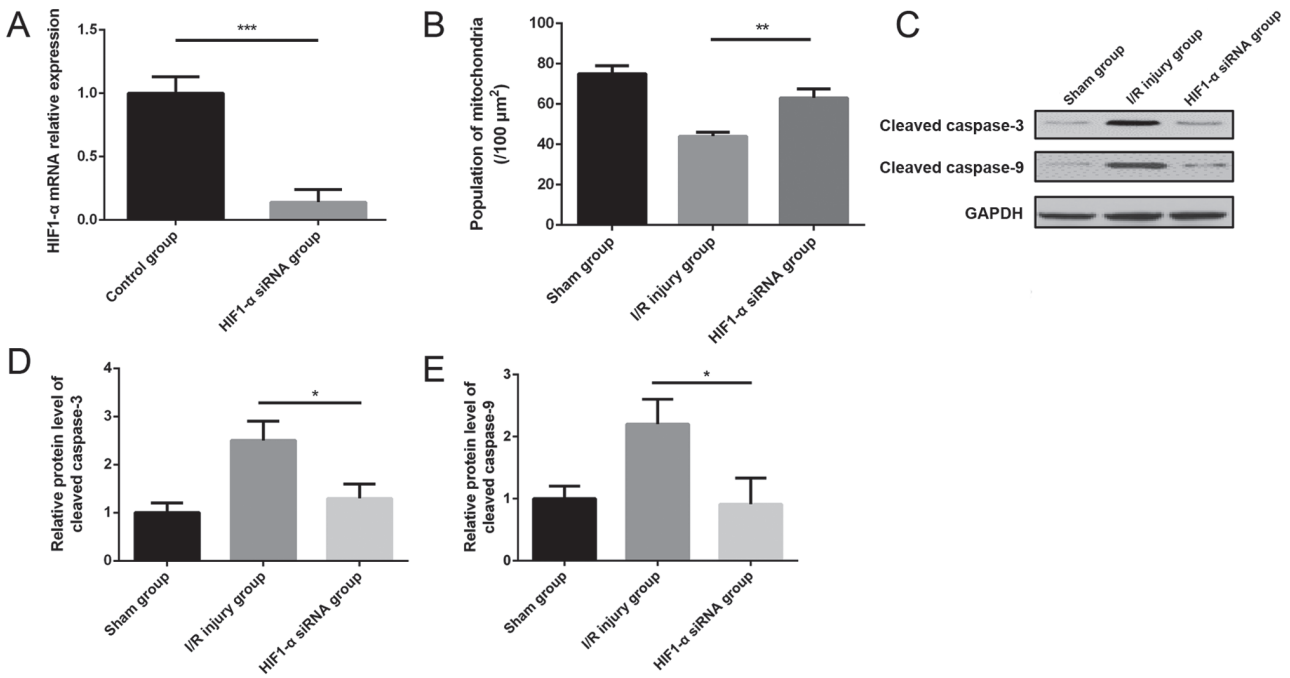

Figure 6. Downregulation of HIF1-α inhibited myocardial I/R-induced mitochondrial apoptosis. (A) HIF1-α mRNA expression measured by RT-qPCR. (B) Effect of HIF1-α siRNA on the number of mitochondria and (C) the expression of cleaved caspase-3 and caspase-9 in the myocardium. Densitometric quantification of (D) caspase-3 and (E) caspase-9 in the myocardium. (*P<0.05, **P<0.01, ***P<0.001). miR, microRNA; siRNA, small interfering RNA; I/R, ischemia-reperfusion; HIF1-α, hypoxia-inducible factor 1-α; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

area was increased significantly in the mouse myocardium of the HIF1-α siRNA group compared with the I/R injury group (Fig. 6B). Notably, western blotting results indicated that the protein expression levels of cleaved caspase-9 and -3 were decreased significantly in the myocardium after HIF1-α siRNA administration compared with the I/R injury group (Fig. 6C-E).

Discussion

CHD is considered to be the leading cause of death worldwide, with approximately 17.5 million people dying because of cardiovascular disease according to the estimates from the World Health Organization (26-28). Acute myocardial I/R usually causes the detrimental effects of CHD (29). Therefore, to explore the molecular mechanism involved in myocardial I/R injury, the authors of the current study conducted functional experiments and identified the critical role of miR-138 and mitochondria-mediated apoptosis in the myocardial I/R injury model. The results indicated that low expression levels of miR-138 were found in the myocardium with I/R injury compared with that of control myocardium. Furthermore, the level of HIF1-α was significantly upregulated in the myocardium of the myocardial I/R injury model compared with control myocardium. Overexpression of miR-138 reduced the myocardial I/R-induced increase in infarct size and myocardial enzymes by targeting HIF1-α to inhibit myocardial I/R-induced mitochondrial apoptosis. These findings suggest that miR-138 may prevent damage of the myocardium after I/R injury.

miRNAs are a type of small non-coding RNAs that bind to their target mRNAs specifically, and subsequently cause the downregulation of the target gene by repressing degradation or translation (30). In recent years, miRNAs have been implicated in many processes related to the heart, such as cardiac hypertrophy, cardiac development and heart failure (31). miRNAs function as endogenous intracellular regulators of mRNA translation, but the significance of miR-138 in the process of myocardial I/R injury has not been reported previously, particularly its role in myocardial I/R-induced mitochondrial apoptosis. In the current study, the results indicated that miR-138 was downregulated in myocardium with I/R injury compared with control myocardium. The results also showed that the infarct size and serum CTn I and CK-MB levels of the myocardium with I/R injury were significantly decreased in the miR-138 mimic group, but were significantly increased in the miR-138 inhibitor group compared with the control group.

Apoptosis is known as a critical pathological process in the course of myocardial I/R injury and the amount of apoptosis determines the severity of myocardial I/R injury (32,33). Therefore, understanding the mechanism involved in cardiomyocyte apoptosis in myocardial I/R injury is critically important in the development of effective treatment methods. Prior research has shown that myocardial I/R-induced mitochondrial dysfunction and apoptosis are responsible for the exacerbation of cardiac ischemic injury in diabetic patients (15). In the present study, the authors found that in the miR-138 mimic group, the mitochondria per area were significantly increased, while the number of mitochondria was significantly decreased in the miR-138 inhibitor group. Furthermore, the expression levels of cleaved caspase-9 and -3 were both significantly increased in the myocardial I/R injury model compared with control mice. Additionally, after injection of the miR-138 mimic, their expression levels were

decreased. After treatment with the miR-138 mimic, protein expression levels of Drp1 and Fis1, proteins related to mitochondrial morphology, were both significantly decreased, while their expression levels were increased in the miR-138 inhibitor group. Moreover, HIF1-α was confirmed as the target of miR-138. Therefore, the aforementioned results showed that miR-138 might lessen myocardial ischemia reperfusion injury by inhibiting mitochondria-mediated apoptosis, due to its associated targeting of HIF1-α.

There are some limitations in the current study. The mitochondrial membrane potential generated by proton pumps is an essential component in the process of energy storage during oxidative phosphorylation, and it is associated with cells' capacity to generate ATP by oxidative phosphorylation (34,35). Several fluorescent lipophilic cationic dyes (including, tetramethylrhodamine methyl ester and tetramethylrhodamine ethyl ester, Rhodamine 123, 3,3'-dihexyloxacarbocyanine iodide and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) have become important tools for directly measuring the mitochondrial membrane potential (36). Furthermore, apoptosis inducing factor (AIF), such as Cyt-C, Smac and Apaf-1, are proteins that trigger chromatin condensation and DNA fragmentation in a cell in order to induce programmed cell death (37,38). The mitochondrial AIF protein was found to be a caspase-independent death effector that can allow independent nuclei to undergo apoptotic changes (39). Therefore, future studies should detect the change of mitochondrial membrane potential and apoptosis factors to detect mitochondrial apoptosis.

This study indicated that overexpression of miR-138 reduces the myocardial I/R-induced increase in infarct size and myocardial enzymes by targeting HIF1-α to inhibit myocardial I/R-induced mitochondrial apoptosis. These results demonstrated a cardioprotective effect of miR-138 and suggested the potential to become a promising target to alleviate myocardial I/R injury.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

QZ conceptualized the idea; YL and JFZ performed the experiments; YL and XYL searched the literature; YL, XYL and JFZ analyzed the data; YL and JFZ designed and made the figures; YL created the tables; YL and QZ wrote the manuscript. QZ reviewed the paper.

Ethics approval and consent to participate

This current study was approved by the Clinical Ethics Committee of Affiliated Hospital of Weifang Medical University (Shandong, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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