

Effects of miR-103a-3p on the autophagy and apoptosis of cardiomyocytes by regulating *Atg5*

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Abstract. Autophagy and apoptosis are associated with cardiovascular diseases. Emerging evidence shows that microRNAs (miRs) are critical in the development of pathological processes underlying cardiovascular diseases by regulating the induction of apoptosis and autophagy. The present study aimed to investigate the role of miR-103a-3p in cardiomyocyte injury through autophagy and apoptosis. H9c2 cells were cultured under hypoxia and reoxygenation (H/R) conditions and were used to mimic cells under ischemia. The transfection of cells with miR-103a-3p (mimics and inhibitors) was performed to examine its function in cardiomyocytes. The expression levels of miR-103a-3p were evaluated by reverse transcription-quantitative polymerase chain reaction analysis. Cell viability was determined using an MTT assay, and the lactate dehydrogenase assay (LDH) was used to investigate cell injury. The expression levels of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein, Beclin-1, autophagy-related 5 (*Atg5*), cleaved caspase-3 and cleaved caspase-9 were detected using western blotting. Immunofluorescence assays were performed to detect the expression of LC3 as a marker of autophagy. The target gene of miR-103a-3p was identified using dual-luciferase reporter assays. The results revealed that the expression levels of miR-103a-3p were significantly downregulated in cardiomyocytes under H/R conditions. Injury of the cardiomyocytes was evaluated under H/R conditions. Following transfection of the cells with miR-103a-3p inhibitors, cell injury was increased, as determined by LDH and MTT assays. The expression levels of apoptotic proteins were consistent with the results obtained in the LDH and cell viability assays. The induction of autophagy was increased in cells under H/R conditions and cells with miR-103a-3p inhibitor transfection, whereas the induction of autophagy was decreased in cells transfected with miR-103a-3p mimics. In addition, the data indicated that miR-103a-3p directly targeted

Atg5, which regulated the induction of autophagy and apoptosis. Taken together, these findings indicate that, following the inhibition of miR-103a-3p, *Atg5* promotes autophagy and apoptosis in cardiomyocytes by directly targeting *Atg5*. Therefore, miR-103a-3p can be considered a potential therapeutic target for myocardial ischemia.

Introduction

The incidence and mortality rates of coronary heart disease are increasing annually. Acute myocardial infarction (AMI) is a disease with a high mortality rate, which can cause significant damage to myocardial tissues and lead to disability or mortality. AMI mainly refers to regional myocardial ischemic necrosis caused by acute coronary artery occlusion and blood flow rupture. Therefore, early opening of the infarct vessel and reperfusion of the ischemic myocardium are the most effective methods to repair extensive myocardial damage (1). However, reperfusion can lead to myocardial cell injury, which is termed reperfusion injury. Reperfusion injury can cause more extensive cell loss and further expand the area of the ischemic myocardium, affecting patient survival rate (2). A previous study showed that the final area of myocardial necrotic tissue, estimated to cover ~50% of the total tissue region, may be associated with reperfusion injury (3). The reduction of reperfusion injury can significantly reduce the extent of the final necrotic area of the myocardial tissue, and thus improve long-term cardiac function in patients with AMI. Therefore, the mechanism of reperfusion injury requires extensive investigation for the development of novel therapeutic approaches for AMI.

Autophagy is the process by which cells use lysosomes to degrade their damaged organelles and macromolecules. It has an important role in cell growth, development and the maintenance of cell homeostasis (4). Autophagy is implicated in cellular housekeeping duties and the maintenance of intracellular homeostasis through the removal of damaged proteins and organelles, however, the chronic upregulation of autophagy may result in an imbalance of homeostatic conditions under pathophysiological conditions (5). In cardiomyocytes in nutrient deprivation conditions, ischemic injury induces autophagy by activating the AMPK signaling pathways (6). However, if the ischemic event is more severe, for example, in acute myocardial infarction and ischemia/reperfusion (I/R) injury, the increase in autophagic flux may lead to cell death, and exacerbate cardiac diseases, including heart failure (7-9).

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MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs with a length of ~22 nucleotides. These molecules are important in the post-transcriptional regulation of various genes. In the human genome, ~1,000 miRNA families have been identified, with each family including hundreds of target miRNAs (10,11). The total number of miRNA molecules in a cell regulates the expression of at least 30% of the genes. Therefore, miRNAs are involved in almost all cell function activities, including cell growth, differentiation and disease progression, acting as major contributors in the regulation of cellular activity (12-14). Previous studies have suggested that miRNAs are important in the regulation of cellular stress, including hypoxia, nutritional deprivation and DNA damage (15-17). Previous studies have indicated that numerous miRNAs, including miRNA (miR)-214 and miR-21, are associated with myocardial protection (18,19). miR-214 and miR-21 exert a cardioprotective effects during cardiomyocyte ischemia. miR-103a-3p has been shown to be overexpressed and to promote malignant transformation in several types of cancer (20-22). Circular RNA expression profiles have revealed the regulatory role of the circTCF25-miR-103a-3p pathway in bladder carcinoma (21). However, there have been no investigations of the interaction between miR-103a-3p and cardiomyocytes.

Autophagy is regulated by the autophagy-related gene (*Atg*), which is involved in the formation of autophagosomes (23). Following mature autophagosome formation, *Atg5-Atg12* conjugates are localized to phagophores and separated (24). The conjugation of *Atg5-Atg12* is activated by the class III PI-3-kinase (Vps34) (25). In addition, the activation of Vps34 is positively regulated by Beclin-1 (26). Under normal conditions, the BH domain of the Beclin-1 protein binds to B-cell lymphoma 2 (Bcl-2) and/or Bcl-2-like protein (Bcl-xL) to inhibit autophagy. However, under stress conditions, including a lack of growth factor supply or starvation, the induction of autophagy is activated when the Bcl-2 or Bcl-xL proteins are separated from Beclin-1 (27). Beclin-1 is required for *Atg5*-dependent or -independent autophagy (28). Microtubule associated protein light chain 3 (MAP-LC3/Atg8/LC3) is conjugated with phosphatidylethanolamine (PE) involved in the second modification. Cytosolic LC3 is cleaved to form LC3-I, LC3-I is conjugated to PE to form LC3-II, and LC3II specifically targets phagophores associated with *Atg5-Atg12*. At present, LC3 is the only protein known to be associated with autophagosomes specifically. Therefore, the levels of LC3II can be used to assess autophagosome number (29).

To the best of our knowledge, the present study is the first to demonstrated the abnormal expression of miR-103a-3p in a myocardial ischemia/reperfusion *in vitro* model. To improve the understanding of the role of miR-103a-3p in the induction of autophagy and apoptosis in cardiomyocytes, the present study investigated the effects of miR-103a-3p on the autophagy and apoptosis of H9c2 cells under hypoxia/reoxygenation (H/R) conditions. The involvement of autophagy-related gene expression in this process was also examined.

Materials and methods

Cell culture and treatment. H9c2 cardiomyocytes were obtained from the Cell Line Bank of the Chinese Academy

of Sciences (Shanghai, China) and cultured in high glucose (4.5 g/l) Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), at 37°C and 5% CO₂. To mimic ischemia, the cells were cultured in low glucose (1.0 g/l) DMEM (Gibco; Thermo Fisher Scientific, Inc.), placed in a hypoxic incubation chamber with 90% N₂ and 5% CO₂ for 24 h. Subsequently, the cells were reoxygenated by incubation with high glucose DMEM supplemented with 10% FBS at 37°C in the presence of 5% CO₂.

Transfection. The H9c2 cells were plated in 6-well plates (1x10⁵ per well) and incubated at 37°C for 24 h. The cells were transiently transfected with a final 20 nM dose of miR-103a-3p mimics (cat. no. miR10000101-1-5), miR-control (miR-con; cat. no. miR1N0000001-1-5), miR-103a-3p inhibitor (cat. no. miR20000101-1-5), inhibitor-con (cat. no. miR2N0000001-1-5), *Atg5* small interfering (si)RNA (cat. no. siB160225095638-1-5) or siRNA-con (cat. no. siN0000002-1-5; all from Guangzhou RiboBio Co., Ltd., Guangzhou, China) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequent analysis of the different cell groups was performed following incubation for 48 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The H9c2 cells were plated in 6-well plates (1x10⁵ per well). Following transfection, the cells were cultured for 48 h and total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration and purity of the RNA were measured using the NanoDrop system. Complementary DNAs (cDNAs) were synthesized using RevertAid first strand cDNA (Fermentas; Thermo Fisher Scientific, Inc.) or a Taqman microRNA reverse transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cDNA samples were amplified using Power SYBR®-Green PCR Master mix or a TaqMan microRNA assay (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacture's protocol. In brief, the PCR amplification program was the following: 95°C for 20 sec, followed by 40 cycles of 95°C for 1 sec and 60°C for 20 sec. In addition, ddH₂O as the non-template control was analyzed for every plate. The primers used were as follows: miR-103a-3p forward, 5'-ACACTCCAGCTGGGAGCAGCATTGTACAGGG-3' and reverse, 5'-TGGTGTCTGGAGTCG-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; *Atg5* forward 5'-TATCAGAGCATGTCACCCTT-3' and reverse, 5'-TTCCTGTCTGGCTTGAGCA; and GAPDH forward, 5'-GGCACAGTCAAGGCTGAGAATG-3' and reverse, 5'-ATGGTGGTGAAGACGCCAGTA-3'. U6 was used for normalization the expression levels of miR-103a-3p. GAPDH was used for normalization the expression levels of *Atg5*. Relative expression was quantified by the 2^{-ΔΔCq} method (30).

MTT assay. The H9c2 cells were plated in 96-well plates (1x10⁴ per well). Following transfection, the cells were cultured for 48 h. A total of 20 μl MTT reagent (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was

added to each well, and the cells were incubated at 37°C for 4 h. Subsequently, 150 μ l of dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) was added, and the absorbance of each well was measured on a microplate reader at 490 nm.

Lactate dehydrogenase (LDH) assay. The H9c2 cells were plated in 24-well plates (1×10^5 per well) and were separately transfected with miR-103a-3p mimics, miR-con, miR-103a-3p inhibitor, inhibitor-con, *Atg5* siRNA or siRNA-Con. The LDH leakage assay was performed to determine cell injury using the LDH cytotoxicity assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. The absorbance was measured at 490 nm.

Western blot analysis. The H9c2 cells were plated in 6-well plates (1×10^5 per well). Following transfection for 48 h, total protein was lysed using the RIPA lysis buffer (Beyotime Institute of Biotechnology). The concentration and purity of the protein were measured using the NanoDrop system. Protein samples of 40 μ g per lane were separated on SDS-PAGE gels (10%) and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked for 2 h in 5% skimmed milk at room temperature and subsequently incubated with primary antibodies (Beclin-1, cat. no. sc-48341, 1:1,000; *Atg5*, cat. no. sc-133158, 1:1,000; Bcl-2, cat. no. sc-7382, 1:1,000; Bax, cat. no. sc-7480, 1:1,000; cleaved caspase-3, cat. no. sc-65496, 1:1,000; cleaved caspase-9, cat. no. sc-56076, 1:1,000; GAPDH, cat. no. sc-47724, 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C. The membranes were then washed with TBST three times, incubated with horseradish peroxidase-labelled secondary antibodies (cat. no. 7074, 1:10,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 2 h at room temperature, and further washed with TBST three times. Subsequently, the blots were detected using the enhanced chemiluminescence kit and analyzed using ImageJ k1.45 software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence. The cells on coverslips were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 in phosphate buffered saline (PBS) for 10 min. The samples were blocked with PBS containing 2% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature and further incubated with primary antibodies (LC3, cat. no. 2775, 1:200; Cell Signaling Technology, Inc.) overnight at 4°C. The following day, the samples were incubated with secondary antibodies (cat. no. 1647, 1:200; Invitrogen; Thermo Fisher Scientific, Inc.) for 2 h at room temperature in the dark. Finally, the cells were stained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich; Merck KGaA) for 5 min. The coverslips were washed with PBS following each step. Images were captured using an Olympus IX50 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Luciferase reporter assay. The H9c2 cells were plated in 24-well plates (1×10^5 per well) and then co-transfected with a pmir-GLO dual-luciferase miRNA target expression vector (Promega Corporation, Madison, WI, USA), containing a wild-type or mutant *Atg5* 3'untranslated region (3'UTR), and

miR-103-3p mimics or miR-con using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following 48 h of incubation, the luciferase activity was measured using a dual luciferase reporter gene assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol.

Statistical analysis. All data are presented as the mean \pm standard deviation. Significant differences were determined using Student's t-test or one-way analysis of variance followed by Tukey's test. Data were analyzed using SPSS version 20.0 (IBM Corp., Armonk., NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of miR-103a-3p are decreased significantly in hypoxia-induced H9c2 cardiomyocytes. To examine the functional role of miR-103a-3p in cardiomyocytes, its levels were examined in the following groups: H9c2 cells or negative control (NC) group (H9c2 cells only), H9c2 H/R group (H9c2 cells under H/R conditions), H9c2 miR-103a-3p mimics group (H9c2 cells transfected with miR-103a-3p-mimics) and/or miR-103a-3p inhibitor group (H9c2 cells transfected with miR-103a-3p inhibitor). The expression levels of miR-103a-3p were significantly lower in the H9c2 cells under H/R conditions compared with those noted in the NC group, as shown in Fig. 1A. To further investigate the role of miR-103a-3p, the H9c2 cells were transfected with miR-103a-3p mimics and miR-103a-3p inhibitors. The miR-103a-3p mimics group exhibited a significantly higher level of miR-103a-3p compared with that in the mimic-con group, whereas the miR-103a-3p inhibitor exhibited a significantly lower level compared with that in the inhibitor-con group, as shown in Fig. 1A. No significant differences were observed between the mimic-con or inhibitor-con and NC groups. These results indicated that the transfection was efficient.

Cell viability is affected by the expression of miR-103a-3p. To examine the effect of miR-103a-3p, the H9c2 miR-103a-3p mimics H/R group (H9c2 miR-103a-3p mimics group cells treated under H/R conditions) was used for cell viability experiments. The viability of H9c2 cells under H/R conditions and of H9c2 cells transfected with miR-103a-3p inhibitor were significantly decreased compared with that of the NC cells, whereas no significant difference was observed between the inhibitor-con and NC groups (Fig. 1B). However, the cell viability of the miR-103a-3p mimics H/R group was significantly higher than that of the H/R group, whereas no significant difference was noted between the NC and mimic-con groups. These results revealed that the viability of H9c2 cells was increased following an increase in the expression of miR-103a-3p.

Expression of miR-103-3p is associated with the induction of H9c2 cardiomyocyte apoptosis. To detect the induction of apoptosis in H9c2 cardiomyocytes, the LDH cytotoxicity assay kit was used. LDH leakage in the H/R and miR-103a-3p inhibitor groups was increased compared with leakage in the NC and the inhibitor-con groups, whereas no significant difference was noted between the inhibitor-con and NC groups. In

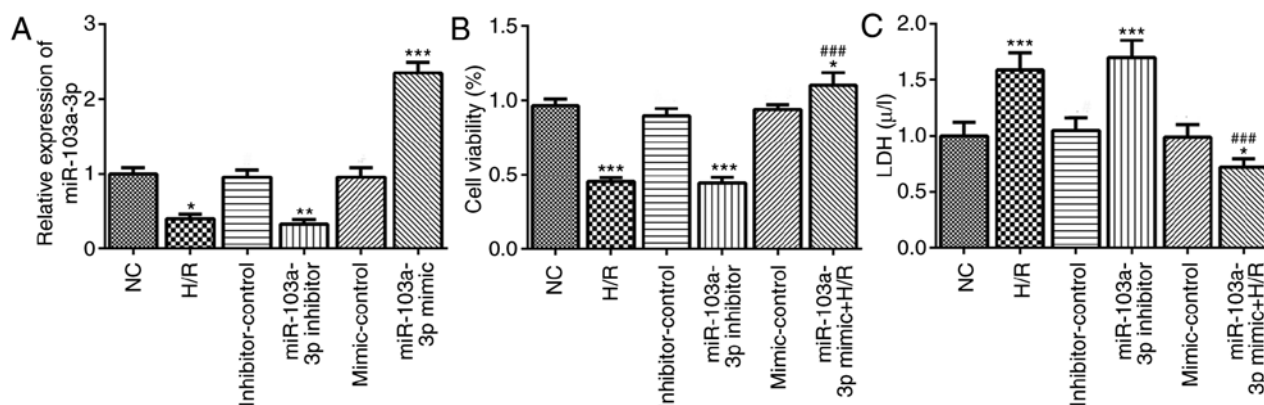


Figure 1. Effects of miR-103a-3p on H9c2 cell viability. (A) Transfection efficiency of miR-103a-3p inhibitor and miR-103a-3p mimics detected via reverse transcription-quantitative polymerase chain reaction analysis. (B) MTT assays for the detection of cell viability. (C) Cell injury was determined via the leakage of LDH. NC, untreated H9c2 cells; H/R, H9c2 cells under H/R; mimic-Control, H9c2 cells transfected with miRNA mimic control; miR-103a-3p mimics, H9c2 cells transfected with miR-103a-3p mimic; miR-103a-3p mimics + H/R, H9c2 cells transfected with miR-103a-3p mimic and then H/R; miR-103a-3p inhibitor, H9c2 cells transfected with miRNA inhibitor; inhibitor-Control, H9c2 cells transfected with miRNA inhibitor control. All data are expressed as the mean \pm standard deviation. * P <0.05, ** P <0.01 and *** P <0.001, vs. NC; ### P <0.001, vs. H/R. miR, microRNA; NC, negative control; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase.

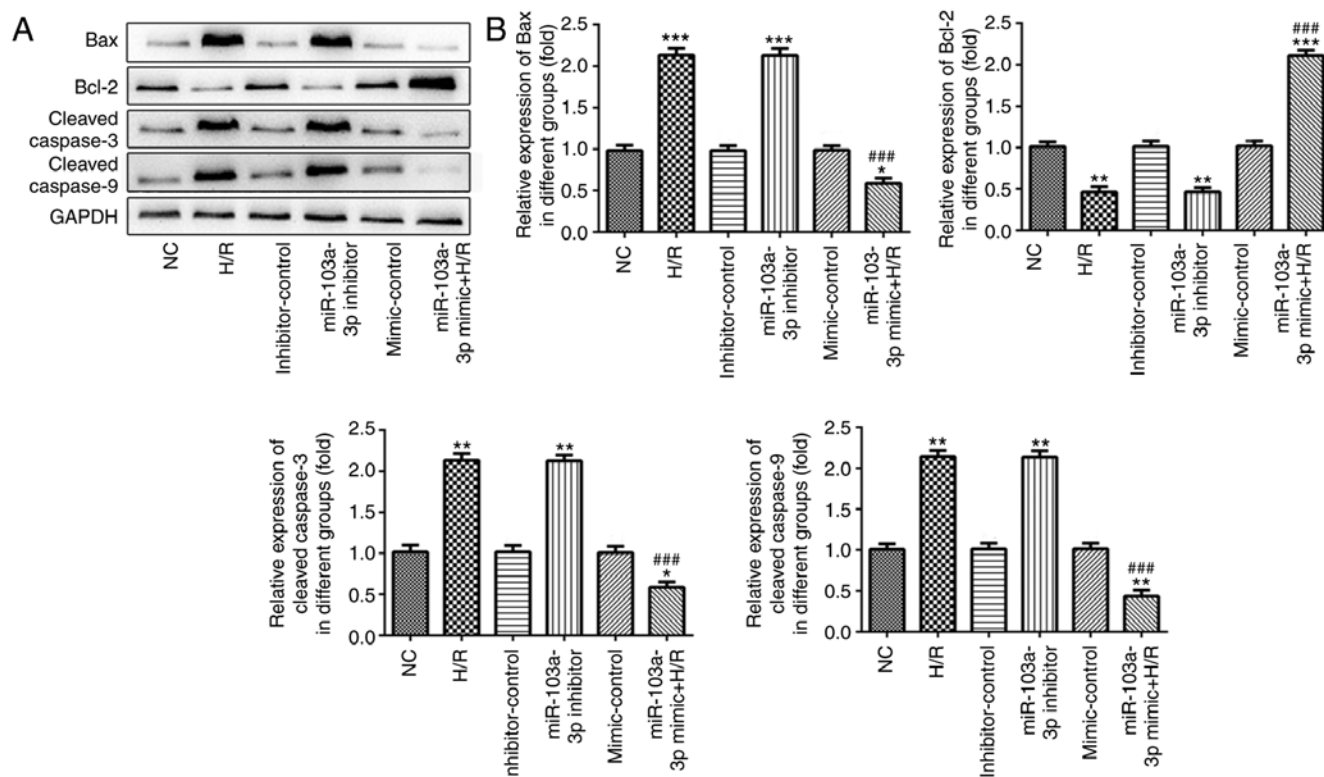


Figure 2. Expression of apoptotic proteins and autophagy-related proteins in H9c2 cells under H/R conditions, in H9c2 cells transfected with miR-103a-3p mimics and/or miR-103a-3p inhibitors. (A) Protein expression levels of Bax, Bcl-2, cleaved caspase-3 and cleaved caspase-9 were determined by western blotting. (B) Quantitative evaluation of the protein expression levels. NC, untreated H9c2 cells; H/R, H9c2 cells under H/R; mimic-Control, H9c2 cells transfected with miRNA mimic control; miR-103a-3p mimics + H/R, H9c2 cells transfected with miR-103a-3p mimic and then H/R; miR-103a-3p inhibitor, H9c2 cells transfected with miRNA inhibitor; inhibitor-Control, H9c2 cells transfected with miRNA inhibitor control. All data are expressed as the mean \pm standard deviation. * P <0.05, ** P <0.01 and *** P <0.001, vs. NC; ### P <0.001, vs. H/R. miR, microRNA; NC, negative control; H/R, hypoxia/reoxygenation; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

addition, the leakage of LDH in the miR-103a-3p mimics H/R group was significantly lower compared with that noted in the H/R group (Fig. 1C).

The detection of the expression levels of apoptotic proteins Bax, cleaved caspase-3 and cleaved caspase-9 by western blotting indicated that their levels were significantly upregulated in

the H/R and the miR-103a-3p inhibitor groups compared with those noted in the NC and inhibitor-con groups. In contrast to the apoptotic proteins, the anti-apoptotic protein Bcl-2 was downregulated in the aforementioned groups, whereas the trend in the miR-103a-3p mimics H/R group was the opposite to that noted in the H/R group (Fig. 2A and B).

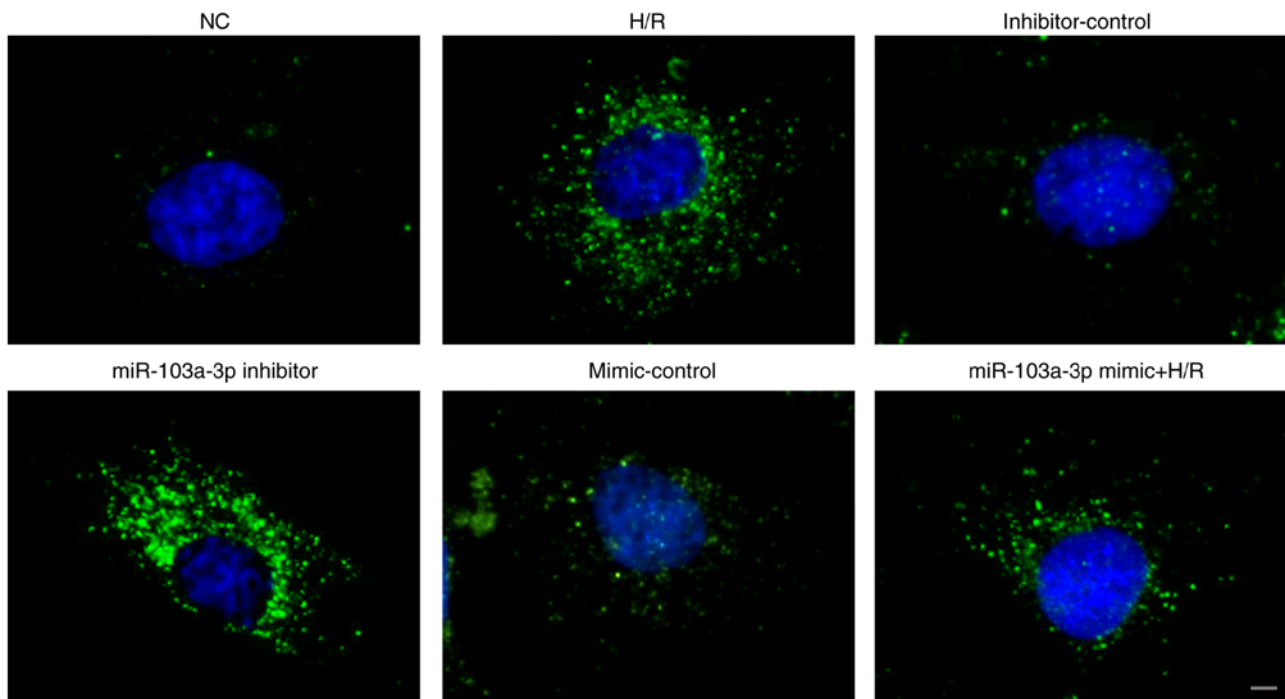


Figure 3. Induction of autophagy is associated with the expression of miR-103a-3p. Immunofluorescence of LC3 was detected in H9c2 cells (scale bar, 10 μ m). NC, untreated H9c2 cells; H/R, H9c2 cells under H/R; mimic-Control, H9c2 cells transfected with miRNA mimic control; miR-103a-3p mimics + H/R, H9c2 cells transfected with miR-103a-3p mimic and then H/R; miR-103a-3p inhibitor, H9c2 cells transfected with miRNA inhibitor; inhibitor-Control, H9c2 cells transfected with miRNA inhibitor control. miR, microRNA; NC, negative control; H/R, hypoxia/reoxygenation.

Induction of autophagy in H9c2 cardiomyocytes. The immunofluorescence staining indicated that LC3 was increased in the H/R and the miR-103a-3p inhibitor groups. The expression levels of LC3II in the miR-103a-3p mimics H/R group were lower than those in the cells cultured under H/R conditions (Fig. 3). The protein expression levels of Beclin-1 and *Atg5*, which are associated with the induction of autophagy, were also detected by western blotting. The levels of these two proteins were significantly increased in the H/R and the miR-103a-3p inhibitor groups compared with those in the NC and inhibitor-con groups, whereas their levels were significantly decreased in the miR-103a-3p mimics H/R group compared with those in the H/R group (Fig. 4A and B). Furthermore, miR-103a-3p was observed to regulate the mRNA expression level of *Atg5* (Fig. 4C). These results indicated that a reduction in the expression levels of miR-103a-3p promoted the induction of autophagy.

miR-103-3p regulates the induction of autophagy and apoptosis via *Atg5*. The present study further examined the association between *Atg5* and miR-103a-3p. The luciferase reporter assays indicated that *Atg5* was a direct and negative target of miR-103a-3p (Fig. 5A and B). *Atg5*-siRNA was used to efficiently knock down the expression of *Atg5* in H9c2 cells (Fig. 5C).

To investigate the effect of *Atg5* on H9c2 cardiomyocytes, the *Atg5*-siRNA H/R group of cells (H9c2 *Atg5*-siRNA cultured under H/R condition) was used for further investigation. The cell viability of the *Atg5*-siRNA H/R group was higher than that of the H/R group, which was significantly decreased compared with that of the NC group (Fig. 6A). LDH leakage was significantly increased in the H/R group

compared with that in the NC group, whereas LDH leakage in the *Atg5*-siRNA H/R group was significantly decreased (Fig. 6B). The expression levels of apoptotic proteins in the NC and siRNA-con groups did not differ significantly. The protein expression levels of Beclin-1, Bax, cleaved caspase 3 and cleaved caspase 9 were significantly upregulated and the levels of Bcl-2 were significantly downregulated in the H/R group compared with those in the NC group (Fig. 7). However, these proteins exhibited an opposite pattern of expression in the *Atg5*-siRNA H/R group compared with that of the H/R group (Fig. 7). The protein expression of LC3II in the H/R group was increased compared with that in the NC group, although the expression in the *Atg5*-siRNA H/R group was lower than that in the H/R group (Fig. 8). These results indicated that *Atg5* is a potential target for miR-103a-3p.

Discussion

Recently, miRNAs have become a major focus of investigation with regard to the development of cardiovascular disease. In the present study, it was demonstrated that cardiomyocytes cultured under H/R conditions were protected from the induction of apoptosis and excessive autophagy by miR-103a-3p. Furthermore, it was demonstrated that miR-103a-3p directly targets *Atg5* which mediates the effects of miR-103a-3p in cardiomyocytes.

The induction of cardiomyocyte apoptosis is a rare event in the healthy myocardium and is the predominant form of cell death in the infarcted myocardium, which is often caused by ischemia (31,32). It has been shown that miRNAs are associated with the induction of cardiomyocyte apoptosis following ischemic injury (33). As apoptosis is essential for cardiac

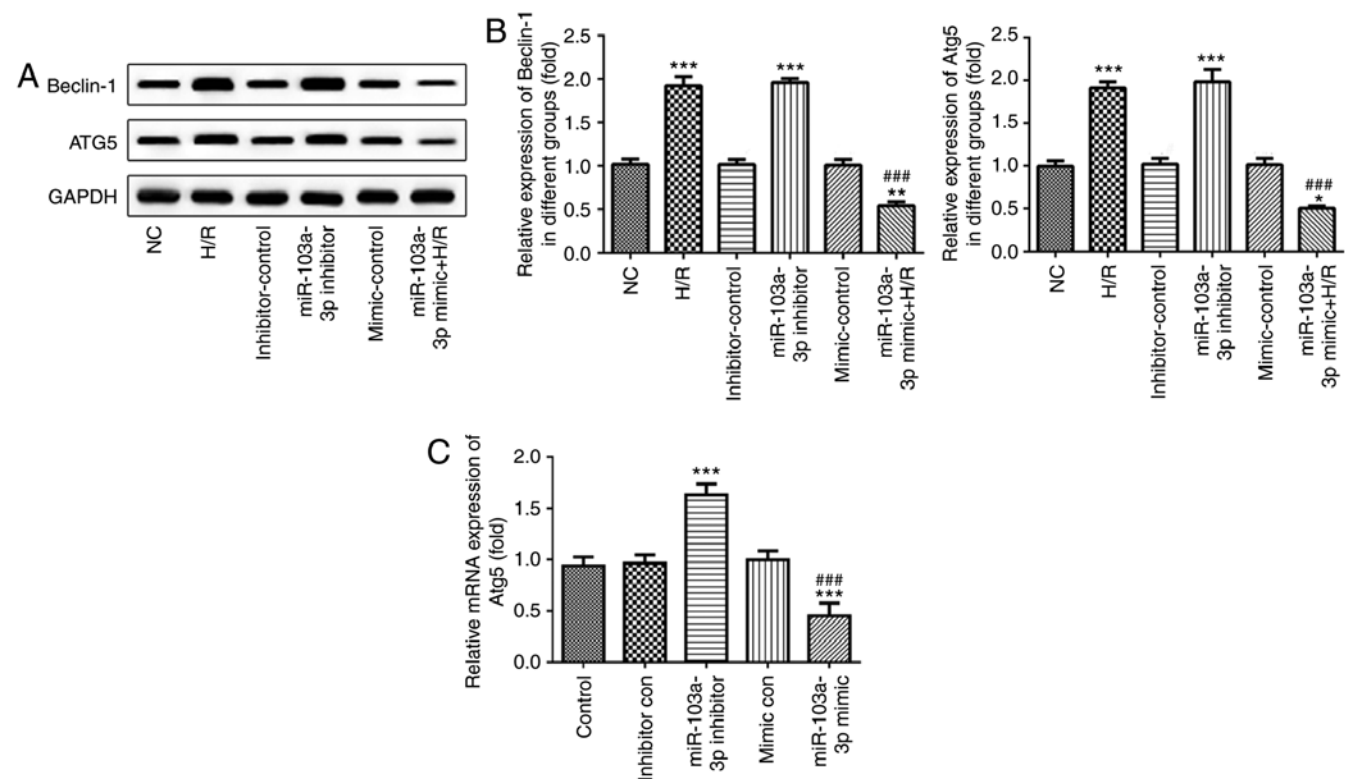


Figure 4. miR-103a-3p regulates the expression of autophagic proteins. Expression levels of Beclin-1 and Atg5 were (A) detected by western blotting and (B) results were quantified. (C) Expression levels of *Atg5* were examined by reverse transcription-quantitative polymerase chain reaction analysis. NC, untreated H9c2 cells; H/R, H9c2 cells under H/R; mimic-Control, H9c2 cells transfected with miRNA mimic control; miR-103a-3p mimic, H9c2 cells transfected with miR-103a-3p mimic; miR-103a-3p mimics + H/R, H9c2 cells transfected with miR-103a-3p mimic and then H/R; miR-103a-3p inhibitor, H9c2 cells transfected with miRNA inhibitor; inhibitor-Control, H9c2 cells transfected with miRNA inhibitor control. All data are expressed as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, vs. NC; *** $P < 0.001$, vs. H/R. miR, microRNA; NC, negative control; H/R, hypoxia/reoxygenation; *Atg5*, autophagy-related 5.

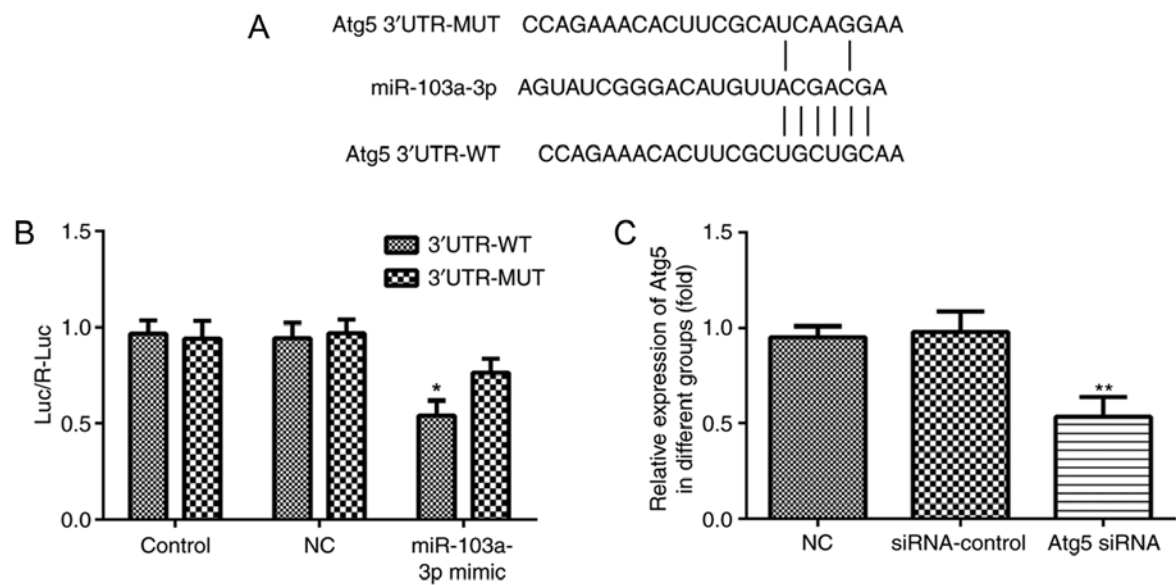


Figure 5. *Atg5* is a potential target for miR-103a-3p. (A) Luciferase reporter assays were used to evaluate the interaction between miR-103a-3p and *Atg5*. (B) Quantification of results. Control, untreated cells; NC, cells co-transfected with miRNA control and 3'UTR WT or MUT; miR-103a-3p mimics, cells co-transfected with miR-103a-3p mimics and 3'UTR WT or MUT. (C) Relative mRNA expression levels of *Atg5* were determined to detect the transfection efficiency. All data are expressed as the mean \pm standard deviation. * $P < 0.01$, vs. control; ** $P < 0.01$, vs. NC. miR, microRNA; NC, negative control; 3'UTR, 3' untranslated region; WT, wild-type; MUT, mutant; siRNA, small interfering RNA; *Atg5*, autophagy-related 5; luc, luciferase.

injury (34), the present study detected the levels of apoptosis in cardiomyocytes and verified that apoptosis was significantly increased in H9c2 cells cultured under H/R conditions. A

previous study showed that miR-103a-3p was associated with the induction of apoptosis in human glioma stem cells (35). Another previous study showed that miR-103a-3p was

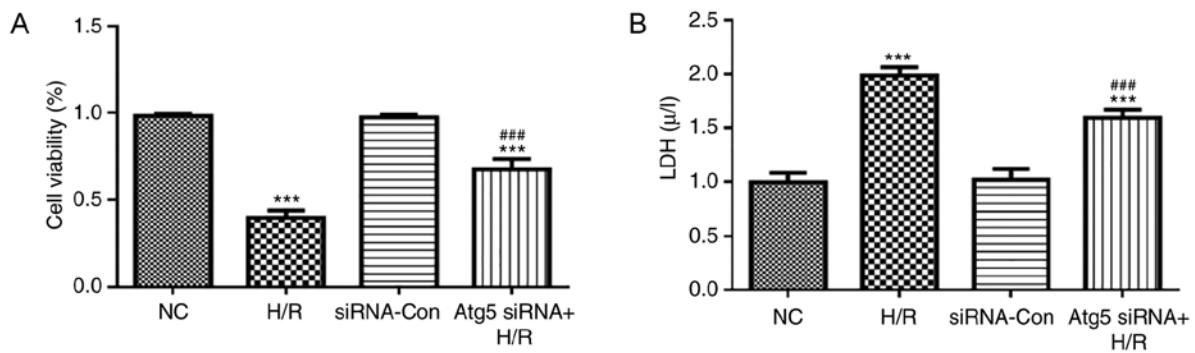


Figure 6. Cell viability is associated with *Atg5*. (A) MTT assay was used to detect cell viability in the four groups. (B) LDH leakage was evaluated to detect cell injury in the four groups. NC, normal H9c2 control cells; H/R, H9c2 cells under H/R; siRNA-Con, H9c2 cells transfected with control siRNA; *Atg5*-siRNA + H/R, H9c2 cells transfected with *Atg5*-siRNA and then H/R. All data are expressed as the mean \pm standard deviation. *** P <0.001, vs. NC; ### P <0.001, vs. H/R. miR, microRNA; H/R, hypoxia/reoxygenation; NC, negative control; siRNA, small interfering RNA; *Atg5*, autophagy-related 5; LDH, lactate dehydrogenase.

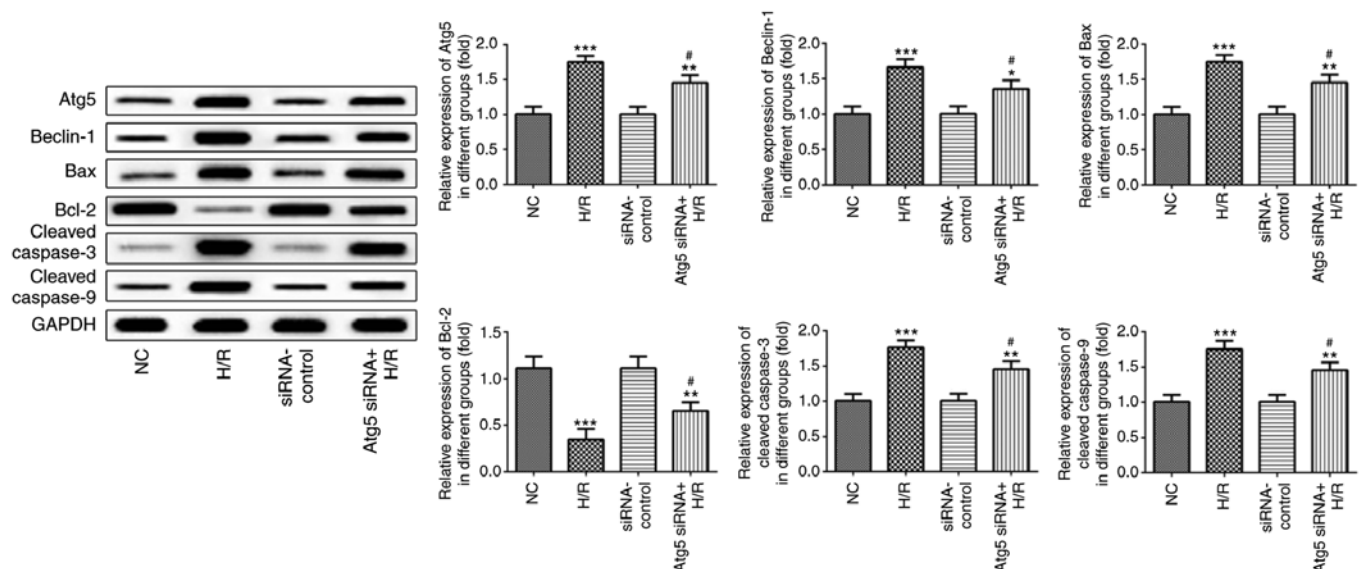


Figure 7. Expression levels of apoptotic proteins and autophagic proteins and their quantitative evaluation in cells cultured under H/R conditions or in cells transfected with *Atg*-siRNA. All data are expressed as the mean \pm standard deviation. * P <0.05, ** P <0.01 and *** P <0.001, vs. NC; # P <0.05, vs. H/R. siRNA-control and NC did not differ significantly. miR, microRNA; H/R, hypoxia/reoxygenation; NC, negative control; siRNA, small interfering RNA; *Atg5*, autophagy-related 5; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

also upregulated in ischemic dilated cardiomyopathy (36). Furthermore, the levels of miR-103a-3p were significantly decreased in H9c2 cells cultured under H/R conditions, which mimic I/R conditions. Therefore, miR-103a-3p may be involved in I/R via the regulation of apoptosis. Further analysis confirmed these conclusions using miR-103a-3p inhibition and overexpression cell models. The results indicated that the expression of miR-103a-3p was inhibited following the induction of apoptosis by H/R injury, whereas the overexpression of miR-103a-3p inhibited this process. This indicated that the upregulation of miR-103a-3p inhibited the induction of apoptosis under H/R conditions. In addition, cell apoptosis is a process of cell death and can be promoted by the release of cytochrome *c*, cleaved caspase-3 and cleaved caspase-9, which are essential in the formation of apoptotic bodies (37,38). Bcl-2 prevents the permeabilization of mitochondria to inhibit Ca^{2+} overload and consequently the activity of cleaved caspases, whereas Bax exerts the opposite effects (39). A previous study showed that the Bcl-2 family proteins are important in

myocardial I/R injury (40). The results of the present study are in accordance with previous investigations and demonstrated the downregulation of Bcl-2 and the upregulation of Bax, cleaved caspase-3 and cleaved caspase-9 proteins. The interaction between Bcl-2, Bax, cleaved caspase-3 and cleaved caspase-9 proteins and I/R injury require further investigation.

Autophagy or 'self-devouring' is the natural, regulated, destructive mechanism of the cell that removes unnecessary or dysfunctional components (41). It is a constitutive house-keeping process involved in the progression of cardiovascular disease (42,43). A number of studies have revealed the role of miRNAs in regulating autophagy in cardiomyocytes (44,45). In the present study, the LC3 protein was expressed at high levels and assembled in the cytoplasm of H9c2 cells under H/R conditions, or in H9c2 cells transfected with miR-103a-3p inhibitor, compared with control cells. These findings revealed that the downregulation of miR-103a-3p promoted autophagy. The induction of cell apoptosis was accompanied with autophagy, as the two processes were promoted in the cell line

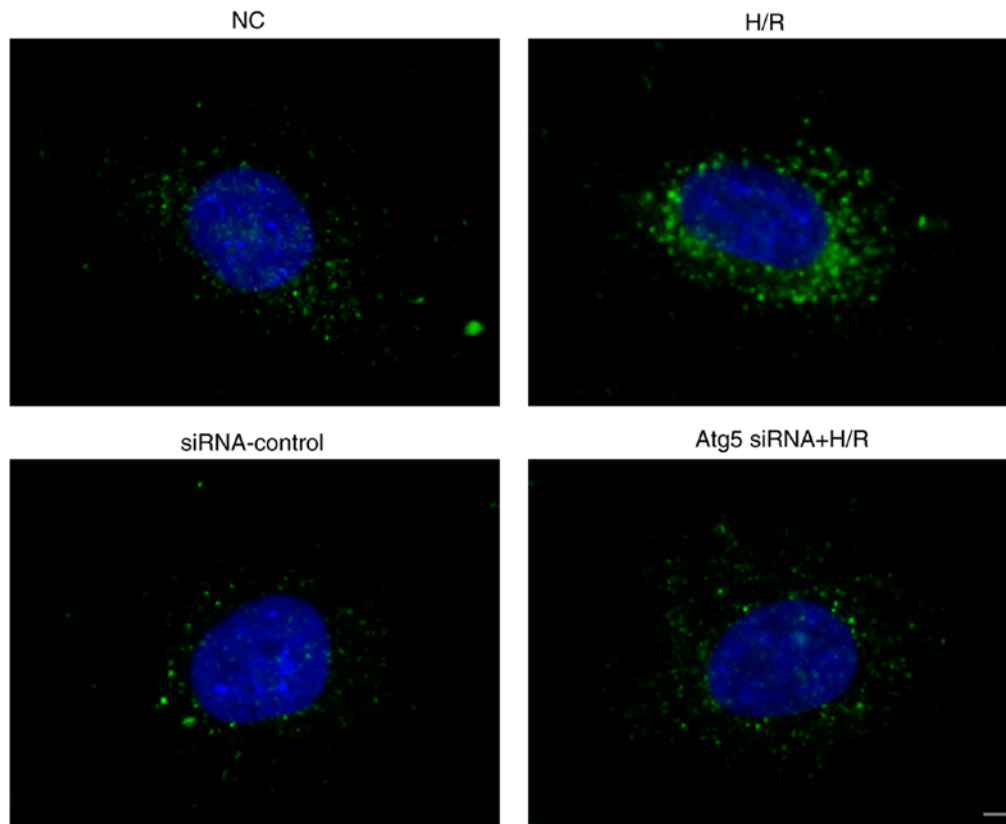


Figure 8. Induction of autophagy is associated with the expression of *Atg5*. Immunofluorescence of LC3 was detected in the four H9c2 cell groups (scale bar, 10 μ m). NC, normal H9c2 control cells; H/R, H9c2 cells under H/R; siRNA-Con, H9c2 cells transfected with control siRNA; *Atg5*-siRNA + H/R, H9c2 cells transfected with *Atg5*-siRNA and then H/R. miR, microRNA; H/R, hypoxia/reoxygenation; NC, negative control; siRNA, small interfering RNA; *Atg5*, autophagy-related 5.

models used. Therefore, the downregulation of miR-103a-3p injures cardiomyocytes by inducing autophagy.

Autophagy and apoptosis are two types of programmed cell death (46). The induction of autophagy depends on the expression of Atg proteins; two of the most important ubiquitination pathways are the *Atg8*(LC3)-PE and *Atg12-Atg5-Atg16L* pathways, which are involved in autophagosome formation. A previous study showed that the same inducible factors can induce autophagy or apoptosis in different cells, as demonstrated by the expression of Beclin-1, *Atg4*, *Atg5* and Bcl-2 (47). *Atg5* is attached to the autophagic vacuole membrane in order to facilitate LC3 binding (48). Evidence suggests that autophagy induces cell death under certain conditions. Additionally, a previous study indicated that *Atg5* promoted apoptotic cell death via the activation of caspase-8 (49). In the present study, the expression of *Atg5* was upregulated by the inhibition of miR-103a-3p. Concomitantly, the levels of miR-103a-3p were not altered when the expression of *Atg5* was inhibited prior to the induction of H/R, indicating that the regulation of *Atg5* by miR-103a-3p was unidirectional (Fig. S1). Furthermore, *Atg5* was downregulated in the miR-103a-3p mimics group compared with that in the H/R group at the mRNA and protein levels, which indicates that miR-103a-3p regulated the expression of *Atg5* through gene expression. miRNAs usually bind to a complementary sequence in the 3'UTR of specific target genes in order to regulate their cellular functions (50). The luciferase reporter assay indicated that miR-103a-3p directly targeted the 3'UTR of the *Atg5* gene. Concomitantly, the mRNA expression

levels of *Atg5* were significantly decreased following the over-expression of miR-103a-3p. Therefore, miR-103a-3p directly targeted *Atg5* to downregulate its levels. Of note, the expression levels of the apoptotic protein was not significantly decreased following the inhibition of *Atg5* under H/R compared with that in the H/R group. A previous report also indicated that *Atg5* promoted autophagy for cell death (51). Further investigation of the induction of apoptosis by the downregulation of miR-103a-3p, and whether this effect is mediated by *Atg5*, is required. It was also found that LC3 was decreased in H9c2 cells transfected with *Atg5* siRNA under H/R conditions compared with that in the H9c2 H/R group, which indicates that autophagy may be mediated by *Atg5*. In the present study, *Atg5* knockdown also inhibited cell death induced by H/R.

It has been shown that Bcl-2 is a well-characterized apoptotic protein, which is considered an important binding partner of Beclin-1 required to regulate autophagy (52). Disruption of the Bcl-2-Beclin interaction induces autophagy (27). In the present study, autophagy was induced in H9c2 cells under H/R conditions and in H9c2 cells transfected with the miR-103a-3p inhibitor. These two types of cell line models exhibited high protein expression levels of Beclin-1. Concomitantly, the expression levels of Bcl-2 were significantly decreased. This indicated that Beclin-1 is involved in the regulation of autophagy in cardiomyocytes.

In conclusion, the data obtained in the present study demonstrated that miR-103a-3p has a protective role in myocardial I/R injury. The mechanisms involve anti-apoptotic and

anti-autophagic effects through increasing the expression of *Atg5*. In addition, miR-103a-3p also promoted the expression of *Belin-1*, which is associated with autophagosome formation. Collectively, these observations provide novel insight into the treatment of myocardial ischemia.

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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

CZ contributed to this article, including the study design, data analysis, data interpretation and drafting of the manuscript. JL and HW collected and analyzed the data. HW and YQ provided suggestions for study design and data analysis. YK and ZG provided key suggestions on study design and edited the language of the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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