Knockdown of MicroRNA-122 Protects H9c2 Cardiomyocytes from Hypoxia-Induced Apoptosis and Promotes Autophagy

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Statistical Analysis C
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Background:
Acute myocardial infarction (AMI) is a severe disease causing heart failure and sudden death. Studies indicate that microRNAs (miRNAs) are involved in the pathophysiology of AMI. In the present study, we carefully explored the effects of miR-122 on myocardial hypoxia injury and its possible underlying mechanism.

Material/Methods:
miR-122 expression was analyzed in H9c2 cardiomyocytes after being transfected with miR-122 mimic, ASO-miR-122, or negative control. Cell viability and apoptosis were investigated by CCK-8 assays and flow cytometry analysis, respectively. Cell migration was analyzed using wound-healing assays. Western blotting was performed to analyze the expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN)/phosphatidylinositol 3-hydroxy kinase (PI3K)/AKT and LC3-II/LC3-I.

Result:
Hypoxia exposure significantly inhibited H9c2 cell viability ($P<0.01$). miR-122 overexpression promoted the hypoxia-induced H9c2 cell proliferation and migration loss ($P<0.05$), and cell apoptosis was increased ($P<0.05$). miR-122 knockdown enhanced cell viability and decreased cell apoptosis ($P<0.05$). Knockdown of miR-122 enhanced PTEN/PI3K/AKT activation and cell autophagy. Overexpression of miR-122 inhibited the PTEN/PI3K/AKT pathway and cell autophagy pathway.

Conclusions:
The expression of miR-122 is involved in hypoxia-induced H9c2 cardiomyocyte injury. Knockdown of miR-122 protects H9c2 cells from hypoxia-induced apoptosis and enhances cell viability.

MeSH Keywords:
Autophagy • Cell Hypoxia • MicroRNAs • Myocardial Infarction • Phosphatidylinositol 3-Kinases

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Background

Acute myocardial infarction (AMI) is a severe ischemic disease responsible for cardiac fibrosis, heart failure, and sudden death [1,2]. It has been reported that smoking is a risk factor for the development of myocardial infarction [3]. Patients with AMI always present severe and persistent pain in the chest, which can be combined with arrhythmia, shock, or heart failure [4,5]. Oxidative stress plays an important role in cardiomyocyte proliferation and differentiation [6]. Hypoxia disrupts microenvironment homeostasis, leading to cardiomyocyte cell apoptosis [6].

MicroRNAs (miRNAs) are small, non-coding RNAs which act as essential regulators for almost all aspects of intracellular signaling pathways in eukaryotic cells. miRNAs regulate protein expression by binding to specific sequences in mRNA molecules, most commonly in the 3'-untranslated regions (UTR) [7]. The action of miRNAs usually leads to downregulation of their target genes by triggering messenger RNA degradation or by inhibiting the translational machinery [8]. The importance of miRNAs in the cardiovascular system is demonstrated by the profound structural and functional abnormalities observed in cardiac-specific Dicer deletion in animal models [8–10].

MicroRNA-122 (miR-122) is abundantly expressed in the developing liver and at high levels in the adult liver. Studies showed that the expression of miR-122 is downregulated in hepatocellular carcinoma (HCC) cells, and overexpression of the same inhibits tumor development and enhances sensitivity to cancer drugs. MiR-122 is considered as a tumor-suppressor miRNA. In addition, miR-122 can be used as a biomarker for AMI in clinical observation [11]. However, the regulatory effect of miR-122 on myocardial hypoxia injury is not clear. The present study explored the effect of miR-122 on hypoxia-induced injury in H9c2 cardiomyocytes.

Material and Methods

Cell culture and treatment

Cardiomyocyte cell line H9c2 (Sigma-Aldrich, St. Louis, MO) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (LifeTechnologies, Carlsbad, CA), supplemented with 10% FBS, 1% Penicillin/Streptomycin (100 U/ml: 100 mg/ml), and 1% GlutaMAX (Life Technologies, Carlsbad, CA), at 37°C in 5% CO_2. Metabolic ischemia was induced by a buffer exchange to an ischemia-mimetic solution (125 mM NaCl, 8 mM KCl, 1.2 mM KH_2PO_4, 1.25 mM $MgSO_4$, 1.2 mM $CaCl_2$, 6.25 mM $NaHCO_3$, 5 mM sodium lactate, and 20 mM HEPES, pH 6.6) and by placing the dishes in hypoxic pouches with an indicator (GasPakTM EZ, BD Biosciences) for 2 h. As certified by the manufacturer,

the Anaerobe Gas Generating Pouch System produces an atmosphere containing 10% carbon dioxide and 1% oxygen.

miRNAs Transfection

miR-122 mimic, ASO-miR-122, and the NC controls were synthesized by GenePharma Co. (Shanghai, China). Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's protocol.

CCK-8 assay

Cells were seeded in 96-well plates with 5000 cells/well, and cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, after stimulation, the CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 h at 37°C in humidified 95% air and 5% CO_2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

Apoptosis assay

Cell apoptosis was detected using propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated Annexin V staining. Briefly, H9c2 cells were washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol. Then, fixed cells were washed twice in PBS and stained in PI/FITC-Annexin V in the presence of 50 µg/ml RNase A (Sigma-Aldrich), and incubated for 1 h at room temperature in the dark. Flow cytometry analysis was done by using a FACS can (Beckman Coulter, Fullerton, CA). The data were analyzed by using FlowJo software.

Migration assay

Cells were plated in 60 mM dishes until confluence. After 3-h pre-treatment of cells with 50 µM mitomycin C, wounds were created by scratching cell sheets with a sterile 200-µl pipette tip. The pictures of a specific position on the scratched areas were taken by an inverted microscope (Leica, Germany) every 24 h. The wound widths were measured and the relative wound widths were calculated. Data are shown as mean ± standard deviation (SD) of 3 independent experiments.

qRT-PCR

Total RNA was extracted from cells and tissues using Trizol reagent (Life Technologies Corporation, Carlsbad, CA) according to the manufacturer's instructions. The TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II with the TaqMan MicroRNA Assay of miR-122 and U6 (Applied Biosystems, Foster City, CA) were used for testing the expression levels of miR-122 in cells.
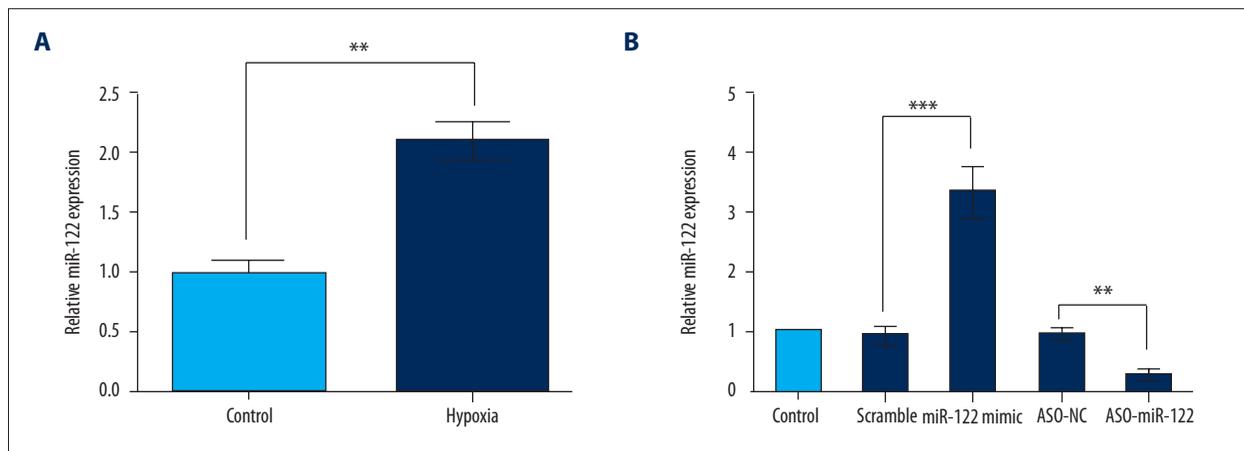
Figure 1. Expression of miR-122. The expression of miR-122 in hypoxic H9c2 cells and the transfection efficiency were assessed. (**A**) Expression of miR-122 was significantly elevated in hypoxic H9c2 cells. (**B**) After transfection, miR-122 expression was significantly increased by miR-122 mimic but decreased by ASO-miR-122. Scramble and ASO-NC were the controls of miR-122 and ASO-miR-122, respectively. Data represent the mean ±SD of 3 independent experiments. ** $P<0.01$; *** $P<0.001$.

Western blotting

H9c2 cells were washed in PBS, then the total proteins were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocol. After quantification using the BCA™ Protein Assay Kit (Pierce, Appleton, WI), an equal amount of protein was loaded and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein in gel was transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA), which were incubated with relevant antibodies. The expression levels of proteins were analyzed with Image Lab™ software (Bio-Rad, Shanghai, China).

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software. The results of multiple experiments are presented as the mean ±SD. P-values were calculated using one-way analysis of variance (ANOVA). $P<0.05$ was considered to indicate a statistically significant result.

Results

Expression of miR-122

To examine the functional role of miR-122 in cardiomyocytes, we first evaluated the expression levels of miR-122 in hypoxic H9c2 cells. As shown in Figure 1A, the expression of miR-122 was significantly higher in hypoxic H9c2 cells compared to the non-treated cells ($P<0.01$). To further confirm the role of miR-122, H9c2 cells were transfected with the miR-122 mimic, ASO-miR-122, or negative control. After transfection, the

miR-122 expression was confirmed by qRT-PCR (Figure 1B). As expected, the expression levels of miR-122 were significantly higher in the miR-122 mimic group compared to the control group ($P<0.001$). miR-122 expression levels were significantly decreased by transfection with ASO-miR-122 ($P<0.01$). These results suggest that the transfection was efficient.

Abnormal expression of miR-122 in cell viability change induced by hypoxia

After transfection with the miR-122 mimic or ASO-miR-122 in H9c2 cells, cell viability was analyzed by CCK-8 assay. As shown in Figure 2, cell viability was significant decreased after hypoxia treatment ($P<0.01$). miR-122 overexpression promoted the hypoxia-induced H9c2 cell viability loss ($P<0.05$), and miR-122 knockdown enhanced the H9c2 cell viability induced by hypoxia treatment ($P<0.05$).

Abnormal expression of miR-122 in cell apoptosis change induced by hypoxia

After transfection with the miR-122 mimic or ASO-miR-122 in H9c2 cells, cell apoptosis was analyzed by flow cytometry analysis. As shown in Figure 3, cell apoptosis was significantly increased after hypoxia treatment ($P<0.011$). miR-122 overexpression promoted the hypoxia-induced H9c2 cell apoptosis. Knockdown of miR-122 protected H9c2 cells from apoptosis induced by hypoxia treatment ($P<0.05$).

Abnormal expression of miR-122 in cell migration change induced by hypoxia

After transfection with the miR-122 mimic or ASO-miR-122 in H9c2 cells, cell migration was measured by wound-healing
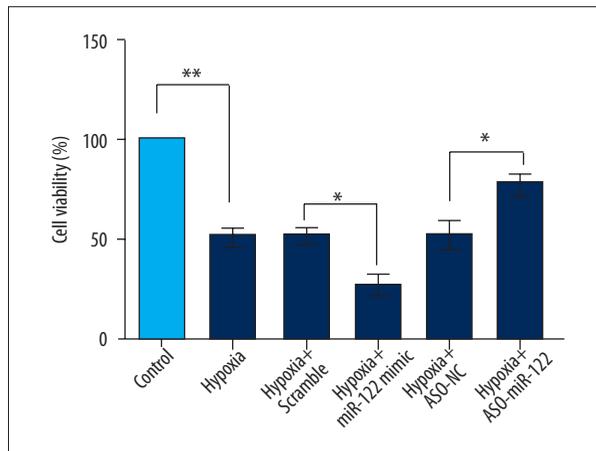
Figure 2. Abnormal expression of miR-122 in cell viability change induced by hypoxia. H9c2 cell viabilities were analyzed by CCK-8 assay after transfection with miR-122 mimic or ASO-miR-122. Scramble and ASO-NC were the controls of miR-122 and ASO-miR-122, respectively. Data represent the mean ±SD of 3 independent experiments. * *P*<0.05; ** *P*<0.01.
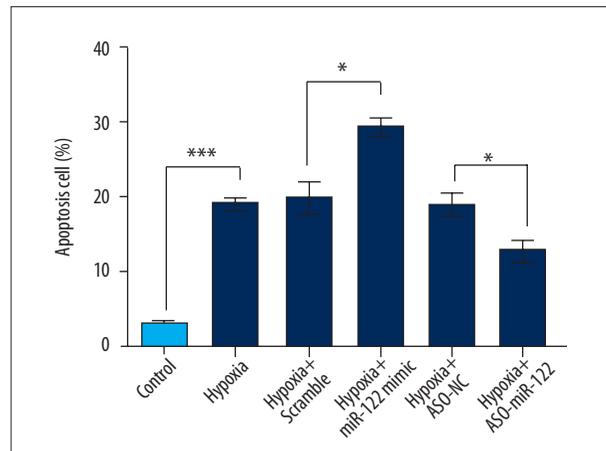

Figure 3. Abnormal expression of miR-122 in cell apoptosis change induced by hypoxia. After transfection with the miR-122 mimic or ASO-miR-122 in H9c2 cells, cell apoptosis was analyzed by flow cytometry. Scramble and ASO-NC were the controls of miR-122 and ASO-miR-122, respectively. Data represent the mean ± SD of 3 independent experiments. * *P*<0.05; *** *P*<0.001.

assays. As shown in Figure 4, cell migration abilities were significantly decreased in the hypoxia-treated group (*P*<0.01 or *P*<0.001). miR-122 overexpression promoted hypoxia-induced H9*c*2 cell migration loss (*P*<0.05), and knockdown of miR-122 enhanced the H9ce cell migration (*P*<0.05 or *P*<0.01).

Knockdown of miR-122 promoted cell proliferation by regulating phosphatase and tensin homolog deleted on chromosome 10 (PTEN)/phosphatidylinositol 3-hydroxy kinase (PI3K)/AKT pathway

To investigate the effect of miR-122 on the PTEN/PI3K/AKT pathway, the protein levels of PI3K, AKT, and PTEN were analyzed by Western blotting. As shown in Figure 5, hypoxia significantly decreased the expression of p-PI3K and p-AKT (*P*<0.01), but significantly increased the PTEN levels, suggesting that hypoxia inhibits the PTEN/PI3K/AKT pathway (*P*<0.01). Overexpression of miR-122 led to inhibition of the PTEN/PI3K/AKT pathway (*P*<0.05), but knockdown of miR-122 significantly activated the PTEN/PI3K/AKT pathway (*P*<0.01 or *P*<0.001).

Knockdown of miR-122 increased cell autophagy induced by hypoxia

To investigate the effect of miR-122 on cell autophagy, the protein levels of LC3-II/LC3-I, Beclin-1, and p62 were analyzed by Western blotting. As shown in Figure 6, hypoxia significantly increased the rate of LC3-II/LC3-I and expression levels of Beclin-1, but significantly decreased the levels of p-62 (all *P*<0.01), indicating that hypoxia induced cell autophagy.
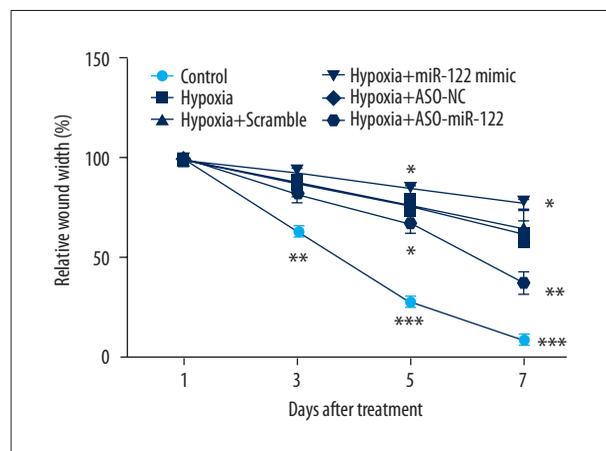

Figure 4. Abnormal expression of miR-122 in cell migration change induced by hypoxia. After transfection with the miR-122 mimic or ASO-miR-122 in H9c2 cells, cell migration was measured by wound-healing assays. Scramble and ASO-NC were the controls of miR-122 and ASO-miR-122, respectively.

Overexpression of miR-122 led to inhibition in hypoxia-induced autophagy (all *P*<0.05), but knockdown of miR-122 significantly activated autophagy (all *P*<0.05).

Discussion

In the present study, we observed that miR-122 was significantly increased in hypoxic H9c2 cells. Overexpression of miR-122 further aggravated the effects of hypoxia on reduced cell
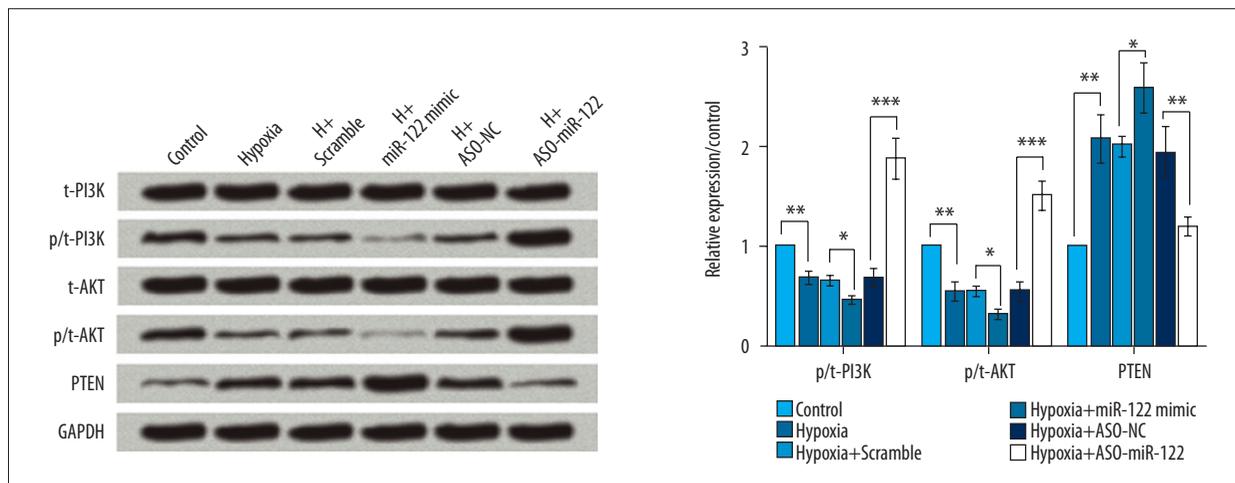
Figure 5. Knockdown of miR-122 promoted cell proliferation by regulating the PTEN/PI3K/AKT pathway. To investigate the effect of miR-122 on the PTEN/PI3K/AKT pathway, the protein levels of PI3K, AKT, and PTEN were analyzed by Western blotting. Hypoxia significantly inhibited the PTEN/PI3K/AKT pathway, overexpression of miR-122 further inhibited the PTEN/PI3K/AKT pathway, and knockdown of miR-122 significantly activated the PTEN/PI3K/AKT pathway. Scramble and ASO-NC were the controls of miR-122 and ASO-miR-122, respectively. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.
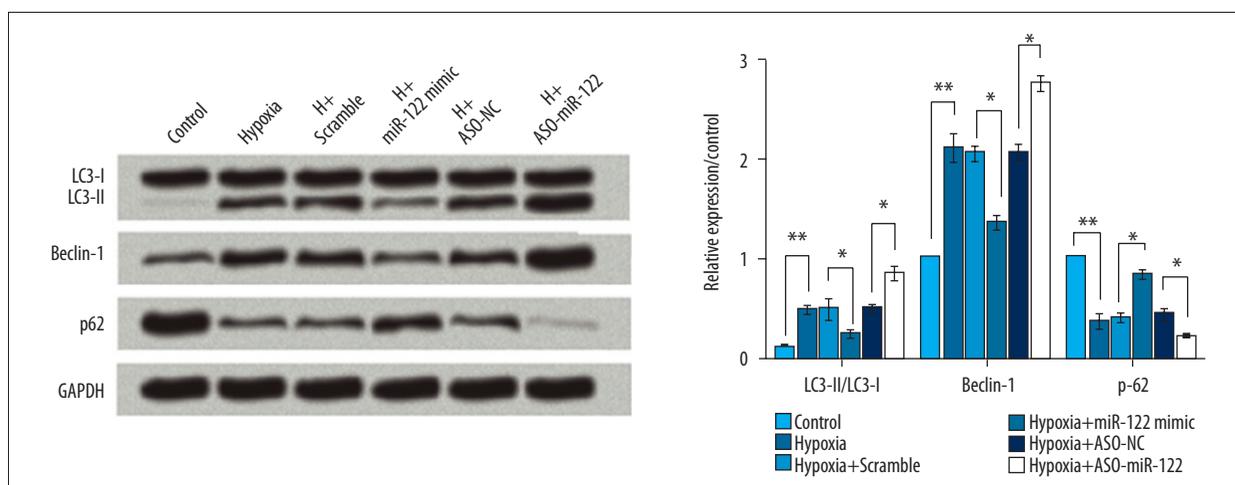

Figure 6. Knockdown of miR-122 increased cell autophagy induced by hypoxia. Hypoxia induced autophagy, overexpression of miR-122 inhibited autophagy, and knockdown of miR-122 activated autophagy. Scramble and ASO-NC were the controls of miR-122 and ASO-miR-122, respectively. * $P<0.05$; ** $P<0.01$.

viability, increased cell apoptosis, and decreased cell migration ability. In addition, the findings suggested that miR-122 expression is associated with the inhibition of PTEN/PI3K/AKT pathway and autophagy. Our results indicate that knockdown of miR-122 protects H9c2 cardiomyocytes from hypoxia.

AMI is a major cause of morbidity and mortality worldwide and needs to be addressed urgently [12]. Apoptosis and necrosis are 2 pivotal events of AMI, which are known to play important roles during the process [13,14]. Myocardial apoptosis is followed by necrosis, which occurs during sustained and severe ischemia, and it is an unregulated irreversible response to a fatal challenge which usually involves myocardial

pump failure [15]. Hence, reducing the magnitude of apoptosis and necrosis in the myocardium might be helpful in prevention of such mortalities. Many studies have been conducted on genes and gene products that are involved in the regulation of myocardial apoptosis during AMI, but the underlying molecular mechanism remains elusive. Increasing evidence indicate that in addition to genetic alteration, miRNAs also play an important role in the regulation of myocardial apoptosis [16]. It has been reported that the expression levels of some miRNAs are distorted in the myocardium of AMI rats, which subsequently regulates the myocardial apoptosis [17–19]. In the present study, we focused on the functional role of miR-122 in AMI. The roles of miR-122 in heart

diseases have been investigated previously. For instance, Gao et al. suggested that serum levels of miR-122 were associated with coronary artery disease [20]. Li et al. reported that plasma miR-122 levels are potentially novel biomarkers for acute coronary syndrome [21]. In addition, miR-122 has been reported to be a biomarker for AMI [11]. However, the exact role of miR-122 in AMI has not been elucidated. Thus, the effects and mechanisms of miR-122 on myocardial hypoxia injury were explored in our study. We observed that miR-122 was dramatically increased in hypoxic H9c2 cells. Therefore, we assumed that miR-122 might play a significant role in AMI. To confirm this assumption, we overexpressed and downregulated the expression of miR-122 in H9c2 cells treated with hypoxia. As expected, miR-122 knockdown enhanced cell viability and cell migration and inhibited the cell apoptosis that was induced by hypoxia. However, overexpression of miR-122 showed opposite results. These results indicate the downregulation of miR-122 protected against hypoxia-induced cell injury. Our results were similar to those of some previous studies. For example, Dong et al. reported that multiple miRNAs are unusually expressed in the early phase of AMI, and miR-21 has a protective effect on myocardial infarction by reducing cardiac cell apoptosis via its target, PDCD4 [22]. On the other hand, a study by Ren et al. used a well-established cardiac ischemia/reperfusion (I/R) model to determine the miRNA expression in ischemic hearts, and found that knockdown of endogenous miR-320 protected against I/R-induced cardiomyocyte death and apoptosis by targeting a well-studied cardioprotector, HSP20 [23]. In another study, by Xu et al., muscle-specific miRNAs, miR-1 and miR-133, regulated myocardial apoptosis in opposite ways, with miR-1 being pro-apoptotic and miR-133 being anti-apoptotic [24]. In short, miRNAs play a fundamental role in the pathophysiology of AMI.

It is well-acknowledged that PTEN/PI3K/AKT is an important signaling pathway regulating multiple biological processes, such as cell proliferation, apoptosis, and metabolism [25]. PTEN is a dual lipid and protein phosphatase, which primarily targets the lipid phosphatidylinositol-3, 4, 5- triphosphate (PIP3) [26]. PIP3 is the product of PI3K. Loss of PTEN function leads to the activation of PI3K and AKT [25]. Moreover, PI3K/AKT pathway plays a key role in heart diseases [27]. Therefore, we hypothesized that the effects of miR-122 might be involved in the regulation of the PTEN/PI3K/AKT pathway. Interestingly, our results showed that hypoxia significantly elevated the levels of PTEN but decreased the levels of p-PI3K and p-AKT, indicating that hypoxia inactivated the PI3K/AKT pathway. Furthermore, overexpression of miR-122 also inactivated the PI3K/AKT pathway, and knockdown of miR-122 reversed the results. Our study results were in line with a previous study reporting that attenuation of miR-22 derepressed PTEN and then could effectively protect cardiomyocytes from hypertrophy [28].

Autophagy is an evolutionarily conserved mechanism involving degradation of cytoplasmic components [29]. Deregulation of autophagy has been confirmed to occur in a wide range of cardiovascular pathologies. For example, increased autophagy is often found in hearts with acute and chronic ischemia and heart failure [30–34]. The rate of LC3-II/LC3-I is related to the extent of autophagosome formation [35] and beclin-1, which is a mammalian autophagy gene [36]. Moreover, p62 is a critical mediator in controlling cell death and survival, playing a significant role in autophagic degradation [37]. Our results revealed that hypoxia significantly increased the ratio of LC3-II/LC3-I and levels of beclin-1, but decreased the levels of p62, indicating that hypoxia induced the autophagy. These results were in line with some previous studies [29, 33]. In addition, our results also demonstrated that overexpression of miR-122 inhibited the autophagy pathway, while miR-122 knockdown enhanced autophagy, indicating that the cardioprotective effects of miR-122 inhibition increase the autophagy.

Concusions

To conclude, knockdown of miR-122 protects H9c2 cardiomyocytes from hypoxia-induced cardiomyocytes H9c2 injury. These effects might be by regulating the PTEN/PI3K/AKT signaling pathway and promoting autophagy. However, further studies should be performed to confirm our results.

Conflict of interest

The authors declare that there are no conflicts of interests.

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