Research Article

miR-19a protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis via PTEN/PI3K/p-Akt pathway

Guochao Sun1,*, Ying Lu2,*, Yingxia Li3, Jun Mao1, Jun Zhang4, Yanling Jin5, Yan Li6, Yan Sun1, Lei Liu1 and Lianhong Li1

1 Department of Pathology and Forensics, Dalian Medical University, No.9 West Section, Lvshun Road, Dalian 116044, China; 2Teaching Laboratory of Morphology, Dalian Medical University, No.9 West Section, Lvshun Road, Dalian 116044, China; 3Department of Spine Surgery, The Second Affiliated Hospital of Dalian Medical University, Dalian 116011, China; 4Teaching Affairs Department, Dalian Medical University, No.9 West Section, Lvshun Road, Dalian 116044, China; 5Department of Pathology, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, China; 6Department of Anatomy, Dalian Medical University, No.9 West Section, Lvshun Road, Dalian 116044, China

Correspondence: Lianhong Li (lilianhong9177@163.com)

miRNAs have been implicated in processing of cardiac hypoxia/reoxygenation (H/R)-induced injury. Recent studies demonstrated that miR-19a might provide a potential cardioprotective effect on myocardial disease. However, the effect of miR-19a in regulating myocardial ischemic injury has not been previously addressed. The present study was to investigate the effect of miR-19a on myocardial ischemic injury and identified the potential molecular mechanisms involved. Using the H/R model of rat cardiomyocytes H9C2 in vitro, we found that miR-19a was in low expression in H9C2 cells after H/R treatment and H/R dramatically decreased cardiomyocyte viability, and increased lactate dehydrogenase (LDH) release and cardiomyocyte apoptosis, which were attenuated by co-transfection with miR-19a mimic. Dual-luciferase reporter assay and Western blotting assay revealed that PTEN was a direct target gene of miR-19a, and miR-19a suppressed the expression of PTEN via binding to its 3′-UTR. We further identified that overexpression of miR-19a inhibited the expression of PTEN at the mRNA and protein levels. Moreover, PTEN was highly expressed in H/R H9C2 cells and the apoptosis induced by H/R was associated with the increase in PTEN expression. Importantly, miR-19a mimic significantly increased p-Akt levels under H/R. In conclusion, our findings indicate that miR-19a could protect against H/R-induced cardiomyocyte apoptosis by inhibiting PTEN /PI3K/p-Akt signaling pathway.

Introduction

Ischemic heart disease causes myocardial infarction, increasing the loss of cardiomyocytes, and has long been a leading cause of morbidity and mortality worldwide. Hypoxia affects mitochondrial oxidative metabolism, leading to a heart remodeling process. Reperfusion strategies are the current standard therapy for myocardial ischemia [1,2]. However, they may result in myocardial cell dysfunction and worsen tissue damage, ultimately causing a process known as ‘reperfusion injury’ [3,4]. Molecular, cellular, and tissue alterations such as cell death inflammation, neurohumoral activation, and oxidative stress are considered to have association with reperfusion injury development [5,6]. But, the exact mechanisms of reperfusion injury are not fully known [7]. Thus, the exploration of novel strategies to protect myocardial cells following ischemia/reperfusion (I/R) is necessary to improve the clinical prognosis of the ischemic heart disease patients.
Recent evidence has indicated that myocardial apoptosis contributes to myocardial I/R injury [8]. I/R induces cardiacmyocyte apoptosis, which is an important feature in the deterioration of ischemic heart disease [9,10]. Therefore, we have reason to believe that attenuating the apoptosis of cardiomyocytes effectively reduces the extent of myocardial I/R injury and the degree of heart failure [11,12].

miRNAs are small (19–25 nts), noncoding, regulatory RNAs that can negatively regulate gene expression by complementary base pairing with the 3’-UTR of target mRNAs, leading to their degradation or repressing mRNA translation [13,14]. Close to 90% of mammalian genes are considered to be regulated by miRNAs [15]. It is widely demonstrated that miRNAs play a key role in several biological processes and are major regulators of cell biology, such as cell growth, differentiation, and apoptosis, as well as fibrosis [16-18].

Recent studies have shown that the roles of miRNAs in the pathogenesis of heart diseases have attracted more attention. It has been shown that overexpression of miR-138 inhibits the activity of PI3K/Akt signaling pathways, a major cardiac gap junction protein in mice, resulting in alleviating human coronary artery endothelial cell (HCAEC) injury and inflammatory response [19]. Moreover, Ball et al. [20] reported on the role and regulation of miR-21 in aldosterone-mediated cardiac injury and dysfunction in male rats. The researchers found that miR-21 down-regulation aggravated aldosterone/salt-mediated cardiac hypertrophy, fibrosis markers gene expression, interstitial and perivascular fibrosis, and cardiac dysfunction.

miR-19a is previously confirmed to be an important regulator in heart development. Chen and Li [21] found that the plasma concentrations of miR-19a were significantly higher in pulmonary arterial hypertension (PAH) patients than in controls; circulating miR-19a was found to be a potential, independent biomarker for diagnosis of PAH. However, little information is available regarding the effects of miR-19a on hypoxic adaptation in cardiomyocytes.

In the present study, we confirmed that miR-19a expression was dramatically down-regulated in H9C2 cells during hypoxia/reoxygenation (H/R) injury. Therefore, our aim was to unravel the effects of miR-19a on apoptosis, and to investigate the underlying mechanisms in H9C2 cells.

Materials and methods
Reagents
Dulbecco’s modified Eagle’s medium (DMEM), FBS, and penicillin/streptomycin (pen/strep, 10000 U/ml each) were purchased from the Gibco Company (Life Technologies, Shanghai, China). The precursor of miR-19a, the negative control miRNA, and the transfection kit (Lipofectamine 2000) were purchased from Invitrogen (Carlsbad, CA).

Cell culture
The H9C2 was maintained in DMEM supplemented with 10% FBS and a 1% pen/strep solution at 37°C in a 5% CO2 incubator (Thermo, Waltham, MA, U.S.A.). To establish the H/R model, the culture medium was then changed to serum-free DMEM and placed into an anaerobic chamber that was purged with 94% N2, 5% CO2, and 1% O2 for 24 h. To simulate reperfusion, the cells were again treated with culture medium with a gas mixture of 95% O2, 5% CO2 at 37°C, for 3 h. Cells under normoxia throughout the experiments were included as a control [22].

miRNA transfection
Cells in exponential phase of growth were plated in six-well plates at 2 × 10^5 cells/plate and cultured overnight. Then, the cells were transfected with the miR-19a mimic (50 nM) or a negative control RNA (50 nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols. The transfected cells were harvested for RNA isolation at 24 h after transfection and protein extraction/apoptosis analysis at 48 h after transfection.

Cell count kit-8 assay
Cell viability was assessed with the cell count kit-8 (CCK-8, Sigma), according to the manufacturer’s instructions. H9C2 cells were plated in 96-well plates at 5 × 10^3 cells per well. After transfection and H/R treatment, the culture medium was replaced with 100 μl of CCK-8 solution (containing 90 μl of serum-free DMEM and 10 μl of CCK-8 reagent) and incubated for 2 h at 37°C, and then the absorbance at 450 nm was measured.

Lactate dehydrogenase assay
Cell death was assessed by the release of lactate dehydrogenase (LDH), a cytoplasmic enzyme, and a marker of membrane integrity. After treatment, samples were centrifuged at 10000 g for 10 min, and supernatants were collected for analysis using an LDH assay kit (Beyotime, China). The results were divided by the value of maximal LDH release in each group, which were expressed as cell death rate.
Table 1 Sequences for real-time qRT-PCR

| miRNA | Primers sequence |
|-------|------------------|
| miR-19a | RT: 5’-GTCGTATCCAGTGAGGTAGTATCCGCACGTCACTGATTTG-3’  (forward) 5’-CTGAGACTGGAACGCTTCACGAATTTG-3’  (reverse) 5’-GTGAGGGTTCAGTGGCGAACTT-3’ |
| U6     | RT: 5’-AAA ATATGGAAAGGGTCAAGAATTTG-3’  (forward) 5’-CTCGCTTCGGCAGGCCACCATACT-3’(reverse) 5’-ACGCTTCCGGTGTCG-3’ |

Table 2 Primer sequences for PTEN and GAPDH used for real-time qRT-PCR

| Name   | Sequence ID     | Forward sequence | Location     | Reverse sequence         | Location     | Length (bp) |
|--------|-----------------|------------------|--------------|--------------------------|--------------|-------------|
| PTEN   | XM_006526769    | CAATGGTCACAGTGAGGAATTTG  | c.1519-1533  | GGCCAATGGCTGAGGGACT      | c.1651-1636  | 163         |
| GAPDH  | XM_017321385    | ACCCCTCTTGGGACTCACTGA  | c.539-560    | TCTCCTCTGGGAAGATGTGCA    | c.677-656   | 177         |

RNA extraction and real-time quantitative RT-PCR analysis

The transfected cells were harvested for RNA isolation at 24 h post transfection. Total RNA was extracted with TRIzol Reagent (Invitrogen). For miRNA quantitative analysis, cDNA was synthesized using TaqMan MicroRNA Reverse Transcription Kit (Life Technology) using miR-19a-specific RT primers. The real-time quantitative RT-PCR (qRT-PCR) analysis for miRNAs was performed with the TaqMan Universal PCR Master Mix II by using TaqMan Small RNA Assay according to the manufacturer's instructions (Life Technology). U6 was used as an endogenous control. Primers were used as indicated in Table 1.

For mRNA quantitative analysis, cDNA was synthesized using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa). SYBR Premix Ex Taq II (TaKaRa) was used for relative quantitative real-time PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal control. Real-time qRT-PCR analysis was performed on an MX3000P real-time PCR system (Agilent). Primers were used as in Table 2.

The expression levels of miR-19a were calculated as \( \Delta C_T \) values normalizing to the U6. PTEN expression levels were normalized to the control gene(GAPDH) and were calculated by \( \Delta C_T \) method. The relative quantitation (RQ) values were plotted.

Protein extraction and Western blotting analysis

For the ectopic expression of miRNAs, the protein was extracted at 48 h after transfection. Parental and transfected cells were washed with prechilled PBS and lysed in 1× RIPA buffer (Sigma). Then extraction and solubilization were performed. Equal amounts of protein extract (50 μg) were denatured with SDS/PAGE (10%) gel. Protein abundance of GAPDH (1:500, Abgent) served as a control for protein loading. Each sample was treated with rabbit monoclonal anti-PTEN (phosphatase and tensin homolog deleted on chromosome ten) (1:500, Abcam), rabbit polyclonal anti-p-Akt (1:200, Abgent), rabbit polyclonal anti-Akt (1:200, Abgent), rabbit polyclonal anti-Bcl-2 (1:200, Abgent), rabbit polyclonal anti-BAX (1:200, Abgent), rabbit polyclonal anti-caspase-3 (1:200, ProteinTech), primary antibodies at 4°C overnight. Membranes were incubated with secondary antibody, HRP-conjugated rabbit/mouse anti-IgG (LI-COR Biosciences), diluted at 1:16000 in TBST, for 2 h at room temperature. Protein bands were detected by Odyssey infrared imaging system (LI-COR Biosciences).

Apoptosis analysis

The Annexin V–FITC/PI apoptosis detection kit was used to determine the cell apoptosis, according to the manufacturer’s instructions. After treatment, cells were harvested, washed twice with PBS, resuspended with 500 μl binding buffer, and stained with 5 μl of FITC–Annexin-V (BD Biosciences, San Jose, CA, U.S.A.) and 10 μl of propidium iodide (50 μg/ml, BD Biosciences) for 15 min at room temperature protected from light. Cells were analyzed by flow cytometry using the Aria cell sorter (BD, Franklin Lakes, NJ, U.S.A.). This experiment was repeated in triplicate.

 Hoechst 33342 staining

Morphological detection of apoptotic cells was observed using Hoechst 33342 staining. H9C2 cells were plated in six-well plates. After transfection, cells were harvested and washed twice with PBS. Ten microliter of Hoechst 33342
(10 μg/ml; Keygen Biotech, Nanjing, China) was added to each well and incubated for 30 min at 37°C protected from light, and then imaged using an inverted fluorescence microscope. Each treatment was performed in triplicate.

Vector construction and dual-luciferase reporter assay

For luciferase assays, the potential miR-19a-binding site in the PTEN 3′-UTR was predicted by miRNA target prediction databases, including Miranda, TargetScan, and PicTar. dsDNA oligonucleotides containing the miR-19a-binding sequence (wild-type) or a mismatch sequence (mutant) of the 3′-UTR of PTEN mRNAs and the HindIII and SpeI restriction site overhangs were amplified using PCR method. After annealing, double-stranded oligonucleotides were inserted into the pMIR-REPORT plasmid, downstream of the firefly luciferase reporter. Twenty-four hours before transfection, H9C2 cells were plated in the 96-well plates at 1.5 × 10⁵ cells/well in triplicate. pMIR-REPORT constructs (100 ng) together with 1 ng of Renilla luciferase plasmid phRL-SV40 (Promega) and 50 nM of miR-19a were transfected by Lipofectamine 2000 (Invitrogen). Thirty hours after transfection, cells were lysed and luciferase activity was measured by the dual-luciferase reporter assay system (Promega). Firefly luciferase activity for each condition was normalized by dividing to Renilla internal control and then compared with empty vector pMIR-REPORT.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde, blocked with 10% normal goat serum (Gibco Laboratories), and incubated with antibody anti-PTEN (dilution 1:100) or anti-p-Akt (dilution 1:100) overnight at 4°C in a humidified container. Immunolabeling was revealed by PE-conjugated or FITC-conjugated secondary antibodies. Immunofluorescence was observed under a laser scanning confocal microscope (Leica, Solms, Germany).

Statistical analysis

All experiments were performed in triplicate at a minimum. The data were presented as the means ± S.D. and analyzed by one-way ANOVA, followed by all pair-wise multiple comparison procedures using Bonferroni’s test. A P-value of <0.05 was considered statistically significant. The statistical analysis was performed with SPSS version 20.0 (SPSS, Chicago, IL, U.S.A.).

Results

miR-19a expression in H9C2 after H/R

To identify the potential effect of miR-19a in myocardial I/R injury, we measured the expression of miR-19a in H9C2 cells after 24-h hypoxia and 3-h reoxygenation. Expression of miR-19a was overtly reduced by reaching an inhibition of 40.48% in H9C2 after H/R treatment compared with normoxia group (P < 0.05) (Figure 1). This finding raised the possibility that miR-19a may play an important role in H/R injury of H9C2 cells.

Effect of miR-19a on cell survival in H/R-induced cardiomyocyte injury

We examined the role of miR-19a up-regulation in H/R-induced cardiomyocyte injury. CCK-8 assay was used to test cell growth and LDH release is a sign of cellular injury. We observed that cell viability was decreased in the H/R group,
Figure 2. Effects of miR-19a on H/R-induced injury in H9C2 cells

(A) After transfection with miR-19a mimic, cell viability was measured by CCK-8. (B) The level of LDH release. Data are expressed as mean ± S.D. (n=3). *P<0.05 compared with the control group; †P<0.05 compared with the H/R + NC group.

as shown in Figure 2A, and the H/R group had a lower percentage of viable cells compared with the control group (57.7 ± 9.6% compared with 101.2 ± 9.7%, *P<0.05), whereas cells treated with the miR-19a mimics efficiently promoted H9C2 cell growth and exhibited an amelioration of the cell loss induced by H/R injury (*P<0.05) (Figure 2A). As shown in Figure 2, the (H/R + miR-19a) group had a higher percentage of viable cells compared with the H/R group (85.9 ± 7.6% compared with 56.2 ± 5.6%, P<0.05). The results of the CCK-8 assay confirmed the anti-H/R-induced cardiomyocyte injury role of miR-19a.

Cell death rate was measured by LDH release. After 24-h exposure to hypoxia and 3-h reoxygenation, the LDH release was increased in the culture medium (Figure 2B), the relative amount of LDH release reached 133.6% of that in control group (*P<0.05) (Figure 2B), and the miR-19a up-regulation group significantly decreased LDH release (*P<0.05 compared with H/R + NC group). Together, these data indicated that miR-19a ameliorates H/R-induced cardiomyocyte injury.

miR-19a reduces H/R-induced cell apoptosis

Because miR-19a expression was inhibited by H/R in H9C2 cells and we observed that cell viability was decreased in the H/R group, whereas overexpression of miR-19a inhibited the reduction in the cell induced by H/R injury, we wondered whether miR-19a protected cardiomyocyte against H/R-induced cell apoptosis. Flow cytometry revealed greater apoptosis with H/R than control treatment in H9C2 cells; transfection with miR-19a mimic significantly decreased the apoptosis rate induced by H/R as compared with H/R alone (Figure 3A).

As similar to flow cytometry results, with Hoechst 33342 staining, changes in cell morphology indicating apoptosis were observed in cells. Treatment with H/R resulted in the characteristic morphologic indicators of apoptosis, including nuclear condensation and fragmentation (Figure 3B), but the ratio of cells with nuclei stained by Hoechst 33342 was reduced in the miR-19a mimic group (*P<0.05 compared with H/R + NC group).

To further investigate the molecular mechanisms involved, the apoptosis-related proteins Caspase-3, Bcl-2, and Bax were detected by Western blotting. As shown in Figure 3C, the H/R group showed an approximate 1.67-fold increase in Caspase-3 expression compared with the control group (*P<0.05 compared with control group), which was reduced to 1.46-fold when the cells were treated with the miR-19a mimics (*P<0.05 compared with H/R + NC group). In addition, H/R stimulation increased the expression of Bax and down-regulated the expression of Bcl-2. Overexpression of miR-19a markedly reversed the effect on the expression levels of Bcl-2 and Bax in the cells exposed to H/R. Figure 3C shows a higher ratio of Bcl-2 to Bax in the (miR-19a + H/R) group than in the (H/R + NC) group (P<0.05).

PTEN is a potential target of miR-19a

In order to elucidate the underlying molecular mechanism, we performed a bioinformatic analysis using ‘TargetScan’. miRNA target prediction program revealed PTEN as one of the possible target genes of miR-19a. We found that PTEN contained theoretical miR-19a-binding site in its 3’-UTR (Figure 4A). To confirm this hypothesis, we constructed a reporter vector consisting of the luciferase-coding sequence followed by the 3’-UTR of PTEN (wild-type and mutant type) and co-transfected miR-19a mimics with the vector in 293T cells. As shown in Figure 4B, miR-19a mimics was significantly reduced the activity of the luciferase reporter fused with the PTEN 3’-UTR compared with control group (*P<0.05). We also constructed a vector in which miR-19a-binding sites were all mutated; miR-19a mimics failed to decrease the activity of luciferase gene with mutant 3’-UTR (Figure 4B,C). Furthermore, transfection of miR-19a
resulted in significant reduction in PTEN mRNA and protein expression by real-time RT-PCR and Western blotting analysis (Figure 4D,E). All these findings suggested that miR-19a inhibited PTEN expression by directly binding to its 3′-UTR.

**Effect of miR-19a on PTEN/p-Akt pathway in H/R cardiomyocytes**

PTEN is traditionally known to generate effects via suppression of p-Akt. We sought to examine the significance of miR-19a-mediated regulation of the PTEN/PI3K/p-Akt signaling pathway in H9C2 cells during H/R injury. The proteins about PTEN/PI3K/p-Akt signaling pathway including PTEN, p-Akt expressions were quantitated by qRT-PCR and Western blotting analysis. As Figure 5 shows, H/R stimulation induced a distinct increase in the expression of PTEN both in protein and mRNA levels. Moreover, we observed that the expression of p-Akt protein level was restrained after H/R stimulation. After transfection of miR-19a mimic, the expression of PTEN decreased, while the p-Akt expression was increased (Figure 5A,B). Furthermore, we detected the expression of PTEN and p-Akt in the cells by double color immunofluorescence. As the Figure shows, the expression of PTEN was decreased and p-Akt increased after miR-19a up-regulation (Figure 5C). In summary, the results implied that miR-19a can signal through the PTEN/Akt axis in the H9C2 cells during H/R injury.

**Discussion**

Abnormal expression of various miRNAs has been reported in the pathogenesis of cardiovascular diseases. Recent evidence suggests that miRNAs are involved in apoptosis and other injuries in myocardial cells induced by H/R [23–25]; miRNAs such as miR-1, miR-15b, and miR-21 have been implicated in modulating the survival and recovery of myocardial I/R injury due to their effects on key genes associated with apoptosis [26–29]. In addition, Du et al. [30]
Figure 4. **miR-19a targets PTEN**

(A) The potential binding site for **miR-19a** in the 3’-UTR of PTEN mRNA. (B) Luciferase reporter assay was performed by co-transfection of 293T cells with luciferase reporter containing the 3’-UTR of rat PTEN with **miR-19a** mimic. (C) Transfection of **miR-19a** inhibited luciferase activity of 3’-UTR-PTEN (wild-type, wt) and such inhibition was absent with mutations in the **miR-19a**-binding site (mutant, mut). The impact of **miR-19a** on PTEN expression was normalized and compared with the negative miRNA (*n*=3), *P*<0.05 compared with the wt + negative miRNA group. (D) Western blot analysis of protein level of PTEN with **miR-19a** mimic. (E) qRT-PCR analysis of mRNA level of PTEN with **miR-19a** mimic. Data are expressed as mean ± S.D. (*n*=3). *P*<0.05 compared with the control group.

demonstrated that up-regulation of **miR-22** contributed to I/R-induced mitochondrial oxidative damage and cell damage through targeting Sirt1 and PGC1α in vitro. Furthermore, they first clarified the protective effect of **miR-22** inhibition against myocardial I/R injury by modulating mitochondrial oxidative damage both in vitro and in vivo. He et al. [31] discovered that **miR-138** was up-regulated by hypoxia in cardiomyocytes and its up-regulation was beneficial for cardiomyocyte survival, and they also indicated that modulation of MLK3/JNK/c-jun signaling pathway was one potential mechanism by which it attenuated hypoxia-induced apoptosis effects.

Here, we explored the expression changes of **miR-19a** during myocardial H/R injury. Zhong et al. [32] revealed that the levels of plasma **miR-19a** in acute myocardial infarction (AMI) were 120-fold higher than control group
Figure 5. Regulation of the PTEN/p-Akt pathway by miR-19a

(A) Real-time PCR analysis of PTEN in H9C2 cells. The cells under different conditions. (B) Protein levels of PTEN, p-Akt, and total Akt were measured by Western blot analysis in H9C2 cells, accompanied by a quantitative bar graph. As an internal control, GAPDH was used for normalization. (C) The expression of PTEN and p-Akt in H9C2 cells detected by immunofluorescence. *P<0.05 H/R group compared with control group. #P<0.05 H/R + miR-19a group compared with H/R + NC group. Values are means ± S.E.M.

and reached the level of a highly detectable. Therefore, there is a close association of circulating miR-19a levels with susceptibility to AMI, and has a highly predictive and distinguishing ability.

In our studies, it was found that H/R injury resulted in reduction by 47.5% of the expression of miR-19a after 24-h hypoxia and 3-h reoxygenation as compared with controls. Therefore, miR-19a is an H/R-induced myocardial cell injury related miRNA in cardiomyocyte.

Further, we explored the potential role of miR-19a in H/R-induced myocardial cell injury. We investigated the protective effect of miR-19a against H/R-induced cell injury by CCK-8 assay and LDH assay. Results from the experiments showed that H/R treatment significantly decreased cell viability and increased LDH release in the culture medium, whereas overexpression of miR-19a efficiently promoted cell growth and decreased LDH release. Our study demonstrated that H/R-induced injury of H9C2 cells was significantly inhibited by miR-19a pretreatment.

Accumulating investigation indicates that cardiomyocyte apoptosis contributes critically to cardiac H/R injury [33]. In addition to working as the primary pathologic factors that lead to cardiomyocyte damage, apoptotic cell death plays a crucial role during myocardial H/R injury. Moreover, immature myocardial cells are more likely to cause hypoxia injury [34]. It is well known that apoptosis is regulated by apoptosis-related proteins, which are mainly divided into two categories: proapoptotic substrates (such as Bax and Bak) and anti-apoptotic substrates (such as Bcl-2 and Bcl-xL).
After a series of injuries, the destruction of the balance between proapoptotic and anti-apoptotic proteins is the key factor for cell survival or death [35]. In addition, caspase-3 is the most critical apoptotic protease and is also one of the important markers of apoptosis [36].

In the present study, we found that the protein levels of caspase-3 were increased and the proportion of Bcl-2/Bax was decreased by H/R injury. Moreover, flow cytometry revealed that the H/R-treated cells showed greater apoptosis than control, whereas miR-19a pretreatment reversed these effects. So, our results showed that H/R injury resulted in increase in apoptosis and overexpression of miR-19a may be responsible for decreasing apoptosis and protecting cardiomyocyte during H/R injury, which is consistent with the results of other studies [37,38].

Signal transduction pathways involving PTEN, PI3K, and p-Akt are known to be associated with apoptosis and play an important role in the control of cardiomyocyte survival and function [39]. Activation of this pathway may be useful in promoting myocyte survival in the damaged heart [40]. The PI3K has the ability to phosphorylate PIP3 into the secondary messenger PIP2 and activate Akt. The activation of the Akt-related signaling pathway has been related to myocardial cell apoptosis [41,42]. Recent studies demonstrated that the activation of PI3K and its downstream target Akt could inhibit cardiomyocyte apoptosis and promote cell survival in ischemic heart [43,44]. In 2000, Fujio et al. [45] found that PI3K/AKT activation was involved in left ventricular functional reserve, reduction in apoptotic cardiomyocytes, and limitation of infarct size.

Moreover, the activation of Akt may produce its anti-apoptotic function via activating the downstream substrates such as Bcl-2, Bax, caspase, eNOS, and p70s6k [46]. While the most important mechanism of Akt activated is the absence of PTEN expression [47,48].

PTEN is a frequently inactivated tumor suppressor in human cancer, depresses the PI3K pathway, and prevents Akt activation [49]. Previously, studies have found that PTEN plays an important role in myocardial remodeling, myocardial fibrosis, cardiac hypertrophy, and myocardial ischemia reperfusion injury [50-52].

According to bioinformatic analyses, PTEN was regarded as a target of miR-19a. In our experiment, such a prediction was affirmed by a dual luciferase reporter assay. Therefore, we can assume that PTEN is a functional target gene of miR-19a involved in protecting cardiomyocyte injury in myocardial I/R. We verified that overexpression of miR-19a inhibited PTEN expression and initiated the PI3K/Akt pathway, resulting in down-regulation of apoptosis. Moreover, in the present study we found that the protein and mRNA levels of PTEN were increased and the p-Akt was decreased by H/R injury, whereas miR-19a pretreatment reversed these effects. So, our results showed that miR-19a can regulate via a PTEN/Akt pathway in H9C2 cells, which inhibits cardiomyocyte apoptosis during H/R injury.

In summary, the present study provided evidence that miR-19a can decrease cardiomyocyte apoptosis induced by H/R via inhibiting PTEN and increasing nuclear AKT. PTEN may be the target gene of miR-19a against H/R-mediated cardiomyocyte apoptosis. miR-19a may be a potential drug target for treating cardiomyocyte I/R injury. Further research in human clinical trials is necessary to pave the way for miR-19a ultimate application in the clinic to benefit myocardial I/R patients.

Competing interests
The authors declare that there are no competing interests associated with the manuscript.

Author contribution
G.S. and Ying L. conceived of and performed all the experiments, analyzed the data, and drafted the manuscript. Yingxia L. was responsible for the molecular biology experiments. J.M. performed the cell experiments and analyzed the data. J.Z. revised the manuscript. Y.J. contributed essential reagents and tools. Yan L. analyzed the data. Y.S. and Lei L. revised the manuscript. Lianhong L. conceived of the research, co-ordinated the study, interpreted the data, and revised the manuscript. All authors read and approved the final manuscript.

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Abbreviations
AMI, acute myocardial infarction; CCK-8, cell count kit-8; Bax, Bcl-2 Associated Protein; Bcl-2, B-cell lymphoma-2; DMEM, Dulbecco’s modified Eagle’s medium; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H/R, hypoxia/reoxygenation; HRP, Horseradish Peroxidase; I/R, ischemia/reperfusion; LDH, lactate dehydrogenase; PAH, pulmonary arterial hypertension; PGC1α, Peroxisome proliferator-activated receptor coactivator-1α; pen/strep, penicillin/streptomycin; PI, Propidium Iodide; qRT-PCR, quantitative RT-PCR; p70s6K, p70 ribosomal protein S6 kinase;
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