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Original Article

Upregulation of miR-335 ameliorates myocardial ischemia reperfusion injury via targeting hypoxia inducible factor 1-alpha subunit inhibitor

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Abstract: MicroRNA-335 (miR-335) is implicated in several pathophysiological processes, including tumorigenesis, lipid metabolism and ischemic stroke; however, whether miR-335 plays a role in modulating myocardial ischemia reperfusion injury (MIRI) is still unknown. This study is aimed to explore the role and mechanism of miR-335 in the pathophysiological process of MIRI. Specifically, miR-335 mimics or a chemically modified agomiR-335 were transfected or injected into H9c2 cells and Wistar rats to upregulate miR-335 expression *in vitro and in vivo*, respectively. The effects of miR-335 overexpression on hypoxia/reoxygenation (H/R)-treated cardiomyocytes and ischemia/reperfusion (I/R)-exposed heart samples were investigated by a Cell Counting Kit-8 assay, flow cytometry, TTC staining and a TUNEL assay. The target of miR-335 was identified using a luciferase reporter assay. The expression of heme oxygenase 1 (HO-1) and inducible nitric oxide synthase (iNOS) was detected by reverse transcription-quantitative polymerase chain reaction and western blotting. The results showed that miR-335 expression in cardiomyocytes and the myocardium was downregulated during MIRI but was induced by hypoxic/ischemic postconditioning. MiR-335 overexpression led to an increase in cell viability and a reduction in the apoptosis of H/R-treated cardiomyocytes. Meanwhile, myocardial infarct size and the apoptosis of I/R-exposed heart tissues were decreased in response to miR-335 upregulation. Furthermore, we identified that hypoxia inducible factor 1-alpha subunit inhibitor (HIF1AN), a suppressor of hypoxia inducible factor 1-alpha (HIF-1α) stabilization and transcriptional activity, is a novel target of miR-335. MiR-335 overexpression enhanced the transcriptional activity of HIF-1α, increased the expression of HO-1 and iNOS, and inhibited mitochondrial permeability transition pore (MPTP) opening. In conclusion, we are the first to demonstrate that upregulation of miR-335 ameliorates MIRI by targeting HIF1AN. Thus, miR-335 may be a new therapeutic target for the treatment of MIRI.

Keywords: MicroRNA, myocardial ischemia reperfusion injury, hypoxia inducible factor 1-alpha, mitochondrial permeability transition pore, apoptosis

Introduction

Acute myocardial infarction (AMI) is a leading cause of death and disability worldwide [1]. When a patient suffers from acute ST-segment elevation myocardial infarction (STEMI), one of the most serious types of AMI, timely and effective myocardial reperfusion using thrombolytic therapy or primary percutaneous coronary intervention is considered as an optimal approach to reduce acute myocardial ischemic injury and save ischemic myocardium [2]. However, the reperfusion of ischemic myocardium can induce cardiomyocyte death and aggravate myocardial ischemic injury, which is termed as myocardial ischemia reperfusion injury (MIRI) [3]. Although the phenomenon of MIRI has been reported in the past few decades, there is still no effective therapy for preventing MIRI [3].

MicroRNA (miRNA/miR), a small noncoding RNA molecule of approximately 22 nucleotides in length, can regulate gene expression at the post-transcriptional level [4]. It is estimated that over thirty percent of genes in the human genome are controlled or regulated by miRNAs [5]. Interestingly, certain miRNAs have been found to be dysregulated in the process of MIRI.

Meanwhile, the gain or loss of function of miRNAs could alleviate or aggravate MIRI [6]. For instance, the expression of miR-29 is significantly increased in ischemia-reperfusion-injured myocardium and the downregulation of microRNA-29 by an antisense inhibitor provided protection against MIRI [7]. In contrast, the expression of miR-1 [8], miR-133a [8] and miR-320 [9] are significantly decreased; upregulation of theses miRNAs protects against MIRI. Therefore, miRNAs appear to be a novel therapeutic target for the treatment of MIRI.

MiR-335, a well-reported miRNA, has been implicated in several pathophysiological processes. Lynch *et al.* reported that miR-335 is a master regulator of neuroblastoma cell migration and invasion by directly targeting the formin family of actin nucleators [10]. Nakanishi *et al.* found that miR-335 is related to lipid metabolism in the liver and white adipose tissue of genetically obese mice [11]. Of note, Zhao *et al.* demonstrated that miR-335 regulates the pathophysiological process of ischemic stroke by targeting calmodulin [12]; however, whether miR-335 plays a role in the pathophysiological process of MIRI remains unknown.

In the present study, we explored the regulatory role and mechanism of miR-335 in the pathophysiological process of MIRI. The results showed that miR-335 was significantly downregulated in cardiomyocytes and heart tissues under the condition of MIRI, and overexpression of miR-335 alleviated MIRI in cardiomyocytes and isolated rat hearts. Mechanistically, we demonstrated that overexpression of miR-335 enhanced the transcriptional activity of hypoxia inducible factor (HIF)-1α, upregulated the gene expression of heme oxygenase 1 (HO-1) and inducible nitric oxide synthase (iNOS), and inhibited the opening of mitochondrial permeability transition pore (MPTP) by targeting HIF-1α subunit inhibitor (HIF1AN).

Materials and methods

Cell culture and dimethyloxalyl glycine (DMOG) treatment

A rat myocardial cell line (H9c2) obtained from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China) was cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; TBD, Tianjin, China), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere at 37°C with 5% CO_2. Cells were treated with 0.5 mM DMOG, a known activator of hypoxia inducible factor 1-alpha (HIF-1α) [13], 24 h before hypoxia to stabilize HIF-1α protein expression levels as previously described [14, 15].

Cell transfection

Specifically, miR-335 mimics and mimics NC (negative control) were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were transfected with the miRNAs using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cells were harvested and processed for further analysis after 24 h or 48 h of transfection.

Generating the hypoxia/reoxygenation (H/R) model

H9c2 cells were subjected to H/R to induce a model of MIRI *in vitro*. Specifically, cells were treated upon attaining 80-90% confluence. The normal culture medium was removed and replaced with Earle's medium without glucose and FBS, and cells were cultured in a tri-gas incubator with 90% N_2, 5% CO_2 and 5% O_2 at 37°C for 6 h to induce hypoxia. Subsequently, Earle's medium was removed and the cells were cultured with normal medium in an incubator with 5% CO_2 at 37°C for 3 h of reoxygenation. Hypoxic postconditioning (HPoC), comprising 3 cycles of 5 min of reoxygenation and 5 min of hypoxia, was immediately performed after 6 h of hypoxia, followed by 2.5 h of reoxygenation.

CCK-8 assay

Cell viability was determined with a CCK-8 assay using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. The absorbance was detected at 450 nm using an ultramicro microporous plate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA).

Lactate dehydrogenase (LDH) assay

LDH released from cardiomyocytes into the culture medium was determined by using an LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols. The absorbance value was detected at 450 nm by an ultramicro microporous plate spectrophotometer (Biotek Instruments, Inc.).

Animals

A total of 30 healthy male Wistar rats, weighting 250 ± 20 g, were purchased from Changsheng Bio-Technology Co., Ltd. (Shenyang, China). All rats were treated and employed according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA). The experimental protocol was approved by the Institutional Ethics Committee of China Medical University (Shenyang, China).

Overexpression of miR-335 in rat myocardium using agomiR-335

Chemically modified agomiR-335 and a negative control, designed and synthesized by Shanghai GenePharma Co., Ltd., were used to upregulate miR-335 expression *in vivo*. Specifically, agomiR-335 and the negative control, at a dose of 80 mg/kg per day, were injected into rats through the tail vein for 3 consecutive days. After 24 h following the last injection, miR-335 expression in the left ventricular myocardium was detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to examine the effects of agomiR-335.

Induction of the MIRI model

Heart samples were prepared as described previously [16]. In brief, pentobarbital sodium at a dose of 100 mg/kg was administered intravenously to anesthetize rats. Meanwhile, heparin (1,500 IU/kg) was injected intravenously to prevent intracoronary clot formation. After opening the thoracic cavity, the heart was swiftly removed and immediately immersed in ice cooling heparinized Krebs-Henseleit (K-H) solution [17]. Then, the isolated heart was suspended on a Langendorff apparatus from the root of the aorta followed by perfusion with K-H solution saturated with 95% O_2+5% CO_2 at a constant temperature of 37°C. All isolated rat hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion to induce a model of MIRI. Ischemic postconditioning (IPoC), comprising 3 cycles of 0.5 min of reperfusion and 0.5 min of ischemia, was immediately performed after 30 min of ischemia, followed by 117 min of reperfusion.

Damage to myocardial submicroscopic structure as determined by electron microscopy

At 120 min of reperfusion, 1 mm × 1 mm × 1 mm of cardiac tissue was isolated from the left ventricle and prepared into sections for electron microscopy as previously described [17]. The changes in myocardial submicroscopic structure were observed by transmission electron microscopy (JEM-1200EX, JEOL, Japan).

HE staining

At the end of reperfusion, the heart was collected for analysis. The heart samples were subject to routine fixation, dehydration and paraffin embedding and were subsequently prepared into 4 um paraffin sections. For HE staining, the paraffin sections were successively de-waxed with xylene, hydrated by a descending series of ethanol and stained with hematoxylin-eosin. The sections were dehydrated via an ascending series of ethanol, cleared by xylene and finally mounted by neutral balsam. The dyed sections were observed under a light microscope (Olympus BX51, Olympus Corporation Tokyo, Japan) to assess the pathological changes of the myocardium.

Measurement of infarct size

The infarct size of the myocardium was determined by TTC staining as described previously [16]. Briefly, at the end of reperfusion, the hearts were removed and frozen at -20°C for 1 h. The frozen samples were rapidly cut into 1-2 mm sections and incubated in 1% TTC at 37°C for 20 min, followed by fixation in methanol overnight. Viable myocardium was stained red and infarcted myocardium was unstained. The myocardial sections were then photographed using a digital camera. Myocardial infarct size was expressed as the ratio of infarct areas to that of the whole area.

Table 1. The information of primer sequences

Name	Sequences (5' to 3')
miR-335 forward	GCGGTCAAGAGCAATAACGAA
miR-335 reverse	GTGCAGGGTCCGAGGTATTC
HIF1AN forward	TGCAGCAAACACTCAATGACACCG
HIF1AN reverse	AGTGAGCAGGTGTCACATTCCCTT
HO-1 forward	ACAGGTTGACAGAAGAGGCTAA
HO-1 reverse	AACAGGAAGCTGAGAGTGAGG
iNOS forward	CAGGACCACACCCCCTAGGA
iNOS reverse	AGCCACATACCGAGCCATGC

Measurement of apoptosis

Myocardial apoptosis was detected by a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the In Situ Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. Apoptotic cells were observed under a light microscope and photographed.

Cell apoptosis was determined using an Annexin V-Fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) kit (Nanjing KeyGen Biotech Co., Ltd., Jiangsu, China) according to the manufacturer's instructions for flow cytometry analysis (BD Biosciences, Franklin Lakes, NJ, USA).

Sensitivity of MPTP to calcium

The mitochondria were isolated from the cell lysates using the Cell Mitochondria Isolation Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. The sensitivity of MPTP to calcium was determined using the Purified Mitochondrial Membrane Pore Channel Colorimetric Assay kit (GENMED, Shanghai, China) according to the manufacturer's instructions.

RT-qPCR

Total RNA was extracted from cardiomyocytes and heart tissues with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). To detect mRNA expression, the PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan) and SYBR Premix Ex Taq II (Takara Bio, Inc.) were used for reverse transcription and quantitative PCR according to the manufacturer's instructions. β actin was used as an internal control. To detect miR-335 expression, Mir-X™ miRNA First Strand Synthesis Kit and Mir-X™ miRNA qRT-PCR SYBR® Kit were used for reverse transcription and quantitative PCR according to the manufacturer's instructions. U6 was used as an internal control. All oligonucleotide primers were designed by Sangon Biotech Co., Ltd. (Shanghai, China) (**Table 1**). The relative expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Luciferase activity assay

Luciferase reporter plasmids (pmirGLO-HIF1-AN-wt and pmirGLO-HIF1AN-mut) were obtained from Shanghai GenePharma Co., Ltd. The cells were seeded in a 24-well plate and transfected with pmirGLO-HIF1AN-wt or pmirGLO-HIF1AN-mut, together with miR-335 mimics or a negative control. At 48 h post-transfection, the luciferase activity was analyzed using the Dual-Glo Luciferase Assay System (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

Measurement of HIF-1α activity

To measure the activity of HIF-1α, cells were harvested after transfection with miR-335 mimics and mimics NC, and the nuclear extract lysates were obtained from harvested cells using the Nuclear Extraction kit (cat. no. ab113474; Abcam, Cambridge, UK), according to the manufacturer's protocols. The activity of HIF-1α in nuclear extract lysates was detected using the HIF-1 alpha Transcription Factor Assay Kit (cat. no. ab133104; Abcam) according to the recommended experimental protocol.

Immunohistochemistry (IHC) analysis

The heart tissues were fixed in 4% polyoxymethylene, embedded in paraffin, and cut into 3.5 μm-thick sections. The sections were immersed in 0.01 M citrate buffer (pH=6.0) and antigen retrieval was performed using a microwave. After inhibiting endogenous peroxidase activity with 3% H_2O_2 and blocking nonspecific reactions with 10% goat serum, the sections were incubated with HIF1AN (1:1000; Abcam), anti-HO-1 (1:1000; Abcam) and anti-iNOS (1:1000; Abcam) primary antibodies overnight at 4°C, followed by incubation with an horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The sections were stained with 3,3-diaminobenzidine (DAB) solution (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and counterstained with hematoxylin.

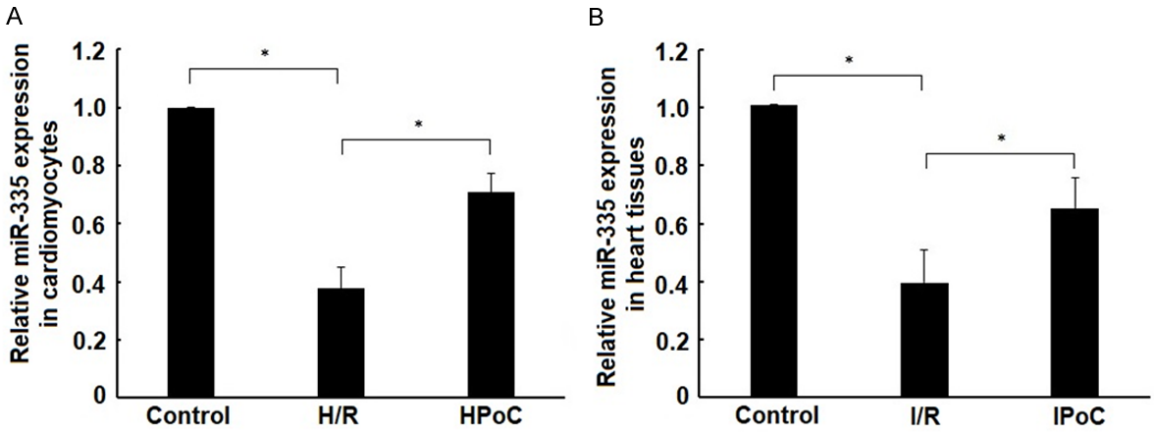

Figure 1. MicroRNA (miR)-335 expression during myocardial ischemia reperfusion injury. A. H9c2 cells were subjected to 6 h of hypoxia, followed by 3 h of reoxygenation (H/R). Hypoxic postconditioning (HPoC), comprising 3 cycles of 5 min of reoxygenation and 5 min of hypoxia, was immediately performed after 6 h of hypoxia, followed by 2.5 h of reoxygenation. Cells underwent 9 h of normoxia as a control. The relative expression of miR-335 in cardiomyocytes was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). B. The isolated rat hearts were subjected to 30 min of global ischemia, followed by 120 min of reperfusion (I/R). Ischemic postconditioning (IPoC), comprising 3 cycles of 0.5 min of reperfusion and 0.5 min of ischemia, was immediately performed after 30 min of ischemia, followed by 117 min of reperfusion. The isolated rat hearts were continuously perfused with K-H solution without ischemia as a control. The relative expression of miR-335 in heart tissues was determined by RT-qPCR (n=6 per group). $*P < 0.05$.

Western blot analysis

The proteins were extracted from cell lysates using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology) and the protein concentration was measured by an Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Heat-denatured proteins were separated by SDS-PAGE and then transferred to PVDF membranes, followed by blocking with 1% bovine serum albumin (BSA) solution for 1 h. Subsequently, the membranes were incubated with primary antibodies, including anti-HIF1AN (1:1000; Abcam), anti-HO-1 (1:1000; Abcam), anti-iNOS (1:1000; Abcam), anti-cytochrome c (1:1000; Abcam), anti-cleaved caspase-9 (1:1000; Abcam), anti-cleaved caspase-3 (1:1000; Abcam) and anti-β actin (1:2000, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) at 4°C overnight. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin G (1:4000; Earth-Ox Life Sciences, Millbrae, CA, USA) at room temperature for 30 min. The detection of protein bands was performed by using an enhanced chemi-luminescence (ECL) for western blotting kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. β actin was used as an internal control. Relative expression levels were determined using Image J2x analysis software (National Institutes of Health).

Statistical analysis

The data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed with SPSS software, version 17.0 (SPSS, Inc., Chicago, USA). Differences between groups were first evaluated using one-way analysis of variance (ANOVA); if the differences were significant, multiple comparison analysis was then performed using Fisher's Least Significant Difference (LSD) test. All *P* values less than 0.05 were considered statistically significant.

Results

The expression of miR-335 in hypoxia/reoxygenation (H/R)-treated cardiomyocytes and ischemia/reperfusion (I/R)-exposed heart tissues

The results of RT-qPCR showed that the expression of miR-335 was significantly downregulated in H/R-exposed cardiomyocytes (**Figure 1A**) and I/R-treated heart tissues (**Figure 1B**) compared with normoxic cardiomyocytes and non-ischemic myocardium, respectively ($P < 0.05$). Moreover, we found that the expression of miR-335 in cardiomyocytes and heart tissues was

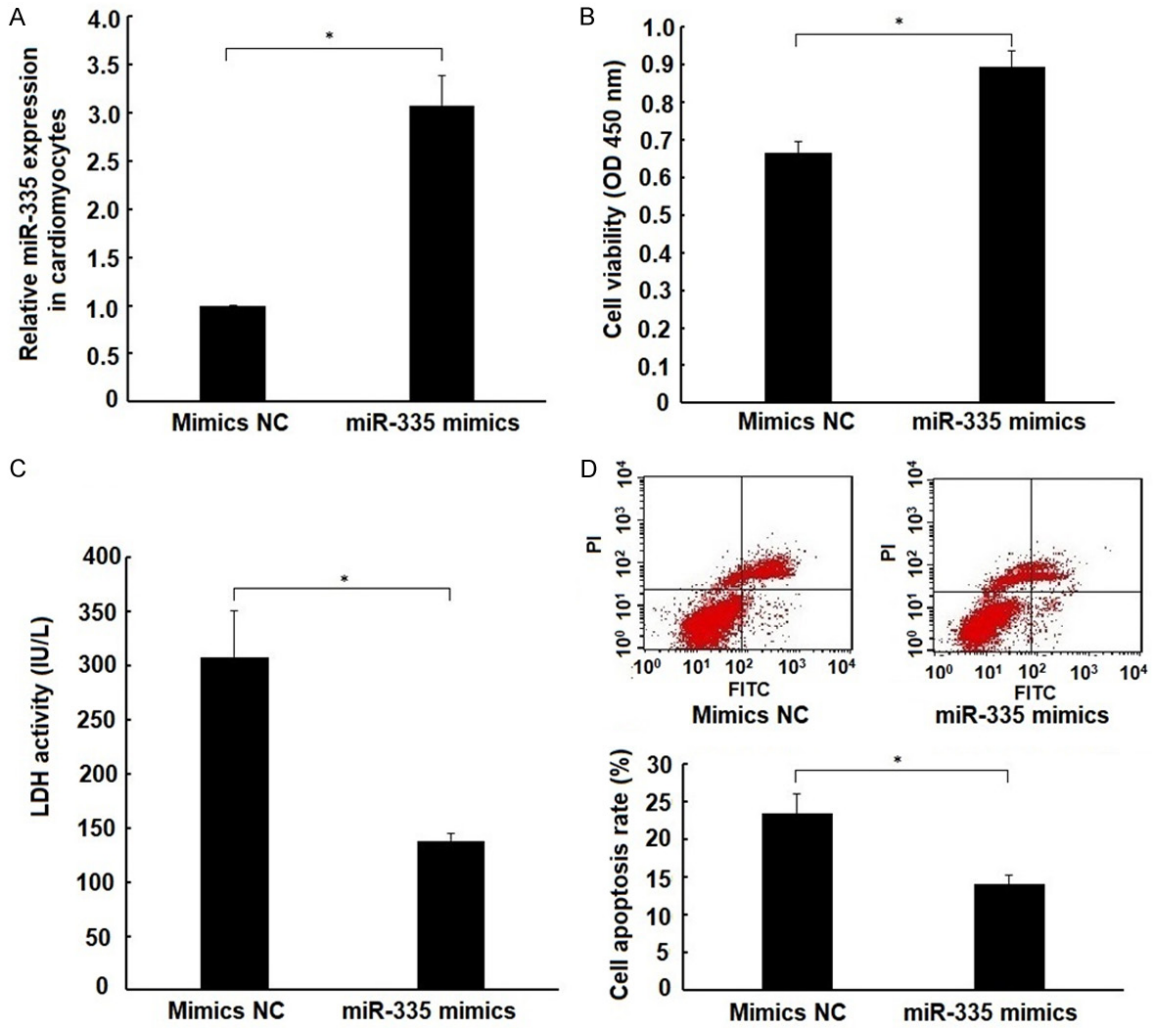

Figure 2. The effect of microRNA (miR)-335 overexpression on hypoxia/reoxygenation (H/R)-induced cardiomyocyte injury *in vitro*. (A) The expression of miR-335 in H9c2 cells was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) after transfection with miR-335 mimics or a negative control (mimics NC). After 24 h of transfection, cells were subjected to 6 h of hypoxia, followed by 3 h of reoxygenation. Then, cell viability was determined by a Cell Counting Kit-8 (CCK-8) assay (B). (C) The lactate dehydrogenase (LDH) activity in culture medium was measured by spectrophotometry. (D) Cell apoptosis was detected by flow cytometry. Data are presented as the mean ± standard deviation (SD) from three independent experiments. *$P < 0.05$.

relatively upregulated by HPoC and IPoC compared with H/R-treated cardiomyocytes and I/R-exposed heart tissues, respectively ($P < 0.05$) (**Figure 1A**, **1B**).

Overexpression of miR-335 protected against H/R-induced cardiomyocyte injury in vitro

We found that miR-335 expression was increased by nearly 3-fold in rat myocardial cells (H9c2 cells) after transfection with miR-335 mimics compared with transfection of negative control (NC), suggesting that miR-335 mimics effectively induced miR-335 expression in cardiomyocytes (**Figure 2A**). Additionally, we observed that overexpression of miR-335 in H9c2 cells led to an increase in cell viability (**Figure 2B**), and reductions in lactate dehydrogenase (LDH) activity (**Figure 2C**) and the rate of apoptosis (**Figure 2D**) ($P < 0.05$). Taken together, the results suggested that overexpression of miR-335 protected against H/R-induced cardiomyocyte injury *in vitro*.

Overexpression of miR-335 protected against I/R-induced myocardial injury in isolated rat hearts

We found that miR-335 expression in rat myocardium was significantly increased following

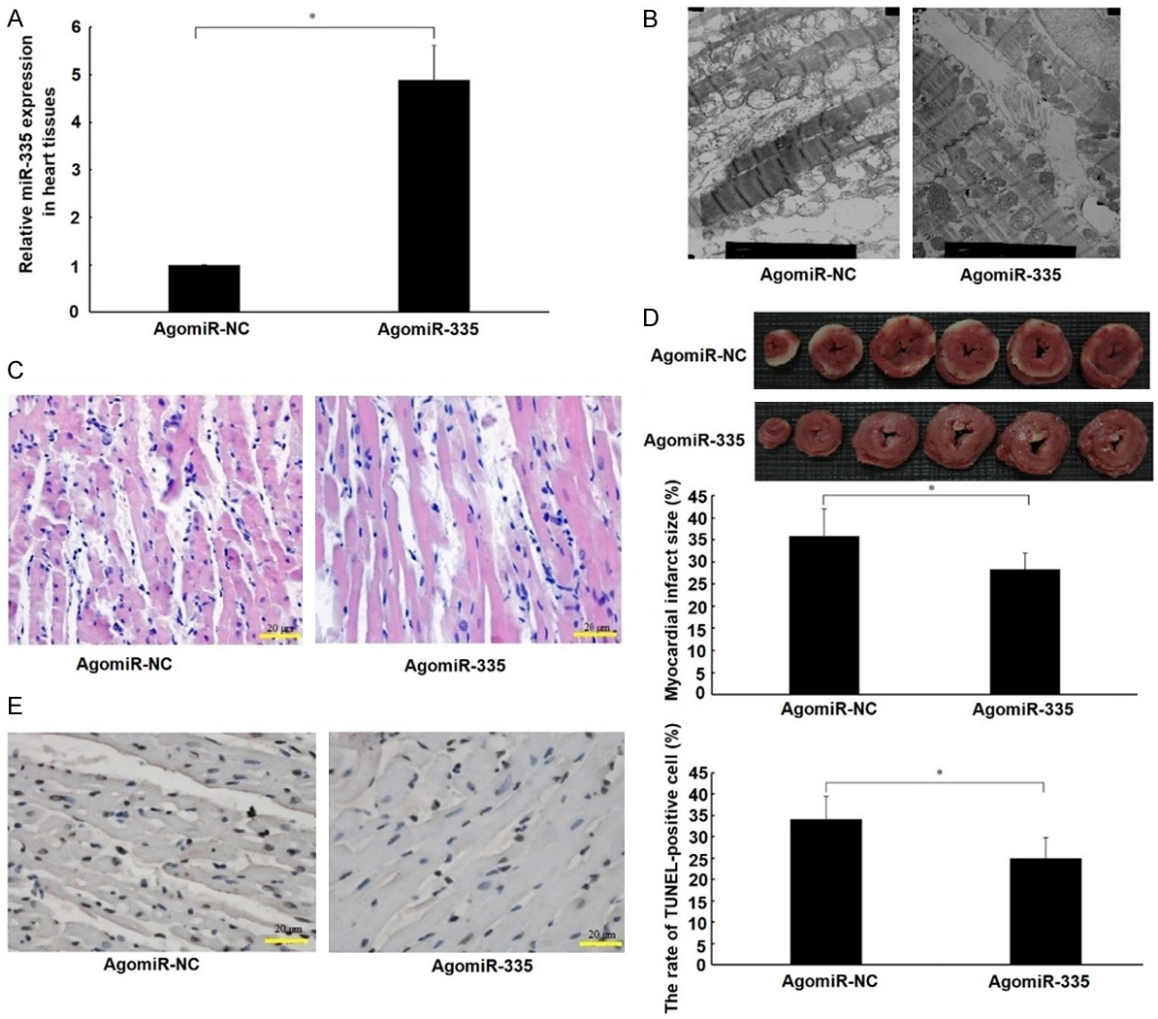

Figure 3. The effect of microRNA (miR)-335 overexpression on ischemia/reperfusion (I/R)-induced myocardial injury in isolated rat hearts. A. The expression of miR-335 in the left ventricular myocardium was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) after treatment with agomiR-335 mimics or a negative control (agomiR-NC), for 3 consecutive days. 24 h after the last injection, the hearts were isolated and underwent 30 min of ischemia, followed by 120 min of reperfusion. B. Myocardial submicroscopic structure was observed by transmission electron microscope (× 5000). C. Myocardial microscopic structure was observed with an optical microscope (× 400). D. The effect of miR-335 overexpression on myocardial infarct size measured by TTC staining (n=6 per group). The viable myocardium was stained red and the infarct myocardium was unstained. E. The effect of miR-335 overexpression on apoptosis as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (× 400) (n=6 per group). The apoptotic cell nuclei were stained brown and the nuclei of viable cells were stained blue. Data are presented as the mean ± standard deviation (SD). *$P < 0.05$.

treatment with agomiR-335 compared with the NC (**Figure 3A**). The damage to the myocardial microstructure was ameliorated by agomiR-335 treatment as observed by reductions in vacuolization and breaks in mitochondria via transmission electron microscopy (**Figure 3B**); fewer ruptures in myocardial fibers were detected in the agomiR-335 group via optical microscopy (**Figure 3C**). In addition, there was a significant reduction in infarct size (**Figure 3D**) and apoptosis (**Figure 3E**) in myocardium treated with agomiR-335 compared with the NC group ($P < 0.05$). Taken together, the results suggested that overexpression of miR-335 protected against I/R-induced myocardial injury in an isolated rat heart model.

HIF1AN was a target of miR-335

The binding site between the HIF1AN 3'UTR and miR-335 was shown in **Figure 4A**. The results of the luciferase reporter assay showed

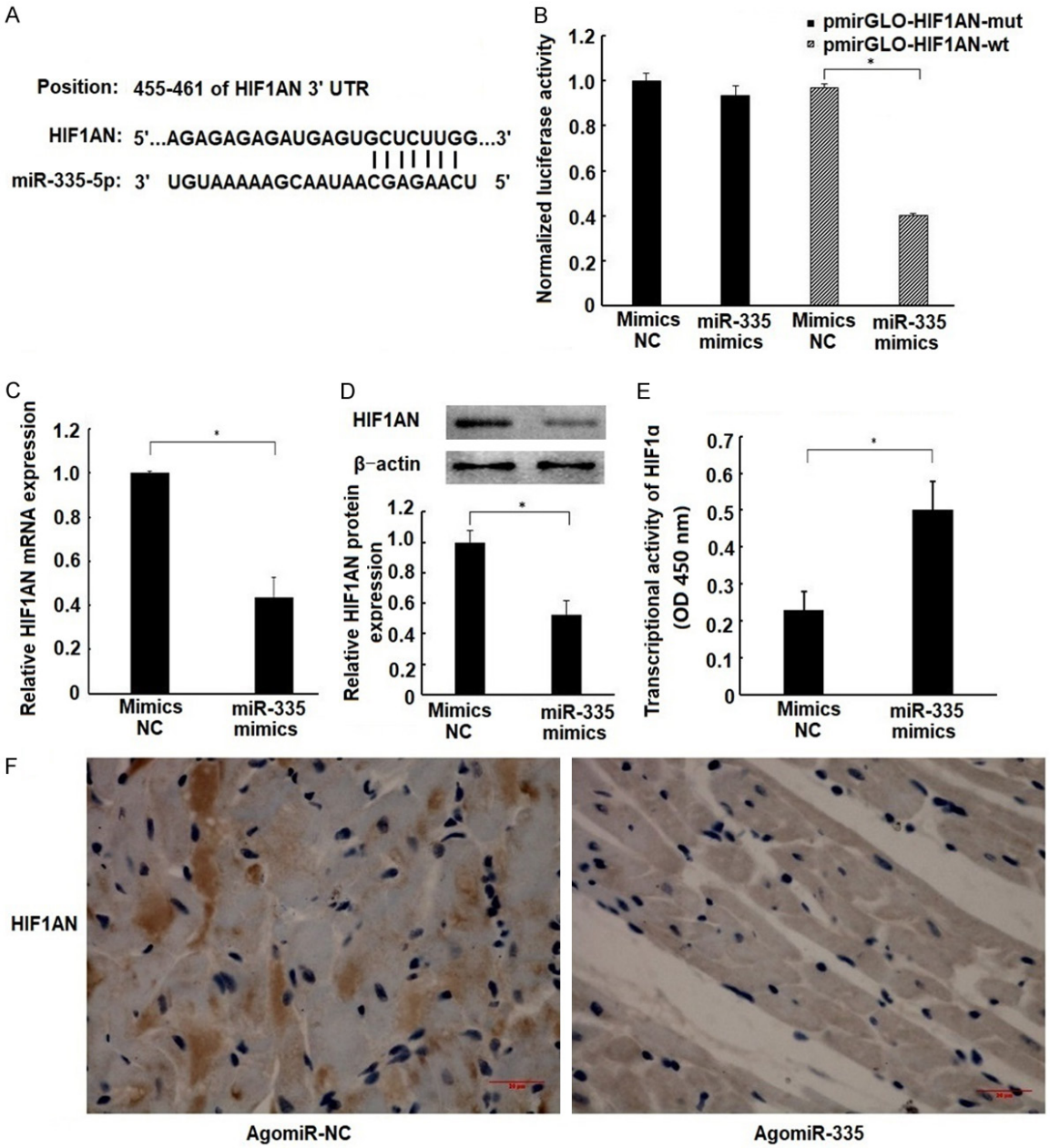

Figure 4. Hypoxia inducible factor 1-alpha subunit inhibitor (HIF1AN) is a target of microRNA (miR)-335. (A) The binding site between HIF1AN 3'-untranslated region (3'UTR) and miR-335. (B) A luciferase reporter gene assay validated the interaction between HIF1AN 3'UTR and miR-335 in H9c2 cells by transfection with a luciferase vector containing HIF1AN 3'UTR (pmirGLO-HIF1AN-wt) or HIF1AN 3'UTR mutant vector (pmirGLO-HIF1AN-mut), together with miR-335 mimics or negative control (mimics NC). H9c2 cells were harvested after transfection with miR-335 mimics or mimics NC. The mRNA and protein expression levels of HIF1AN were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (C) and western blotting (D). The transcriptional activity of HIF-1α was measured by an enzyme-linked immunosorbent assay (ELISA) (E). (F) The rats were injected with agomiR-335 mimics or agomiR-NC for 3 consecutive days. 24 h after the last injection, the hearts were harvested and HMGB1 expression was analyzed by immunohistochemical analysis. Data are presented as the mean ± standard deviation from three independent experiments. *$P < 0.05$.

that luciferase activity was reduced by nearly 60% in the presence of miR-335 mimics relative to the NC in the pmirGLO-HIF1AN-wt group; miR-335 mimics did not exert any inhibitory effects on luciferase activity compared with the NC in the pmirGLO-HIF1AN-mut group (**Figure**

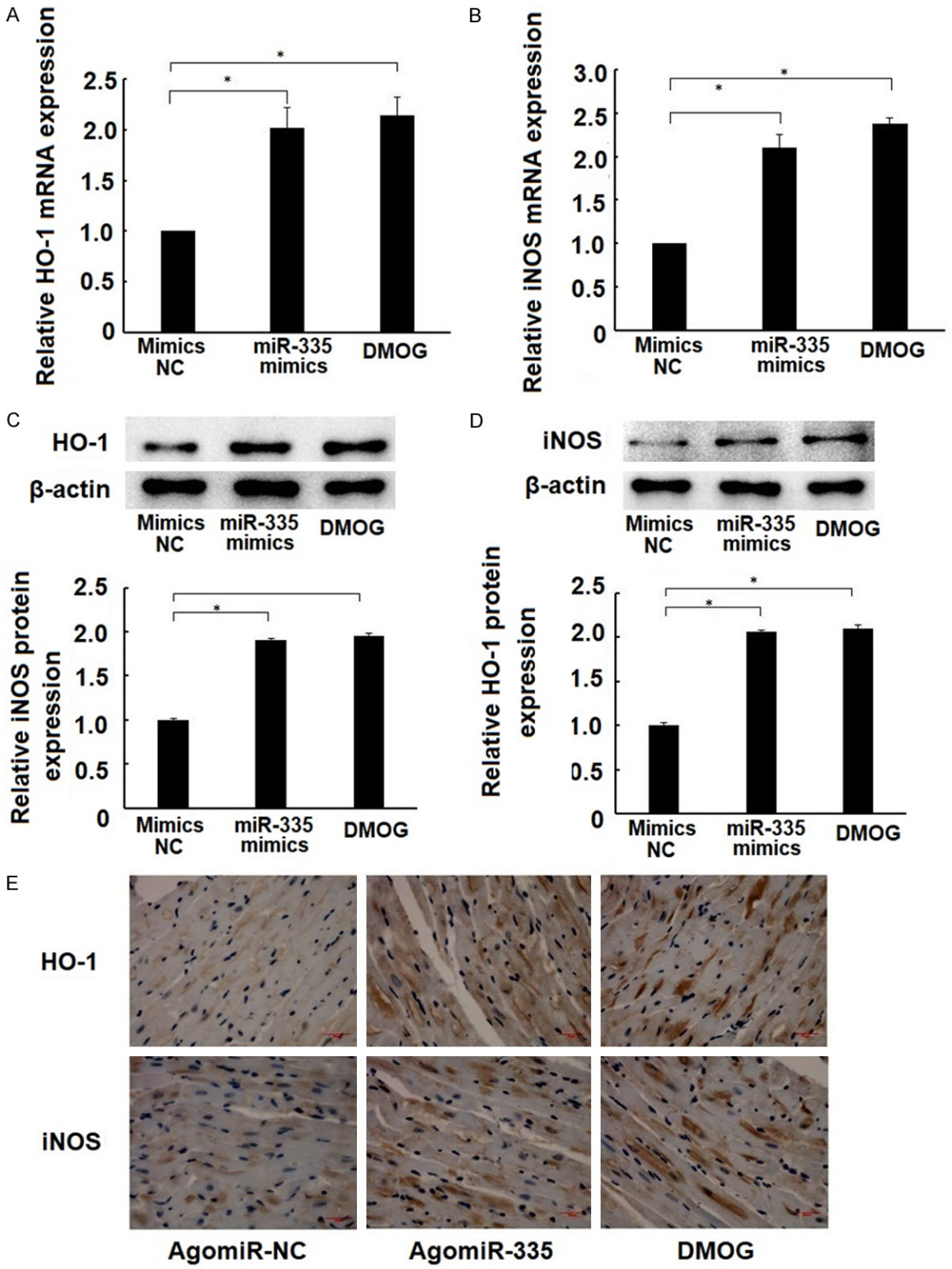

Figure 5. The effect of miR-335 overexpression on expressions of heme oxygenase 1 (HO-1) and inducible nitric oxide synthase (iNOS). H9c2 cells were transfected with miR-335 mimics or a negative control (mimics NC) or treated with 0.5 mM dimethyloxalylglycine (DMOG) for 24 h. Then, cells were harvested and the mRNA expression of HO-1 (A) and iNOS (B) was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and the protein expression of HO-1 (C) and iNOS (D) was analyzed by western blotting. (E) The rats were injected with agomiR-335 mimics or agomiR-NC for 3 consecutive days, or administered an intraperitoneal injection of DMOG (40 mg/kg) 24 h before cardiotomy. 24 h after the last injection, the hearts were harvested and analyzed HO-1 and

iNOS expression by immunohistochemical analysis. Data are presented as the mean ± standard deviation from three independent experiments. *$P < 0.05$.

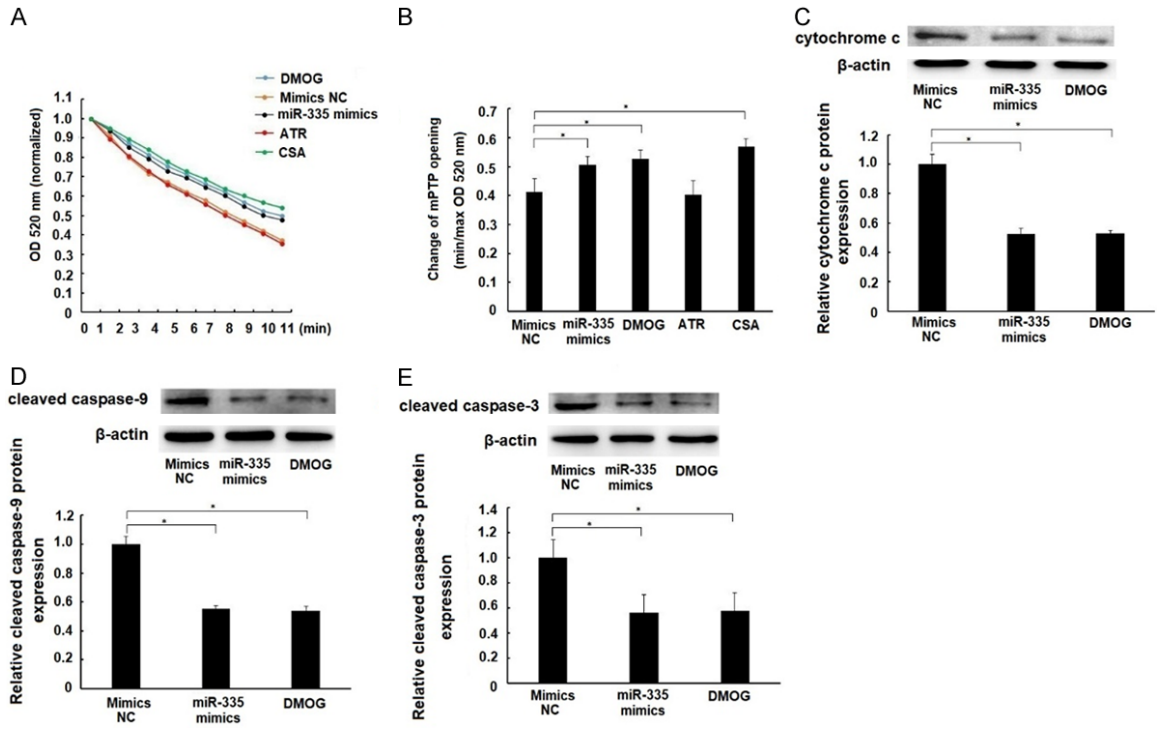

Figure 6. The effect of microRNA (miR)-335 overexpression on mitochondrial permeability transition pore (MPTP) opening. H9c2 cells were transfected with miR-335 mimics or a negative control (mimics NC), or treated with 0.5 mM dimethyloxalylglycine (DMOG) for 24 h. A. MPTP opening was induced by $CaCl_2$. The decrease in optical density (OD) reflected the extent of MPTP opening. B. Statistical analysis for MPTP opening. Minimum optical density (min OD) represents the OD value recorded at the onset of the experiment (0 min); maximum optical density (max OD) represents the OD value recorded at the end of the experiment (10 min). Min/max OD is negatively associated with the extent of MPTP opening. C. The protein expression of cytochrome c was measured by western blotting. D. The protein expression of cleaved caspase-9 was measured by western blotting. E. The protein expression of cleaved caspase-3 was measured by western blotting. Data are presented as the mean ± standard deviation from three independent experiments. *$P < 0.05$.

4B). These results indicated that miR-335 specifically bound with HIF1AN. We also found that overexpression of miR-335 in cardiomyocytes downregulated HIF1AN mRNA (**Figure 4C**) and protein expression (**Figure 4D**). Additionally, overexpression of miR-335 enhanced the transcriptional activity of HIF-1α (**Figure 4D**). Moreover, the results of IHC showed a significant reduction in HIF1AN protein expression (**Figure 4F**) in myocardium treated with agomiR-335 compared with the agomiR-NC group. Therefore, these results demonstrated that HIF1AN was a target of miR-335.

Overexpression of miR-335 upregulated the expression of HO-1 and iNOS

Given that HIF1AN negatively regulates the stability and transcriptional activity of HIF-1α [18], we explored the effect of miR-335 on the expression of HO-1 and iNOS, two candidate downstream target genes of HIF-1α, by RT-qPCR and western blotting. The results showed that the mRNA and protein expression levels of HO-1 and iNOS were increased in miR-335 mimics group compared with the NC group. Meanwhile, similar effects of miR-335 mimics on the expression levels of HO-1 and iNOS were reported in response to dimethyloxalylglycine (DMOG), a known activator of HIF-1α (**Figure 5A-D**). Moreover, the results of IHC showed a marked increase in HIF1AN protein expression (**Figure 5E**) in myocardium treated with agomiR-335 compared with the agomiR-NC group. Therefore, we suggested that overexpression of miR-335 could induce the expression of HO-1 and iNOS, similar to the effects of the HIF-1α activator.

MiR-335 regulated the opening of MPTP

Considering that the pharmacological stabilization of HIF-1α protects against MIRI by inhibiting MPTP opening [19], we explored the effect of miR-335 on the opening of MPTP by measuring the sensitivity of mitochondria to calcium, a marker of MPTP opening [20]. The results showed that the sensitivity of mitochondria to calcium was significantly attenuated in the miR-335 mimics and DMOG group compared with the NC group (**Figure 6A**, **6B**). In addition, MPTP opening could lead to the activation of mitochondrial apoptosis-related proteins [21, 22]. Thus, we further detected the effect of miR-335 on the expression of cytochrome c, cleaved caspase-9 and cleaved caspase-3 by western blotting. As expected, the results showed that the protein expression levels of cytochrome c, cleaved caspase-9 and cleaved caspase-3 were downregulated in the miR-335 mimics and DMOG group compared with the NC group (**Figure 6C-E**). Taken together, the results suggested that overexpression of miR-335 could suppress MPTP opening as HIF-1α is activated.

Discussion

In the present study, we reported that the expression of miR-335 in cardiomyocytes or myocardium was downregulated during MIRI, but was induced by HPoC or IPoC. These findings were consistent with that of Varga *et al*. [23], and further suggested that miR-335 may mimic or mediate the cardio-protection of HPoC/IPoC. As expected, we demonstrated that overexpression of miR-335 in cardiomyocytes protected against H/R-induced cardiomyocyte injury and a similar protective effect against MIRI was also confirmed in isolated rat hearts. To the best of our knowledge, the current study is the first to report the role of miR-335 in the pathophysiological process of MIRI and proposes a new therapeutic target for the treatment of MIRI.

MiRNAs are highly conserved across species and are thought to act as vital regulators of gene expression [24]. The biological function of miRNAs appears to be achieved by the modulation of their target genes. A given miRNA may have hundreds of different conserved targets [25]. Similar findings were reported as many target genes of miR-335 were determined in previous studies. For instance, Gao *et al*. reported that miR-335 inhibits the migration of breast cancer cells by targeting oncoprotein c-Met [26]. Wang *et al*. demonstrated that miR-335 suppresses the migration and invasion of osteosarcoma cells by targeting rho-associated, coiled-coil-containing protein kinase 1 [27]. In this study, we identified HIF1AN as a novel target of miR-335. This finding provides insight as to how miR-335 is involved in the pathophysiological process of MIRI. HIF1AN, an asparaginyl hydroxylase, has been demonstrated to negatively regulate HIF-1α stability and transcriptional activity by interacting with HIF-1α [18]. HIF-1α is a principal transcriptional regulator of the cellular and developmental response to hypoxia [28], and may confer protection against MIRI by triggering the activation of its downstream protective genes [29], such as HO-1 [14] and iNOS [30]. Considering that miR-335 targets HIF1AN suppressing expression, thereby enhancing the transcriptional activity of HIF-1α, we inferred that miR-335 might also affect expression of the target genes of HIF-1α. In this study, we found that overexpression of miR-335 increased the expression levels of HO-1 and iNOS, which was similar to the effects following treatment with a HIF-1α activator. Taken together, we suggest that the protective effects of miR-335 on MIRI are mediated, at least partly, by modulating the transcriptional activity of HIF-1α.

MPTP, a non-specific pore protein located in the mitochondrial inner membrane, remains closed during ischemia, but rapidly opens once reperfusion occurs [31, 32]. The opening of MPTP could lead to mitochondrial depolarization, swelling, cell apoptosis and necrosis [21, 22]. MPTP has also been identified as a terminal effector of ischemic preconditioning or postconditioning [33]. Thus, MPTP is a key regulator in the pathophysiological process of MIRI [34]. More importantly, MPTP opening has been demonstrated to be regulated by HIF-1α; Ong *et al*. reported that HIF-1α stabilization by a pharmacological or genetic approach protected against MIRI by inhibiting MPTP opening [19]. Considering that miR-335 could regulate the transcriptional activity of HIF-1α, we further inferred that miR-335 may have an effect on MPTP opening. As expected, we found that overexpression of miR-335 or activation of HIF-1α by its activator reduced the sensitivity of

mitochondria to calcium, a marker of MPTP opening [20], and subsequently suppressed the expression of its downstream apoptosis-related proteins. Therefore, we suggest that miR-335 may regulate MPTP opening through, at least partially, the modulation of HIF-1α.

However, we must acknowledge that there was a limitation in our study. Although we demonstrated that miR-335 could inhibit HIF1AN and lead to the activation of HIF-1α, thereby ameliorating MIRI, whether the protective effects of miR-335 on MIRI are affected by overexpression of HIF1AN or inhibition of HIF-1α activity is unknown and should be investigated in the future.

In conclusion, we demonstrated that miR-335 is downregulated under the conditions of MIRI. Overexpression of miR-335 could increase the expression of HO-1 and iNOS, and inhibit opening of MPTP by targeting HIF1AN, thereby reducing MIRI. Thus, miR-335 may be a new therapeutic target for the treatment of MIRI.

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Disclosure of conflict of interest

None.

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