Downregulation of miR-16 protects H9c2(2-1) cells against hypoxia/reoxygenation damage by targeting CIAPIN1 and regulating the NF-κB pathway

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Abstract. The aim of the present study was to determine the function of microRNA-16 (miR-16) in myocardial hypoxia/reoxygenation (H/R)-induced cardiomyocyte injury and the possible mechanism underlying its involvement. An H/R model was constructed using H9c2(2-1) cells *in vitro*. The results of reverse transcription-quantitative PCR demonstrated that the expression levels of miR-16 were significantly upregulated in H9c2(2-1) cells in the H/R group compared with the sham group (1.53±0.09 vs. 1.0±0.08; P=0.0019). Cell Counting Kit-8 assays revealed that the relative proliferative ability of H9c2(2-1) cells was significantly decreased in the H/R + negative control (NC) group compared with the sham group (0.53±0.05 vs. 1.0±0.08; P=0.00005). Upregulation of miR-16 using miR-16 mimics further decreased the proliferative ability of cells (0.31±0.03 vs. 0.53±0.05; P=0.0097), whereas downregulation of miR-16 using an miR-16 inhibitor increased the proliferative ability of cells compared with the H/R+NC group (0.89±0.08 vs. 0.53±0.05; P=0.000385). Flow cytometric analysis found that the apoptotic rate of H9c2(2-1) cells was increased significantly following H/R compared with the sham group (25.86±2.62% vs. 9.29±0.82%, P=0.000014). Upregulation of miR-16 further increased the apoptotic rate (38.62±2.04% vs. 25.86±2.62%; P=0.000099), whereas downregulation of miR-16 decreased the apoptotic rate compared with the H/R+NC group (15.14±0.92% vs. 25.86±2.62%; P=0.000343). miR-16 directly bound to the 3'-untranslated region of cytokine-induced apoptosis inhibitor 1 (CIAPIN1) and negatively modulated CIAPIN1 expression. Overexpression of CIAPIN1 reversed the changes in the expression of apoptosis-associated proteins caused by H/R. Western blot analysis revealed that the levels of phospho-(p-) nuclear factor-κB (NF-κB) and p-NF-κB inhibitor α (IκBα) were upregulated following H/R (1.82±0.11 vs. 1.0±0.08; P=0.000152; and 1.77±0.07 vs. 1.0±0.00; P=0.000024, respectively), and these changes were further enhanced when miR-16 expression levels were increased (3.10±0.14 vs. 1.82±0.11; P=0.000006; and 2.19±0.10 vs. 1.77±0.07; P=0.0017, respectively). Downregulation of miR-16 exhibited the opposite effect on p-NF-κB and p-IκBα expression levels. The present study illustrates that downregulation of miR-16 may protect against H/R-induced injury partially by targeting CIAPIN1 and the NF-κB signaling pathway.

Introduction

Hypoxia may result in an insufficient supply of blood and nutrients to the heart, resulting in cardiomyocytes undergoing apoptosis, which may potentially injure the cardiac tissue (1). Myocardial infarction, a normal ischemic heart disease, is one of the primary causes of disability and the leading cause of mortality worldwide (2). At present, the most effective therapy for treating patients following a myocardial infarction is reperfusion, a process of rapidly restoring the blood flow through the occluded coronary artery (3-5). Reperfusion will result in myocardial ischemia/reperfusion (MI/R) injury, which will result in additional death of cardiomyocytes and will enlarge the size of the infarction (4,6,7). A deeper understanding of the molecular mechanism underlying MI/R injury may improve treatments and reduce MI/R-associated damage.

There is increasing evidence illustrating the roles of micro-RNAs (miRNAs) in the modulation of numerous biological processes in diseases, including MI/R injury (8-10). miRNAs, which are members of the non-coding small RNA family, are involved in gene silencing by binding to the 3'-untranslated region (3'-UTR) of its target genes (11,12). Several differentially expressed miRNAs, including miR-21 (13), miR-24 (14) and miR-29 (15), have been demonstrated to be involved in I/R-induced injury. miR-16 was identified as an anti-apoptotic factor in glioblastoma multiforme (11).

In the present study, the mRNA expression levels of miR-16 were significantly increased in H9c2(2-1) cardiac myoblast

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cells following simulation of H/R, suggesting an important role of miR-16 in H/R-injury response. In addition, the potential target genes of miR-16 were examined. It was demonstrated that miR-16 may reduce the damage of cardiac myoblast cells caused by H/R, partially by downregulating the expression of cytokine-induced apoptosis inhibitor 1 (CIAPIN1). To the best of our knowledge, the present study is the first to study the specific role of miR-16 in H/R injury.

Materials and methods

Cell culture and construction of an H/R model. The H9c2(2-1) cardiac myoblasts cell line (henceforth referred to as H9c2) was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modi-fied Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 95% atmosphere and 5% CO_2. To construct the H/R model, H9c2 cells were transferred into DMEM without FBS before being subjected to hypoxia. The cells were incubated at 37°C for 6 h in an anaerobic chamber containing 95% N_2 and 5% CO_2. Subsequently, the cells were moved to a normoxic incubator (5% CO_2 and 95% atmosphere) with DMEM and incubated for 24 h for re-oxygenation. The same treatment was performed in the sham group without the hypoxic stimulus.

Transfection of miR-16 mimics/inhibitor. To investigate the role of miR-16, the miR-16 mimics, miR-16 inhibitor and their corresponding negative controls (NC) were all synthesized by GenePharma Co., Ltd. (Shanghai, China). The sequences of miR-16 mimics and the negative control (NC) were: 5'-UAG CAGCACGUAAAUAUUGGUG-3' and 5'-UACACCGAU CGAGUCAGGUTT-3', respectively. The sequences of the miR-16 inhibitor and its NC were 5'-CACCAAUAUUUACGU GCUGCUA-3' and 5'-UCGAGACACGUACGCAGAATT-3', respectively. A pcDNA3.1-CIAPIN1 plasmid was constructed by GenePharma Co., Ltd. to overexpress CIAPIN1, and an empty pcDNA3.1 vector (GenePharma Co., Ltd.) was used as the NC. H9c2 cells were seeded into 24-well plates and grown to ~60% confluence. The miR-16 mimics, miR-16 inhibitor, pcDNA3.1-CIAPIN1 and their corresponding NCs were transfected into the cells using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham MA, USA) according to the manufacturer's protocol. The final concentration of miR-16 mimics and its NC was 50 nM, and the final concentration for miR-16 inhibitor and NC was 100 nM. The expression level of miR-16 was detected by reverse transcription-quantitative (RT-q)PCR 24 h after transfection.

Dual-luciferase reporter gene assay. The potential targets of miR-16 were predicted using the TargetScan website (http://www.targetscan.org/vert_72/). CIAPIN1 was predicted as a target of miR-16. In order to confirm that miR-16 directly bound to CIAPIN1, a dual-luciferase reporter gene assay was performed. The 3'-UTR of CIAPIN1 containing the miR-16-binding sites (CIAPIN1-WT) and 3'-UTR of CIAPIN1 containing the mutant miR-16-binding sites (CIAPIN1-Mut) were synthesized. The CIAPIN1-WT and CIAPIN1-Mut were cloned into pmiR-RB-REPORT™ plasmid (Guangzhou RiboBio Co., Ltd.). Each recombinant vector,

pmiR-CIAPIN1-WT or pmiR-CIAPIN1-Mut, along with miR-16 mimics or miR-16 mimics NC, were co-transfected into the H9c2 cells using Lipofectamine™ 3000 reagent, according to the manufacturer's protocol. After 48 h of transfection, the total protein was extracted, and the luciferase activity was detected using a Dual-Luciferase Reporter assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. The activity of *Renilla* luciferase was used for normalization.

Cell Counting Kit-8 (CCK-8) assay. A CCK-8 assay (Beyotime Institute of Biotechnology, Haimen, China) was used to examine the proliferative ability of H9c2 cells after 24 h trans-fection. Single cell suspensions were prepared and seeded into 96-well plates with $1x10^3$ cells/well. The plates were incubated at 37°C with 5% CO_2. After a 24 h incubation, 10 μl CCK-8 reagent was added to each well and incubated at 37°C for 1.5 h. Subsequently, the absorbance was detected at 450 nm using an ELx800 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

RT-qPCR. Cells were harvested 24 h after transfection and the total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For mRNA expression analysis, the isolated RNA was reverse-transcribed to cDNA using a PrimeScript™ RT Reagent kit (Takara Bio, Inc.). The RT procedure was: 37°C for 15 min and then 85°C for 15 sec. qPCR was performed using SYBR Premix Ex Taq II (Takara Bio, Inc.) on a 7500 Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). To determine the level of miR-16, Mir-X™ miRNA First Strand Synthesis kit (Takara Bio, Inc.) was used to reverse-transcribe the RNA to cDNA according to the supplier's instruction. qPCR was then performed using SYBR PrimeScript™ miRNA RT-PCR kit (Takara Bio, Inc.). The PCR steps including 95°C for 5 min, followed by 40 cycles of 5 sec at 95°C, 34 sec at 60°C, and then 72°C for 30 min. The expression of GAPDH and U6 small nuclear RNA were used as internal references for mRNA and miRNA accord-ingly. The relative expression of CIAPIN1 and miR-16 was calculated using the $2^{-\Delta\Delta Cq}$ method (16). All the primers used for qPCR are presented in Table I. The U6 small nuclear RNA primers and uni-miR qPCR primer were included with the SYBR PrimeScript™ miRNA RT-PCR kit.

Western blotting. Total cellular proteins were extracted from H9c2 cells using the RIPA lysate with protease inhibi-tors (CWBIO, Beijing, China. http://www.cwbiotech.com/) after 48 h transfection. Proteins (20 μg each well) were then separated by SDS-PAGE with a 10% gel. Subsequently, the proteins from the gel were transferred to a PVDF membrane (EMD Millipore, Billerica, MA, USA). Then the membranes were blocked by 5% skimmed milk for 1 h at room tempera-ture. Then the membranes were incubated with primary antibodies for 12 h, and then the secondary antibody for 1 h at 4°C. The signals were visualized using enhanced chemilu-minescent reagent, and the expression of β-actin was used for normalization. The following primary antibodies were used, Bcl-2 (Abcam, Cambridge, UK; cat. no. ab196495; 1:1,000), Bax (Abcam; cat. no. ab53154; 1:1,000), cleaved caspase-3

Table I. Primer sequences.

A, mRNA primer sequences

Primer	Sequence
CIAPIN1 F	5'-GCTTGTGGCAGTGTTCTGTG-3'
CIAPIN1 R	5'-CACAGAACACTGCCACAAGC-3'
GAPDH F	5'-GCCAGCCTCGTCTCATAGAC-3'
GAPDH R	5'-AGTGATGGCATGGACTGTGG-3'

B, miRNA primer sequences

Primer	Sequence
miR-16 F	5'-GCGTAGCAGCACGTAAAT-3'
miR-16 R	Uni-miR qPCR Primer

miR, microRNA; CIAPIN1, cytokine-induced apoptosis inhibitor 1; F, forward; R, reverse; qPCR, quantitative PCR.

(Abcam; cat. no. ab49822; 1:1,000), CIAPIN1 (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. PA5-59903; 1:1,000), NF-κB (Abcam; cat. no. ab16502; 1:1,000), p-NF-κB (CST Biological Reagents Co., Ltd., Shanghai, China; cat. no. 3039, 1:1,000), IκBα (Abcam; cat. no. ab7217; 1:1,000), p-IκBα (CST, cat. no. 2859; 1:1,000) and β-actin (CST Biological Reagents Co., Ltd., cat. no. 4970; 1:1,000). The anti-rabbit immuno-globulin G, horseradish peroxidase-linked antibody of CST Biological Reagents Co., Ltd. (cat. no. 7074; 1:2,000) was used as the secondary antibody. Quantity One 4.6.2 software (Bio-Rad Laboratories, Inc.) was used for densitometry analysis.

Cell apoptosis analysis. Flow cytometry was used to evaluate the apoptosis of H9c2 cells. Cells were harvested by centrifu-gation (300 x g, 5 min, 4°C) and washed using pre-cooled PBS. Cells were resuspended in binding buffer(Sigma-Aldrich; Merck KGaA) at a final concentration of $1.5x10^6$ cells/ml. Double staining was performed with an Annexin V-fluorescein isothiocyanate (FITC)/propidium staining (PI) kit, according to the manufacturer's protocol (Sigma-Aldrich; Merck KGaA; Darmstadt, Germany). Staining was measured using flow cytometry (BD Biosciences) and analyzed with FlowJo software 7.2 (FlowJo LLC).

Statistical analysis. All the results are presented as the mean ± standard deviation. Comparisons between two groups were analyzed using a Student's t-test. Comparisons between multiple groups were analyzed using a one-way ANOVA, followed by a post hoc Tukey's or Dunnett's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-16 expression is upregulated in H9c2 cells following hypoxia. The mRNA expression levels of miR-16 in H9c2 cells increased 1.53-fold following hypoxic treatment compared with the H9c2 cells in the sham group (P=0.0019; Fig. 1A). To determine the possible biological functions of miR-16, miR-16 mimics and miR-16 inhibitor were used to upregulate or downregulate the expression of miR-16 in H9c2 cells, respectively. The mRNA expression levels of miR-16 were significantly increased in H9c2 cells transfected with miR-16 mimics (1.59±0.10 vs. 0.98±0.08; P=0.000026) and significantly decreased in H9c2 cells transfected with miR-16 inhibitor (0.56±0.05 vs. 1.05±0.09; P=0.000299; Fig. 1B) compared with the respective NC. When H/R was simulated in the H9c2 cells, the expression of miR-16 was also increased in cells transfected with miR-16 mimics (1.98±0.15 vs. 1.53±0.01; P=0.002205) and decreased by miR-16 inhibitors (1.16±0.09 vs. 1.52±0.01; P=0.006857 Fig. 1C) compared with the respective NC.

miR-16 regulates the proliferation and apoptosis of H9c2 cells following hypoxia. A CCK-8 assay and flow cytometry were performed to examine the role exerted by miR-16 on the proliferation and apoptosis of H9c2 cells following hypoxia. The viability of H9c2 cells was significantly reduced in the H/R+NC group compared with the sham group (0.53±0.05 vs. 1.00±0.08; P=0.000003; Fig. 2). Upregulation of miR-16 using miR-16 mimics (H/R+miR-16 mimics group) further decreased proliferation of H9c2 cells compared with the H/R+NC group (0.31±0.03 vs. 0.53±0.05; P=0.0097; Fig. 2). Downregulation of miR-16 using the miR-16 inhibitor significantly increased the proliferation of H9c2 cells compared with the H/R+NC group (0.89±0.08 vs. 0.53±0.05; P=0.000385; Fig. 2).

Furthermore, flow cytometric analysis demonstrated that that the apoptotic percentage was significantly increased in the H/R+NC group compared with the sham group (25.86±2.62% vs. 9.29±0.82%; P=0.000014; Fig. 3). Compared with the H/R+NC group, the apoptotic percentage was increased in the H/R+miR-16 mimics group (38.62±2.04% vs. 25.86±2.62%; P=0.000099; Fig. 3), whereas in the H/R+miR-16 inhibitor group the apoptotic rate was significantly decreased (15.14±0.92% vs. 25.86±2.62%; P=0.000343; Fig. 3) compared with the H/R+NC group. The expression levels of apop-tosis-associated proteins were determined by western blotting (Fig. 4A). The results demonstrated that the expression of Bcl-2 (0.49±0.05 vs. 1.00±0.03; P=0.000004; Fig. 4B) was decreased; whereas the levels of Bax (1.60±0.06 vs. 1.00±0.05; P=0.000005; Fig. 4C) and cleaved caspase-3 (1.54±0.07 vs. 0.96±0.03; P=0.00001; Fig. 4D) were increased in the H/R+NC group compared with the sham group. Compared with the H/R+NC group, the expression of Bcl-2 (0.27±0.07 vs. 0.49±0.05; P=0.00193; Fig. 4B) was decreased, whereas the levels of Bax (2.20±0.04 vs. 1.60±0.06; P=0.000005; Fig. 4C) and cleaved caspase-3 (2.17±0.09 vs. 1.54±0.07; P=0.000012; Fig. 4D) were increased in the H/R+miR-16 mimics group. In the H/R+miR-16 inhibitor group, the expression of Bcl-2 (0.73±0.02 vs. 0.49±0.05; P=0.000983; Fig. 4B) was increased, whilst the levels of Bax (1.26±0.07 vs. 1.60±0.06; P=0.000394; Fig. 4C) and cleaved caspase-3 (1.27±0.08 vs. 1.54±0.07; P=0.001939; Fig. 4D) were declined compared with the H/R+NC group.

miR-16 negatively regulates the expression of CIAPIN1. To further examine how miR-16 affected cell proliferation
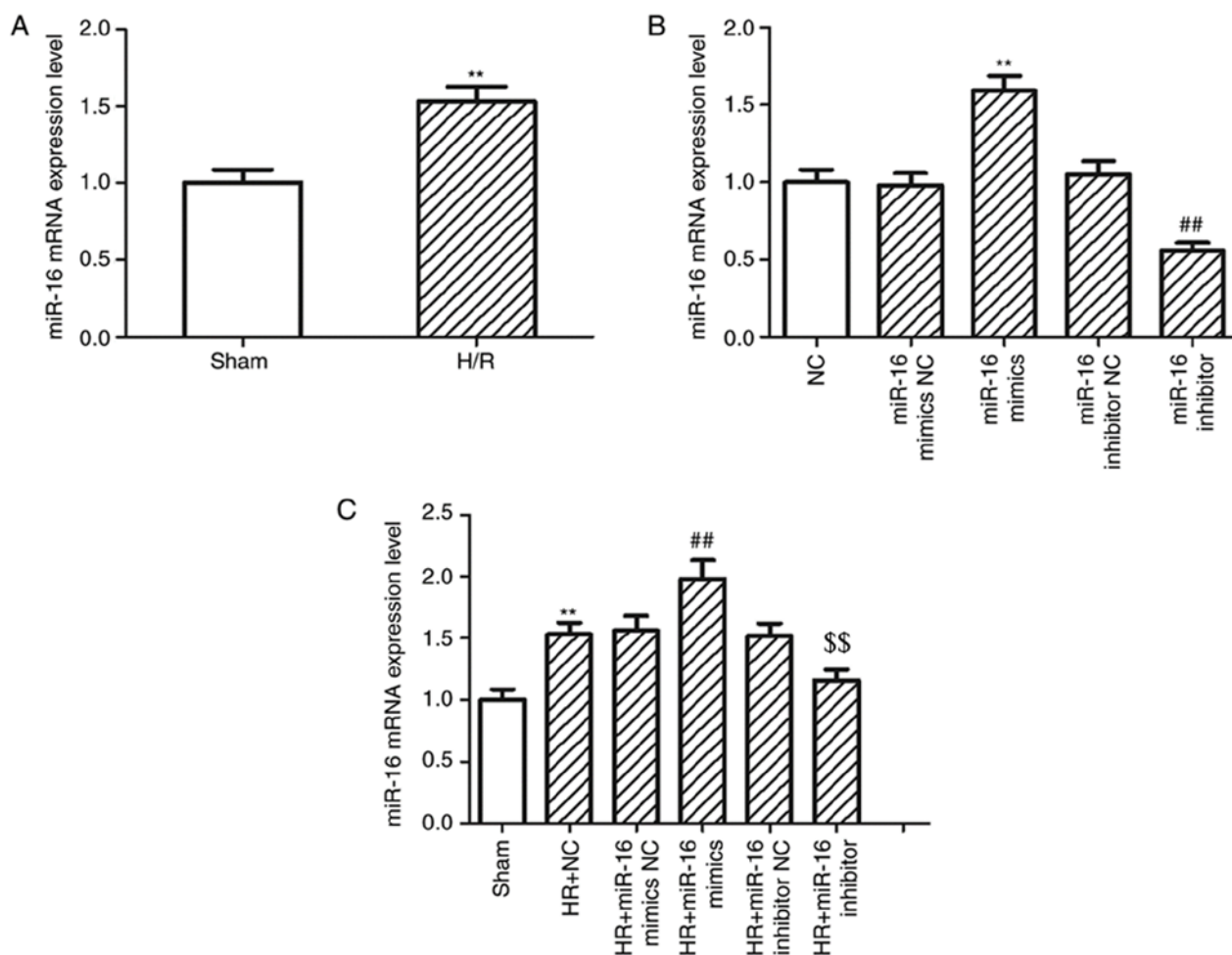
Figure 1. mRNA expression levels of miR-16 in H9c2 cells following H/R. (A) mRNA expression levels of miR-16 were significantly upregulated in H9c2 cells following H/R compared to the sham treatment. **P<0.01. (B) mRNA expression levels of miR-16 were increased or decreased in H9c2 cells following transfection with miR-16 mimics or miR-16 inhibitor, respectively. **P<0.01 vs. miR-16 mimics NC, $^{##}$P<0.01 vs. miR-16 inhibitor NC. (C) mRNA expression levels of miR-16 were increased or decreased by miR-16 mimics or the miR-16 inhibitor, respectively, in the H/R model. **P<0.01 vs. sham; $^{##}$P<0.01 vs. miR-16 mimics NC, $^{\$\$}$P<0.01 vs. miR-16 inhibitor NC. miR, microRNA; NC, negative control; H/R, hypoxia/reoxygenation.
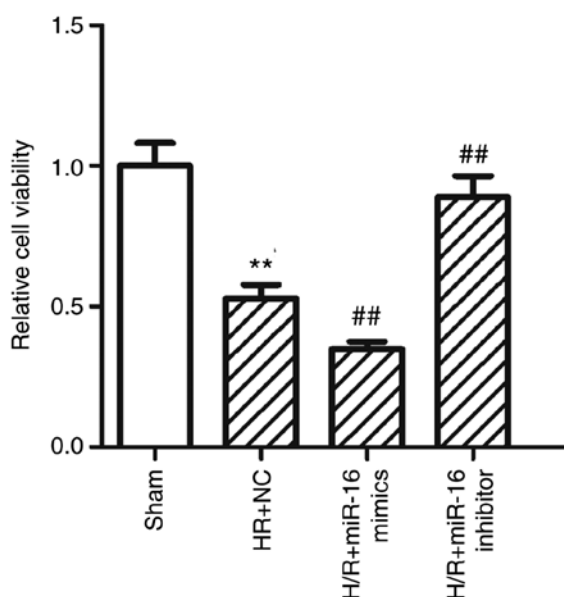

Figure 2. Effect of miR-16 expression on the viability of H9c2 cells following H/R, determined using a cell counting kit-8 assay. **P<0.01 vs. sham; $^{##}$P<0.01 vs. H/R+NC. miR, microRNA; NC, negative control; H/R, hypoxia/reoxygenation.

and apoptosis in H/R injury, the target was predicted using TargetScan. CIAPIN1 was one of the targets of miR-16, and the predicted binding sites are shown in Fig. 5A. The results of the luciferase reporter assay revealed that the relative luciferase activity of CIAPIN1-WT was dramatically decreased in the H/R+miR-16 mimics group compared with the H/R+miR-16 mimics NC group (P<0.01; Fig. 5B). However, upregulation of miR-16 did not significantly influence the luciferase activity of CIAPIN1-Mut (P>0.05; Fig. 5B). Therefore, the effect of miR-16 on CIAPIN1 expression was determined by qPCR and western blotting. The mRNA and protein expression levels of CIAPIN1 were decreased significantly in the H/R+NC group compared with the sham group (both P<0.01; Fig. 6). Upregulation of miR-16 using miR-16 mimics further decreased CIAPIN1 expression at both the mRNA (Fig. 6A) and protein expression levels (Fig. 6) in comparison with H/R+NC group (both P<0.01). CIAPIN1 expression was enhanced in the H/R+miR-16 inhibitor group compared with the H/R+NC group (P=0.000858).

Overexpression of CIAPIN1 reverses the changes of expression of apoptosis-associated proteins caused by H/R. CIAPIN1
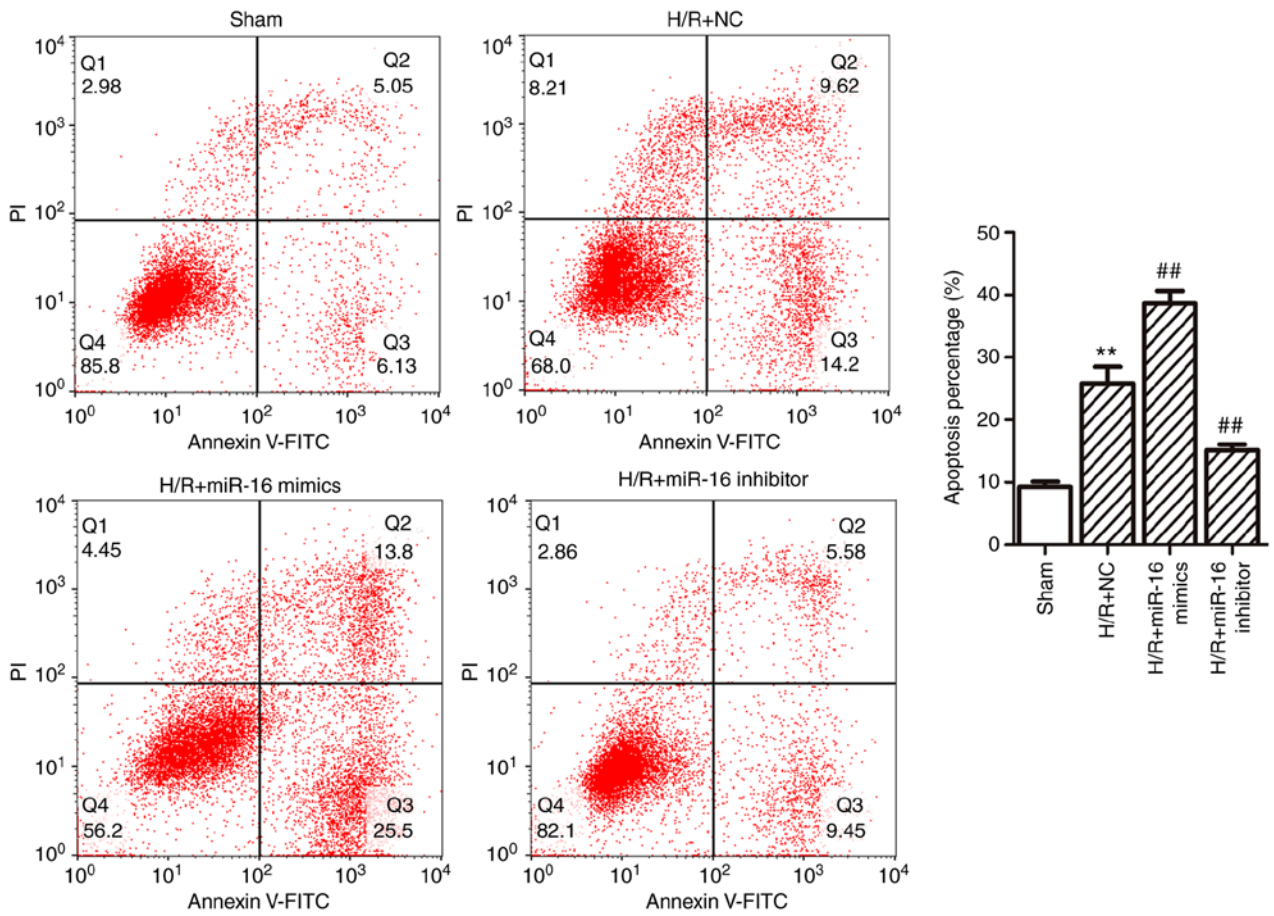
Figure 3. miR-16 regulates the apoptosis of H9c2 cells following H/R. Effect of miR-16 expression on apoptosis of H9c2 cells following H/R was determined using flow cytometry. **P<0.01 vs. sham; ##P<0.01 vs. H/R+NC. miR, microRNA; NC, negative control; H/R, hypoxia/reoxygenation.
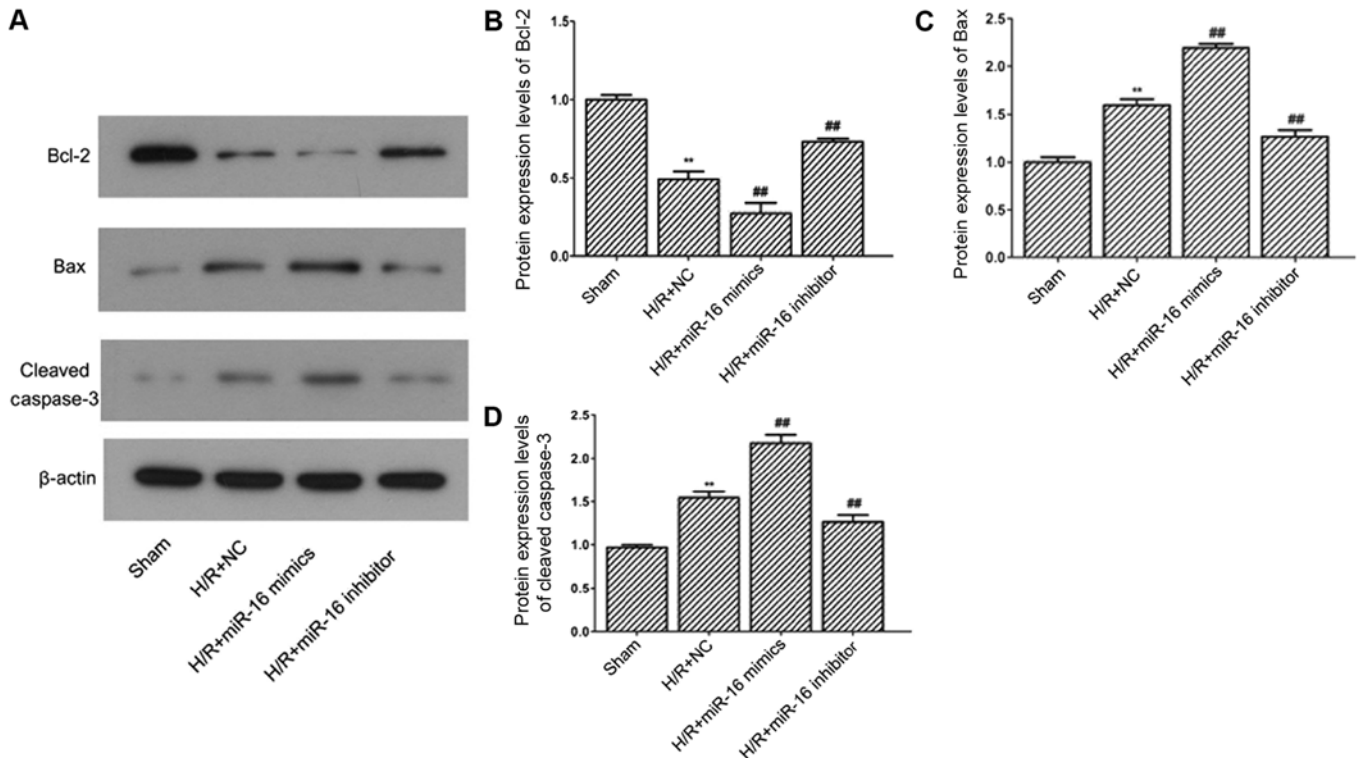

Figure 4. Effect of miR-16 on the expression of apoptosis-associated proteins in H9c2 cells following H/R. (A) Protein expression levels of Bcl-2, Bax and cleaved-caspase-3 were determined by western blotting. Relative expression levels of (B) Bcl-2, (C) Bax and (D) cleaved caspase-3. **P<0.01 vs. sham; ##P<0.01 vs. H/R+NC. miR, microRNA; NC, negative control; H/R, hypoxia/reoxygenation.

A CIAPIN1 WT   5'-GGCAGUGGGGUGUGGUGCUGCUA- 3'

miR-16   3'-GCGGUUAUAAAUGCACGACGAU- 5'
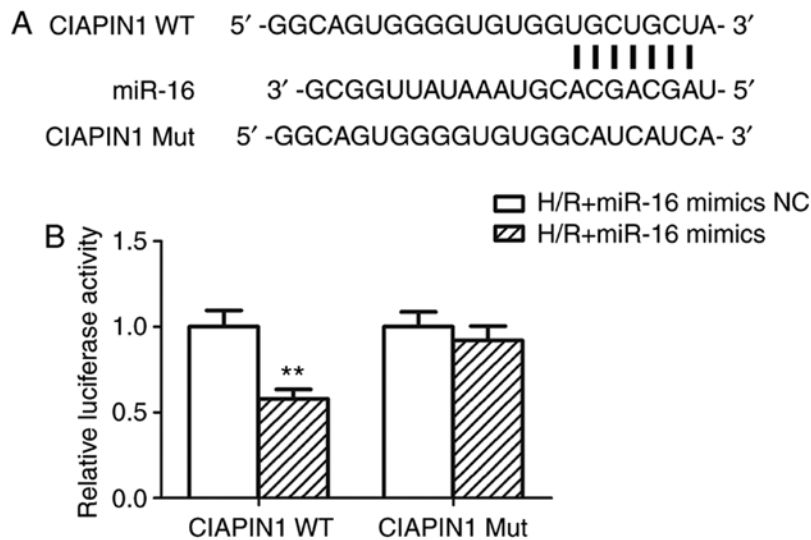
CIAPIN1 Mut   5'-GGCAGUGGGGUGUGGCAUCAUCA- 3'

Figure 5. miR-16 directly binds to the 3'-untranslated region of CIAPIN1. (A) Predicted binding site of miR-16 with CIAPIN1 and the mutated binding site used in the present study. (B) Luciferase activity in cells transfected with miR-16 mimics + WT or miR-16 mimics + Mut compared with cells transfected with miR-16 mimics NC + WT and miR-16 mimics NC + Mut. $^{**}P<0.01$ vs. H/R+miR-16 mimic NC. CIAPIN1, cytokine-induced apoptosis inhibitor 1; MUT, mutated; WT, wildtype; miR, microRNA; NC, negative control; H/R, hypoxia/reoxygenation.
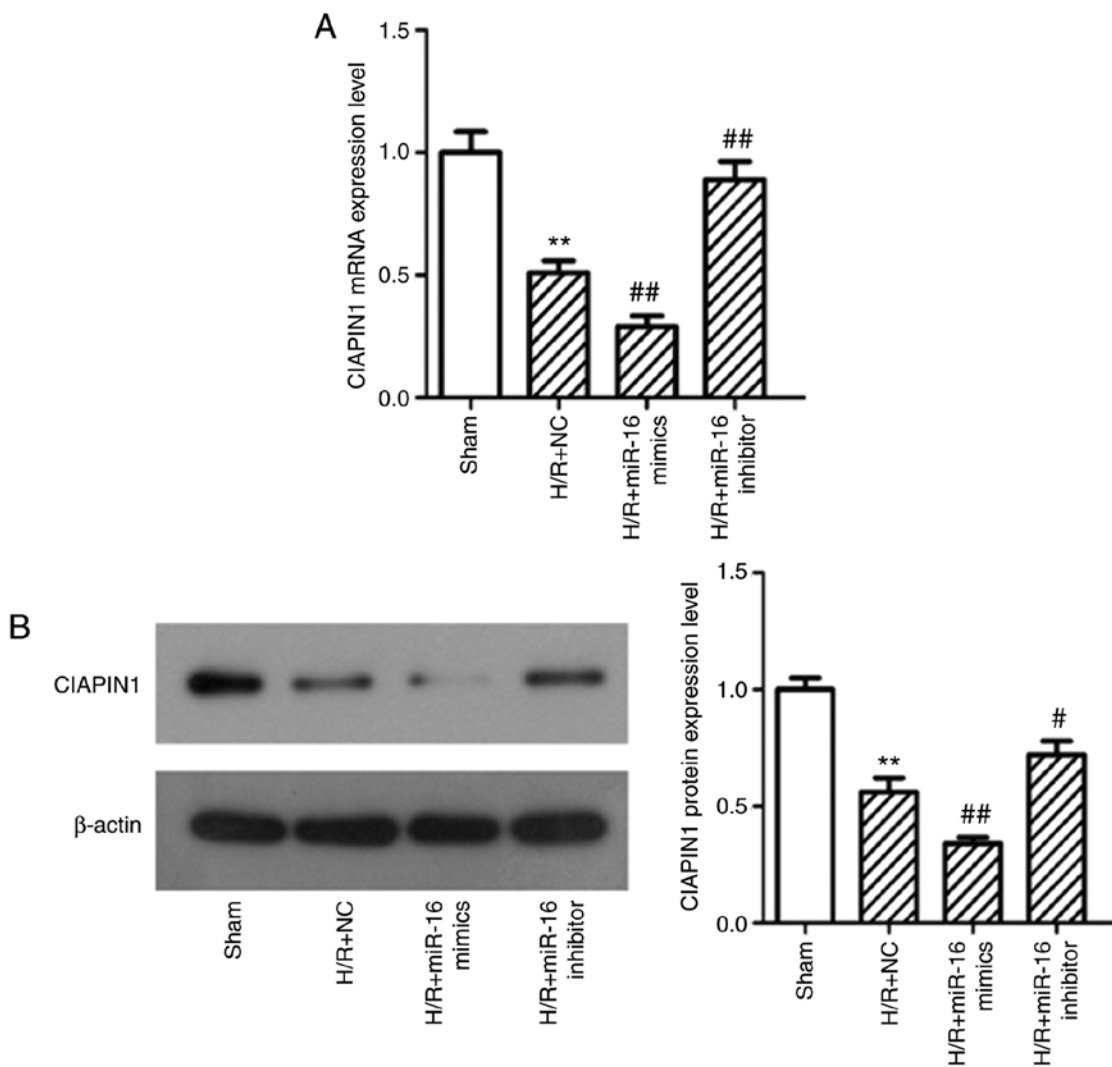

Figure 6. Transfection with miR-16 mimics decreases the expression levels of CIAPIN1 following H/R. (A) mRNA expression levels of CIAPIN1 in H9c2 cells were detected by qPCR. $^{**}P<0.01$ vs. sham; $^{\#\#}P<0.01$ vs. H/R+NC. (B) Protein expression levels of CIAPIN1 in H9c2 cells were determined by western blotting. $^{**}P<0.01$ vs. sham; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. H/R+NC. CIAPIN1, cytokine induced apoptosis inhibitor 1; miR, microRNA; NC, negative control; H/R, hypoxia/reoxygenation.
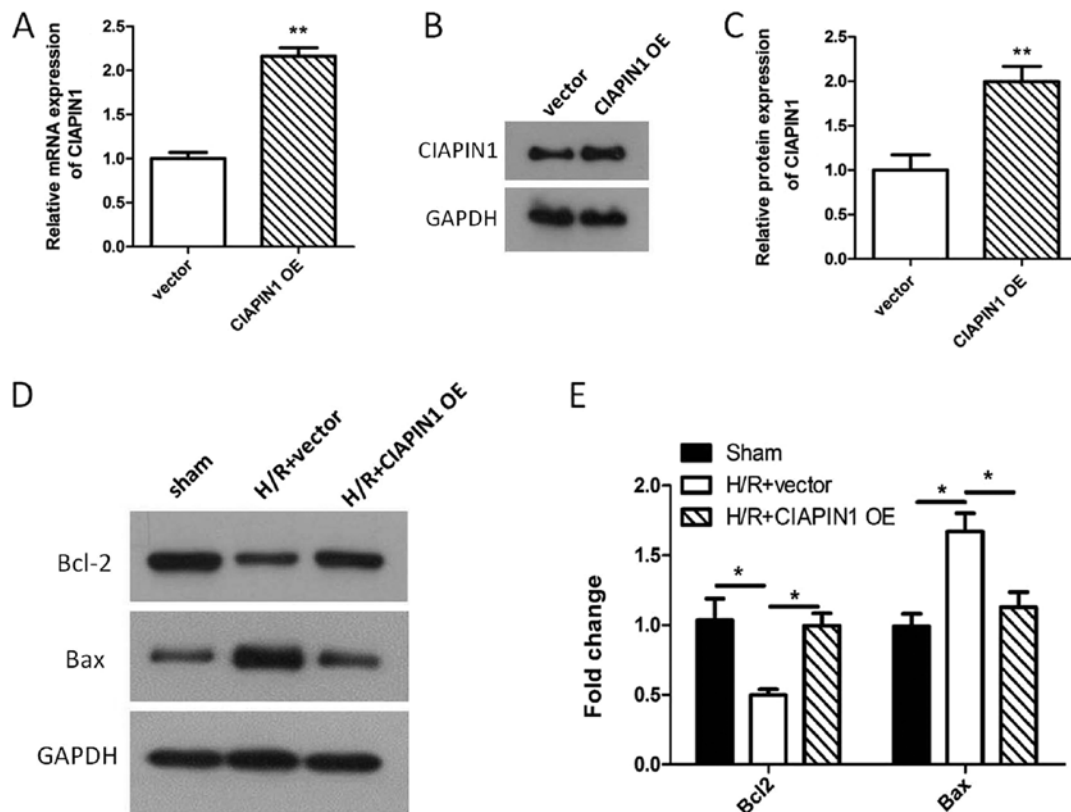
Figure 7. Effect of CIAPIN1 overexpression on the expression of apoptosis-associated proteins in H9c2 cells following H/R. (A) mRNA expression levels of CIAPIN1 in H9c2 cells transfected with pcDNA3.1-CIAPIN1 compared with cells transfected with the empty vector. **P<0.01. (B) Protein expression levels of CIAPIN1 in H9c2 cells was detected by western blotting. (C) Relative protein expression levels of CIAPIN1 in H9c2 cells transfected with pcDNA3.1-CIAPIN1 compared with cells transfected with the empty vector. **P<0.01. (D) Expression levels of Bcl-2 and Bax were determined by western blotting. (E) Relative expression levels of Bcl-2 and Bax. *P<0.05. CIAPIN1, cytokine induced apoptosis inhibitor 1; H/R, hypoxia/reoxygenation; OE, overexpression; vector, empty vector.

was overexpressed in H9c2 cells to determine its effect on apoptosis. As shown in Fig. 7A-C, the mRNA and protein expression levels of CIAPIN1 were significantly increased in H9c2 cells in the CIAPIN1-overexpression group compared with the control (P<0.01). The effect of CIAPIN1 overexpression on the expression of Bcl-2 and Bax in H9c2 cells following H/R was determined by western blotting. The results showed that the expression levels of Bcl-2 were significantly increased, whereas the expression of Bax was significantly decreased in H/R-damaged H9c2 cells transfected with CIAPIN1-OE compared with the H/R+ CIAPIN1-OE group (Fig. 7D and E; P=0.035; P=0.032). These data suggested that overexpression of CIAPIN1 reversed the changes in expression of apoptosis-associated proteins caused by H/R, suggesting that overexpression of CIAPIN1 reduced apoptosis induced by H/R injury.

The NF-κB pathway may be involved in miR-16-mediated protective effects following myocardial ischemia. Western blotting was performed to determine the influence of miR-16 expression on the NF-κB signaling pathway. The results demonstrated that the expression levels of NF-κB and inhibitor of NF-κB alpha (IκBα) remained relatively unchanged despite the change in miR-16 expression. However, the expression levels of p-NF-κB and p-IκBα were significantly upregulated in H9c2 cells following H/R (Fig. 8; 1.82±0.11 vs. 1.00±0.08, P=0.000152; and 1.77±0.07 vs. 1.00±0.00, P=0.000024,

respectively). Upregulation of miR-16 using miR-16 mimics further increased the levels of p-NF-κB and p-IκBα compared with the H/R+NC group (Fig. 8; 3.10±0.14 vs. 1.82±0.11, P=0.000006; and 2.19±0.10 vs. 1.77±0.07, P=0.0017, respectively). In contrast, the expression levels of p-NF-κB and p-IκBα were decreased in the cells transfected with the miR-16 inhibitor group compared with the H/R+NC group (Fig. 8; 1.26±0.13 vs. 1.82±0.11, P=0.002; and 1.45±0.12 vs. 1.77±0.06, P=0.009).

Discussion

miRNAs have been demonstrated to serve important roles in the regulation of MI/R injury (17). In the present study, miR-16 expression was enhanced in H9c2 cells following simulation of H/R. Upregulation of miR-16 expression enhanced the inhibitory effects on H9c2 cell proliferation caused by H/R. Downregulation of miR-16 expression reduced the suppressive effects of H/R on cell proliferation as well as cell apoptosis. These observations suggested that downregulation of miR-16 may mitigate H/R-induced injury. Higher expression levels of miR-16 have been observed in the urine of patients with acute kidney injury (AKI). The researchers identified that miR-16 was transactivated by C/EBP-β, which aggravated the I/R-induced AKI (18), consistent with the results of the present study.

To date, the majority of the research surrounding miR-16 has been focused on its role in cancer, and there are
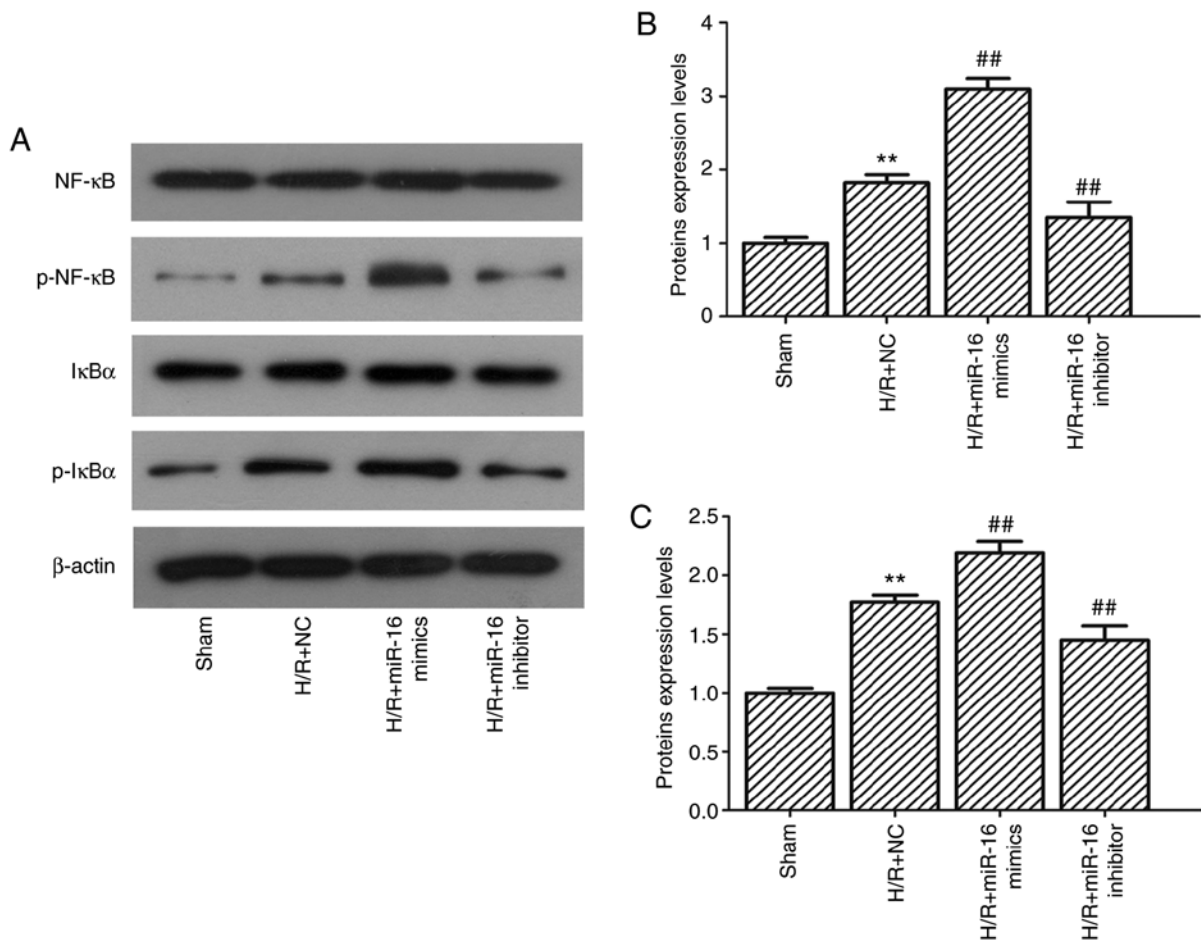
Figure 8. Effect of miR-16 on the expression of NF-κB, p-NF-κB, IκBα and p-IκBα. (A) Protein expression levels of NF-κB, p-NF-κB, IκBα and p-IκBα were determined by western blot. (B) Relative protein expression levels of p-NF-κB in cells following H/R and/or transfection of the miR-mimics or inhibitors. **P<0.01 vs. sham; ##P<0.01 vs. H/R+NC. (C) Relative protein expression levels of p-IκBα in cells following H/R and/or transfection of the miR-mimics or inhibitors. **P<0.01 vs. sham; ##P<0.01 vs. H/R+NC. miR, microRNA; H/R, hypoxia/reoxygenation; p-, phospho; NC, negative control; NF-κB, nuclear factor-κB; IκBα, NF-κB inhibitor α.

conflicting reports on its function based on the type of cancer studied (19-22), suggesting that it may target distinct genes in different organs resulting in varied effects (18). Among the numerous targets of miR-16, a focus was placed on the CIAPIN1 gene in the recent study. CIAPIN1 is a novel apoptosis inhibitor with no association with apoptosis regulators in the Bcl-2 and caspase families (23). Furthermore, it has been identified as an important downstream effector of the Ras signaling pathway (24). At present, numerous studies have shown that CIAPIN1 exhibits different functions based on the physiological and pathological conditions (25-27). In large B-cell lymphoma and ovarian cancer, CIAPIN1 exhibited anti-apoptotic effects and facilitated proliferation (26). However, in esophageal cancer and renal cell carcinoma, CIAPIN1 exhibited an inhibitory effect on tumor cell viability (27). In hypoxia-induced cardiomyocytes, CIAPIN1 has been illustrated to be a target of miR-182-5p, and the expression of CIAPIN1 was dramatically decreased following hypoxia (1). Furthermore, CIAPIN1 overexpression reduced the H/R-induced damage in H9c2 myocytes (28). In the present study, CIAPIN1 was predicted as one of the targets of miR-16. Using a luciferase reporter assay, it was confirmed that miR-16 directly regulated the expression of CIAPIN1 by binding to its

3'-UTR. Expression of CIAPIN1 was downregulated in H9c2 cells following H/R. Increasing expression of miR-16 using miR-16 mimics further decreased both the mRNA and protein expression levels of CIAPIN1 following H/R compared with the H/R+NC group. In contrast, downregulation of miR-16 using an inhibitor resulted in a significant increase in the expression of CIAPIN1 compared with the H/R+NC group. These observations illustrate that CIAPIN1 may be negatively regulated by miR-16.

MI/R is associated with a serious inflammatory response. NF-κB transcription factors take part in a number of physiological processes, including inflammation, immunity and tumorigenicity (29). In the non-activated state, NF-κB exists as a heterotrimer composed of p50, p65 and IκB subunits in the cytoplasm. Upon activation, IκBα is phosphorylated, and subsequently degraded, allowing p65 to translocate to the nucleus and bind to the DNA, leading to gene transcription (29). Numerous studies have suggested the involvement of the NF-κB signaling pathway in the pathogenesis of MI/R (30-32). In myocardial tissues of MI/R mice, the expression of IκBα was significantly increased, and it was reported that downregulation of miR-27a protected mice against MI/R injury, partially by modulating the NF-κB

signaling pathway (31). In rats, myocardial NF-κB expression was significantly increased following MI/R, highlighting its potential role in MI/R (30). In the present study, the relative expression levels of p-NF-κB and p-IκBα were upregulated in H9c2 cells following H/R. Upregulation or downregulation of miR-16 significantly increased or decreased the expression levels of p-NF-κB and p-IκBα levels. Therefore, it was hypothesized that downregulation of miR-16 may reduce H/R injury, at least partially through suppression of the NF-κB signaling pathway, which results in decreased levels of inflammatory cytokines, and subsequently reduced apoptosis. In ulcerative colitis, miR-16 was demonstrated to participate in regulating the NF-κB signaling pathway by modulating the expression of the adenosine A2a receptor (33). Upregulation of miR-16 increased activation of the NF-κB signaling pathway in ulcerative colitis (33), and this result was consistent with the results of the present study. In the bladder cancer cell line, T24, upregulation of miR-16 resulted in deactivation of the NF-κB signaling pathway (34). In addition, miR-16 was reported to decrease glioma malignancy by downregulating NF-κB (35). These seemingly opposite results may result from differences in the tissues used and the diseases studied. Reports regarding the association between CIAPIN1 and the NF-κB signaling pathway are rare. In K562 chronic myeloid leukemia cells, depletion of CIAPIN1 led to relatively lower levels of p-IKKα/β and p-IκBα compared with CIAPIN1 scramble-transfected K562 cells (24). However, in the present study, the expression levels of CIAPIN1 were relatively higher in the miR-16 inhibitor group, whereas the expression levels of p-IκBα and p-NF-κB were relatively lower in this group. Additional studies are required to determine how miR-16 and CIAPIN1 interact with the NF-κB signaling pathway.

In conclusion, the present study highlights the potential of targeting the miR-16/CIAPIN1 axis for treating MI/R-induced injury. However, additional *in vivo* studies are required to verify the present results. The association between miR-16 and other targets, such as serotonin transporter (SERT) (36) and B lymphoma mouse Moloney leukemia virus insertion region (Bmi-1) (37), in MI/R injury may also be worth studying to improve our understanding of the molecular mechanisms underlying the role of miR-16 in MI/R-induced injury.

Availability of data and materials

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

Authors' contributions

HJZ and YNZ conceived the study. HJZ and ZYT conducted the experiments. HJZ wrote the manuscript. YNZ helped interpret the results and revised the manuscript. All authors 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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