MicroRNA-145-5p inhibits hypoxia/reoxygenation-induced apoptosis in H9c2 cardiomyocytes by targeting ROCK1

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Abstract. There is increasing evidence that microRNAs (miRs) play critical roles in the pathological and physiological processes associated with myocardial ischemia reperfusion (I/R). miR-145 has been extensively studied in the cardiovascular system; however, the role of miR-145 in myocardial I/R remains unclear. Therefore, the present study aimed to investigate the role and mechanism of miR-145-5p in myocardial I/R by establishing a hypoxia/reoxygenation (H/R) model using H9c2 cardiomyocytes. The expression of miR-145-5p was regulated by transfection and the potential target of miR-145-5p was identified. In addition, apoptosis of the cardiomyocytes was evaluated using flow cytometry and the detection of cleaved caspase-3 by western blotting. The results revealed that miR-145-5p expression was decreased while cell apoptosis and Rho-associated coiled-coil-containing kinase 1 (ROCK1) expression were increased in H/R-stimulated H9c2 cardiomyocytes. The upregulation of miR-145-5p reduced apoptosis and the expression of ROCK1 in H/R-stimulated H9c2 cardiomyocytes. Furthermore, the overexpression of ROCK1 significantly attenuated the miR-145-5p-induced reduction of apoptosis following H/R. In conclusion, the present study indicates that the overexpression of miR-145-5p inhibits H/R-induced cardiomyocyte apoptosis by targeting ROCK1.

Introduction

The most effective treatment for acute myocardial infarction (AMI) is prompt reperfusion of the ischemic myocardium. However, the restoration of blood flow to the ischemic myocardium may result in arrhythmias, myocardial stunning, microvascular obstruction and lethal myocardial reperfusion injury (1), known as myocardial ischemia/reperfusion injury (I/R). Although several critical factors that act to mediate the damaging effects of myocardial I/R have been identified, there is as yet no effective therapeutic strategy for the prevention of myocardial I/R injury in patients (2).

MicroRNAs (miRs/miRNAs) are endogenous, small, non-coding RNAs that serve crucial roles in the regulation of gene expression via sequence-specific interaction with the 3'-untranslated region (3' UTR) of target messenger RNA (mRNA) (3). miRNAs are highly conserved and ubiquitously expressed in all species. There is evidence to suggest that miRNAs regulate necrotic, apoptotic and autophagic cardiomyocyte death by altering key signaling in myocardial I/R injury (4,5). For example, miR-494 targets pro- and anti-apoptotic proteins to provide cardioprotective effects against I/R-induced injury via activation of the Akt pathway (6). miR-145 is highly expressed in the vasculature and its role in the cardiovascular system has been extensively investigated (7-9). A few studies have explored the role of miR-145-5p in myocardial I/R (10-12). However, the results of these studies were inconsistent and the role of miR-145-5p in myocardial I/R remains elusive.

Rho-kinases (Rho-associated coiled-coil-containing kinases; ROCK) have important roles in numerous cellular functions, including contraction, motility, proliferation and apoptosis (13). Additionally, these kinases have been demonstrated to serve a role in the pathogenesis of vasospasm, arteriosclerosis, heart failure, hypertension, pulmonary hypertension and myocardial I/R injury (14-19). There are two isoforms of ROCK in humans and mice: ROCK1 and ROCK2 (20). Although ROCK1 and ROCK2 are expressed in cardiomyocytes, there are some differences in their functions. A number of studies have indicated that ROCK1 may be a key molecule in the mediation of apoptotic signaling in cardiomyocytes (21-24).

The present study established a hypoxia/reoxygenation (H/R) H9c2 cell model to investigate the effects of miR-145-5p on myocardial I/R injury. The effect of H/R on the expression of miR-145-5p in H9c2 cardiomyocytes was investigated. In addition, the effect of overexpressing miR-145-5p on H/R-induced cardiomyocyte apoptosis and whether the underlying mechanism involves the targeting of ROCK1 were examined. The findings of the present study may reveal novel information to assist in the therapy of myocardial I/R injury.

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Materials and methods

H/R cell model. H9c2 cardiomyocytes were purchased from the American Type Culture Collection (cat. no. CRL-1446) and cultured in DMEM (Hyclone; Cytiva) containing glucose (25 mmol/l), 10% fetal bovine serum (FBS; GE Healthcare) and 1% penicillin/streptomycin at 37˚C with 5% CO_2. Prior to hypoxia, the culture medium was changed to FBS-free DMEM. Hypoxia was induced by culturing the H9c2 cardiomyocytes in FBS-free DMEM without glucose in 95% N_2 and 5% CO_2. Following hypoxia treatment for 6 h, H9c2 cardiomyocytes were cultured in normoxic conditions (with glucose; 5% CO_2) for 6 h for reoxygenation. H9c2 cardiomyocytes cultured in normal conditions served as controls.

Cell transfection. Transfection of the H9c2 cardiomyocytes was performed prior to hypoxia treatment. miR-145-5p mimic (miR10000851), miR mimic negative control (miR mimic-NC; miR1N0000001-1-5), miR-145-5p inhibitor (miR20000851) and miR inhibitor-NC (miR2N0000001-1-5) were purchased from Guangzhou RiboBio Co., Ltd. and transfected into H9c2 cardiomyocytes using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The H9c2 cardiomyocytes were seeded in 6-well plates ($5x10^5$ cells/well) and transfected with 100 pmol miR-145-5p mimic, miR-145-5p inhibitor or the respective controls in 500 μl serum-free media at 37˚C for 24 h. After 24 h of transfection, western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were performed to select the most efficient mimic and inhibitor sequences. The sequences of the miR-145-5p mimic, miR-145-5p inhibitor and controls were as follows: miR-145-5p mimic, 5'-GUCCAGUUUUCCCAGGAAUCCCU-3'; miR-145-5p inhibitor, 5'-AGGGAUUCCUGGGAAAACUGGAC-3'; miR mimic-NC, 5'-UUCUCCGAACGUGUCACGUTT-3'; miR inhibitor-NC, 5'-CAGUACUUUUGUGUAGUACAA-3'. The ROCK1 sequence was sub-cloned into a pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) to generate a ROCK1-expression vector (1 μg/μl) and transfected into the H9c2 cardiomyocytes using Lipofectamine® 2000 at 37˚C for 48 h. Cells transfected with the empty pcDNA3.1 vector were used as the negative control.

Extraction of total RNA and RT-qPCR. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The RNA concentration and purity were quantified using a NanoDrop™ ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc.). The final concentration of RNA was adjusted to 200 ng/μl in all samples. The specific stem-loop primer of miR-145-5p was used in RT-qPCR and the U6 gene was used as an endogenous control when quantifying miR-145-5p; β-actin served as the endogenous control for ROCK1. RT of the RNA was conducted using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was performed with Fast Start Universal SYBR Green I kit (Roche Diagnostics). Fold-changes were calculated using the $2^{-\Delta\Delta Cq}$ method (25) and each data was performed in triplicate. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec. The primer sequences used for RT-qPCR are listed in Table I.

Western blotting. Cells were harvested and lysed with lysis buffer (Beyotime Institute of Biotechnology). The protein concentration of the cell lysate was determined using a BCA assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Protein samples (50 μg/lane) were subjected to 12% SDS-PAGE and transferred to PVDF membranes (GE Healthcare). The membranes were then blocked with 5% defatted milk in TBST (0.5% Tween-20 in TBS) for at 37˚C 1 h and incubated with primary antibodies at 4˚C overnight. The primary antibodies used were as follows: ROCK1 (1:1,000; 158 kDa; cat. no. ab219587; Abcam), pro-caspase-3 (1:1,000; 32 kDa; cat. no. ab90437; Abcam), cleaved caspase-3 (1:1,000; 17 kDa; cat. no. ab49822; Abcam) and β-actin (1:1,000; 42 kDa; cat. no. ab8227; Abcam). Subsequently, the membranes were washed in TBST and then incubated with HRP-conjugated secondary antibody (1:10,000; cat. no. ab205718; Abcam) at room temperature for 2 h. Immunoreactive bands were visualized with the SuperSignal West Pico enhanced chemiluminescent substrate (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Band intensities were quantified using Quantity One software v4.6.6 (Bio-Rad Laboratories, Inc.).

Flow cytometric analysis. To detect cell apoptosis, the cardiomyocytes were digested with 0.25% trypsin at 37˚C for 5 min, washed and double-stained using an Annexin V-FITC/PI Apoptosis Staining/Detection kit (Nanjing KeyGen Biotech Co., Ltd.). The percentage of apoptotic cells was detected using a CytoFLEX flow cytometer (Beckman Coulter, Inc.). Data were analyzed with FlowJo software (10.0.7; TreeStar).

Dual luciferase reporter gene assay. TargetScan online bioinformatics software (http://www.targetscan.org/vert_72/) was used to predict target genes that may be regulated by miR-145-5p, and ROCK1 was identified as a target. H9c2 cardiomyocytes were transfected with the wild-type or mutant ROCK1 reporter plasmids (Shanghai GenePharma, Inc.) together with either miR-145-5p mimic or miR mimic-NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The resulting firefly and *Renilla* luciferase activities were measured 24 h post-transfection using the Dual-Glo™ Luciferase Assay System, according to the manufacturer's instructions (Promega Corporation) The firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis. All experiments were performed at least six times, and representative data are shown. All descriptive variables are expressed as the mean ± SD. The Shapiro-Wilk test was used for testing normality. Comparisons between two groups were conducted using an unpaired Student's t-test when the variables were normally distributed, and Mann-Whitney U test when they were abnormally distributed. Differences among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc tests. Statistical analyses were performed using SPSS 20.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primers used in quantitative PCR.

Primer name	Forward	Reverse
miR-145-5p	ACACTCCAGCTGGGGTCCAGTTTTCCCAGGA	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGT TGAGAGGGATTC
ROCK1	GGAAACGCTCCGAGACACTG	CTGTTCTCACTGGGATTTGCTG
β-actin	TGCTATGTTGCCCTAGACTTCG	GTTGGCATAGAGGTCTTTACGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

miR, microRNA; ROCK1, Rho-associated coiled-coil-containing kinase 1.

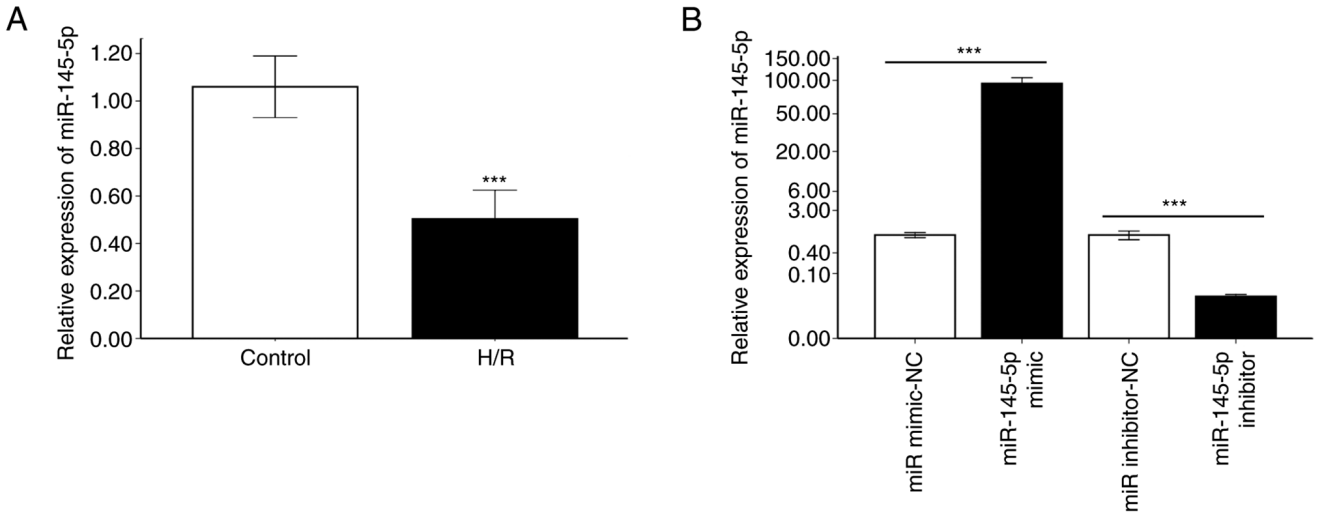

Figure 1. Expression of miR-145-5p in H9c2 cells. (A) miR-145-5p expression is downregulated in cardiomyocytes subjected to H/R. ***P<0.001 vs. control (n=6). (B) Following transfection with miR-145-5p mimic, miR-145-5p inhibitor, miR mimic-NC and miR inhibitor-NC for 24 h, transfection efficiency was verified via reverse transcription-quantitative PCR. The expression of miR-145-5p is significantly increased in the miR-145-5p mimic group and significantly decreased in the miR-145-5p inhibitor group compared with that in the respective control group. ***P<0.001 (n=6). miR, microRNA; H/R, hypoxia/reoxygenation; NC, negative control.

Results

miR-145-5p expression is downregulated in cardiomyocytes following H/R. To assess the changes of miR-145-5p expression in cardiomyocytes subjected to H/R, RT-qPCR was performed on H9c2 cells. miR-145-5p expression in H9c2 cardiomyocytes after H/R was significantly downregulated compared with that in cells cultured under normal conditions (Fig. 1A; P<0.001). miR-145-5p mimic and miR-145-5p inhibitor were then used to transfect H9c2 cardiomyocytes. RT-qPCR analysis revealed that miR-145-5p expression was significantly increased after transfection with miR-145-5p mimic compared with miR mimic-NC, indicating that the miR-145-5p mimic significantly induced miR-145-5p expression in the H9c2 cardiomyocytes (Fig. 1B; P<0.001). In addition, transfection with miR-145-5p inhibitor resulted in lower levels of miR-145-5p expression compared with those in cells transfected with the miR inhibitor NC (Fig. 1B; P<0.001). These results confirm the successful transfection of the H9c2 cells with miR-145-5p mimic and inhibitor.

Upregulation of miR-145-5p reduces H/R-induced cardiomyocyte apoptosis. The proportion of apoptotic cells following H/R injury was determined by flow cytometry with Annexin V-FITC/PI staining (Fig. 2A). The H9c2 cardiomyocytes in the H/R group exhibited a significant increase in apoptosis compared with those in the control group (Fig. 2B; P<0.001). Transfection with miR-145-5p mimic significantly decreased the H/R-induced apoptosis rate compared with that in the H/R + miR mimic-NC group (Fig. 1B; P<0.01), and transfection with miR-145-5p inhibitor significantly increased the apoptosis rate compared with that in the H/R + miR inhibitor-NC group. The activation of caspase-3 is a hallmark of apoptotic cell death (26). Therefore, caspase-3 cleavage in the cells was investigated. Western blotting revealed that transfection of the H9c2 cells with miR-145-5p mimic significantly reduced the H/R-induced increase in caspase-3 cleavage compared with that in the H/R + miR mimic-NC group. In addition, caspase-3 cleavage in the miR-145-5p inhibitor group was significantly increased compared with that in the H/R + miR inhibitor NC group (Fig. 2C and D). The aforementioned findings indicate that miR-145-5p suppressed H/R-induced cardiomyocyte apoptosis.

miR-145-5p directly targets ROCK1 and negatively regulates its expression. TargetScan was used to predict target genes that

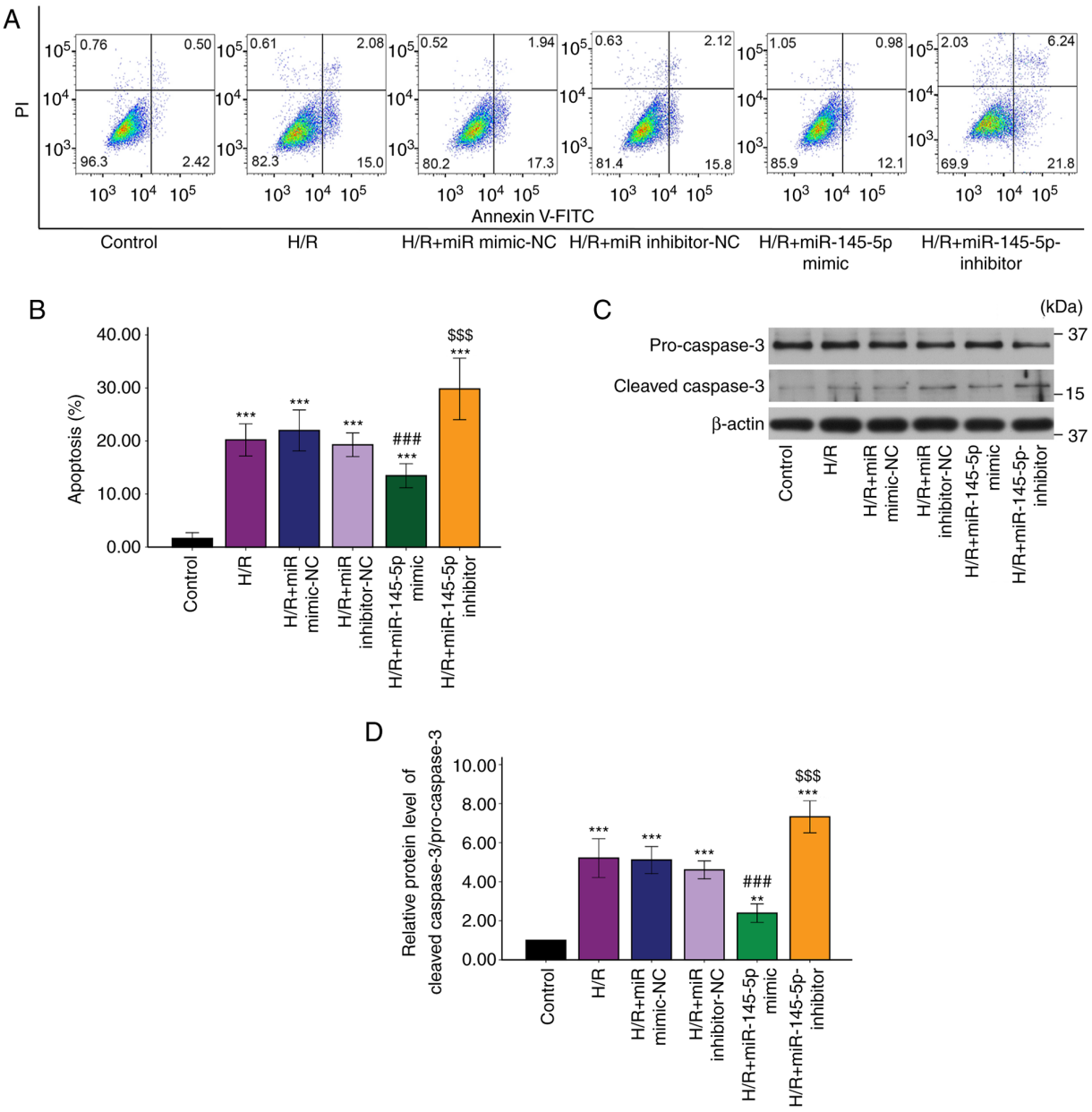

Figure 2. Overexpression of miR-145-5p protects H9c2 cells from H/R-induced apoptosis. (A) Representative flow cytometry plots showing the apoptosis of H9c2 cells analyzed using an Annexin V-FITC/PI staining assay. (B) Statistical representation of the apoptosis rates. (C) Western blot analysis of pro-caspase-3, cleaved caspase-3 and β-actin. (D) Quantitative analysis of the cleaved caspase 3/pro-caspase 3 ratio. **P<0.01 and ***P<0.001 vs. the control group; $^{###}$P<0.001 vs. the H/R + miR mimic-NC group; $^{\$\$\$}$P<0.001 vs. the H/R + miR inhibitor-NC group (n=6). miR, microRNA; H/R, hypoxia/reoxygenation; NC, negative control.

may be regulated by miR-145-5p, and ROCK1 was revealed as a possible target (Fig. 3A). The potential regulation of ROCK1 by miR-145-5p was investigated using a luciferase reporter assay. The results demonstrated that luciferase activity was significantly repressed in the presence of miR-145-5p mimic compared with the miR mimic-NC in cells transfected with the wild-type ROCK1 3' UTR, while the miR-145-5p mimic exhibited no inhibitory effects on luciferase activity in cells transfected with the mutant ROCK1 3' UTR (Fig. 3B). In addition, western blot and RT-qPCR analysis revealed that the protein and mRNA levels of ROCK1 were significantly upregulated in H9c2 cells following H/R compared with those in H9c2 cells maintained under normal conditions (Fig. 3C and D). Furthermore, the transfection of H9c2 cells with miR-145-5p mimic significantly downregulated ROCK1 protein and mRNA expression (Fig. 3E and F). These results indicate that miR-145-5p specifically targeted ROCK1.

miR-145-5p protects H9c2 cells subjected to H/R by targeting ROCK1. To investigate the mechanism underlying the effects of miR-145-5p 1, further experiments were conducted using cells transfected with a ROCK1 overexpression vector (Fig. 3G). Annexin V-FITC/PI double staining demonstrated that the H9c2 cardiomyocyte apoptosis rate significantly decreased following transfection with miR-145-5p mimic and increased following transfection with miR-145-5p inhibitor. However, the apoptosis rate of H9c2 cardiomyocytes transfected with miR-145-5p mimic + pcDNA-ROCK1 was significantly increased compared with that of the H9c2 cardiomyocytes transfected with miR-145-5p mimic alone (Fig. 4A and B). Similarly, transfection with

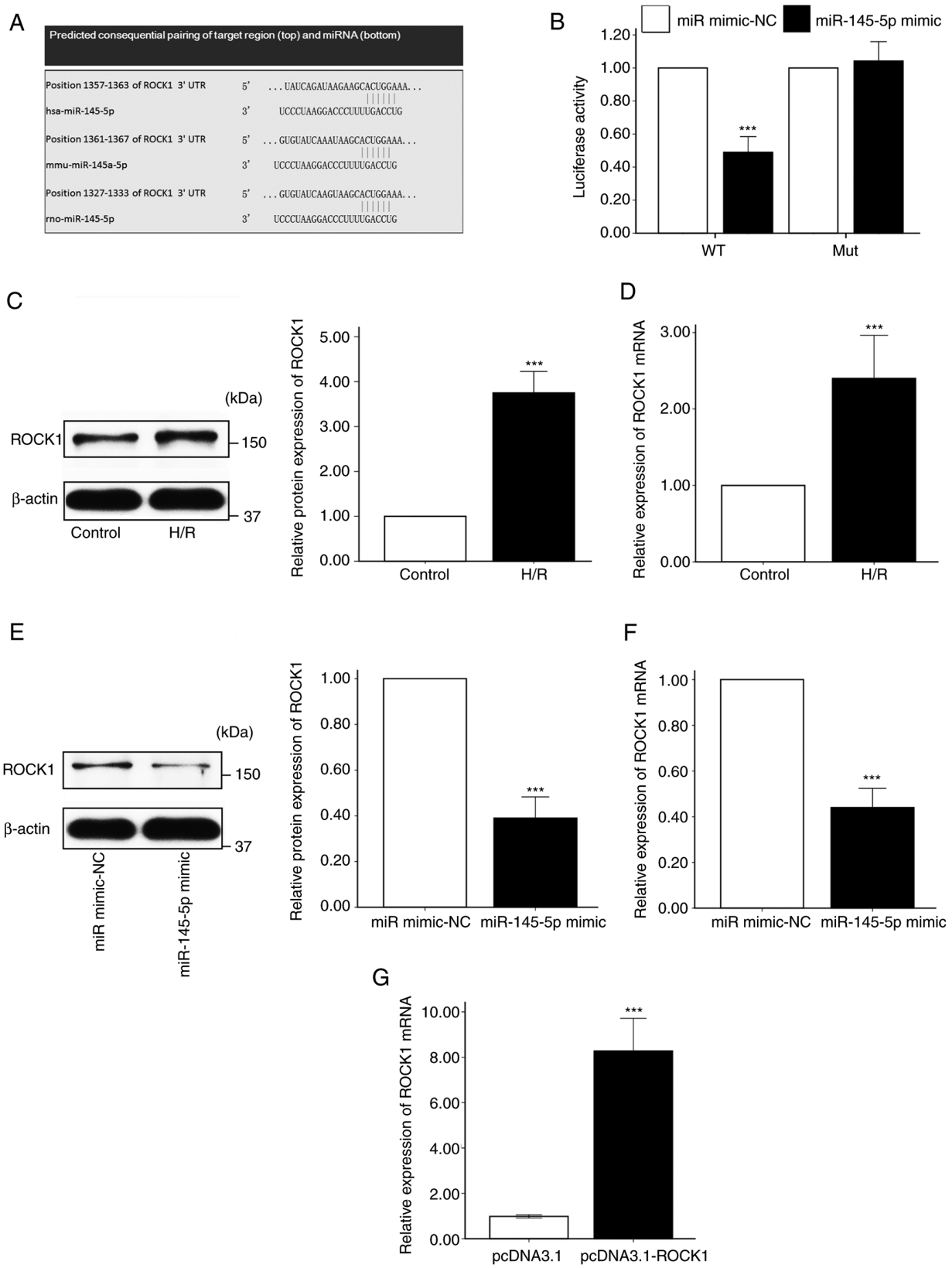

Figure 3. ROCK1 is a target gene of miR-145-5p. (A) The predicted target sequences for miR-145-5p in the ROCK1 3' UTR. (B) Luciferase reporter assays were conducted to demonstrate that miR-145-5p directly binds to the WT target site of ROCK1. (C) ROCK1 protein expression following the exposure of H9c2 cells to H/R. (D) The mRNA levels of ROCK1 following exposure of H9c2 cells to H/R. ROCK1 (E) protein expression and (F) mRNA levels after transfection with miR-145-5p mimic. (G) ROCK1 mRNA levels following transfection with pcDNA3.1 ROCK1 overexpression vector. ***P<0.001 vs. the respective control (n=6). miR, microRNA; H/R, hypoxia/reoxygenation; NC, negative control; ROCK1, Rho-associated coiled-coil-containing kinase 1; WT, wild type; Mut, mutant; UTR, untranslated region.

miR-145-5p mimic + pcDNA-ROCK1 significantly increased caspase-3 cleavage compared with that in the miR-145-5p mimic group (Fig. 4C and D). These results demonstrate that miR-145-5p was unable to protect H9c2 cardiomyocytes against

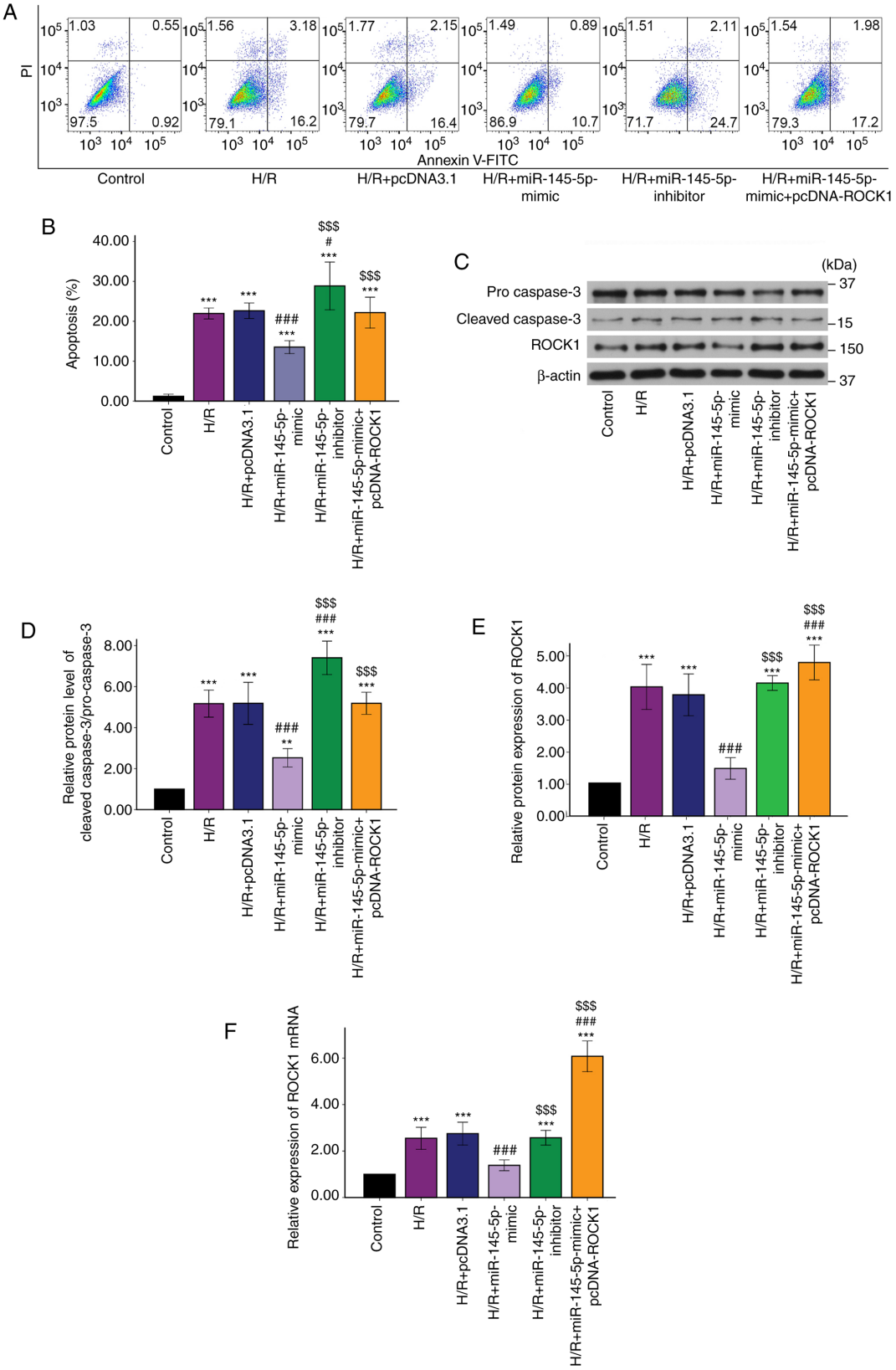

Figure 4. miR-145-5p protects H9c2 cells against H/R-induced apoptosis by targeting ROCK1. (A) Flow cytometric analysis of the Annexin V-FITC/PI staining of H9c2 cells exposed to H/R. (B) Statistical representation of the apoptosis rates. (C) Western blot analysis of caspase-3, cleaved caspase-3, ROCK1 and β-actin. (D) Quantitative analysis of the cleaved caspase 3/pro-caspase 3 ratio. Quantitative analysis of (E) ROCK1 protein expression and (F) ROCK1 mRNA levels. **P<0.01 and ***P<0.001 vs. the control group; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. the H/R and H/R + pcDNA3.1 groups; $^{\$\$\$}$P<0.001 vs. the H/R + miR-145-5p mimic group (n=6). miR, microRNA; H/R, hypoxia/reoxygenation; ROCK1, Rho-associated coiled-coil-containing kinase 1.

H/R-induced apoptosis when ROCK1 was overexpressed. The H/R treatment induced a significant increase in the expression of ROCK1 in the cardiomyocytes at the protein and mRNA levels. Following transfection with miR-145-5p mimic, ROCK1 protein

and mRNA expression levels were significantly decreased in the H9c2 cells subjected to H/R. By contrast, transfection with the miR-145-5p inhibitor resulted in ROCK1 protein and mRNA expression levels comparable with those in the H/R group (Fig. 4E and F). In addition, when the cardiomyocytes were co-transfected with miR-145-5p mimic and pcDNA-ROCK1 vector, the effects of the miR-145-5p mimic on ROCK1 protein and mRNA levels were abolished (Fig. 4E and F). Based on these results, it was concluded that miR-145-5p reduced H/R-induced cardiomyocyte apoptosis by targeting ROCK1.

Discussion

miRNAs are non-coding RNAs that regulate gene expression by inhibiting transcription or degrading mRNA (27). The expression of miRNAs undergoes changes in numerous disease states, including myocardial I/R injury (5). Studies have shown that some miRNAs promote fibrosis and apoptosis while others have opposite effects during myocardial I/R injury. The aim of the present study was to investigate the effects of miR-145-5p on cell apoptosis in a H9c2 cardiomyocyte H/R model. The findings confirmed that miR-145-5p has a protective effect against H/R injury in H9c2 cardiomyocytes. Additionally, the present study demonstrated that ROCK1 is a target of miR-145-5p.

Increasing evidence, summarized in a recent review, has shown that miR-145-5p is associated with vascular biology and the pathophysiology of cardiovascular disease (28). For example, a previous study reported that miR-145-5p promoted the apoptosis of cardiomyocytes after myocardial I/R in rats (12). However, other studies demonstrated that miR-145-5p protected against myocardial I/R injury in murine models via anti-apoptotic, anti-inflammatory and autophagy pathways (10,11,29). Hence, the role of miR-145-5p in myocardial I/R injury remains unclear. In the present study, after subjecting H9c2 cells to H/R, RT-qPCR was performed to detect the expression of miR-145-5p and the results revealed that its expression in the H/R-treated cells was significantly decreased. Following the discovery that miR-145-5p was repressed in H9c2 cardiomyocytes subjected to H/R, a miR-145-5p mimic and inhibitor were each transfected into H9c2 cells to assess the role of miR-145-5p in cardiomyocytes exposed to H/R. Flow cytometry was used to detect the apoptosis rate of the H9c2 cardiomyocytes. Transfection with miR-145-5p mimic significantly reduced the H/R-induced apoptosis of the H9c2 cardiomyocytes. This finding suggests that miR-145-5p plays a protective role against myocardial I/R injury.

Our previous studies indicated that ROCK has an important role in myocardial I/R injury. One of these studies revealed that ROCK inhibition reduced infarct size and the extent of cardiomyocyte apoptosis induced by myocardial I/R injury *in vivo* (30), while another identified increased ROCK activity in the peripheral blood leukocytes of patients with AMI undergoing primary percutaneous coronary intervention, suggesting that ROCK activity is increased in patients with myocardial I/R (31). ROCK has two isoforms, ROCK1 and ROCK2, which are ~64% similar in sequence and have overlapping substrates (32). However, the two isoforms may have different roles in various types of cells and tissues (33,34). For example, a study indicated that ROCK1 protects the heart against pressure overload-induced heart failure with postcapillary pulmonary hypertension while ROCK2 compromises it (34).

Based on a TargetScan database search and double-luciferase reporter assay, the present study demonstrated that miR-145-5p directly targets ROCK1. A previous study showed that ROCK1 is targeted by miR-145-5p in laryngocarcinoma cells, and the suppression of ROCK1 displays effects similar to those of miR-145-5p (35). Consistent with this, the present study detected a negative interaction between miR-145-5p and ROCK1 expression in H9c2 cardiomyocytes. ROCK1 expression in cardiomyocytes was significantly increased by H/R compared with that in control myocytes cultured under normal conditions. In addition, the levels of ROCK1 mRNA and protein were reduced in H9c2 cardiomyocytes transfected with miR-145-5p mimic compared with H9c2 cardiomyocytes without transfection or transfected with miR-145-5p inhibitor. These results indicate that ROCK1 expression is negatively regulated by miR-145-5p in H9c2 cardiomyocytes. The present findings also indicate a positive association of ROCK1 expression with apoptosis in H9c2 cardiomyocytes subjected to H/R. Elevated miR-145-5p expression may rescue cardiomyocytes from apoptosis during H/R injury via the inhibition of ROCK1. Notably, the overexpression of ROCK1 significantly attenuated the miR-145-5p-induced inhibition of cell apoptosis following H/R, which further confirms the relationship between miR-145-5p and ROCK1.

It should be noted that the experiments in the present study were conducted only in H9c2 cardiomyocytes, and the significance and implications of the results require verification in another cardiac cell line or stem cell-derived cardiomyocytes. Moreover, only *in vitro* experiments were performed. Further studies with *in vivo* experiments are required.

In conclusion, the present study showed that miR-145-5p is significantly downregulated in H/R, and indicated that the overexpression of miR-145-5p reduced H/R-induced H9c2 cardiomyocyte apoptosis by targeting ROCK1. These results demonstrate a cardioprotective effect of miR-145-5p and suggested its potential as a therapeutic approach for the alleviation of myocardial I/R injury.

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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

JZ designed the experiments and drafted the manuscript. CC and DLX performed the experiments and analyzed the data. XBL and SJB analyzed and interpreted the data. JZ and CC confirm the authenticity of all the raw data. 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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