Original Article
miR-190 protects cardiomyocytes from apoptosis induced by H_2O_2 through targeting MAPK8 and regulating MAPK8/ERK signal pathway

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Abstract: MicroRNAs (miRs) have been demonstrated to regulate physiological and pathological processes. Numerous miRsprotect against cardiomyocyte injury induced by oxidative stress. However, the function of miR-190 still remains unclear. Here, we determined the expression level of miR-190 in H9c2 cells under H_2O_2 treatment and found that miR-190 expression was significantly inhibited by H_2O_2. Further study indicated that miR-190 significantly reduced cell apoptosisand the LDH and MDA levels of H9c2 cells induced by H_2O_2. Luciferase activity assay, quantitative real-time-PCR, and Western blot demonstrated that miR-190 directly targets MAPK8. Rescue experiment confirmed this hypothesis. Further study has revealed that miR-190 protects H9c2 cells from oxidative stress injury through inhibiting the MAPK8/ERK signal pathway. In conclusion, these data suggest that miR-190 protects against oxidative stress injury of H9c2 cells induced by H_2O_2 through inhibiting MAPK8 expression and the MAPK8/ERK pathway. Our findings provide a potential therapeutic target to promote functional recovery after cardiac ischemia/reperfusion.

Keywords: MiR-190, cardiomyocytes, apoptosis, MAPK8

Introduction

Acute myocardial infarction (AMI) is the most common cause of cardiovascular death all over the world [1]. Apoptotic cell death has been reported in a large number of research studiesof the pathogenesis of AMI and apoptosis was frequently found in the infarct myocardium [2-4]. During the process of myocardial ischemia or reperfusion injury, oxidative stress induces the generation of large amounts of reactive oxygen species which breaks the balance between pro-apoptosis and anti-apoptosis proteins such as bax and bcl-2, therefore inducing cell apoptosis [4, 5]. Elucidating the molecular mechanisms of cardiomyocyte apoptosis would be of great importance in tackling apoptosis related heart disease.

microRNAs (miRs) are 20-23 nucleotides, endogenous, non-coding RNAs which regulate target gene expression directly leading to the degradation of mRNAs or inhibiting the translation of their target genes [6-8]. According to this kind of gene function, miRs are involved in various biological processes such as cell proliferation, differentiation, apoptosis, autophagy, and cell cycle. Recent studies have demonstrated that miRs participate in regulating cadiomyocyte apoptosis [9, 10]. miR-486 regulates cardiomyocyte apoptosis by p53-mediated BCL-2 associated mitochondrial apoptotic pathway [11]. miR-122 regulates caspase-8 and promotes theapoptosisof mouse cardiomyocytes [12]. miR-142-3p inhibits hypoxia/reoxygenation-induced apoptosis and fibrosis of cardiomyocytes by targeting high mobility group box 1 [13]. miR-322 protects hypoxia-inducedapoptosisin cardiomyocytes via BDNF gene [14]. However, the potential roles of miR-190 in H_2O_2-induced cardiotoxicity have not been well illustrated.

In our present study, we established a cardiomyocyte injury model to identify whether miR-190 could reduce cardiomyocyte apoptosis induced by H_2O_2. Furthermore, we investigated the molecular mechanism including the target gene of miR-190 and the downstream signal pathway regulated by miR-190 during the process of cardiomyocyte apoptosis. Our findings provide a novel potential biomarker for the treatment or diagnosis of ischemic heart disease.

Methods and materials

Cell culture and treatment

The H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, UT, USA), 2 mM l-glutamine, and penicillin/streptomycin (Invitrogen, CA, USA) in an atmosphere of 95% air and 5% CO_2. The miR-190 mimic and mimic negative control were synthesized by Genepharma (Shanghai, China). Cells were transfected with the miR mimic or plasmids using Lipofectamine2000 (Thermo Fisher Scientific), following the manufacturer's protocols.

Quantitative real-time PCR

Total RNA was extracted with TRIzolReagent (Invitrogen, CA, USA) according to the manufacturer instructions. 2 μg total RNA were reverse-transcribed into cDNA using the SuperScript First Strand cDNA System (Invitrogen, CA, USA). The cDNA was used as the template and quantitative PCR was conducted using a SYBR Green PCR kit (Qiagen, Shanghai, China) and determined on an ABI PRISM 7700 system (Applied Biosystems, CA, USA). All of the experiments were carried out in triplicate. GAPDH and U6 were utilized as the internal control for the mRNA and miR respectively.

Western blot

Total protein of the H9c2 cells were extracted using RIPA lysis buffer (Beyotime, Shanghai, China). The protein concentration was determined on a nanodrop system (Thermo fisher, CA, USA). 40 μg protein was separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). The blots were blocked with 5% non-fat milk in TBST buffer for 1 hour and incubated with the primary antibodies at 4°C overnight. The membranes were then incubated by horseradish peroxidase-conjugated (HRP) conjugated secondary antibody at room temperature for another 2 hours. The blots were visualized and signals were detected by an enhanced ECL-based imaging system.

Flow cytometry

Cell apoptosis was assessed by FACS analysis of annexin V/propidium iodide (PI) staining. The H9c2 cells were digested with 0.20% trypsin-EDTA solution (Invitrogen, CA, USA) for 5 minutes at 37°C and resuspended. Cells at 1×10^6 cells/mL were added into 1× binding buffer (Sigma, USA) and incubated with annexin V-fluorescein isothiocyanate (FITC) and PI (Sigma-Aldrich) in the dark. Fifteen minutes later, cell apoptosis was analyzed by flow cytometry (FACS Calibur, BD Biosciences, USA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

Cellular apoptosis was analyzed using TUNEL assay. After treatment, cells were fixed by 4% paraformaldehyde and TUNEL assay was carried out using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany), following the manufacturer's protocols. The apoptotic rate was calculated as the percentage of TUNEL-positive cells divided by the total number of cells.

Luciferase activity assay

TargetScan and Pictar were used to predict the 3'UTR sites miR-190 binds to MAPK8. The wild or mutant miR-190 binding sequences predicted before were cloned into the pGL3 reporter vector (Promega, WI, USA). For luciferase assay, H9c2 cells were plated in 12-well plates and then transfected with wild-type or mutant pGL3 plasmid with or without miR mimic or mimic negative control along with Renilla luciferase reporter plasmid. The luciferase activity was evaluated using the Dual-Luciferase Assay System (Promega, WI, USA), according to manufacturer instructions. Firefly luciferase activity was normalized by the corresponding Renilla luciferase activity.
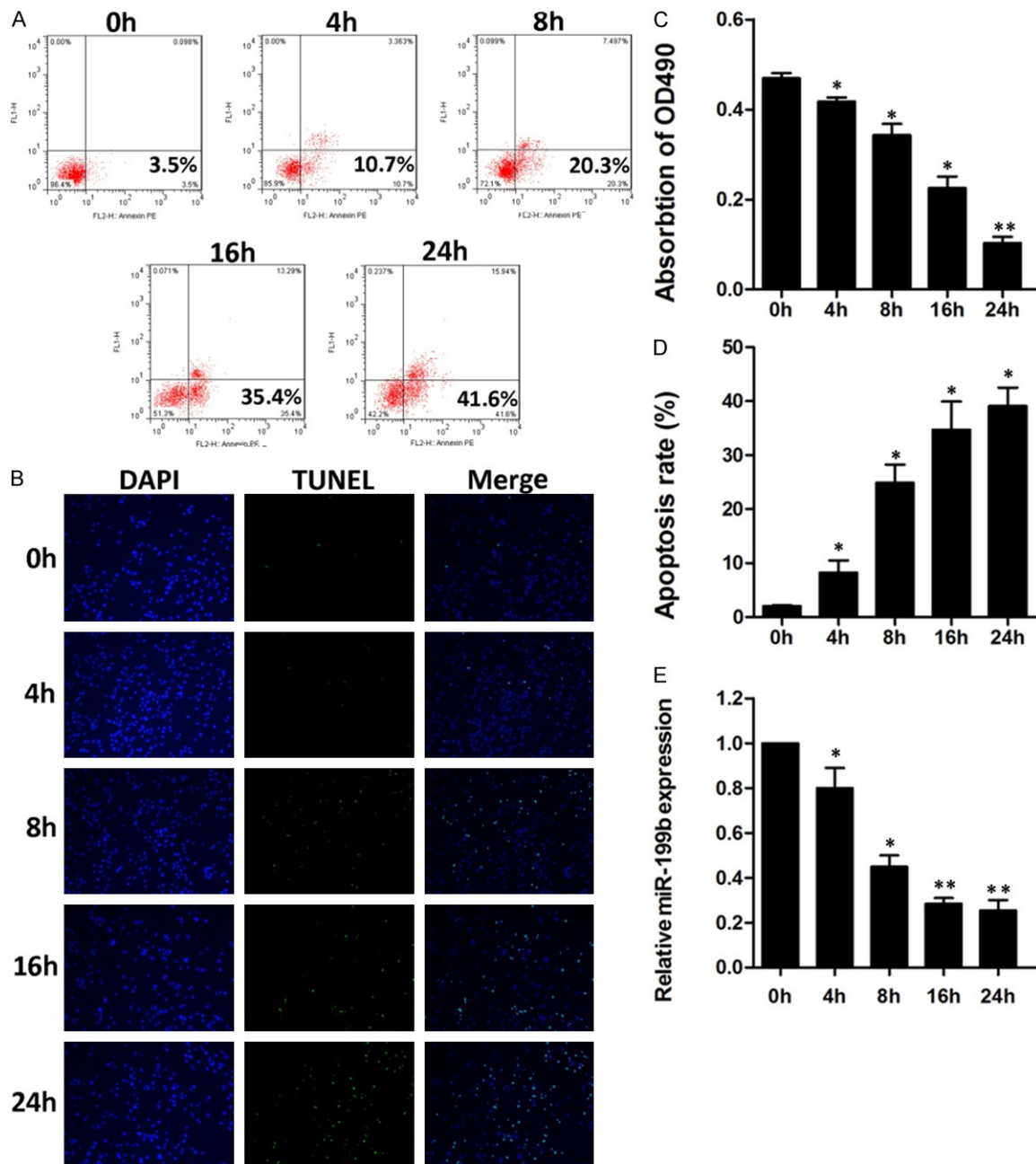
Figure 1. H_2O_2 inhibits the proliferation and induced apoptosis of H9c2 cells and inhibitsthe expression level of miR-190. A, D: Flow cytometry was used to evaluate the cell apoptosis of H9c2 cells. B: TUNEL assay was carried out to evaluate the apoptosis of H9c2 cells. C: MTT was used to detect the cell viability of H9c2 cells under treatment of different concentrations of H_2O_2. E: Quantitative real-time PCR was used to detect the expression level of miR-190. Data represent the mean ± SD, n=3 independent experiments. *P<0.05, **P<0.01.

Immunofluorescence staining

The H9c2 cells were seeded on 12-well plates and cultured for 24 hours at 37°C. After transfection and treatment with H_2O_2, the cells were washed twice in PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. The cells were then permeabilizedwith 0.1% Triton X-100, washed, and incubated with blocking buffer for 1 hour at room temperature. The cells were then incubated overnight with primary antibodies against AC-tubulin (6-11B-1; ab24610) at 1:200 dilution and Nrf2 (both from Abcam) at a 1:200 dilution in a hu-
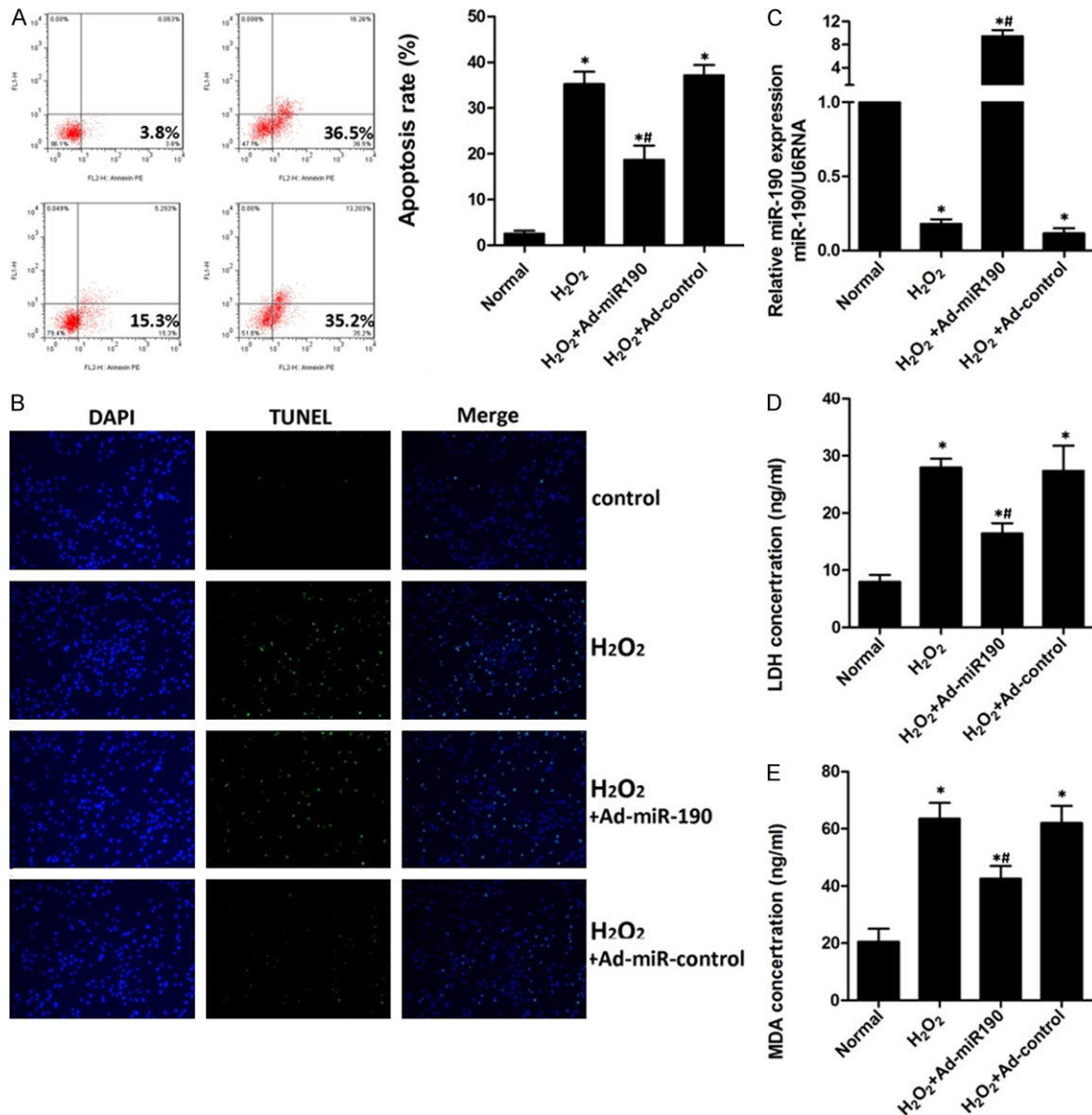
Figure 2. miR-190 over-expression significantly inhibitsoxidative stress and H9c2 cell apoptosis induced by H_2O_2. A: Flow cytometry was used to evaluate the cell apoptosis of H9c2 cells. B: TUNEL assay was carried out to evaluate the apoptosis of H9c2 cells. C: Quantitative real-time PCR was used to detect the expression level of miR-190. D: The level of LDH under H_2O_2 treatment and at the same time along with Ad-miR-190 or Ad-miR-control. E: The level of MDA under H_2O_2 treatment and along with Ad-miR-190 or Ad-miR-control. Data represent the mean ± SD, n=3 independent experiments. *P<0.05, **P<0.01.

midified chamber at 4°C. Subsequently, the cells were washed with PBS and incubated with secondary antibodies conjugated to either FITC or TRITC at a dilution of 1:500 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 hour at room temperature in the dark. They were then counterstained with DAPI dye to show the nuclear morphology. After the slides were rinsed with PBS, coverslips were mounted on slides and images of the labeled cells were vis-

ualized and photographed using a confocal fluorescence microscope (TCS SP2; Leica, Wetzlar, Germany).

Statistics analysis

SPSS 19.0 (SPSS, IL, USA) was used for data analysis. All data were expressed as the mean ± SD. Differences between groups were determined by ANOVA one-way analysis. Differences were considered statistically significant at P<0.05.
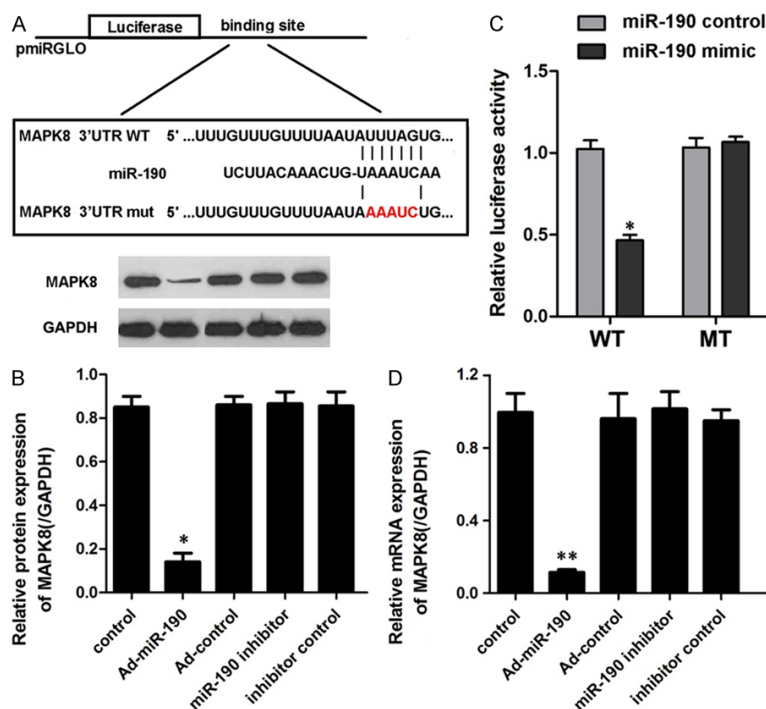
Figure 3. miR-190 directly targets MAPK8 in H9c2 cells. A: Schematic representation of 3'UTR of MAPK8 mRNA reporter with and without the miR-190 seed-binding site. B: Luciferase activity assay of H9c2 cells transfected with plasmids containing wild type 3'UTR and mutant 3'UTR of MAPK8 as well as miR-190 mimic or mimic control. C: Western blot was used to detect the expression level of MAPK8. D: Quantitative real-time PCR was used to evaluate the mRNA expression level of MAPK8. Data represent the mean ± SD, n=3 independent experiments. *P<0.05, **P<0.01.

Results

H_2O_2 exposure induces cardiomyocyte oxidative damage and inhibits miR-190 expression

H9c2 cell was treated with 200 Mm H_2O_2 for 2, 4, 8, 16, 24 hours. Flow cytometry and TUNEL analysis indicated that H_2O_2 treatment significantly induced H9c2 cell apoptosis. The apoptosis rate was positively correlated with the concentration of H_2O_2 (**Figure 1A-C**). We next investigated the miR-190 level of H9c2 cells under H_2O_2 treatment. The qPCR results revealed that H_2O_2 treatment significantly inhibited miR-190 expression and the inhibition effect was also positively correlated with the concentration of H_2O_2 (**Figure 1E**).

miR-190 significantly reduces H9c2 cell oxidative damage induced by H_2O_2

To investigate the role of miR-190 in the oxidative damage, we overexpressed miR-190 by transfection with miR-190 mimics. FSC and TUNEL analysis revealed that miR-190 ove expression also inhibited the apoptosis compared to that in H9c2 cell induced by H_2O_2 (**Figure 2A**, **2B**). In addition, miR-190 significantly reduced the LDH and MDA levels of H9c2 cell subjected to H_2O_2 (**Figure 2D**, **2E**). Moreover, Western blot was used to detect the protein expression and we found that proapoptosis proteins such as cyto C, bax, cleaved caspase9, and cleaved-caspase3 were all upregulated, however, anti-apoptosis protein bcl-2 was significantly downregulated.

miR-190 directly targets MAPK8 in H9c2 cells

miRs play key roles in various biological processes through binding to the target genes. To illustrate the mechanism of miR-190 effects in H9c2 cells, we carried out luciferase activity assay. Target scan and Pictar were used to predict the potential binding sites of the target gene of miR-190 (**Figure 3A**). Luciferase activity assay revealed that miR-190 reduced the activity of the luciferase reporter fused with the wild MAPK8 3'-UTR but not the mutant MAPK8 3'-UTR and miR mimic did not change the luciferase activity of neither reporter fused with the wild nor mutant MAPK8 3'-UTR (**Figure 3C**). Western blot and quantitative realtime PCR were used to confirm the prediction. As expected, miR-190 mimic transfection significantly reduced MAPK8 expression level (**Figure 3B**, **3D**).

MAPK8 restoration reverses the protective effect of miR-190 on oxidative stress injury

To further confirm the role of miR-190 and MAPK8 in H_2O_2 induced oxidative stress injury, we established a rescue experiment. H9c2 cell were transfected with miR-190 and pcDNA3.1-MAPK8 plasmid. FSC, and TUNEL analysis indicated that MAPK8 restoration increased cell
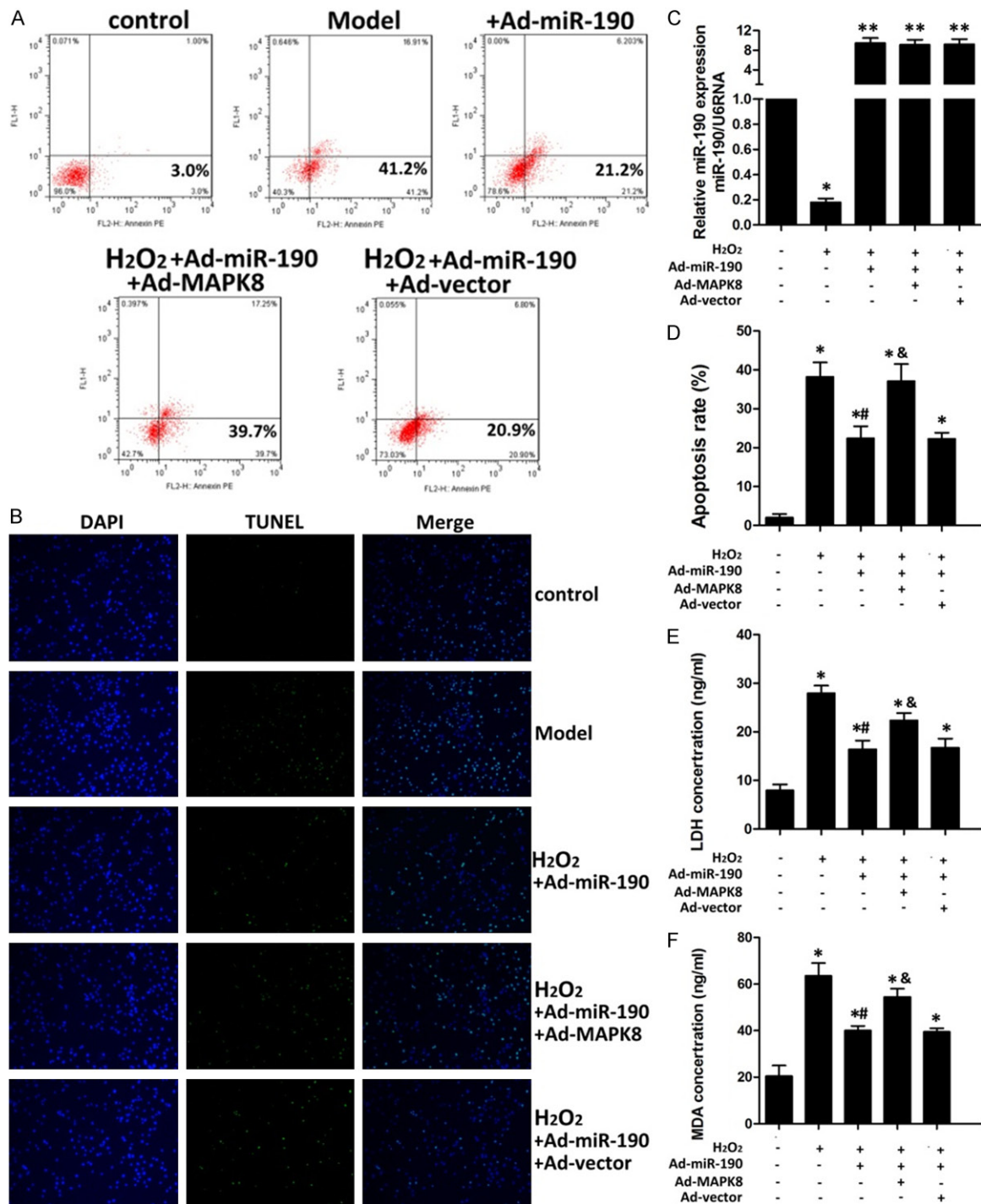
Figure 4. MAPK8 overexpression reverses the protective effect of miR-190 on H9c2 cell apoptosis induced by H_2O_2 treatment. A, D: Flow cytometry was used to detect the apoptosis of H9c2 cells under different treatments. B: TUNEL assay was carried out to evaluate the apoptosis of H9c2 cells under different treatments. C: Quantitative real-time PCR was used to detect the expression level of miR-190. E: The level of LDH under different treatment. F: The level of MDA under different treatment. Data represent the mean ± SD, n=3 independent experiments. *P<0.05, **P<0.01.

apoptosis rate compared to that in miR-190 transfection group (**Figure 4A**, **4B**, **4D**). In addi-

tion, miR-190 significantly reduced the LDH and MDA levels of H9c2 cell subjected to H_2O_2
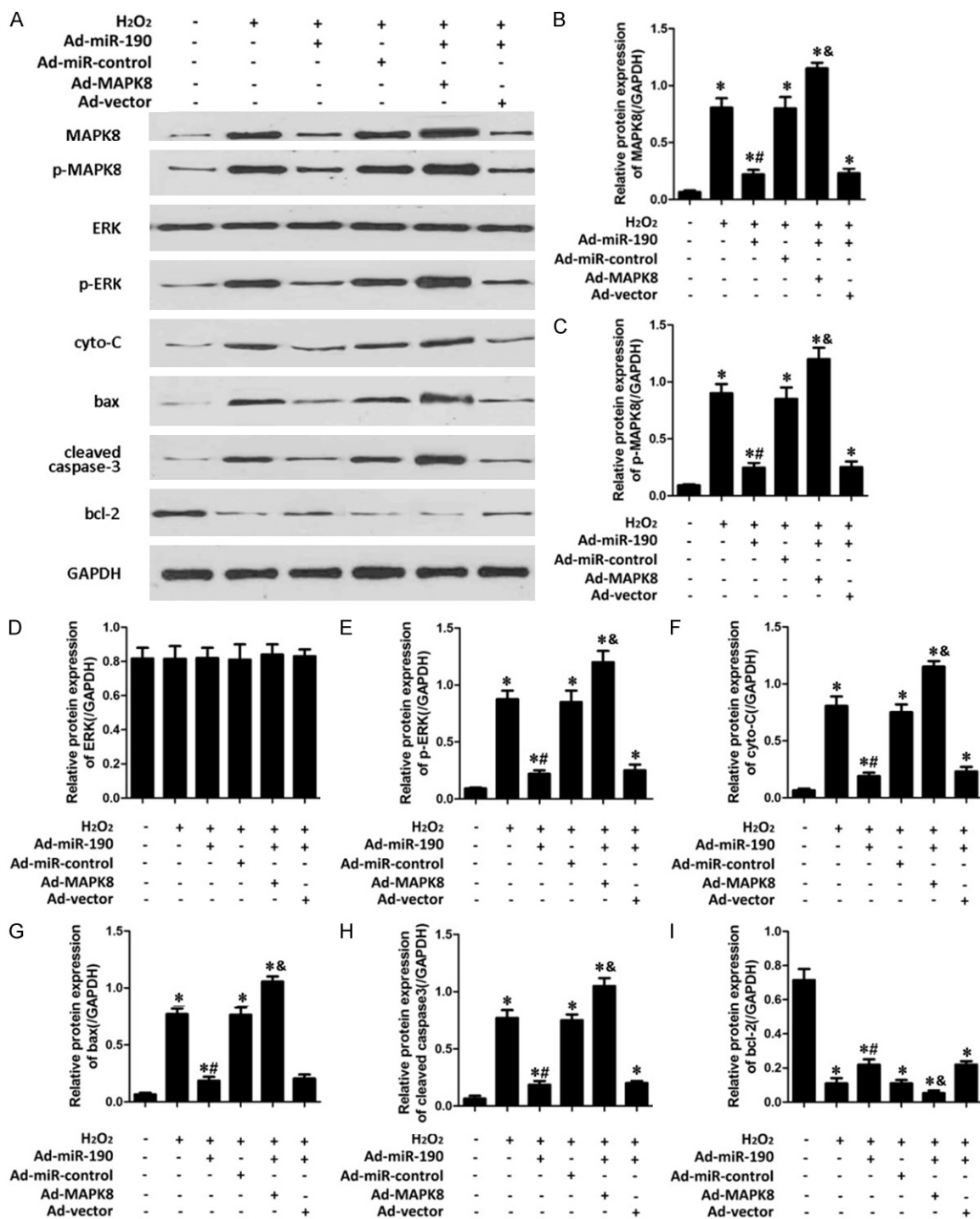
Figure 5. miR-190 regulates the MAPK8/ERK signal pathway and downstream apoptotic proteins. A-I: Western blot was carried out to evaluate the protein expression level of MAPK8, p-MAPK8, ERK, p-ERK, cyto-C, bax, cleaved-caspase3, and bcl-2. Data represent the mean ± SD, n=3 independent experiments. *P<0.05, **P<0.01.

(**Figure 4E**, **4F**). Moreover, Western blot was used to detect protein expression and we found that pro-apoptosis proteins such as cyto C, bax,

cleaved-caspase9, and cleaved-caspase3 were all upregulated, however, anti-apoptosis protein bcl-2 was significantly downregulated.

miR-190 protects H9c2 cells from oxidative stress injury through regulating MAPK8/ERK signal pathway

We then discovered the deep mechanism through which miR-190 protect H9c2 cells from oxidative stress injury. Western blot was carried out to investigate the protein expression level. The results showed that miR-190 inhibited the expression and phosphorylation of MAPK8 at the same time. Furthermore, miR-190 inhibited the phosphorylation but not the expression level of ERK. Apoptotic protein expression was then evaluated. miR-190 significantly inhibited pro-apoptosis protein expression such as cyto-C, bax, and caspase and promoted the anti-apoptosis protein levels such as bcl-2. In addition, the overexpression of MAPK8 by Ad-MAPK8 infection significantly reversed the effect of miR-190 on the protein expression or phosphorylation (**Figure 5A-I**).

Discussion

Extensive apoptosis and necrosis of cardiomyocytes at the earliest stage is a crucial clinical indicator of reperfusion injury [15]. Apoptosis is characterized by cell shrinkage, plasma membrane blebbing, chromatin compaction, and nuclear fragmentation which play a key role in myocardial I/R injury [16]. Apoptosis can be divided into extrinsic pathway which was mediated by the binding of TNF and the Fas ligand and intrinsic pathway which is controlled by the mitochondrial enzymes [17-21]. During mitochondrial-mediated apoptosis, oxidative stress increased intracellular ROS lead to loss of the MMP. Cytochrome C is then released from mitochondrial into cytosol and subsequently activates caspase-9 and the downstream protein such as caspase3 to start cell apoptosis [22, 23]. Lipid peroxidation in cells is due to the oxidative degradation of lipid which results in cell damage. MDA is a common indicator in identifying the level of lipid peroxidation and LDH could be used to evaluate the cytopathic effect of pathogen which is released into the cell culture medium through damaged cell membrane. Both are key factors indicating oxidative stress [24].

Increasing evidence has indicated that miRs participate in the regulation of ischemic reperfusion injury [25-28]. miR-190 has been report-ed to promote gastric tumor growth through binding to FOXP2 and enhances HIF-dependent responses to hypoxia in drosophila by inhibiting the Prolyl-4-hydroxylase Fatiga [29, 30]. However, the role of miR-190 in ischemic injury has not been studied previously.

In our present study, we found that overexpression of miR-190 could significantly reduce the MDA and LDH release and cell apoptosis induced by H_2O_2 treatment through mitochondrial mediated pathway.

Mitogen-activated protein kinase 8 (MAPK8; also known as JNK1) is a member of the mitogen-activated protein kinases (MAPK) family which is critical for cellular function through regulating numerous signaling pathways. MAPK8 is activated by many environmental stimuli including myocardial reperfusion. As a pro-apoptotic kinase, activation of JNK is believed to be important in the induction of cardiomyocyte apoptosis in various pathologies [31, 32]. For instance, IL-33 attenuates anoxia/reoxygenation-induced cardiomyocyte apoptosis by inhibition of PKCβ/MAPK8 pathway [33]. mIGF-1/JNK1/SirT1 signaling confers protection against oxidative stress in the heart [34]. Akt mediates 17 beta-estradiol and/or estrogen receptor-alpha inhibition of LPS-induced tumor necrosis factor-alpha expression and myocardial cell apoptosis by suppressing the JNK1/2-NFkappaB pathway [35]. The main mechanism of JNK activation is cell stress including the reactiveoxygen species (ROS) which is consistent with our findings.

In our current work, we found that miR-190 reduced MAPK8 expression level. The further luciferase activity assay and rescue experiment firstly confirmed that miR-190 directly target MAPK8 in H9c2 cell. Interestingly, we found that miR-190 overexpression promotes the phosphorylation of MAPK8. We speculate that the reduced protein level of MAPK8 lead to the decreased phosphorylated substrate. It is well known that MAPK/ERK pathway is involved in various pathology process such as cell proliferation, metastasis, and apoptosis [36-38]. Our findings suggest that miR-190 could reduce oxidative stress and cell apoptosis induced by H_2O_2 treatment in cardiomyocytes through directly targeting MAPK8 and regulating the MAPK8/ERK pathway. This understanding of

miR-190 and MAPK8 could potentially contribute to novel strategies for cardioprotection against ischemia injury.

Disclosure of conflict of interest

None.

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