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Inhibition of microRNA-802-5p inhibits myocardial apoptosis after myocardial infarction *via* Sonic Hedgehog signaling pathway by targeting PTCH1

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Abstract. – OBJECTIVE: The incidence of acute myocardial infarction (AMI) has increased significantly in recent years, seriously threatening human life and health. This paper focused on the role of microRNA-802-5p (miR-802-5p) in myocardial infarction (MI) and its underlying mechanisms.

MATERIALS AND METHODS: Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) was employed to detect miR-802-5p expression. Western blot was performed to detect protein expression. Flow cytometry and terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining were performed to observe myocardial apoptosis. Hematoxylin-eosin (HE) staining was used to observe the morphology of myocardial tissue. The cardiac function of rats was detected using echocardiography.

RESULTS: The expression of miR-802-5p was increased in hypoxic-treated H9c2 cells and infarcted myocardium in MI rats. Hypoxia treatment reduced the viability of cardiomyocytes and increased the level of lactate dehydrogenase (LDH) in the cell supernatant. Hypoxia treatment increased Bax expression in myocardial cells while Bcl-2 expression decreased, and the number of apoptotic cells increased. MiR-802-5p silencing reversed these effects. Moreover, miR-802-5p silencing reduced myocardial damage in MI rats, and significantly improved cardiac function. Through the Luciferase activity assay, we proved that miR-802-5p could directly target PTCH1. The knockdown of PTCH1 reversed the protective effect of miR-802-5p silencing on hypoxic myocardium.

CONCLUSIONS: MiR-802-5p expression was increased in hypoxia-treated H9c2 cells and infarcted myocardium in MI rats. MiR-802-5p silencing could inhibit apoptosis after MI via activating Sonic Hedgehog signaling pathway by targeting PTCH1, thereby reducing myocardial injury and improving cardiac function of MI rats.

Key Words:
Acute myocardial infarction, MicroRNA-802-5p, Apoptosis, PTCH1, Sonic Hedgehog signaling pathway.

Introduction

Acute myocardial infarction (AMI) is caused by human or non-human factors, resulting in rapid and continuous hypoxic-ischemic phenomenon in the coronary arteries, which eventually leads to myocardial necrosis, which is one of the common clinical emergencies[1,2]. Due to its high mortality rate and poor prognosis, it has caused a serious threat to people's health. At present, the treatment of myocardial infarction (MI) is diversified, including drug therapy, coronary artery bypass and interventional therapy, and stem cell transplantation therapy[3,4]. Conventional treatments, such as drug therapy and coronary intervention, although alleviating the patient's pain to a certain extent, cannot fundamentally recover the damaged myocardium.

The main manifestation of AMI is myocardial cell apoptosis. Coronary artery occlusion causes local myocardial tissue ischemia and hypoxia, which leads to myocardial cell apoptosis[5-7]. Inhibiting cardiomyocyte apoptosis can delay ventricular remodeling and reduce the area of MI. Myocardial cell apoptosis caused by myocardial infarction is the cytological basis for cardiovascular diseases such as heart failure[8]. Therefore, how to reduce cardiomyocyte apoptosis has become one of the main research directions.

MicroRNA (miRNA) is a non-coding single-stranded small molecule RNA with tissue specificity. MiRNA is highly conservative and consists

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of 19 to 25 nucleotides; it regulates protein expression at the post-transcriptional level. MiRNAs play an important role in the pathophysiology of various cardiovascular diseases, such as myocardial hypertrophy, arrhythmia, and myocardial ischemia[9,10]. They are widely expressed in eukaryotes, participate in regulating the growth and differentiation of cells, and significantly contribute to the growth and development of the body and the pathogenesis of diseases[11]. The expression level of miRNAs is closely related to cell apoptosis[12,13]; however, the role of miR-802-5p in MI has not been studied.

In this study, we established cell and animal models of MI to detected miR-802-5p expression. Moreover, the role of miR-802-5p in MI was studied by the downregulation of miR-802-5p. Our results suggested that miR-802-5p could be a potential therapeutic target for MI.

Materials and Methods

Rat MI Model

Thirty adult male Sprague Dawley (SD) rats (6-8-week-old, 200-250g) were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). The rats were reared at about 22°C and were given a free diet and water. This study was approved by the Animal Ethics Committee of Shandong First Medical University Animal Center. One day before the operation, 10 rats in MI+antagomir group were injected with miR-802-5p antagomir (RiboBio, Guangzhou, China) through the tail vein to inhibit the function of miR-802-5p, and 10 rats in MI+NC group were injected with negative control (NC) antagomir through the tail vein. The rats in the sham group only had thoracotomy but no ligation.

Rats were anesthetized by intraperitoneal injection of 5% pentobarbital sodium (40 mg/kg), and then, fixed on the operating table. Intercostal thoracotomy was performed in the left thoracic region and the pericardium was removed to expose the heart. The left anterior descending coronary artery was ligated with a 6-0 suture. The color of left ventricular myocardium was whitish, the movement of the ventricular wall was weakened, indicating that the model was made successfully. After operation, rats were injected intramuscularly with 200,000 units of penicillin to prevent infection

Echocardiographic Measurement

On the 7th day after the operation, rats were anesthetized with isoflurane. The left ventricular end-systolic diameter and left ventricular end-diastolic diameter were measured, and the left ventricular ejection fraction (EF) and left ventricular short axis shortening rate (FS) were calculated.

Cell Culture and Transfection

The complete medium which was used to culture H9c2 cells was composed of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA), 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA). H9c2 cells were cultured in a cell incubator containing 5% CO_2 and 95% O_2, which maintained a constant temperature of 37°C.

According to the protocols, synthetic RNAs (miR-802-5p inhibitor, negative control (NC) inhibitor) (RiboBio, Guangzhou, China) were transfected into H9c2 cells using Transfection Kit (RiboBio, Guangzhou, China). To study the role of PTCH1, small interfering RNA-PTCH1 (siR-PTCH1) or siRNA negative control (siR-NC) (RiboBio, Guangzhou, China) was co-transfected with miR-802-5p inhibitor into H9c2 cells. After placing the cells in the incubator for 48 hours, H9c2 cells in the hypoxia treatment group were cultured at 37°C in a cell incubator containing 5% CO_2 and 1% O_2.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

50 mg heart tissue was taken, cut into pieces, and 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added. Then, the tissue samples were homogenized with a tissue homogenizer. For H9c2 cells seeded in a 24-well plate, 0.5 mL of TRIzol reagent was used to lyse the cells and a pipette tip was utilized to grind the cells to make them lyse fully. Then, chloroform was added, and the mixture was shook vigorously for 30 seconds and allowed to stand for 5 minutes, followed by centrifugation at 4°C with a centrifugal force of 12,000 g for 15 minutes. The upper aqueous phase was separated, and isopropyl alcohol was added into the mixture to separate out RNA. After the mixture was mixed fully and allowed to stand for 10 minutes, the RNA was precipitated by centrifugation at 12,000 g at 4°C for 10 minutes. Subsequently, the supernatant was discarded, and the RNA was washed with 75% ethanol, and then, precipitated by centrifugation at 7500 g at 4°C for 10 minutes. Finally, 20 μL of diethyl pyrocarbonate (DEPC)-treated Water (Beyotime, Shanghai, China) was utilized to dissolve the

total RNA. The purity and concentration of the total RNA was detected using Thermo Nanodrop 2100 spectrophotometer. The purity of RNA was judged based on OD260/OD280 (1.8-2.0). All-in-One™ miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, Guangzhou, China) was used to perform reverse transcription of miR-802-5p in accordance with the protocols. All-in-One™ miRNA qPCR Kit (GeneCopoeia, Guangzhou, China) was used to perform RT-PCR. U6 was the internal control of miR-802-5p. The relative expression of the genes was calculated using 2-ΔΔCT method. All the primers were listed in Table I.

Cell Counting Kit-8 (CCK-8) Assay

After H9c2 cells seeded in 96-well plates have undergone the above treatment, Cell Counting Kit (CCK-8; Vazyme, Nanjing, China) was used to detect the viability of H9c2 cells. CCK-8 solution (10 μL) was added to each well. After incubating the cells in the cell incubator, the absorbance at 450 nm was measured using a microplate reader.

Terminal Dexynucleotidyl Transferase(TdT)-Mediated dUTP Nick End Labeling (TUNEL) Staining

The TUNEL BrightRed Apoptosis Detection Kit (Vazyme, Nanjing, China) was used to detect the apoptosis of H9c2 cells. After the cells seeded in 24-well plates were fixed and the membrane was broken, 50 TdT incubation buffer solution was added to each well, and the cells were incubated at 37°C in the dark for 60 minutes. After being washed with phosphate-buffered saline (PBS), 4',6-diamidino-2-phenylindole (DAPI) staining was carried out, and the cells were stained at room temperature for 5 minutes. Then, the apoptosis of the cells could be observed by fluorescence microscope.

Lactate Dehydrogenase (LDH) Contents

LDH contents in the supernatant of H9c2 cells and serum of rats were detected using LDH ELISA kit (DOJINDO, Shanghai, China).

Western Blot

Total protein of H9c2 cells was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). BCA Protein Assay Kit (Beyotime, Shanghai, China) was employed to measure the concentration of protein. Proteins in each group were electrophoresed on the suitable concentration of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel to separate the proteins. Subsequently, we transferred the electrophoresed proteins to polyvinylidene difluoride (PVDF) membranes. 5% skim milk was prepared with tris buffered saline-tween (TBST) to block the non-specific antigens of the proteins. 2 hours later, primary antibodies (Bcl-2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Bax, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; PTCH1, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Gli1, Abcam, Cambridge, MA, USA, Rabbit, 1:50000; GAPDH, Abcam, Cambridge, MA, USA, Rabbit, 1:1000) were utilized to incubate the membranes at 4°C overnight. Then, secondary antibody was used to incubate the membranes for 2 hours. Super enhanced chemiluminescence (ECL) Detection Reagent (YEASEN, Shanghai, China) was used to develop the blots in Image Lab™ Software.

Flow Cytometry

Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme, Nanjing, China) was used to detect the apoptosis rate of H9c2 cells. The cells were collected using trypsin. 100 μL Binding buffer was added to resuspend the cells. Then, 5 μL of Annexin V-FITc and PI staining solution were added and another 400 μL Binding buffer was added. Finally, the mixed solution was incubated at room temperature in the dark for 20 minutes, and then the apoptosis rate was analyzed by flow cytometry.

Hematoxylin-Eosin (HE) Staining

On the 7th day after MI in rats, the hearts were collected and stored in 10% neutral formalin

Table I. Real time PCR primers.

Gene name	Forward (5'>3')	Reverse (5'>3')
miR-802-5p	AGGGCGTCAGTAACAAAGATT	AAGAGAAGGGAGAGGTGGTG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

RT-PCR, quantitative real-time polymerase chain reaction.

fixative. Afterwards, the hearts were dehydrated using alcohol and then embedded in paraffin and sliced. The paraffin sections were then dewaxed and stained, and mounted with neutral resin. Finally, the HE staining was observed under a microscope.

Luciferase Activity Assay

Luciferase reporter plasmids (RiboBio, Guangzhou, China) containing wild-type (WT) or mutant (MUT) 3′-UTR of PTCH1 were constructed. The plasmids and miR-802-5p mimic or NC mimic were co-transfected into HEK293T cells. The activity of Luciferase was detected using Dual-Glo® Luciferase Assay System.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22. 0 software (IBM Corp., Armonk, NY, USA). Data were represented as mean ± Standard Deviation (SD). The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student'' *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated the significant difference.

Results

MiR-802-5p Was Up-Regulated in MI

Through RT-PCR analysis, miR-802-5p was significantly upregulated in hypoxia-treated H9c2 cells (Figure 1A) and in infarcted myocardium of MI rats (Figure 1B).

Downregulation of MiR-802-5p Inhibited Hypoxia-Induced Apoptosis of H9c2 Cells

Then, we downregulated miR-802-5p by transfecting miR-802-5p inhibitor into H9c2 cells. After hypoxia treatment of H9c2 cells, we detected cell viability. Hypoxia treatment notably reduced the viability of H9c2 cells, while the downregulation of miR-802-5p could restore cell viability (Figure 2A). The contents of LDH in the supernatant of H9c2 cells were also detected. Compared with the control group, the LDH content in the cell supernatant of the hypoxic group increased markedly. However, compared with the hypoxia group, the LDH level in the hypoxia + inhibitor group decreased remarkably (Figure 2B). In addition, the expression of apoptosis-related proteins (Bcl-2, Bax) was also detected through Western blot. MiR-802-5p silencing could reverse hypoxia-induced downregulation of Bcl-2 and up-regulation of Bax (Figure 2C). Further, we also detected the apoptosis rate by flow cytometry and TUNEL staining. The results showed that miR-802-5p silencing protected H9c2 cells from hypoxia-induced apoptosis (Figure 2D and 2E).

MiR-802-5p Targets PTCH1 and Regulates Sonic Hedgehog Signaling Pathway

PTCH1 was predicted by the TargetScan prediction tool to have a binding site with miR-802-5p (Figure 3A). Downregulation of miR-802-5p can increase the expression of PTCH1 (Figure 3B). PTCH1 is the receptor of Sonic Hedgehog signaling pathway, so we thought that miR-802-5p was involved in regulating the activation of Sonic Hedgehog signaling pathway. Gli1 is a key

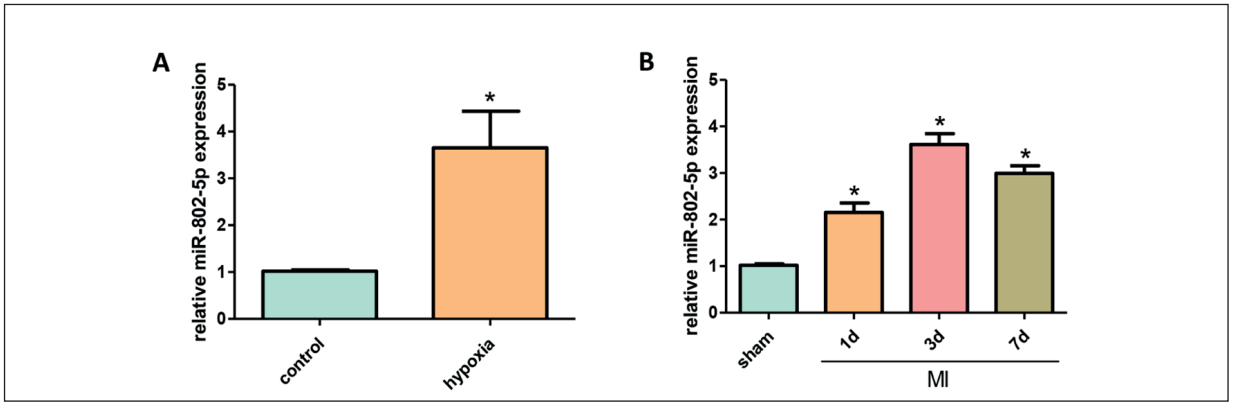

Figure 1. MiR-802-5p was upregulated in MI. **A,** MiR-802-5p expression in hypoxia-treated H9c2 cells was detected by RT-PCR ("*" $p<0.05$ *vs.* control, n = 3). **B,** MiR-802-5p expression in MI rats was detected by RT-PCR ("*" $p<0.05$ *vs.* sham, n = 3).

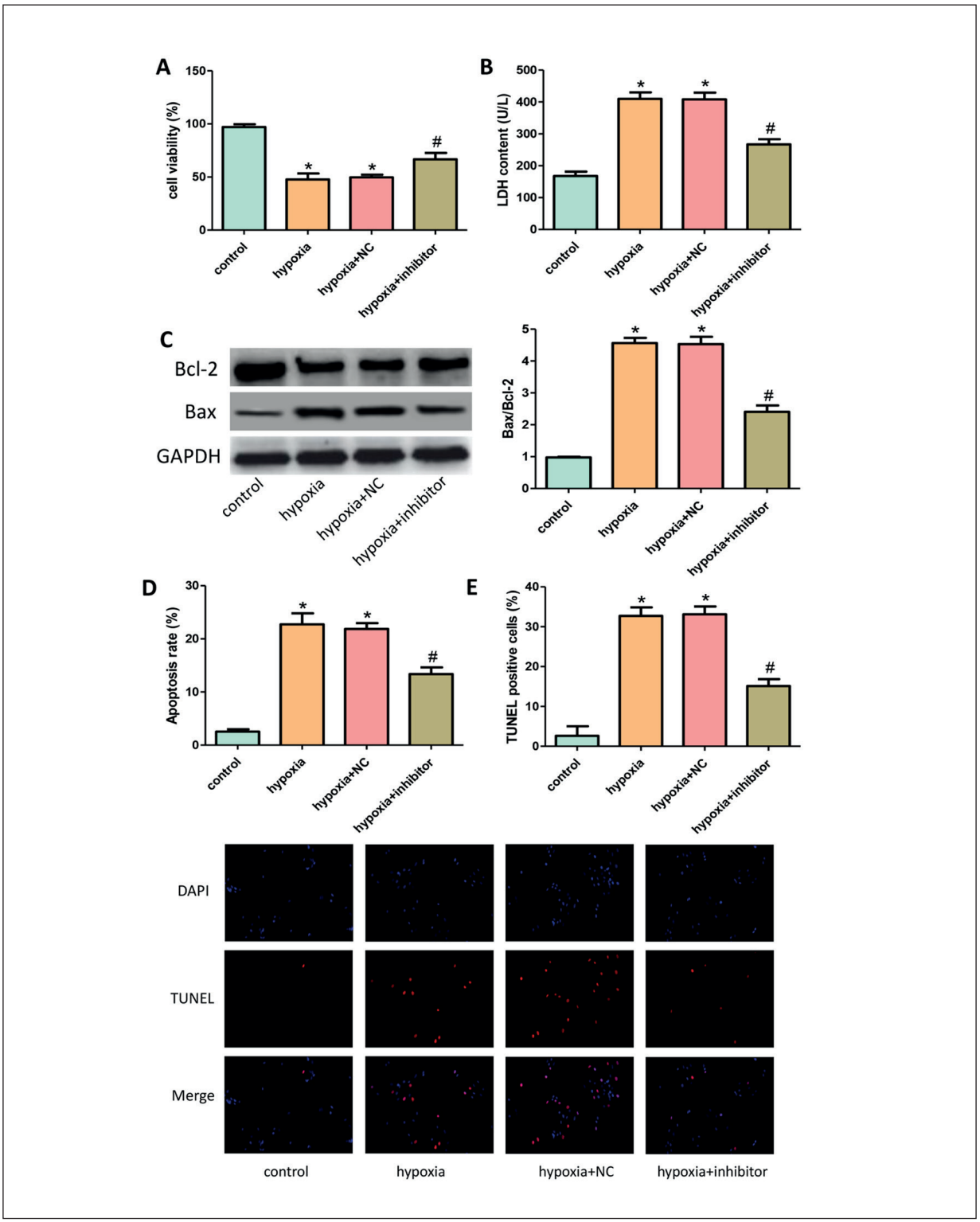

Figure 2. Downregulation of miR-802-5p inhibited hypoxia-induced apoptosis of H9c2 cells. **A,** CCK-8 assay suggested that miR-802-5p silencing restored cell viability ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* hypoxia+NC, n=3). **B,** LDH contents in cell supernatant ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* hypoxia+NC, n=3). **C,** Expression levels of apoptosis-related proteins (Bcl-2, Bax) were detected by Western blot ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* hypoxia+NC, n=3). **D,** Apoptosis rate was detected by flow cytometry ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* hypoxia+NC, n=3). **E,** Results of TUNEL staining of H9c2 cells in each group (magnification: 200×) ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* hypoxia+NC, n=3).

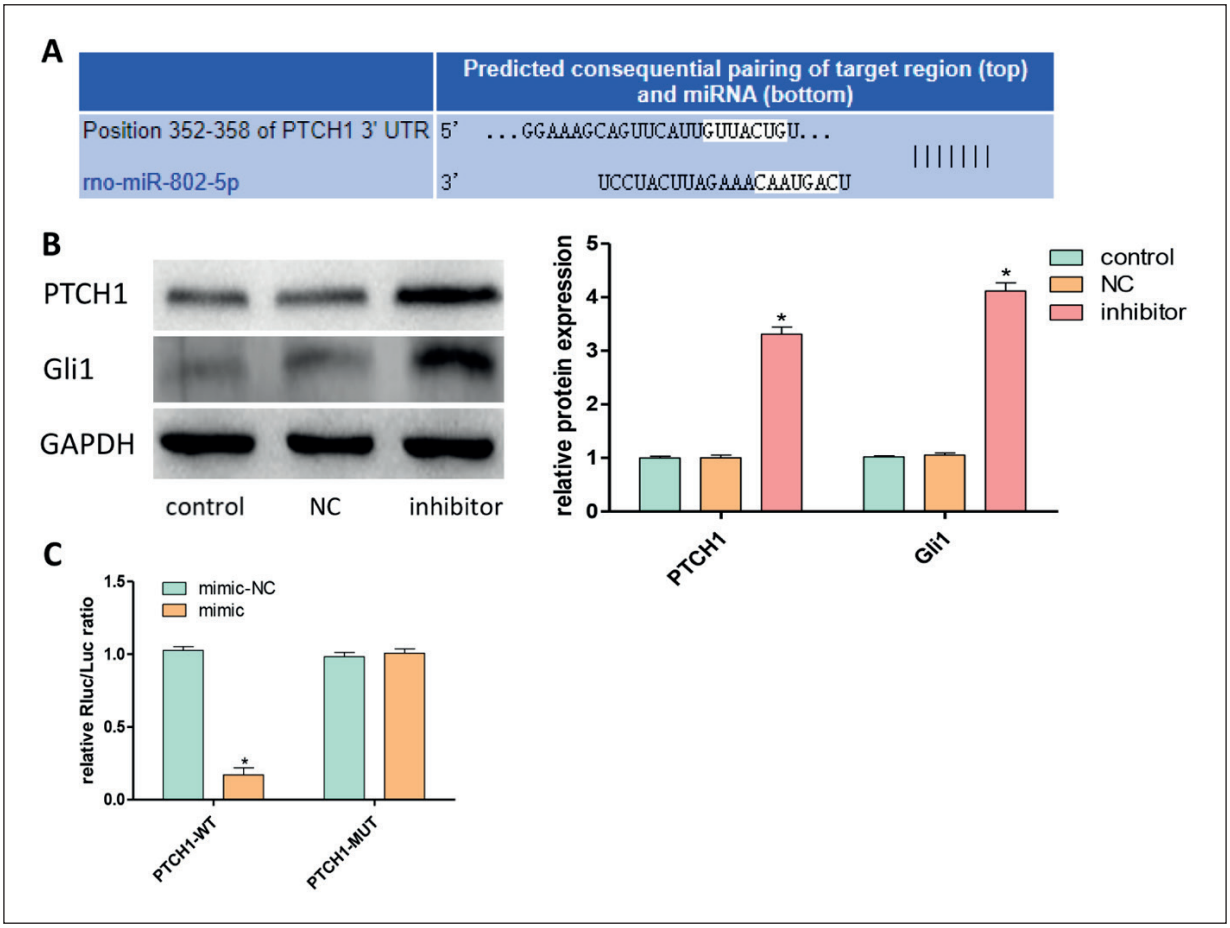

Figure 3. MiR-802-5p targets PTCH1 and regulates Sonic Hedgehog signaling pathway. **A,** Binding site predicted by the TargetScan database. **B,** Western blot showed the expression of PTCH1 and Gli1 ("*" $p<0.05$ *vs.* NC, n=3). **C,** MiR-802-5p mimic significantly decreased the relative luciferase activity in PTCH1-WT group, but did not decrease the relative luciferase activity in PTCH1-MUT group ("*" $p<0.05$ *vs.* mimic-NC, n = 3).

transcription factor in Sonic Hedgehog signaling pathway, and the downregulation of miR-802-5p could also significantly induce its expression (Figure 3B). Therefore, the downregulation of miR-802-5p can activate Sonic Hedgehog signaling pathway. In addition, the Luciferase activity assay proved that miR-802-5p directly targets PTCH1.

Downregulation of MiR-802-5p Inhibited Hypoxia-Induced Apoptosis of H9c2 Cells Via Targeting PTCH1

SiR-PTCH1 or siR-NC and miR-802-5p inhibitor were co-transfected into H9c2 cells. As can be seen from Figure 4A, siR-PTCH1 significantly inhibited the expression of PTCH1, and suppressed Bcl-2 expression and induced Bax expression. Furthermore, the knockdown PTCH1 abolished the protective effect of miR-802-5p silencing on hypoxia-treated H9c2 cells, which was shown by the decrease of cell viability, the increase of LDH content and the increase of apoptosis rate (Figure 4B-4D).

Downregulation of MiR-802-5p Alleviated Myocardial Injury and Improved Cardiac Function of MI Rats

From the HE staining of rat hearts, it can be seen that in the sham group the myocardial fibers were neatly arranged, the sections were uniformly stained and the cell structure was complete, while in the MI+NC group, there was a large amount of myocardial necrosis and fibrosis, and the cell structure was seriously damaged. The downregulation of miR-802-5p greatly reduced myocardial damage of MI rats (Figure 5A). MiR-802-5p silencing significantly reduced the serum LDH content in MI rats (Figure 5B). In addition,

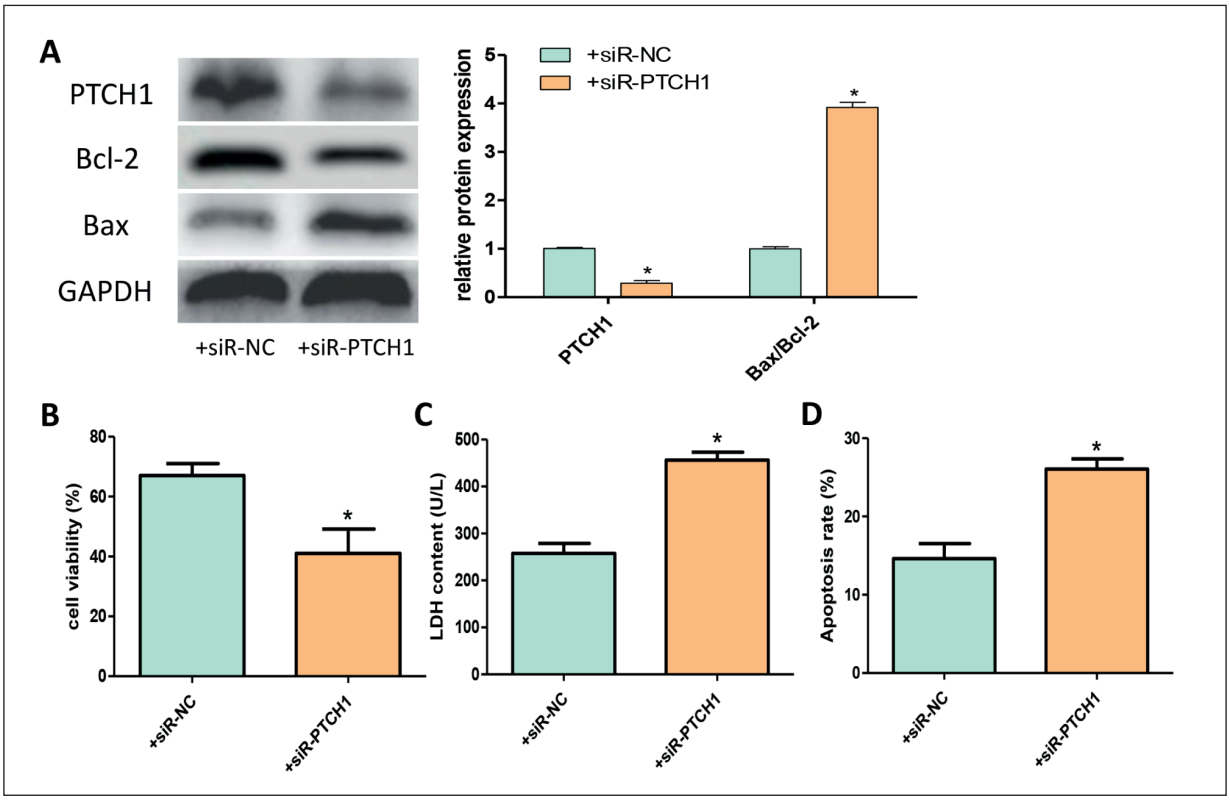

Figure 4. Downregulation of miR-802-5p inhibited hypoxia-induced apoptosis of H9c2 cells *via* targeting PTCH1. **A,** Expression levels of proteins (PTCH1, Bcl-2, Bax) were detected by Western blot ("*" $p<0.05$ *vs.* +siR-NC, n=3). **B,** Viability of H9c2 cells was detected by CCK-8 assay ("*" $p<0.05$ *vs.* +siR-NC, n=3). **C,** LDH contents in cell supernatant ("*" $p<0.05$ *vs.* +siR-NC, n=3). **D,** The apoptosis rate of H9c2 cells was detected by flow cytometry ("*" $p<0.05$ *vs.* +siR-NC, n=3).

on day 7 after MI in rats, cardiac function was detected. The miR-802-5p silencing improved the cardiac function of MI rats, which is manifested by increased EF and FS (Figure 5C and D).

Discussion

MI is mainly due to coronary artery spasm, stenosis or obstruction leading to necrosis of myocardial ischemia and hypoxia in the blood supply area. The pathogenesis is mainly the rupture and bleeding of coronary atherosclerotic plaques, resulting in vascular endothelial damage, collagen exposure, platelet aggregation and adhesion, hemodynamic changes, blood hypercoagulation to form thrombosis, thereby blocking blood flow and causing ischemia and hypoxia[2,14]. The heart is a high-metabolic tissue that has high requirements on blood supply and oxygen supply[15,16]. Myocardial ischemia causes myocardial cell apoptosis and necrosis, promotes the remodeling of the heart structure, and causes serious damage to cardiac function.

Apoptosis is an active cell death in the organism. When a mammal grows, develops, and receives external stimulation, the body eliminates excess, aging, and damaged cells through apoptosis to maintain the balance of the body's internal environment and maintain normal physiological activities[17]. In cell metabolism, once the apoptosis is unbalanced, it will lead to many diseases. Apoptosis is one of the important ways of myocardial cell death in myocardial ischemic injury[18].

MiRNAs are a family of evolutionary conserved small non-coding RNAs processed from much longer primary transcripts that constitute a prevalent gene regulation. By regulating the target genes, miRNAs function as critical mediator of numerous cellular processes, including cell growth, differentiation, proliferation and apoptosis. In this study, we established cell and animal models of MI. Through RT-PCR, we found that miR-802-5p was increased in MI, while down-

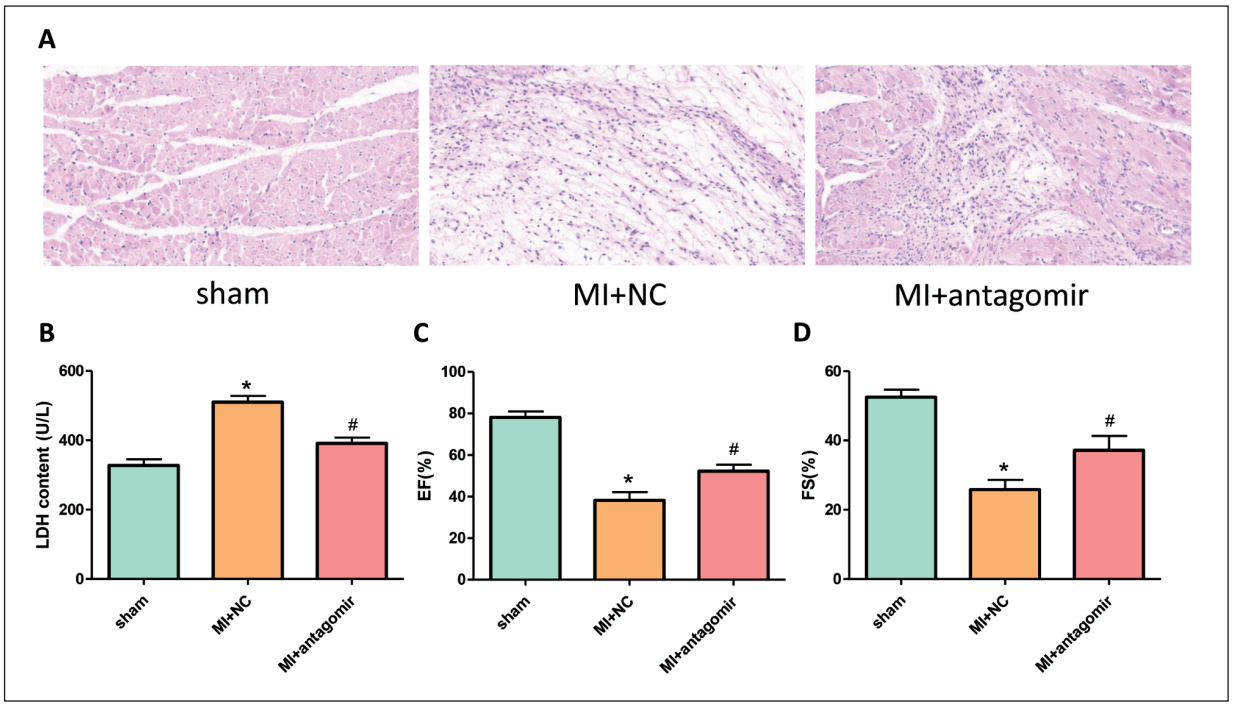

Figure 5. Downregulation of miR-802-5p alleviated myocardial injury and improved cardiac function of MI rats. **A,** Typical HE staining of myocardial tissues (magnification: 200×). **B,** Serum LDH contents of rats ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=6). **C,** and **D,** left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS) ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=6).

regulation of miR-802-5p could significantly inhibit hypoxia-induced cardiomyocyte apoptosis. Through the luciferase activity assay, we found that PTCH1 is the target gene of miR-802-5p, and knocking down PTCH1 can promote myocardial apoptosis.

PTCH1 is a membrane receptor for Sonic Hedgehog ligand in Sonic Hedgehog signaling pathway. Sonic Hedgehog signaling pathway, which is composed of a series of repressive interactions, was initially identified in Drosophila as a crucial mediator of several important processes during embryogenesis[19,20]. Previous research focused on its role in the treatment of various cancers. In recent years, the role of Sonic Hedgehog signaling pathway in myocardial infarction has also been extensively studied. Wang et al[21] found that Sonic Hedgehog ligand can be expressed in the bulbous artery after myocardial damage, which promotes epicardial regeneration. Sonic Hedgehog signaling pathway could significantly increase the survival rate of myocardial cells after myocardial ischemia-reperfusion[22]. Mackie et al[23] also found that Sonic Hedgehog-modified human $CD34^+$ cells significantly reduced myocardial infarction area 4 weeks after myocardial ischemia-reperfusion. In addition, Li et al[24] proved that activation of the Sonic Hedgehog signaling pathway reduced the apoptosis of myocardial cells and improved cardiac functions. In this study, we found that PCTH1 is the target gene of miR-802-5p, and miR-802-5p silencing can overexpress PTCH1 in MI, thereby activating the Sonic Hedgehog signaling pathway, thereby inhibiting myocardial cell apoptosis after MI and improving cardiac function in MI rats.

Conclusions

This research revealed for the first time that the expression of miR-802-5p was increased in myocardial infarction, and reconstructed the expression of miR-802-5p can inhibit hypoxia-induced myocardial cell apoptosis, thereby improving myocardial injury and improving cardiac function of MI rats. In addition, miR-802-5p targets PTCH1, and miR-802-5p silencing can activate the Sonic Hedgehog signaling pathway. However, PTCH1 silencing could deprive miR-802-5p silencing of its inhibitory effect on hypoxia-induced apoptosis of myocardial cells. To sum up,

miR-802-5p was upregulated in MI and downregulation of miR-802-5p could inhibit myocardial apoptosis and improve cardiac function of MI rats *via* activating Sonic Hedgehog signaling pathway by targeting PTCH1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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