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MicroRNA-147 inhibits myocardial inflammation and apoptosis following myocardial infarction *via* targeting HIPK2

C.-G. WU[1], C. HUANG[2]

[1]Department of Ultrasonography, First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China
[2]Department of Medical, First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China

Abstract. – OBJECTIVE: The incidence of acute myocardial infarction (AMI) is increasing year by year, and it has become one of the diseases with the highest mortality in humans. MicroRNAs (miRNAs) are involved in the regulation of many diseases, including AMI. This study aims to investigate the function of miR-147 in myocardial infarction (MI) and its underlying mechanism of action.

MATERIALS AND METHODS: Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) was used to detect miR-147, Bcl-2 mRNA, and Bax mRNA expressions. Enzyme-linked immunosorbent assay (ELISA) kits were used to detect the levels of inflammatory factors (TNF-α, IL-6, IL-β) and lactate dehydrogenase (LDH). Besides, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed to detect cell viability. Cell apoptosis was observed using terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL). Moreover, echocardiography was utilized to measure cardiac function of rats. HIPK2 expression was detected by Western blot.

RESULTS: MiR-147 expression was significantly decreased in rat MI model and H_2O_2 treated H9c2 cells. H_2O_2 treatment increased the expression of inflammatory factors in H9c2 cells and induced apoptosis, while such effects were inhibited by overexpression of miR-147. Overexpression of miR-147 reversed the decrease in Bcl-2 expression and the increase in Bax expression in H9c2 cells caused by H_2O_2. In addition, overexpression of miR-147 could improve cardiac function and reduce serum LDH levels in myocardial infarction rats. Through TargetScan, we found that HIPK2 might have a binding site for miR-147. Moreover, overexpression of miR-147 could inhibit the expression of HIPK2.

CONCLUSIONS: In MI, miR-147 expression is decreased in the myocardium and overexpression of miR-147 can inhibit myocardial inflammation and apoptosis and improve cardiac function in rats *via* targeting HIPK2.

Key Words:
Myocardial infarction, MiR-147, HIPK2, Inflammation, Apoptosis.

Introduction

Myocardial infarction (MI) is one of the main causes of disability and death in the world. About one-third of heart failure patients worldwide are caused by MI, although many treatments are now available[1]. In fact, the occlusion of a major coronary artery can cause severe myocardial ischemia and rapid apoptosis of myocardial cells, leading to progressive fibrosis in lieu of myocardial tissue and expansion of the left ventricle[2]. Ventricular remodeling refers to changes in the morphology and structure of myocardial cells and interstitial cells caused by activation of neurohumoral regulatory mechanisms, inflammation, and cytokines after MI. These factors cause the heart structure and function to reshape according to a certain pattern, resulting in progressive expansion and shape change of the left ventricle, including changes in ventricular volume, shape, wall thickness, and myocardial structure[3]. As a result, the heart morphology and hemodynamics are abnormal, the left ventricle is progressively enlarged, and the contractile function is gradually reduced, eventually leading to heart failure and death[3].

MicroRNA (miRNA) is a type of non-protein-coding RNA that contains approximately 22 nucleotide molecules[4]. Thousands of miRNAs have been found in eukaryotes to regulate the physiological functions of cells. MiRNA inhibits the translation of the target gene mRNA or promotes its degradation by complementary base pairing with the base of the 3' untranslated region

Corresponding Author: Chao Huang, MD; e-mail: huangchao1976make@163.com

(3'-UTR) of the target gene, thereby participating in the regulation of target protein expression[5]. Of note, miRNAs are involved in the pathophysiology of cardiovascular diseases, including endothelial cell dysfunction, changes in cell adhesion, platelet growth and rupture, and myocardial cell proliferation and apoptosis[6,7]. In addition, maintaining and restoring the stable expression of corresponding miRNAs in target organs, such as the heart may become a new target for cardiovascular disease treatment. In the study of MI, myocardial ischemia will lead to inflammatory response and myocardial cell apoptosis, but its underlying mechanism is still unclear, and miRNAs may be involved in regulating this process.

MiR-147 can inhibit apoptosis of L6 myoblasts induced by cyclic mechanical stretch[8]. Down-regulating miR-147 can inhibit the proliferation of gastric cancer cells[9]. However, the role of miR-147 in myocardial infarction is poorly understood. In the present study, we revealed that miR-147 had decreased expression in *in vivo* and *in vitro* models of MI. Overexpression of miR-147 significantly inhibited the apoptosis and inflammation of cardiomyocytes, and improved cardiac function in MI rats.

Materials and Methods

Cell Culture and Transfection

H9c2 cells (COBIOER, Nanjing, China) were cultured with Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA), which contained 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), in an incubator containing 5% CO_2 at 37°C. Then, H9c2 cells were subcultured when the cell confluence reached about 80%.

To study the function of miR-147 *in vitro*, miR-147 mimic or negative control (NC; RiboBio, Guangzhou, China) was transfected into the cells using Lipofectamine 2000 reagent (RiboBio, Guangzhou, China) in line with the protocols. At 48 hours after transfection, H9c2 cells were treated with H_2O_2 (100 μM, 4 h) to construct a model of cardiomyocyte injury *in vitro*.

Rat MI Model

Thirty Sprague Dawley (SD) rats (200 ± 20g) were reared adaptively for one week and randomly divided into three groups: sham, MI + NC (negative control), MI + agomiR-147. All rats were anesthetized using sodium pentobarbital (30 mg/kg), fixed in a supine position on a workbench, and connected to a small animal ventilator after tracheal intubation. The rats in MI group and MI + agomiR-147 group were subjected to thoracotomy and the left anterior descending coronary artery was ligated. The rats in the sham group underwent thoracotomy without left anterior descending coronary artery ligation. The miR-147 agomir (RiboBio, Guangzhou, China) was the same double-stranded RNA analog as mature miR-147. One day before surgery, the rats were injected with agomiR-147 (5 mg/kg) or NC *via* tail vein. The expression of miR-147 in rat's heart was detected on the first, third and seventh days after surgery. Besides, on the seventh day after operation, echocardiography was used to detect the cardiac function of rats. This investigation was approved by the Animal Ethics Committee of Jinzhou Medical University Animal Center.

Echocardiographic Measurement

All rats were subjected to echocardiography on the seventh day after MI. Left ventricular ejection fraction (EF) and fractional shortening (FS) were calculated.

RNA Extraction and Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the cells using TRIzol reagent (MCE, Nanjing, China). Then, the total RNA concentration was measured with a spectrophotometer. MiRNA reverse transcription was performed using miScript RT II kit (Qiagen, Shanghai, China) in accordance with the protocols. Later, mRNA reverse transcription was performed using HiScript™ 1st strand complementary deoxyribose nucleic acid (cDNA) synthesis kit (Vazyme, Nanjing, China) in line with the protocols. The synthesized cDNA was stored in a refrigerator at -20°C.

After the reverse transcription was completed, the cDNA was used as a template for the amplification reaction, which was conducted using miRNA Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and a 20 μL reaction system according to the protocols. The reaction conditions were as follows: pre-denaturation at 95°C for 5 minutes, followed by 40 cycles of reaction at 95°C for 10 seconds and 60°C for 30 seconds. U6 was set as the internal reference of miRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal reference of mRNA.

Real Time-PCR was performed on a Prism 7900 System (Applied Biosystems, Foster City, CA, USA). All the primers were listed in Table I.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

H9c2 cells were seeded in 96-well plates, and the number of cells in each well was 5×10^3. The cells were transfected with miR-147 mimic or NC after 24 hours in a constant temperature incubator. The cells were treated with H_2O_2 at 48 hours after transfection. After 4 hours, 10 μL of MTT (10 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and the 96-well plate was placed in the incubator for another 4 hours. Then, a pipette was utilized to remove the liquid from all the wells and 150 μL of dimethyl sulfoxide was added to each well. Finally, the absorbance at 570 nm was measured with a microplate reader.

Western Blot

Total protein of H9c2 cells was extracted using radioimmunoprecipitation assay (RIPA) Lysis Buffer (Beyotime Biotechnology, Shanghai, China). The culture medium was removed from the 6-well plate, and the 6-well plate was washed twice with phosphate-buffered saline (PBS). Then, 150 μL of RIPA lysis buffer was added to each well to fully lyse the cells. Subsequently, the cell lysates were collected into the Eppendorf (EP; Hamburg, Germany) tubes, shaken thoroughly, and then, let standing on ice for 20 minutes. After centrifugation with a centrifugal force of 12000 g at 4°C for 15 minutes, the supernatant obtained was the extracted total protein. The concentration of total protein was measured using a bicinchoninic acid (BCA) detection kit (KeyGen, Shanghai, China) according to the protocols.

The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was prepared using the SDS-PAGE gel preparation kit (Beyotime Biotechnology, Shanghai, China). 20 μg of protein sample was added to each lane and electrophoresis was conducted at 100 V for approximately 30 minutes. When bromophenol blue reached the junction between the concentrated gel and separated gel, the voltage was set to 150 V for about 60 minutes. When the electrophoresis was completed, the electrophoresed proteins were transferred to the polyvinylidene difluoride (PVDF, EpiZyme, Shanghai, China) membranes. After that, the membranes were put in 5% skim milk and blocked at room temperature for 2 hours. Then, the membranes were incubated with the primary antibodies (HIPK2, Abcam, Cambridge, MA, USA, Mouse, 1:1000; tubulin, Abcam, Cambridge, MA, USA, Mouse, 1:1000) at 4°C overnight. The next day, the membranes were incubated with the corresponding secondary antibodies. After the membranes were washed 3 times for 30 minutes, the protein bands were exposed by Image Lab™ Software.

TNF-α, IL-6 and IL-1β Levels

After H9c2 cells were transfected with miR-147 mimic or NC and treated with H_2O_2, the cell supernatants were collected and centrifuged with a centrifugal force of 200 g for 5 minutes to remove sediment. The content of TNF-α, IL-6 and IL-1β in the cell supernatant was detected by the corresponding enzyme-linked immunosorbent assay (ELISA) detection kits (Bestbio, Shanghai, China).

Lactate Dehydrogenase (LDH) Level

On the seventh day after MI, the serum LDH content in rats was detected by LDH ELISA kit (Dojindo Molecular Technologies, Kumamoto, Japan).

Luciferase Activity Assay

Luciferase reporters (RiboBio, Guangzhou, China) containing wild-type or mutant 3'UTR

Table I. Real time PCR primers.

Gene name	Forward (5'>3')	Reverse (5'>3')
Bax	CAGTTGAAGTTGCCATCAGC	CAGTTGAAGTTACCATCAGC
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTGAGCAGCGTCTTCAGAGACA
miR-147	CGTGTGCGGAAATGCTT	GTTGTTGGTTGGTTGGTTGT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

qRT-PCR, quantitative Reverse-Transcription Polymerase Chain Reaction.

of HIPK2 were constructed. MiR-147 mimic or NC and Luciferase reporters were co-transfected into HEK293T cells. Finally, the Luciferase activities were detected by the Dual-Luciferase Reporter Assay System kit (RiboBio, Guangzhou, China).

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

Four groups of cells were fixed with 4% paraformaldehyde and the cell membrane was disrupted with Triton. Then, the prepared TUNEL reagent (Roche, Basel, Switzerland) was added and co-incubated with the cells. Later, 4',6-diamidino-2-phenylindole (DAPI; Roche, Basel, Switzerland) was applied to stain the nucleus. The results were observed with a fluorescence microscope.

Statistical Analysis

Measurement data were expressed as $\chi \pm s$, tested for normality and analyzed by *t*-test. The differences between the two groups were analyzed by using the Student's *t*-test. Comparison among 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

The Expression of MiR-147 Decreased In Infarcted Myocardium

We examined miR-147 expression in *in vivo* and *in vitro* models of MI, respectively. In the *in vitro* model, miR-147 expression was greatly reduced (Figure 1A). In the *in vivo* model, miR-147 was markedly reduced in the infarcted and border zones of the myocardium, but not significantly in the remote zone (Figure 1B-D).

Overexpression of MiR-147 Inhibited H_2O_2-Induced Cardiomyocyte Inflammation and Apoptosis

After transfection of miR-147 mimic into cardiomyocytes, miR-147 expression increased by more than a hundred times (Figure 2A). We, then, used ELISA kits to detect the levels of inflammatory factors (TNF-α, IL-6, IL-1β) in the

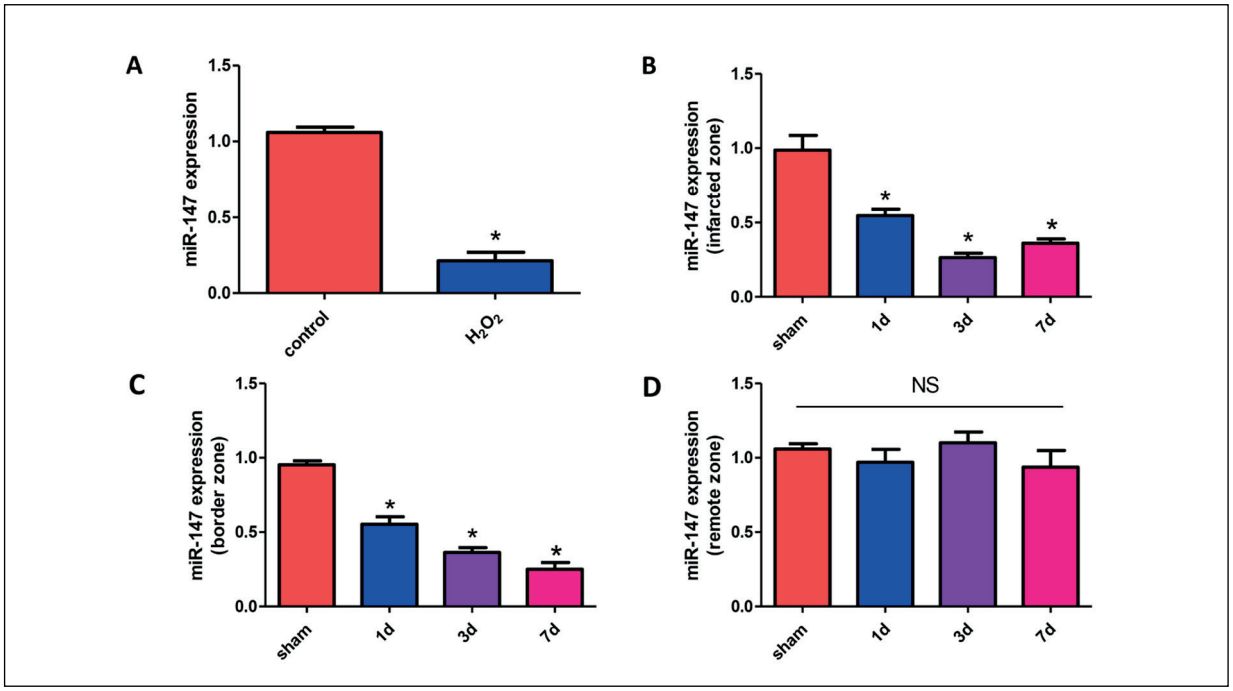

Figure 1. The expression of miR-147 decreased in infarcted myocardium. **A,** qRT-PCR analysis showed that miR-147 expression was decreased in H_2O_2-treated H9c2 cells ("*" $p<0.05$ *vs.* control, n = 3). **B-D,** qRT-PCR analysis showed that miR-147 expression was decreased in the infarcted and border zones, but there was no change in the remote zone ("*" $p<0.05$ *vs.* sham, n = 3).

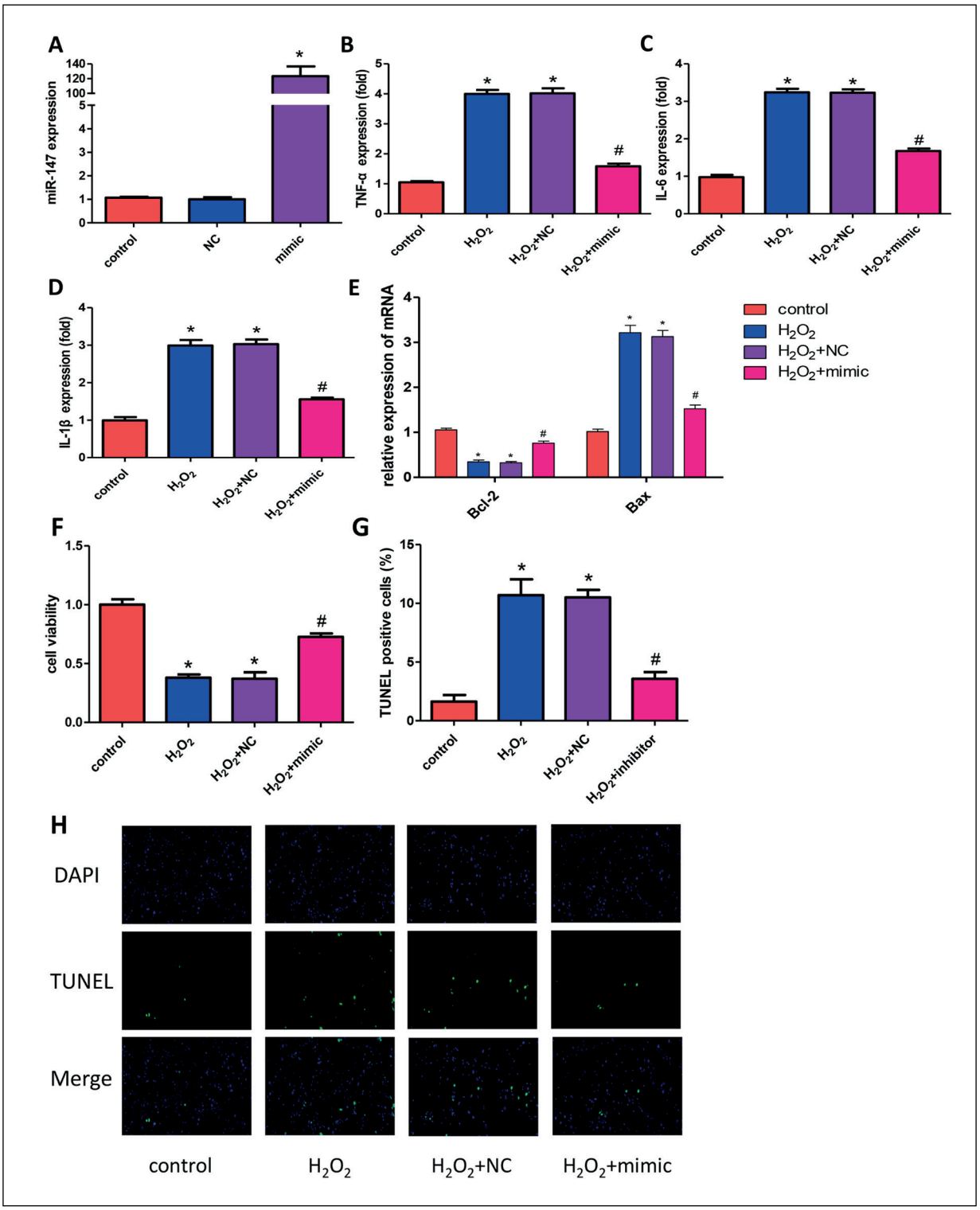

Figure 2. Overexpression of miR-147 inhibited H_2O_2-induced cardiomyocyte inflammation and apoptosis. **A,** Transfection of miR-147 mimic into H9c2 cells significantly increased miR-147 expression ("*" $p<0.05$ *vs.* control, n = 3). **B-D,** The content of inflammatory factors (TNF-α, IL-6, IL-1β) in the cell supernatant was detected ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3). **E,** Expression levels of apoptosis-related genes were detected by qRT-PCR ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3). **F,** MTT assay suggested that miR-147 overexpression restored cell viability after H_2O_2 treatment ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3). **G,** Statistical analysis of TUNEL staining. **H,** Representative images of TUNEL staining (200×, "*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3).

cell supernatant. H_2O_2 significantly promoted the expression of inflammatory factors in cardiomyocytes, while overexpression of miR-147 significantly reduced their expression (Figure 2B-2D). QRT-PCR was used to detect the expression of Bcl-2 and Bax mRNA. It was found that H_2O_2 could inhibit the expression of Bcl-2 mRNA and promote the expression of Bax mRNA, but miR-147 mimic had the opposite effect (Figure 2E). In addition, miR-147 mimic remarkably reversed H_2O_2-induced reduction in myocardial cell viability (Figure 2F). Furthermore, the results of TUNEL staining showed that H_2O_2 could significantly induce cardiomyocyte apoptosis, while the overexpression of miR-147 could markedly inhibit cardiomyocyte apoptosis (Figure 2G and 2H). It can be seen that overexpression of miR-147 can inhibit H_2O_2-induced cardiomyocyte apoptosis and inflammation.

Overexpression of MiR-147 Improved Cardiac Function In MI Rats

Intravenous injection of agomiR-147 greatly increased the level of miR-147 in cardiomyocytes. Echocardiographic results suggested that miR-147 overexpression in the myocardium markedly increased EF and FS, and improved cardiac function in rats (Figure 3A-3D). Moreover, overexpression of miR-147 reduced serum LDH levels in myocardial infarction rats, proving that miR-147 reduces myocardial cell damage caused by ischemia (Figure 3E).

MiR-147 Directly Targeted HIPK2

Using TargetScan software, we found that HIPK2 might be a target gene of miR-147 (Figure 4A). Through Western blot, we found that H_2O_2 could increase the expression of HIPK2, but the overexpression of miR-147 remarkably inhibited the expression of HIPK2 (Figure 4B and 4C). Furthermore, miR-147 mimic noticeably inhibited the Luciferase activity of HIPK2-WT, but did not inhibit the Luciferase activity of HIPK2-MUT (Figure 4D). Collectively, these results indicated that HIPK2 is a target gene of miR-147.

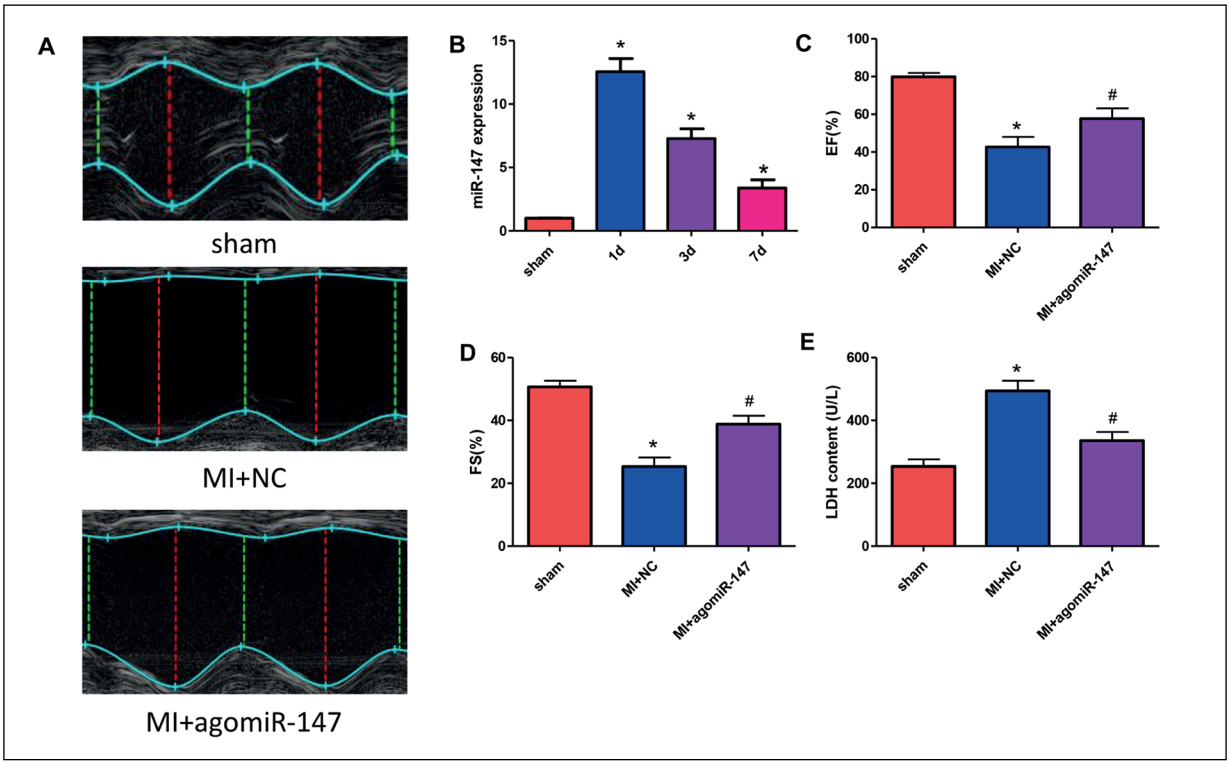

Figure 3. Overexpression of miR-147 improved cardiac function in MI rats. **A,** Representative images of echocardiogram. **B,** qRT-PCR analysis showed that the tail vein injection of agomiR-147 increased the expression of miR-147 in rat heart ("*" $p<0.05$ *vs.* sham, n = 3). **C,** and **D,** Ejection fractions (EF) and Fractional shortening (FS) ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+agomiR-147, n=6). **E,** Rat serum LDH levels were measured ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+agomiR-147, n=6).

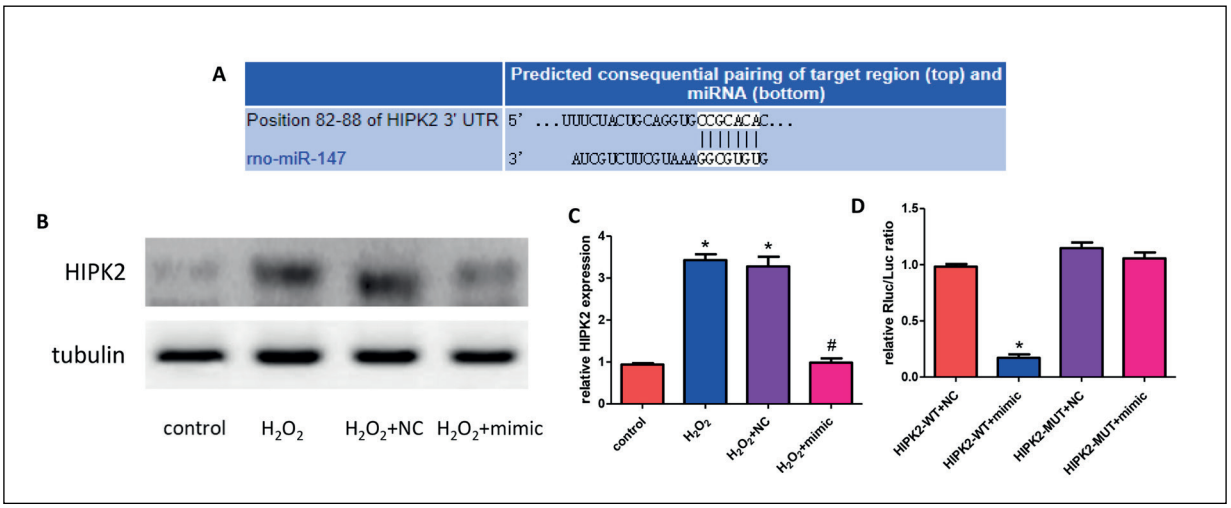

Figure 4. MiR-147 directly targeted HIPK2. **A,** Binding site predicted by the TargetScan database. **B,** The expression of HIPK2 was detected using Western blot. **C,** Statistical analysis of HIPK2 expression ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3). **D,** Luciferase activity assay of HEK293T cells transfected with Luciferase constructs containing WT-3′UTR and Mut-3′UTR of HIPK2 ("*" $p<0.05$ *vs.* WT+NC, n=3).

Discussion

After AMI, a strong inflammatory response is triggered, first with neutrophil infiltration and tthen followed by mononuclear-macrophage and lymphocyte infiltration[10]. Inflammatory factors from neutrophil infiltration play an important role in ventricular remodeling. Macrophages and lymphocytes are also involved in ventricular remodeling[11]. Various inflammatory cells can secrete a variety of cytokines, such as IL-1, IL-6, TNF-α and TGF-β, causing cardiac fibrosis and remodeling[12]. Necrotic cardiomyocytes are engulfed by inflammatory cells. Acute myocardial ischemia only causes a part of myocardial cell necrosis. With the infiltration of a large number of inflammatory cells into the infarcted area of myocardial tissue, the range of myocardial necrosis and apoptosis gradually expands, eventually leading to large-area MI[13]. Thus, the inflammatory response after myocardial infarction is an important cause of cardiac pathological damage.

Cardiomyocyte apoptosis is one of the forms of myocardial death after MI[14]. Cardiomyocyte loss due to apoptosis plays an important role in the development of heart failure after MI[15]. Apoptosis, also known as programmed cell death, is divided into two pathways: endogenous apoptosis and exogenous apoptosis[16]. Exogenous apoptosis is mediated through activation of death receptors on the cell membrane, including Fas, tumor necrosis factor (TNF) and TNF-associated ligands. Endogenous apoptosis is mainly induced by regulating the Bcl-2 protein family, including signaling proteins, such as Bax, Bad, Bcl-2, and Bcl-XL. Bcl-2 is a key regulator of the endogenous apoptotic cascade[17]. Much apoptosis may be induced by inhibiting Bcl-2 which also plays an important regulatory role in cardiomyocyte injury.

MiRNAs are involved in the regulation of inflammation and apoptosis after myocardial infarction. Yang et al[18] found that miR-21 can inhibit excessive inflammation and improve cardiac dysfunction after MI *via* targeting KBTBD7. Sun et al[19] proved that miR-98 could protect cardiomyocytes from apoptosis. In this study, we found that H_2O_2 can induce cardiomyocytes to express a large number of inflammatory factors and promote myocardial cell apoptosis. Moreover, miR-147 expression was decreased in the infarcted myocardium, and overexpression of miR-147 noticeably inhibited myocardial inflammation and apoptosis, thereby improving cardiac function.

HIPK2 (homeodomain-interacting protein kinase 2) belongs to the DYRK kinase (dual specificity tyrosine regulated kinase) family in terms of protein structure[20] and is widely involved in the pathophysiology of diseases, including cell proliferation, cell differentiation, and apoptosis[21-23]. HIPK2 can not only directly induce apoptosis through the phosphorylation of Ser46

in the p53 signaling pathway[24], but also indirectly induce apoptosis by inhibiting the anti-apoptotic transcription inhibitor, C-Terminal Binding Protein (CtBP), a non-p53 pathway[25]. Zhou et al[26] found that miR-27b-3p could inhibit apoptosis of chondrocyte in rheumatoid arthritis by targeting HIPK2. In our study, miR-147 could inhibit myocardial inflammation and apoptosis in MI by targeting HIPK2.

In this article, we elaborated the regulatory role of miR-147 in the pathophysiology of myocardial infarction from both aspects of inflammation and apoptosis. We revealed, for the first time, that the expression of miR-147 is reduced in myocardial infarction, and overexpression of miR-147 can inhibit myocardial inflammation and apoptosis by targeting HIPK2 to improve cardiac function. However, for the role of miR-147 in MI and its molecular mechanism, more research is needed, including the role of miR-147 in the oxidative stress of MI, gain of function and loss of function experiments of HIPK2.

Conclusions

Briefly, miR-147 expression is decreased in infarcted myocardium and overexpression of miR-147 could inhibit myocardial inflammation and apoptosis and improve cardiac function in MI rats through targeting HIPK2. MiR-147 could become a potential therapeutic target for MI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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