Inhibition of microRNA-184 reduces H_2O_2-mediated cardiomyocyte injury *via* targeting FBXO28

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Abstract. – OBJECTIVE: Cardiovascular disease, especially coronary heart disease, is one of the diseases with the highest mortality. A large number of studies have found that microRNAs (miRNAs) are closely related to the occurrence and development of myocardial ischemia. This article mainly focused on the regulation of miR-184 on oxidative stress, inflammation, and apoptosis in myocardial infarction (MI).

MATERIALS AND METHODS: MiR-184 inhibitor or negative control (NC) were transfected into H9c2 cells. Then, H9c2 cells were treated with H_2O_2 to construct a cardiomyocyte injury model. H9c2 cells were divided into 4 groups: control group, $H2_2O_2$ treatment group, H_2O_2 + NC group, and H_2O_2 + miR-184 inhibitor group. The oxidative stress of H9c2 cells was observed by the expression levels of SOD, ROS, and MDA in each group. The inflammatory response of H9c2 cells was reflected by the expression of TNF-α, IL-6, and IL-1β detected by ELISA kits. Western blot was used to detect the expression of cleaved Caspase-3, Bcl-2, Bax and F-box protein 28 (FBXO28). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was utilized to detect miR-184 expression. TdT-mediated dUTP Nick-End Labeling (TUNEL) staining and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay were used to observe the apoptosis and cell viability. The Luciferase reporter experiment was used to prove whether miR-184 could target FBXO28.

RESULTS: MiR-184 expression was significantly increased in H_2O_2-induced H9c2 cell injury model. After H9c2 cells were transfected with miR-184 inhibitor to silence miR-184, the levels of ROS and MDA were markedly reduced, while the expression of SOD was greatly increased. At the same time, the expression of inflammatory factors was greatly reduced. Silencing miR-184 also increased Bcl-2 expression, and reduced the expression of cleaved Caspase-3 and Bax. In addition, compared with the H_2O_2 + NC group, the number of TUNEL positive cells in the H_2O_2 + miR-184 inhibitor group was also significantly reduced, and the cell viability was remarkably increased. The Luciferase reporter experiment proved that FBXO28 is a target gene of miR-184.

CONCLUSIONS: MiR-184 expression was increased in H_2O_2-treated H9c2 cells. Inhibition of miR-184 markedly inhibited oxidative stress and inflammation in cardiomyocytes, thereby inhibiting cardiomyocyte apoptosis, through the regulation of FBXO28.

Key Words:
Myocardial infarction, MicroRNA-184, Inflammation, Oxidative stress, Apoptosis, FBXO28.

Introduction

Cardiovascular disease has surpassed cancer as the disease that poses the greatest threat to human health and life[1]. Acute myocardial infarction (AMI) is an acute cardiovascular disease with high mortality, disability, and high medical costs[2]. The incidence of AMI has been increasing year by year, and it is becoming younger[3]. It has become a prominent public health problem and social problem. Although there are currently clinical methods, such as drug therapy and coronary intervention reperfusion therapy, myocardial necrosis, myocardial remodeling, and irreversible damage to cardiac function are still unsatisfactory in some patients after MI[4]. The main causes of AMI are: coronary atherosclerosis, inflammation, and spasm[5]. Under the same environmental exposure, only a small number of individuals in the population are affected. Large-scale genomic studies have found multiple susceptible sites and segments of MI, suggesting that the occurrence and development of AMI is the result of multiple factors including environmental factors and genetic factors[6]. Therefore, it is of great significance

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to explore the molecular mechanism of AMI and find target for treatment.

Coronary atherosclerosis is a chronic inflammatory process due to the infiltration of inflammatory cells and the accumulation of lipids leading to the gradual thickening of blood vessel walls[7]. This is the main cause of AMI. When AMI occurs, due to the continuous hypoxia and lack of ATP of myocardial cells, the apoptosis cascade is activated and myocardial cells are necrotic[8]. Necrotic cardiomyocytes activate the immune system and produce a severe inflammatory response[8]. Oxidative stress, inflammatory response and apoptosis affect the development of MI and repair of myocardial injury. Proper inflammation facilitates myocardial repair, while excessive inflammatory responses cause secondary myocardial damage[9]. Inhibition of excessive inflammation and apoptosis has become an important part of repair of MI, control of ventricular remodeling after MI, and improvement of cardiac function.

MicroRNA is a non-coding small RNA discovered in recent years[10]. It regulates the expression of its target genes and participates in various biological processes, including cell proliferation, differentiation, and apoptosis[10]. At the same time, microRNA production is also regulated by transcription factors and other factors. In the cell, the microRNA inhibits translation or degradation of the target mRNA through the paired binding of the partial sequence of the 3'-untranslated region (3'-UTR) of the target mRNA[11]. MicroRNAs regulate more than 30% of mRNA in the body at the post-transcriptional level.

MiR-184 is widely conserved among species. Zhang et al[12] found that miR-184 can promote the apoptosis of trophoblast cells and induce spontaneous abortion. Derrick et al[13] found that miR-184 expression was related to conjunctival inflammation caused by Chlamydia trachomatis. Liu et al[14] found that the level of miR-184 was increased in myocardial tissue of MI rats by sequencing. However, there are few studies on the function of miR-184 on MI. In this paper, we established a H_2O_2-induced cardiomyocyte injury model to study the regulatory effects of miR-184 on inflammation, oxidative stress and apoptosis after MI. Our results suggest that miR-184 could be a potential therapeutic target for MI.

Materials and Methods

Cell Culture

H9c2 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM; MCE, Nanjing, China) complemented with 10% fetal bovine serum (FBS; MCE, Nanjing, China) was used to culture H9c2 cells in 37°C incubator under 5% CO_2. When the cells grew to about 80% confluence, we performed cell passage.

MiRNA Transfection

MiR-184 inhibitor or NC (RiboBio, Guangzhou, China) was transfected into H9c2 cells cultured in serum-free medium using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After 48 hours, H9c2 cells were treated with H_2O_2 (100 μM) for 4 hours to establish an *in vitro* model of MI.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (MCE, Nanjing, China) was utilized to extract the total RNA of H9c2 cells. 0.5 ml of TRIzol reagent was added into each well of a 24-well plate, and the liquid was transferred to Eppendorf (EP; Hamburg, Germany) tubes (Eppendorf, Hamburg, Germany) after grinding H9c2 cells. Then, chloroform was added in the tubes. We let the EP tubes shake, and then, let them stand for 5 minutes. After that, the EP tubes were centrifuged with a centrifugal force of 12000 g for about 20 minutes at 4°C. We transferred the upper aqueous phase of the mixture obtained by centrifugation to new EP tubes and added an equal amount of isopropanol to EP tubes, mixed them, and placed the tubes at 4°C for 15 minutes. Then, we centrifuged the mixture at 4°C for 15 minutes with a centrifugal force of 12000 g, after which the supernatant was discarded and 1 ml of a 75% ethanol solution was added to the EP tubes. The solution was centrifuged at 4°C for 5-10 minutes with a centrifugal force of 7500 g, after which the supernatant was discarded, and the EP tubes were dried at room temperature, and 20 μl of ribonuclease free water was added into the EP tubes. Finally, we used NanoDrop™ 8000 to measure RNA concentration.

Reverse transcription was performed using reverse transcriptase kit (MCE, Nanjing, China). QRT-PCR was performed by using Prism 7900 System (La Jolla, CA, USA). We used a 10 μl reaction system in accordance with the protocol. U6 was used to standardize the data. All the primers were listed in Table I.

Superoxide Dismutase (SOD) Activity Assay

When H9c2 cells were transfected with miR-184 inhibitor or NC and treated with H_2O_2, the Total

Table I. Real Time-PCR primers.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
miR-184	GGTGGACGGAGAACTGAT	GAGGAGGAAGAAGGGTAGGA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

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

SOD Assay Kit (Beyotime, Shanghai, China) was used to detect SOD expression in the cells of each group in accordance with the instructions. The absorbance at 450 nm was measured with a microplate reader to calculate the SOD expression.

ROS Quantification

Quantification of Reactive Oxygen Species (ROS) in the cells of each group was performed using the ROS Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol.

Malondialdehyde (MDA) Levels

MDA levels in each group were measured using Lipid Peroxidation MDA Assay Kit (Cambridge, MA, USA) in line with the protocols.

Enzyme Linked Immunosorbent Assay

The supernatants of H9c2 cells in the 4 groups were taken and the contents of TNF-α and IL-6 and IL-1β were detected by commercial kit kits (DOJINDO, Shanghai, China), respectively.

Western Blot

The total protein was obtained using protein extraction kit (KeyGen, Shanghai, China). The concentration was measured in line with the instructions of bicinchoninic acid (BCA) kit (Thermo Fisher, Waltham, MA, USA). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was made using SDS-PAGE gel preparation kit (Beyotime, Shanghai, China). A total of 20 µg of protein was added to the wells of SDS-PAGE for electrophoresis. Then, the electrophoresed protein was transferred to the polyvinylidene difluoride (PVDF, EpiZyme, Shanghai, China) membrane. Then, 5% skim milk was utilized to block the non-specific antigen on the protein bands. After that, the protein bands were incubated by the primary antibodies [cleaved Caspase-3, Abcam (Cambridge, MA, USA) Rabbit, 1:1000; Bcl-2, Abcam, Rabbit, 1:1000; Bax, Abcam, Rabbit, 1:1000; FBXO28, Abcam, Rabbit, 1:1000; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Abcam, Rabbit, 1:1000] overnight. The next day, the protein bands were incubated using the secondary antibody and then washed using TBST. Finally, Image Lab™ Software (Bio-Rad, Life Science, Hercules, CA, USA) was used to expose the bands.

TdT-Mediated dUTP Nick-End Labeling (TUNEL) Staining

H9c2 cells were seeded in 24-well plates. When the cells of each group were treated as described above, TUNEL kit (Roche, Basel, Switzerland) was used as instructed by the manufacturer to detect the apoptotic cells. The nucleus were stained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China). TUNEL staining was showed by a Confocal Laser Scanning Microscope (CLSM).

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

H9c2 cells were seeded in 96-well plate, and then, transfected with miR-184 inhibitor or NC, and then treated with H_2O_2. After 4 hours, 20 µl of MTT solution (YEASEN, Shanghai, China) was added and incubated with the cells for 4 hours at 37°C, and then, 150 µl of dimethyl sulfoxide (DMSO) solution was added to stop the enzyme reaction. Finally, the absorption light intensity at 490 nm was measured with a microplate reader.

Luciferase Activity Assay

To investigate whether miR-184 can directly target and bind to FBXO28 mRNA, Luciferase reporters (RiboBio, Guangzhou, China) containing wild-type and mutant 3'UTR of FBXO28 were constructed. According to the instructions, the Luciferase reporters and miR-184 mimic or mimic negative control (NC; RiboBio, Guangzhou, China) were co-transfected into HEK293T cells. After continuing to culture the cells for 48 hours, we added Luciferase reagent (YEASEN, Shanghai, China) according to the protocols and measured the activity of Luciferase separately using Dual-Glo® Luciferase Assay System (Promega, Madison, WI, USA).

Statistical Analysis

Measurement data is expressed as χ±s. *t*-test was utilized to compare the two groups. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Test level α=0.05. All experiments were repeated 3 times.

Results

MiR-184 Expression was Increased in H_2O_2-Treated H9c2 Cells

First, we treated H9c2 cells with H_2O_2 (100 µM, 4 h) to establish an *in vitro* model of cardiomyocyte injury. After that, we detected the expression difference of miR-184 in the model group and the control group by qRT-PCR, and found that miR-184 in the model group was significantly upregulated (Figure 1A). To study the role of miR-184 in myocardial injury, we transfected miR-184 inhibitor into H9c2 cells to inhibit miR-184 levels. We can find that miR-184 inhibitor significantly inhibited miR-184 expression in cardiomyocytes (Figure 1B).

Down-Regulation of MiR-184 Inhibited H_2O_2-Induced Oxidative Stress and Inflammation in Cardiomyocytes

H9c2 cells were transfected with miR-184 inhibitor or NC, and then, we treated H9c2 cells with H_2O_2. To study the effect of miR-184 on myocardial oxidative stress, we tested the levels of SOD, ROS and MDA in H9c2 cells of each group using commercial kits. We found that compared with the control group, the SOD level in the cardiomyocyte injury model group decreased significantly, while the levels of ROS and MDA increased markedly. When miR-184 was silenced, the expressions of SOD, ROS and MDA were greatly reversed (Figure 2A-C). To investigate the role of miR-184 in the inflammatory response in myocardial injury, we examined the expression of three inflammatory factors. As shown in the figure, silencing miR-184 can greatly inhibit the increase of TNF-α and IL-6 and IL-1β expression in myocardium caused by H_2O_2 (Figure 2D-F). The above results demonstrated that inhibition of miR-184 expression can significantly inhibit oxidative stress and inflammation in cardiomyocytes.

Downregulation of MiR-184 Inhibited H_2O_2-Induced Cardiomyocyte Apoptosis

In order to study the regulation of miR-184 on myocardial apoptosis, we first examined the expression of apoptosis-related proteins. As can be seen from Figure 3A and Figure 3B, the treatment of H_2O_2 could greatly reduce the Bcl-2 expression and increase the expression of Bax and cleaved Caspase-3, but the downregulation of miR-184 could reverse the expression of these three proteins. In addition, TUNEL staining revealed that downregulation of miR-184 remarkably reduced H_2O_2-induced cardiomyocyte apoptosis (Figure 3C, D). Through MTT detection, we found that

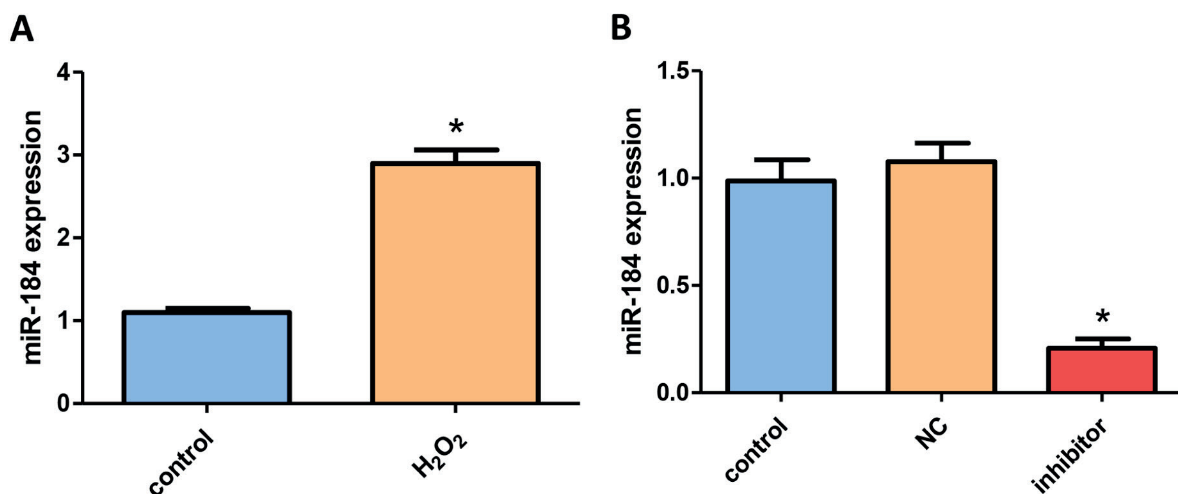


Figure 1. MiR-184 expression was increased in H_2O_2-treated H9c2 cells. **A,** Real Time-PCR analysis showed the upregulation of miR-184 in H9c2 cells treated with H_2O_2 ("*" $p<0.05$ *vs.* control, n = 3). **B,** mMR-184 inhibitor significantly inhibited miR-184 expression in H9c2 cells ("*" $p<0.05$ *vs.* NC, n = 3).

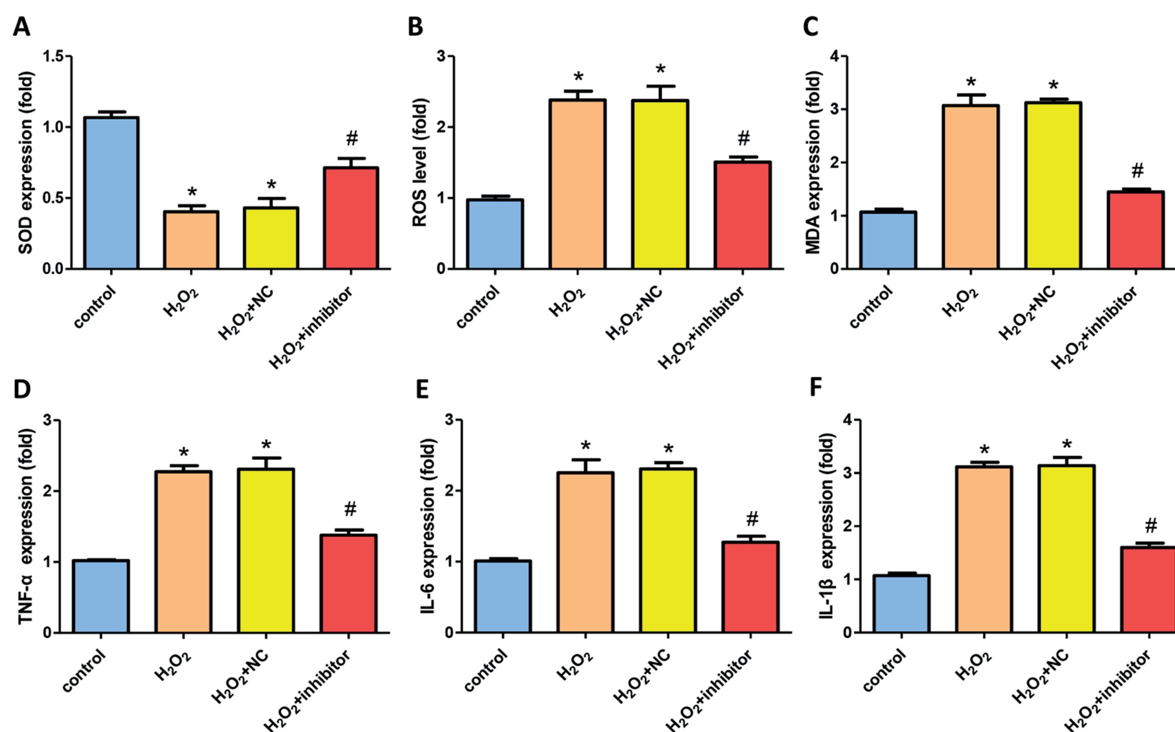


Figure 2. Downregulation of miR-184 inhibited H_2O_2-induced oxidative stress and inflammation in cardiomyocytes. **A,** SOD activity assay showed that H_2O_2 can significantly reduce SOD levels, while miR-184 inhibitor can reverse SOD levels ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3). **B,** The expression of ROS increased in the H_2O_2 treatment group, but decreased significantly in the H_2O_2+inhibitor group ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3). **C,** The levels of MDA in the supernatant were detected by MDA kit ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3). **D-F,** Elisa assay was used to detect the protein expression of TNF-α, IL-6, and IL-1β in the four groups ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3).

H_2O_2 greatly reduced cell viability, but downregulating miR-184 could reverse the decline in cell viability (Figure 3E). These results suggested that the downregulation of miR-184 can inhibit H_2O_2-induced cardiomyocyte apoptosis.

Downregulation of MiR-184 Was Involved in the Protection of Cardiomyocytes by Activating FBXO28

Through the TargetScan database, we found that F-box protein 28 (FBXO28) has a binding site with miR-184 (Figure 4A). Studies have shown that activating FBXO28 can promote cell proliferation by promoting mitosis and can inhibit cell apoptosis. To prove whether miR-184 can directly target FBXO28, we first detected the expression of FBXO28 by Western blot. It can be found that H_2O_2 treatment greatly reduced the expression of FBXO28, while downregulating miR-184 significantly upregulated the expression of FBXO28 (Figure 4B, C). Further, the results of the Luciferase reporter gene experiment showed that miR-184 mimic significantly inhibited Luciferase activity in the WT group but failed to inhibit activity in the mutant group (Figure 4D). These data indicated that FBXO28 was targeted by miR-184.

Discussion

AMI is a serious health problem in the world with a high mortality rate. Current treatments can reconstitute the blood supply to the ischemic areas[15]. However, these treatments do not fully restore the apoptosis and oxidative stress of some cardiomyocytes. In AMI, the myocardial ischemia site produces a large amount of ROS due to oxidative stress, which causes damage to the myocardial cell membrane, and at the same time, produces a large number of inflammatory factors, causing apoptosis of cardiomyocytes[16]. Therefore, in addition to rebuilding the blood supply in the ischemic areas, treatments, such as anti-oxidative stress, anti-apoptosis, and anti-inflammatory are also essential.

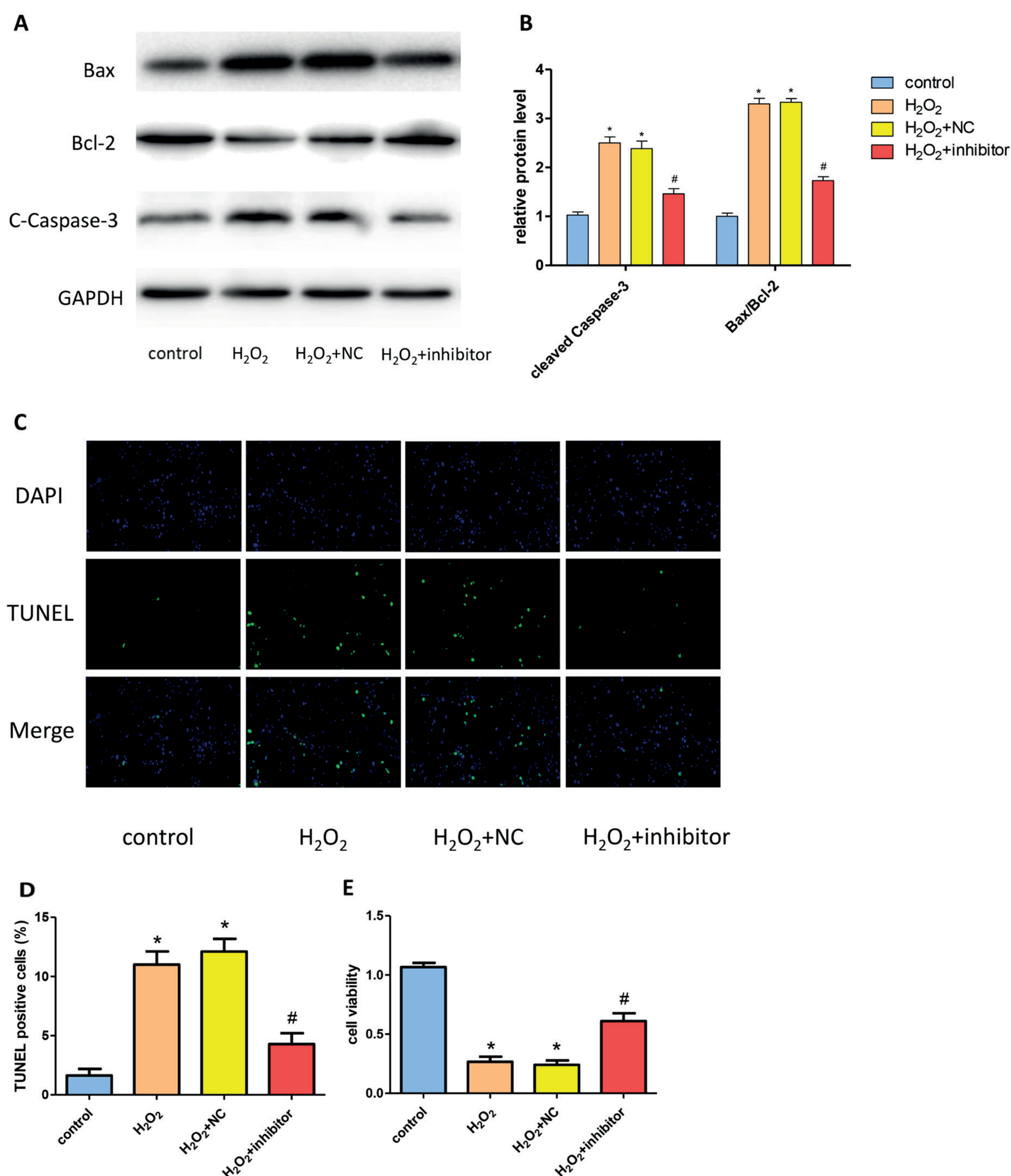


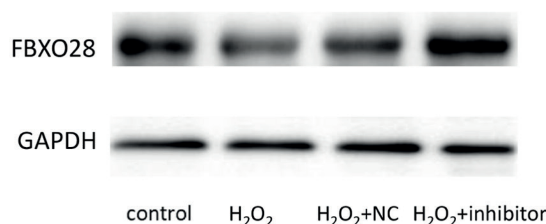
Figure 3. Downregulation of miR-184 inhibited H_2O_2-induced cardiomyocyte apoptosis. **A,** The expression of Bax, Bcl-2, and cleaved Caspase-3 was determined by Western blot analysis. **B,** Statistical results of protein levels ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3). **C,** TUNEL staining showed that miR-184 inhibitor can obviously reduce the increase of H9c2 cell apoptosis caused by H_2O_2 (magnification: 200×). **D,** Statistical analysis of TUNEL staining ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3). **E,** MTT assay suggested that miR-184 inhibitor restored H9c2 cell viability after H_2O_2 treatment ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3).

MiRNA is a kind of non-coding short-stranded RNA with biological activity. MiRNA is a negative regulator of gene expression, which can regulate the stability of the target mRNA and the efficiency of transcription. The generation of miRNA includes two steps of transcription and

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 1785-1792 of FBXO28 3' UTR	5' ...AGCACAAGGUUGUCAUCCGUCCA...
	|||||||
rno-miR-184	3' UGGGAAUAGUCAAGAGGCAGGU

B



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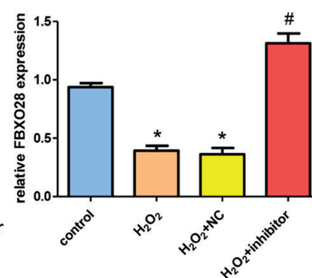

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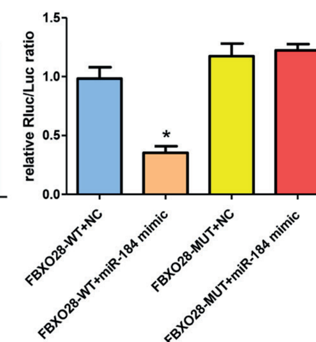

Figure 4. Downregulation of miR-184 was involved in the protection of cardiomyocytes by activating FBXO28. **A,** Binding site predicted by the TargetScan database. **B,** Western blot detected the expression of FBXO28 in the four groups. **C,** Statistical analysis of FBXO28 expression ("$_*$" $p<0.05$ *vs.* control, "$_\#$" $p<0.05$ *vs.* H_2O_2+NC, n=3). **D,** MiR-184 overexpression significantly decreased the relative luciferase activity in WT group, but did not decrease the relative luciferase activity in MUT group ("$_*$" $p<0.05$ *vs.* WT+NC, n = 3).

maturation, which are completed in the nucleus and cytoplasm, respectively[17]. Because miRNAs have a wide range of gene regulatory capabilities and tissue specificity, they may play important regulatory functions in various systems and tissues. MiRNA expression in the heart changes significantly after MI and is closely related to the pathophysiological process of MI[18].

FBXO28 is a conserved F-box protein. Loss of FBXO28 results in metaphase to anaphase progression delay, which in turn leads to several mitotic defects[19]. One study proved that FBXO28 was downregulated in β-cells and in isolated human islets under diabetic conditions, while restoration of FBXO28 protected β-cells from apoptosis of the diabetic milieu[20].

In this study, we found that the expression of miR-184 was greatly increased in an *in vitro* model of MI. In addition, inhibiting miR-184 can inhibit ROS and MDA levels, and can increase the expression of SOD in cardiomyocytes. The levels of inflammatory factors TNF-α, IL-6, and IL-1β in cardiomyocytes were significantly reduced. These indicated that silencing miR-184 can inhibit myocardial inflammation and oxidative stress after MI. Moreover, when miR-184 was inhibited, the expression of pro-apoptotic protein Bax was greatly decreased, and the expression of cleaved Caspase-3 was also markedly reduced, while the expression of anti-apoptotic protein Bcl-2 was markedly increased. Similarly, the results of TUNEL staining suggested that the inhibition of miR-184 inhibited apoptosis of myocardial cells after MI. Therefore, miR-184 plays an important regulatory role in the physiological and pathological process of MI and can be used as a potential target for the treatment of MI.

In order to explore the molecular mechanism of miR-184 in MI, we predicted the target gene of miR-184 through the TargetScan database. We found that FBXO28 mRNA has a binding site with miR-184. Moreover, the expression of FBXO28 in H9c2 cells treated with H_2O_2 was significantly reduced, while inhibition of miR-184 could reconstruct the expression of FBXO28. Through Luciferase reporter gene experiments, we demonstrated that miR-184 directly targets FBXO28.

We revealed the role of miR-184 in MI for the first time *in vitro*, and revealed that downregulation of miR-184 inhibited oxidative stress, inflammation, and apoptosis of myocardium in MI by targeting FBXO28. However, for the role of miR-184 in MI and its molecular mechanism, more research is needed, including the study of the effect of miR-184 on MI *in vivo*, gain of function and loss of function experiments of FBXO28. We will

follow-up the research on miR-184 and FBXO28 in the subsequent experiments.

Conclusions

All together, these data showed that miR-184 expression was elevated in ischemic myocardium. Downregulating miR-184 significantly inhibited myocardial oxidative stress, inflammation, and apoptosis by targeting FBXO28.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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