European Review for Medical and Pharmacological Sciences 2021; 25: 344-352

Upregulation of miR-335 reduces myocardial injury following myocardial infarction *via* targeting MAP3K2

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Abstract. – OBJECTIVE: Acute myocardial infarction (AMI) is a serious cardiovascular disease with a high incidence worldwide and the main cause of sudden cardiac death. The aim of this article was to study the protective role of miR-335 in myocardial infarction (MI) and the underlying molecular mechanism.

MATERIALS AND METHODS: Thirty Sprague Dawley (SD) rats were randomly divided into sham group, MI + NC group and MI + agomiR-335 group. The expression of miR-335 in rat myocardium was detected by quantitative Real Time-Polymerase Chain Reaction (RT-PCR). Western blot was performed to detect the expression of TNF-α, IL-6, IL-1β, Caspase-3, Cleaved Caspase-3 (C-Caspase-3) and MAP3K2 in rat myocardium. On the 7th day of the establishment of the rat MI model, a high-resolution small animal ultrasound system was utilized to detect the cardiac function of the rats, and the heart tissues and blood samples of the rats were collected. The corresponding kits were purchased to detect the contents of LDH, CK-MB, MDA and SOD in rat serum, and HE staining was employed to observe the morphology of rat myocardial tissue.

RESULTS: The expression of miR-335 in myocardial infarcted zones and border zones of MI rats decreased significantly. The upregulation of miR-335 remarkably inhibited myocardial inflammation and apoptosis after MI, thus improving the cardiac function of MI rats. Compared with the sham group, the MAP3K2 expression in the MI + NC group was observably increased, while the overexpression of miR-335 could inhibit the expression of this protein. Through the Luciferase reporter gene experiment, we found that miR-335 could directly target MAP3K2.

CONCLUSIONS: The expression of miR-335 decreased in myocardial tissue after MI, and the overexpression of miR-335 reduced myocardial damage by inhibiting oxidative stress, inflammation, and apoptosis via targeting MAP3K2, thereby improving the cardiac function of MI rats.

Key Words:
Acute myocardial infarction, MicroRNA-335, Oxidative stress, Inflammation, Apoptosis, MAP3K2.

Introduction

Clinically, the incidence of AMI is high, and the prognosis is often poor, which has caused great concern in the medical community. The mortality rate of MI has been rapidly rising in recent years. It is one of the major diseases that threaten human life. It has brought a serious economic burden to families and society, and has become a major public health problem. Although there are currently clinical methods, such as drug therapy and coronary intervention reperfusion therapy, myocardial necrosis and myocardial remodeling lead to irreversible damage to cardiac function after MI, so there are still some patients with poor treatment results[1-4].

The main causes of AMI are: coronary atherosclerosis, inflammation, spasm, etc. Coronary atherosclerosis is a chronic inflammatory process due to infiltration of inflammatory cells

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and gradual thickening of blood vessel walls caused by lipid accumulation, which is the main cause of AMI. During the occurrence of AMI, the apoptotic cascade is activated and the myocardial cells are necrotic due to the continuous hypoxia and lack of ATP. Necrotic cardiomyocytes activate the immune system and produce a severe inflammatory response. Inflammatory response and apoptosis affect the development of MI and myocardial injury repair. Proper inflammation facilitates myocardial repair, while excessive inflammatory responses cause secondary myocardial damage. Inhibition of excessive inflammation and apoptosis has become an important part of repairing MI, controlling ventricular remodeling, and improving cardiac function[5-8].

MicroRNA (miRNA) is an endogenous, small, non-coding RNA molecule. It has been shown that miRNA plays an important regulatory role in cardiovascular disease. Maintaining and restoring stable expression of corresponding miRNA in target organs, such as the heart may become a new target for the treatment of cardiovascular and other diseases[9]. Many miRNAs related to inflammation or apoptosis of cardiomyocytes have been found. Reconstruction of the expression of these miRNAs can inhibit inflammation and apoptosis of myocardium *in vivo* and *in vitro*[10, 11]. MiR-335 has been reported to inhibit chondrocyte inflammation and apoptosis in osteoarthritis[12]. However, its role in MI has not been studied.

In this article, we first constructed a rat model of MI and tested miR-335 expression in myocardial tissue. Afterwards, we injected agomiR-335 *via* the tail vein to upregulate miR-335 in myocardium to study the regulatory role of miR-335 in MI and the underlying molecular mechanism. This article provides a new potential method for the treatment of MI.

Materials and Methods

Rat MI Model

This study was approved by the Animal Ethics Committee of The First People's Hospital of Shangqiu City Animal Center. Thirty female Sprague Dawley (SD) rats (220 ± 10 g) were purchased from Animal Center of Chinese Academy of Medical Sciences (Beijing, China). Before conducting the experiment, the rats were housed in cages with feeding conditions as follows: temperature 20°C-25°C, humidity 40%-60%. Ten of thirty SD rats were randomly selected as the sham group. The other 20 rats were subjected to MI model establishment. Before the model establishment, they were randomly divided into two groups, one group was injected with miR-335 agomir (agomiR-335, 5 mg/kg) (RiboBio, Guangzhou, China) *via* the tail vein and the other group was injected with negative control (NC). The agomiR-335 was the same double-stranded RNA analog as mature miR-335, whose structure was chemically modified and bound to cholesterol molecules to be used *in vivo*. Therefore, SD rats are divided into three groups: sham group, MI + NC group, MI + agomiR-335 group.

All rat models were fasted one night before replication, and the MI model was constructed by ligation of the left anterior descending coronary artery. The rats were intraperitoneally injected with 5% pentobarbital sodium (40 mg/kg) for anesthesia, fixed in the dorsal position, and connected to a small animal ventilator for auxiliary breathing after intubation (the tidal volume is 0.8 mL and the respiration ratio is 2: 1, 70 breaths/min). The left anterior chest area was routinely depilated and sterilized, thorax opened in the third intercostal space, and the left coronary artery was ligated at the lower edge of the junction of the pulmonary artery cone and the left atrial appendage about 1-2 mm. After that, the chest wall was sutured layer by layer and the chest was closed. The operation procedure of the rats in the sham control group was the same as above, but the left coronary artery was not ligated. After operation, the rats were injected with 100,000 units of penicillin intraperitoneally for 3 consecutive days to prevent wound infection. The diagnostic criteria for MI is an electrocardiogram with more than two limb leads or chest leads with ST segment arches raised upward.

Echocardiographic Measurement

The cardiac function was measured on the 7th day after MI in rats. The rats were anesthetized with isoflurane. After the physiological state of the rat is stable, the heart function of the rat was detected by using a high-resolution small animal ultrasound probe. The measured indicators include left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and left ventricular fractional shortening (LVFS), and counted left ventricular ejection fraction (LVEF).

Detection of Serum Myocardial Enzymes (LDH, CK-MB) and Antioxidant Markers (MDA, SOD)

After cardiac function testing, we collected 5-10 mL of blood from the abdominal aorta in a normal blood collection tube and allowed it to stand for 30 minutes at room temperature. After the blood was completely coagulated, it was centrifuged at 3 000 r/min for 15 minutes at low temperature to separate serum. Afterwards, serum myocardial enzymes (LDH, CK-MB) and antioxidant markers (MDA, SOD) were detected according to the kit instructions (Beyotime, Shanghai, China).

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

Appropriate amount of myocardial tissue in infarcted zone, border zone, and remote zone was taken, and grinded under liquid nitrogen. Then, TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to extract total RNA, and the total RNA concentration and purity were determined by microplate reader. Afterwards, the total RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using reverse transcription kit (Roche, Basel, Switzerland) for quantitative PCR amplification. RT-PCR is operated by ABI step one fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA). MiR-335 expression was normalized using U6. The relative expression of the genes was calculated using $2^{-\Delta\Delta}$CT method. The primers were listed in Table I.

Western Blot

Appropriate amount of myocardial tissue in the border zone of the left ventricular was fully ground. After adding radioimmunoprecipitation assay (RIPA) lysate and protease inhibitor (Beyotime, Shanghai, China), the tissue was crushed with a rapid tissue cell disruptor, centrifuged at 12000 r/min for 10 minutes at low temperature, the total protein was separated, and the protein was quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The proteins were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Then, the non-specific antigens of the protein bands were blocked with 5% skim milk for 2 hours. After that, primary antibodies (TNF-α, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IL-6, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IL-1β, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Caspase-3, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Cleaved Caspase-3, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; MAP3K2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; GAPDH, Abcam, Cambridge, MA, USA, 1:1000) were used to incubate the protein bands overnight at 4°C. The membranes were washed 3 times with tris buffered saline-tween (TBST) solution, incubated with secondary antibody at room temperature for 1 hour, and then, washed 3 times with phosphate buffered saline-tween (PBST) solution again. Finally, the membranes were exposed using enhanced chemiluminescence (ECL) luminous fluid and the gray value of the protein band was analyzed with Image J software (NIH, Bethesda, MD, USA).

Hematoxylin-Eosin (HE) Staining

On the 7th day after MI, the rat heart was collected, the atrium was cut along the coronary sulcus, and the left ventricle was transected into two parts below the ligation point. The tissue was fixed by paraformaldehyde, dehydrated, paraffin embedded, sectioned, stained with HE (RiboBio, Guangzhou, China), and the pathological changes of myocardium were observed under the microscope.

Luciferase Activity Assay

The recombinant Dual-Luciferase reporter plasmid (RiboBio, Guangzhou, China) served as the tool vector and were inserted by the fragments of wild-type 3'UTR (WT-MAP3K2-3'UTR)

Table I. Real Time-PCR primers.

Gene name	Forward (5'>3')	Reverse (5'>3')
miR-335	AGGGTCAAGAGCAATAACGAA	GAGATGGGAGAGGGAGTAGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

qRT-PCR, quantitative Real Time-Polymerase Chain Reaction.

or mutant 3'UTR (MUT-MAP3K2-3'UTR) of rat synthetic MAP3K2 gene for constructing the double Luciferase vectors of WT-MAP3K2-3'UTR and MUT-MAP3K2-3'UTR respectively. The HEK293T cells were plated in a 24-well culture plate (the number of cells was about 10,000 per well) and cultured at 37°C in an incubator containing 5% CO_2 until the cell confluence was about 60%. The recombinant Luciferase reporter plasmids and miR-335 mimic (RiboBio, Guangzhou, China) or negative control (NC) were co-transfected into HEK293T cells, and the Luciferase activity was detected and the targeted regulatory effect of miR-335 on MAP3K2 was analyzed.

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. The differences between two groups were analyzed by using the Student's *t*-test. The 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-335 Expression Was Reduced in Infarcted Myocardium

We constructed rat MI models by ligating the left anterior descending coronary artery. The hearts of rats were collected on the 1st, 3rd and 7th days after the operation, and miR-335 expression in the infarcted zone, border zone and remote zone was detected by RT-PCR. Compared with the sham group, miR-335 expression in the infarcted zone and border zone was markedly reduced, while there was no significant change in the remote zone (Figure 1A-1C).

Overexpression of MiR-335 Reduced Myocardial Damage in MI Rats

In order to study the function of miR-335, we injected agomiR-335 *via* tail vein to increase the level of miR-335 in rat myocardium (Figure 2A). On the 7th day after MI in rats, we collected the blood samples. We tested the content of serum LDH and CK-MB in the three groups of rats through commercial kits, and found that compared with the sham group, the serum LDH and CK-MB content in the MI + NC group increased greatly, but the overexpression of miR-335 can notably reduce the release of these two myocardial enzymes (Figure 2B and 2C). We also compared the levels of serum SOD and MDA, the difference was statistically significant ($p<0.05$). The serum SOD level in MI + NC group was lower than that in sham group ($p<0.05$), and the MDA level was higher than that in sham group ($p<0.05$). However, the serum SOD level in MI + agomiR-335 group was higher than that in MI + NC group ($p<0.05$), and the MDA level was lower than that in MI + NC group ($p<0.05$) (Figure 2D and 2E). The expression of inflammatory factors (TNF-α, IL-6, IL-1β) in the myocardium of the three groups of rats was also analyzed by Western blot. MiR-335 overexpression significantly reversed the high expression of 3 inflammatory factors in infarcted myocardium (Figure 2F). In addition, we also detected the changes in the expression of pro-apoptotic proteins in

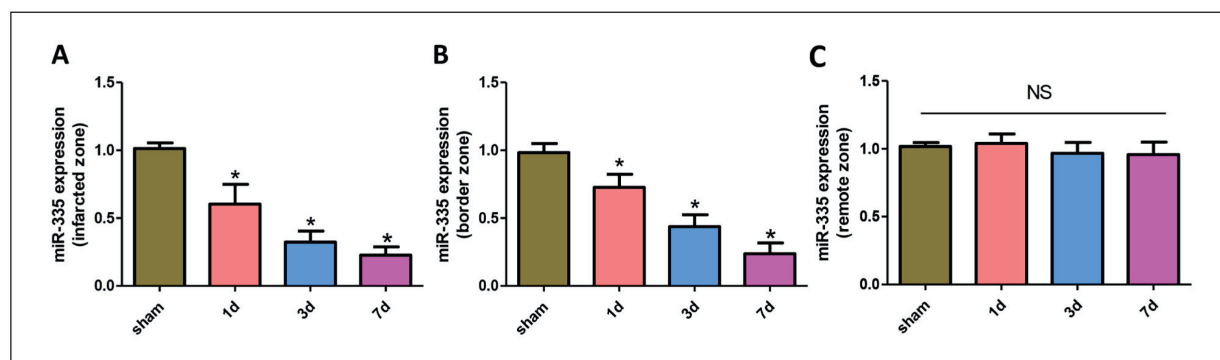

Figure 1. MiR-335 expression was reduced in infarcted myocardium. The expression of miR-335 in infarcted zone (**A**), border zone (**B**) and remote zone (**C**) of hearts of MI rats were detected by RT-PCR ("*" $p<0.05$ *vs.* sham, n = 3).

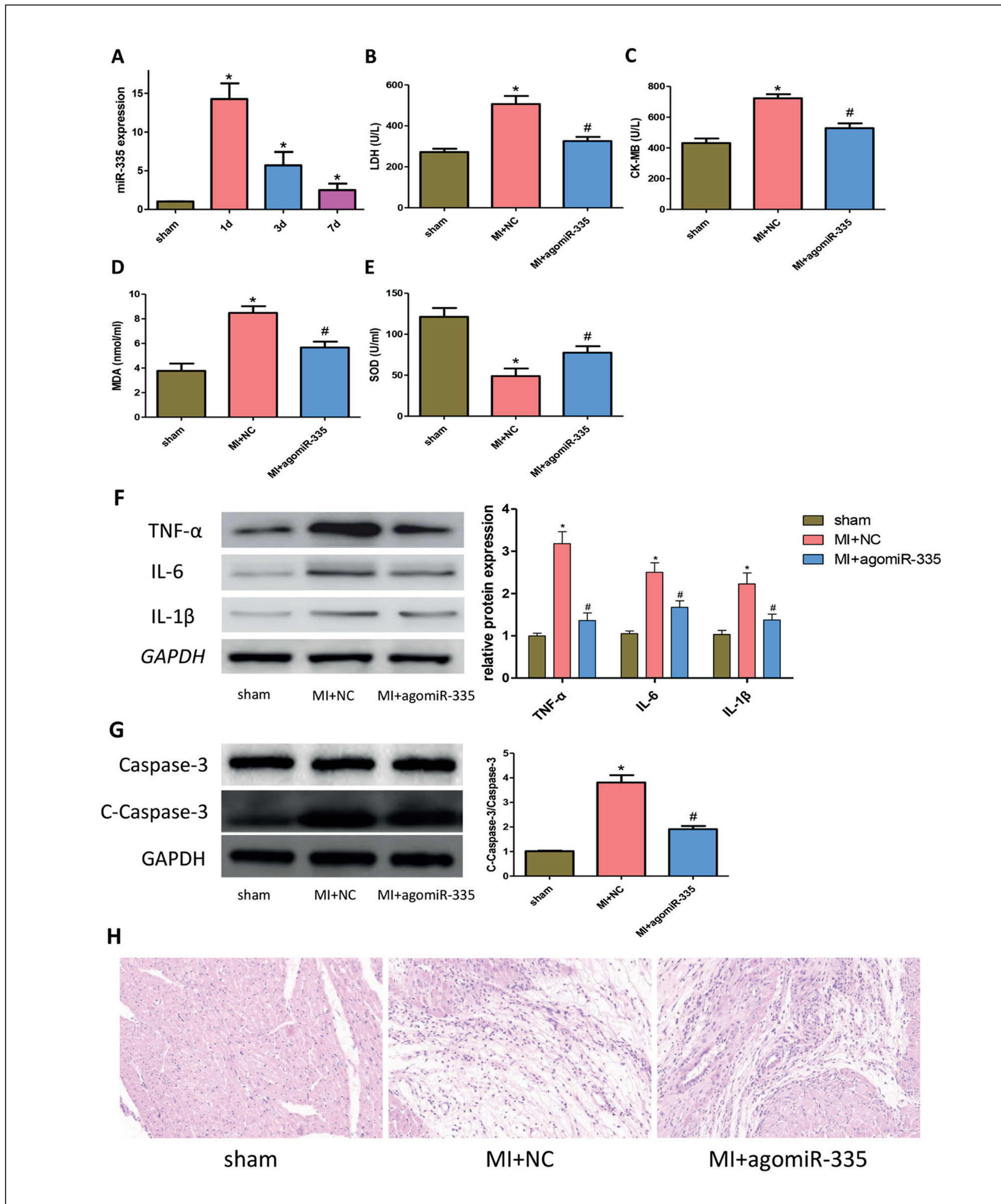

Figure 2. Overexpression of miR-335 reduced myocardial damage in MI rats. **A,** Intravenous injection of agomiR-335 significantly increased the expression of miR-335 in rat myocardium ("*" $p<0.05$ *vs.* sham, n = 3). **B,** Serum LDH content of rats in 3 groups ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=6). **C,** Serum CK-MB content of rats in 3 groups ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=6). **D,** Serum MDA content of rats in 3 groups ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=6). **E,** Serum SOD content of rats in 3 groups ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=6). **F,** The expression of inflammatory cytokines (TNF-α, IL-6, IL-1β) detected by Western blot ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=6). **G,** The expression of Cleaved Caspase-3 detected by Western blot ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=6). **H,** Typical HE staining of myocardial tissues (magnification: 200×).

infarcted myocardium. Intravenous injection of agomiR-335 can protect the myocardium of MI rats from apoptosis (Figure 2G). It can be seen from Figure 2H that the myocardial fibers in the sham group are neatly arranged, the horizontal stripes are clear, and the overall shape is normal. The intima cells and the myocardium are intact, the nucleus is centered, the cytoplasm is uniformly stained, there is no edema in the interstitium, and there is no inflammatory cell infiltration and cell edema, and no evident histological changes were detected. However, in the MI + NC group, more fibrotic areas and myocardial cell necrosis, interstitial edema, and inflammatory cell infiltration were seen under the muscular layer, and the surrounding myocardial cells were hypertrophic and disordered. Compared with MI + NC group, the lesion area in MI + agomiR-335 group was greatly improved. The above results indicate that the overexpression of miR-335 could inhibit oxidative stress, inflammation, and apoptosis of myocardium after MI, thereby protecting the integrity of myocardial cell membrane and reducing myocardial cell damage.

Overexpression of MiR-335 Improved Cardiac Function in MI Rats

Within 7 days after MI, there were no dead rats in the sham group, while 3 mice died in the MI + NC group and 1 died in the MI + agomiR-335 group (Figure 3A). Moreover, compared with the sham group, the average weight of the rats in the MI + NC group decreased, while the average weight of the rats in the MI + agomiR-335 group was higher than that of the MI + NC group, and the difference was statistically significant ($p<0.05$) (Figure 3B). Through echocardiographic testing, we found that the differences in LVEF, LVFS, LVEDD and LVESD at 7 days after surgery in each group were statistically significant (Figure 3C-3F). LVFS and LVEF in MI + NC group were lower than those in the sham group ($p<0.05$), and LVEDD and LVESD were higher than those in sham group ($p<0.05$). LVFS and LVEF in MI + agomiR-335 group were higher than those in MI + NC group ($p<0.05$), and LVEDD and LVESD were lower than those in MI + NC group ($p<0.05$). In summary, the

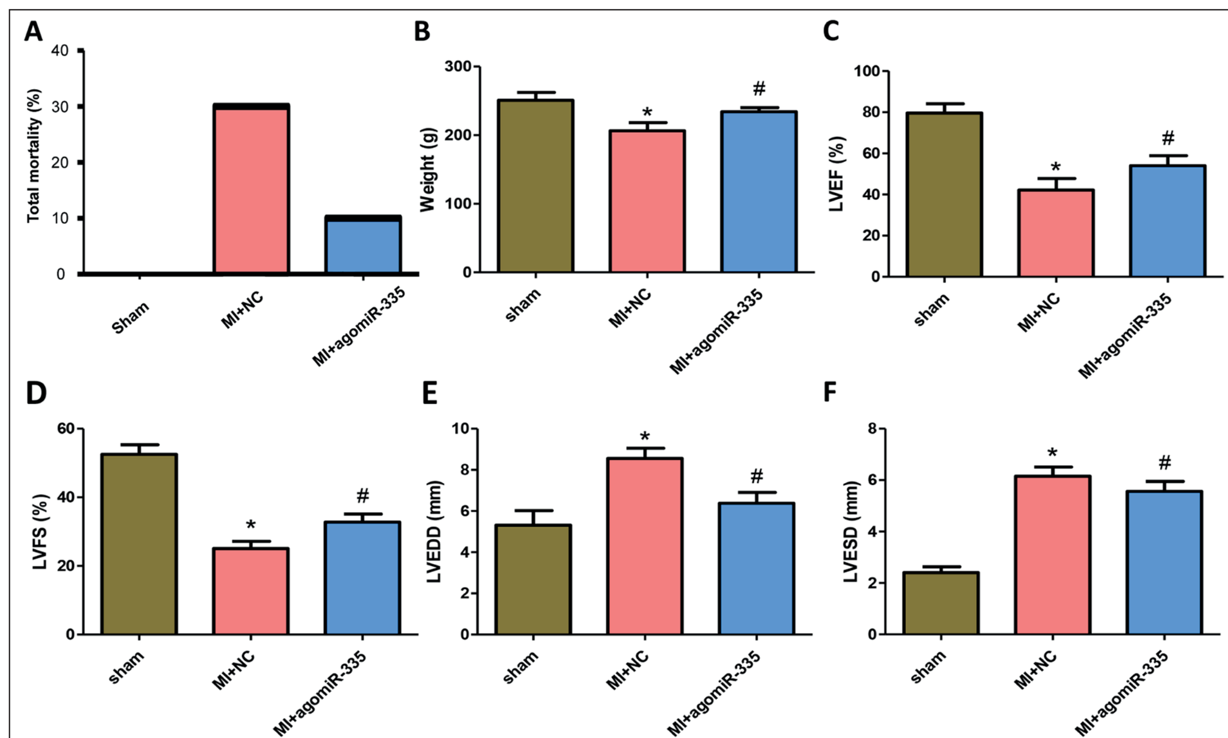

Figure 3. Overexpression of miR-335 improved cardiac function in MI rats. **A,** Rat mortality in 3 groups. **B,** Average body weight of rats in 3 groups ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=6). **C-F,** left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=6).

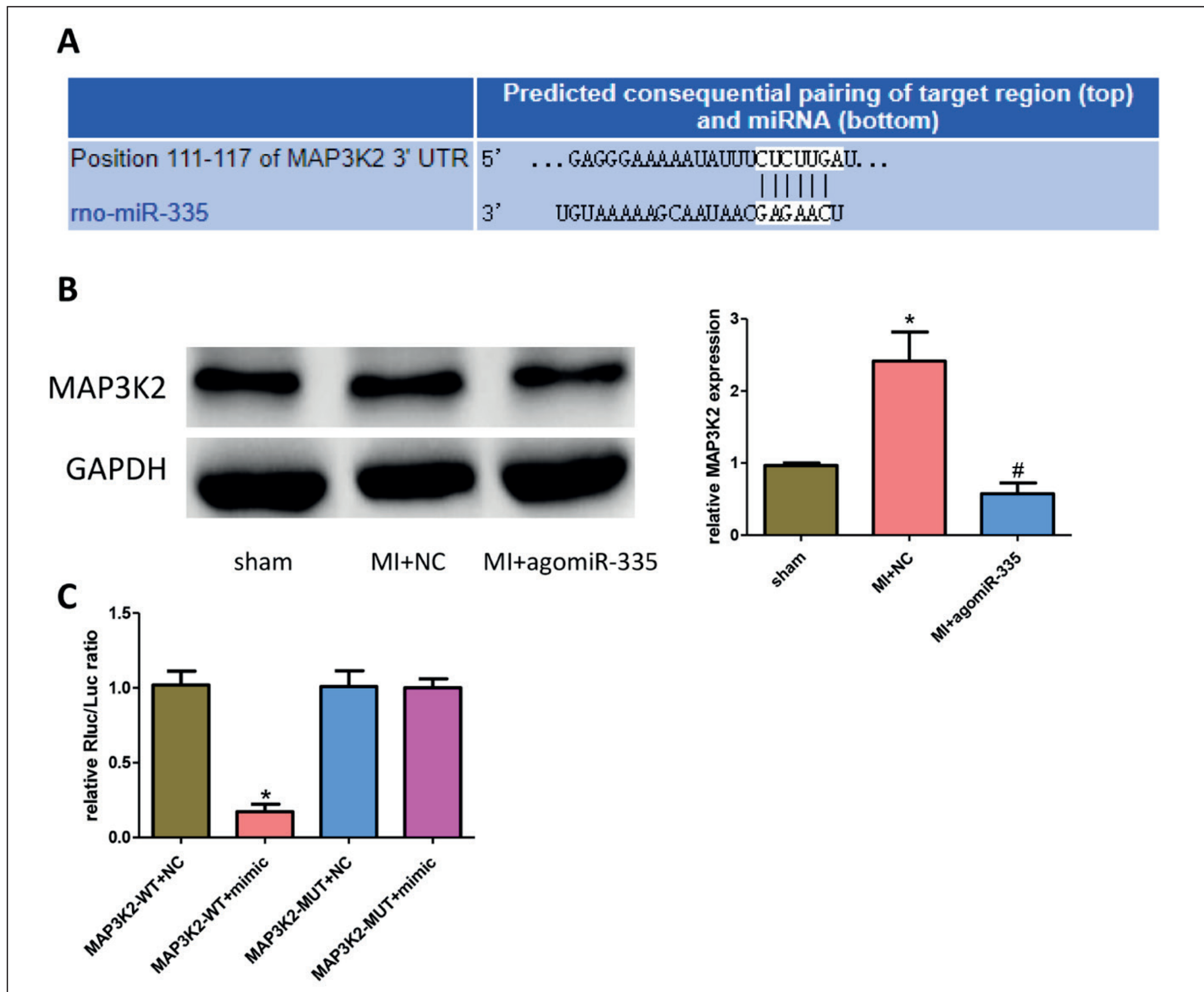

Figure 4. MiR-335 directly targets MAP3K2. **A,** Binding site predicted by the TargetScan database. **B,** The expression of MAP3K2 was detected using Western blot ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=3). **C,** Luciferase activity assay of HEK293T cells transfected with luciferase constructs containing WT-3′UTR and MUT-3′UTR of MAP3K2 ("*" $p<0.05$ *vs.* WT+NC, n=3).

overexpression of miR-335 can significantly improve the cardiac function of MI rats.

MiR-335 Directly Targets MAP3K2

The TargetScan website predicted that miR-335 had a binding site to the 3'UTR of MAP3K2 mRNA (Figure 4A). Western blot showed that MAP3K2 expression increased in infarcted myocardium, and the overexpression of miR-335 greatly inhibited the expression of this protein (Figure 4B). MiR-335 overexpression notably inhibited Luciferase activity in the WT group but failed to inhibit activity in the MUT group (Figure 4C). These data indicated that MAP3K2 was targeted by miR-335.

Discussion

MI refers to the necrosis of myocardial cells caused by the heart after ischemia and hypoxia, and is one of the main causes of death of patients with cardiovascular disease[13]. With the changes in people's lifestyles and the aging population, the morbidity and mortality of MI have been increasing year by year, which has seriously threatened the health of people around the world[14]. MI causes oxidative stress injury, inflammation response, myocardial cell apoptosis, myocardial tissue damage, and ventricular remodeling, and eventually develops heart failure[15]. Therefore, reducing oxidative stress and inflammation, and

then, reducing myocardial cell necrosis and apoptosis is an important way to prevent ventricular remodeling after MI.

Various mechanisms activate the immune system after MI, causing an inflammatory response. The infiltration of the inflammatory cells produces a large amount of cytokines at the same time, which participates in the inflammatory response process after MI, including cell death, cell infiltration, and extracellular remodeling stimulated by cytokines. Appropriate inflammatory response can promote myocardial tissue repair, but overreaction leads to the formation of a large amount of scar tissue and fibrosis, causing ventricular remodeling and ultimately affecting heart function[16]. Oxidative stress refers to the pathological process of excessive production of oxidative active substances in the body and/or weakened antioxidant capacity and insufficient removal of oxygen free radicals, resulting in oxidative damage to cells and tissues. SOD and MDA levels in the body can reflect the body's oxidative stress state. After MI, the generation of oxygen free radicals increases, and the activity of important antioxidant enzyme SOD in the body decreases, destroying the structure of myocardial cells, damaging the membranes of myocardial cells, and leading to the necrosis of myocardial cells[17]. Apoptosis, also known as programmed cell death, is a way of triggering cell death by pre-existing cell death procedures triggered by internal and external factors. After MI, due to ischemia and hypoxia, cardiomyocytes initiate apoptosis-related mechanisms, form an apoptosis complex, and induce apoptosis through the activation of various proteases, which play an important role in the process of MI to heart failure[18].

The research of miRNA in the field of cardiovascular diseases shows that it plays an important role in the occurrence and development of MI. In-depth study of the function of miRNA in the process of MI will enrich the understanding of MI, broaden the thinking and methods of diagnosis and treatment of MI, and provide new targets for future miRNA intervention in the treatment of MI. This study found that miR-335 expression in infarcted zone and remote zone of the heart of MI rats was observably reduced. By injecting miR-335 into the tail vein of rats, miR-335 expression in the rat heart was increased. The up-regulation of miR-335 remarkably promoted the expression of SOD and reduced the level of MDA, indicating that it inhibited the oxidative stress of myocardial tissue. At the same time, miR-335 overexpression also inhibited the production of inflammatory factors (TNF-α, IL-6, IL-1β) and pro-apoptotic protein (Cleaved Caspase-3), thereby inhibiting inflammation and apoptosis of myocardial tissue. Eventually, the release of myocardial enzymes in the serum of MI rats is reduced, and the cardiac function is restored to a certain extent. We also found that in infarcted myocardium, MAP3K2 expression was markedly increased, and the overexpression of miR-335 could remarkably inhibit MAP3K2 expression. Further, through the Luciferase reporter gene experiment, we proved that miR-335 could directly target MAP3K2.

Conclusions

The results of this present study revealed that miR-335 expression was decreased in infarcted zone and border zone of hearts of MI rats. While restoration of miR-335 expression significantly inhibited oxidative stress, inflammation, and apoptosis of infarcted myocardium, thereby reducing myocardial injury and improving cardiac function in MI rats. In addition, we found that MAP3K2 was upregulated in infarcted myocardium, but the overexpression of miR-335 could reduce the expression of MAP3K2. Further, MAP3K2 was proved to be the target gene of miR-335. In conclusion, miR-335 reduced myocardial damage by inhibiting oxidative stress, inflammation and apoptosis *via* targeting MAP3K2, thereby improving the cardiac function of MI rats.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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