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MicroRNA-298 regulates apoptosis of cardiomyocytes after myocardial infarction

Q. ZHANG[1], N. YU[2], B.-T. YU[3]

[1]Department of Cardiology, Coal General Hospital, Beijing, China
[2]Department of Cardiovascular Medicine, The First People's Hospital of Jinan, Jinan, China
[3]Department of Emergency, The Affiliated Central Hospital of Qingdao University, Qingdao, China

Abstract. – OBJECTIVE: To investigate the role and mechanism of micro ribonucleic acid (miR)-298 in myocardial apoptosis after myocardial infarction.

MATERIALS AND METHODS: *In vivo* experiments, the rat model of myocardial infarction was established, and miR-298 was up-regulated via lentivirus with miR-298 overexpression. Cardiac function of rats was detected via echocardiography, Bcl-2 associated X protein (BAX) expressions in infarction border zone were detected via Real-time Quantitative PCR (qT-PCR) and Western blot, and TUNEL assay was used to detect the myocardial apoptosis. *In vitro* experiments, myocardial cells were isolated and cultured, an oxygen-glucose deprivation (OGD) model was established to mimicking the ischemic condition, the relationship between miR-298 and BAX was verified using luciferase reporter vector, lentivirus and small-interfering RNA (siRNA) in BAX.

RESULTS: *In vivo* experiments showed that the miR-298 expression was down-regulated at 2 and 4 weeks after myocardial infarction. The up-regulation of miR-298 significantly improved the cardiac function, decreased the expressions of BAX, reduced the myocardial apoptosis and inhibit the apoptosis proteins expression including cytochrome-c and cleaved caspase-3. *In vitro* experiments revealed that BAX was a target gene of miR-298 and further proof that miR-298 could inhibit the cytochrome-c and cleaved caspase-3 expression and myocardial apoptosis through BAX.

CONCLUSIONS: MiR-298 can improve the myocardial apoptosis through the target gene BAX.

Key Words:
miR-298, Bcl-2 associated X protein (BAX), Myocardial infarction, Apoptosis.

Introduction

With the rapid economic development and improvement of people's living standards, cardiovascular disease (CVD), especially the coronary heart disease (CHD) has become the main cause of human death, whose morbidity and mortality are increased year by year[1]. Ischemic heart disease (IHD), especially acute myocardial infarction is one of those diseases with high morbidity and mortality in the world[2]. When coronary artery occlusion occurs based on lesion, the supply of myocardial oxygen and nutrient substance in the blood supply area will be stopped, thus leading to acute myocardial infarction. The development and wide application of emergency percutaneous coronary intervention (PCI) technology has significantly decreased the mortality of the acute myocardial infarction in recent years. However, as the myocardial cells nearly lose all the regenerative capacity, the heart will not be repaired by the regeneration of myocardial cells after myocardial infarction, and ventricular remodeling will occur in the heart, which will lead to heart failure finally. Therefore, the maintenance of the survival of ischemic myocardial cells is critical for the prognosis of acute myocardial infarction during acute myocardial ischemia.

As a form of cell death, apoptosis is the cell death controlled by genes in an autonomous order[3]. Apoptosis is a process of highly regulated programmed cell death. It plays an important role under many heart pathological conditions[4,5]. The inhibition of apoptosis has become a potential therapeutic strategy. Some studies[6-8] have found that apoptosis is involved in the acute and chronic loss of myocardial cells in various heart diseases, including myocardial infarction, ischemic heart disease, myocardial reperfusion injury, various types of cardiomyopathy and acute or chronic heart failure. Myocardial cell apoptosis also plays an important role in subsequent processes of cardiac remodeling and progress to heart failure. As a result, the loss of myocardial cells is the most important determinant affecting the prognosis after myocar-

Corresponding Author: Qi Zhang, MM; e-mail: fengyeqiqi9999@163.com

dial infarction, thus reducing myocardial cell loss is a key issue in the treatment of myocardial infarction. MicroRNA (miRNA) is a type of small molecule non coding RNA that binds to three prime untranslated region (3'-UTR) of the target messenger RNA (mRNA) to achieve post-transcriptional level control of gene expression, thus participating in the pathophysiological process of many diseases[9]. More and more attention has been paid to the important roles of miRNA in the cardiovascular field gradually. Recently, it has been proven that several miRNAs participate in regulating the apoptosis of myocardial cells in acute myocardial infarction and in affecting the prognosis of acute myocardial infarction[10-14]. MicroRNA 298 (miR-298) is an important component of the microRNA regulatory network. Lately, it has been widely certified that miR-298 exert critical regulatory effects on many diseases[15-17], but the effects of miR-298 on the myocardial infarction have not been researched yet. In this work, the expression of miR-298 in the myocardial cells after myocardial infarction was detect, its myocardial protection effect and mechanism after myocardial infarction were investigated.

Materials and Methods

Establishment of the Rat MI Model

Male Sprague-Dawley (SD) rats (200-250 g) aged 8-10 weeks old were fed freely at room temperature for 1 week for testing. Then rats were divided into 4 groups: sham-operation group (Sham group, n=16), myocardial infarction group (MI group, n=6), miR-298 group (MI + Lv-miR 298 group, n=16) and blank lentivirus control group (MI + Lv-NC group, n=16). The myocardial infarction model was established via the ligation of left anterior descending coronary artery; in sham-operation group, the left anterior descending branch was not ligated, and the remaining operations were the same as those in myocardial infarction group. After ligation, rats were observed for 20 min. The lentivirus with miR-298 overexpression was given to miR-298 group, and the blank lentivirus (GenePharma) was given to blank lentivirus control group[18]. Briefly, the aorta and pulmonary artery were identified. A 23 G catheter containing 300 μL of the lentivirus was inserted from the left ventricle into the aortic root. Three rats chosen randomly from each group were sacrificed to detect the expression of miR-298 in 14 day and 28 day after infarction. The rest rats were fed until the 4th week after operation for further study. This investigation was approved by the Animal Ethics Committee of Qingdao University Animal Center.

Cardiac Function and Histology

At 4 weeks after operation, left ventricular end-systolic diameter and left ventricular end-diastolic diameter were detected via the cardiac M-mode ultrasound images of parasternal left long-axis section using a small animal ultrasonic apparatus, and left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were calculated. After color Doppler ultrasound examination, rats were executed; the heart was removed for other examination.

Cell Culture

SD neonatal rats aged 1-2 days old were taken, disinfected with 75% ethanol and executed. The heart was removed on the aseptic table, and connective tissues and atrial tissues were removed; the ventricular tissues were cut into pieces (1 mm^3) and cells were digested and separated with 0.25% trypsin. The resulting cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) in an incubator with 5% CO_2 at 37°C for 60-90 min. myocardial cells were obtained and continued to be cultured in an incubator with 5% CO_2 at 37°C for 2-3 days before experiments.

Cell Transfection and Treatment

Cardiac fibroblasts were pre-cultured in a 24-well plate for 24 h, Then, three group were established to study the potential relevance between miR-298 and cardiac fibroblast cell: NC group (negative control), miR-298 mimics (cardiac fibroblast cell transfected by miR-144 mimics) and mimics + BAX (cardiac fibroblast cell transfected by miR-298 mimics and siBAX). All the stuff was purchased form RiboBio (Guangzhou, China) and were transfected by using lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. After cultured for another 24 h, an oxygen-glucose deprivation (OGD) model was established to mimicking the ischemic condition[19], OGD was performed by placing plates containing cardiac fibroblasts in Dulbecco's Modified Eagle Medium (DMEM) without medium in an anaerobic chamber (BD Anaerobe Pouch System). After incubation for 8 h at 37°C, cells were collected for further analysis.

Luciferase Reporter Assays

In TargetScan, miRDB and microRNA websites, it was found that BAX is the target gene of miR-298. The binding sequence of miR-298 at the 3'-end of BAX was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA), and the mutated BAX (Mut-type) and non-mutant BAX (WT-type) were connected with the pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA). PGL3-Basic vector with mutant BAX was transfected into myocardial fibroblasts after lentivirus intervention on the 12-well plate. The same treatment was performed on the pGL3-Basic vector connected with the non-mutant BAX according to steps in the Luciferase Reporter Gene Assay Kit. Then the luciferase activity was detected in a multi-function microplate reader.

QPCR

Total RNA was procured by TRIzol Reagent in accordance with the manufacturer's protocol. SYBR green qPCR assay was used to measure the level of BAX expression and endogenous controlled by GAPDH. TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) was used to measure the level of miR-298 expression normalized to miRNA U6.

Western Blot Analysis

Myocardial tissues in the infarction border zone and myocardial cells after transfection were selected for protein analysis. Radioimmunoprecipitation assay (RIPA) tissue lysate (Beyotime, Shanghai, China) was added into the specimen, and the protein supernatant was extracted after homogenate and centrifugation. The protein level was determined using bicinchoninic acid (BCA) method. According to the level, 20 μg protein was taken for loading, followed by 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was removed and transferred onto the polyvinylidene difluoride (PVDF) membrane. The membrane was sealed with 5% skim milk powder at room temperature for 2 h, and added with anti-BAX, anti-cytochrome c and anti-cleaved caspase-3 antibodies (Abcam, Cambridge, MA, USA) for incubation at 4°C overnight. After washing, the horseradish peroxidase-labeled secondary antibody was added for incubation at room temperature for 1 h, followed by color development via chemiluminescence instrument and analysis via ImageJ software. β-actin was used as the internal reference.

TUNEL

The apoptosis of myocardial cells was detected by TUNEL assay according to the manufacturer (Roche, Basel, Switzerland). Horseradish peroxidase (HRP)-mediated diaminobenzidine reaction was used to visualize the TUNEL-positive cells, following the counterstain. Fields were photographed at a magnification of 200× and were randomly selected. The apoptosis index was used to measure the degree of apoptosis.

Statistical Analysis

All results are presented as the means ± standard deviation (SD). The statistical analyses were performed using both Graph Pad Prism 5.01 (Graph Pad Software, Inc., La Jolla, CA, USA) and PASW Statistics 18.0 (SPSS Inc., Fayetteville, NC, USA). One-way ANOVA was used to compare differences among groups. An unpaired *t*-test was used to compare was used to compared between 2 groups. A value of $p<0.05$ was considered statistically significant.

Results

miR-298 was Downregulated in Myocardium Border Zone After Infarction

Compared with that in sham-operation group, the expression of miR-298 in the myocardial infarction border zone in MI groups at 2 and 4 weeks was decreased. However, treated with miR-298 lentivirus could increase the expression of miR-298, demonstrating that the lentivirus with miR-298 overexpression can up-regulate the expression of miR-298 in myocardium (Figure 1).

miR-298 Improved the Cardiac Function After Infarction

LVEF and LVFS in myocardial infarction group at 4 weeks were significantly lower than those in sham-operation group, but LVEF and LVFS in miR-298 group were significantly higher than those in blank lentivirus control group (Figure 2).

miR-298 Inhibited the Expressions of BAX and Cell Apoptosis After I/R

The mRNA levels of BAX in the myocardial infarction border zone in myocardial infarction groups at 4 weeks were higher than those in sham-operation group. The mRNA levels of BAX in miR-298 group were significantly lower than those in blank lentivirus control group. Similar results to PCR were obtained via Western blotting,

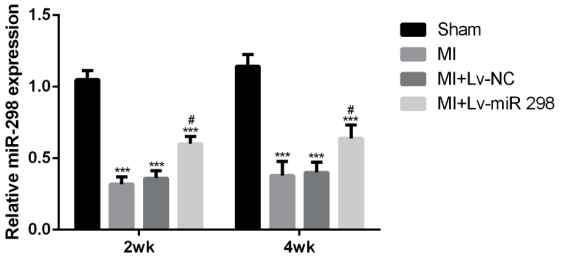

Figure 1. The expressions of miR-298 in in myocardium border zone after infarction. ($^{***}p<0.001$ compared with Sham group; $^{\#}p<0.05$ *vs.* MI group).

indicating that miR-298 inhibits the expressions of BAX in the myocardial infarction border zone (Figure 3A). As detection by Western blotting, we could see the release level of cytochrome-c and cleaved caspase-3 were significantly increased in MI groups by comparing with Sham group. cytochrome-c and cleaved caspase-3 are characteristic signs of apoptosis[20]. Intervened with miR-298 could prevent cytochrome-c and cleaved caspase-3 activation (Figure 3C), TUNEL staining was performed to further evaluation. The TUNEL positive cells were shown as dark brown particles as apoptotic cells and the apoptotic index inmiR-298 group had reduced remarkable when compared with the blank lentivirus control group (Figure 3B).

BAX was a Target Gene of miR-298

To search potential target of miR-298, we checked it in three publicly available algorithms, TargetScan, miRDB and microRNA to elucidate the putative and possible targets of miR-298. Finally, we found the BAX was checked a supposed target of miR-298 (Figure 4A). In *in vitro* experiments, the luciferase reporter gene assay showed that the lentivirus transfected with miR-298 significantly reduced the fluorescence expression of pGL3-Basic vector with WT-BAX, but did not reduce the fluorescence expression of pGL3-Basic vector with Mut-BAX, indicating that BAX is a target gene of miR-298 (Figure 4B). The transfection with miR-298 mimics could reduce the mRNA and protein levels of BAX in myocardial cells after treated with OGD. Furthermore, the apoptosis level measured by TUNEL positive-cell and the protein expression of cytochrome-c and cleaved Caspase-3 decreased much more than Sham group. However, after the BAX siRNA was increased, the apoptosis index and the protein levels of cytochrome-c and cleaved caspase-3 in myocardial cells were also increased, suggesting that miR-298 can down-reg-

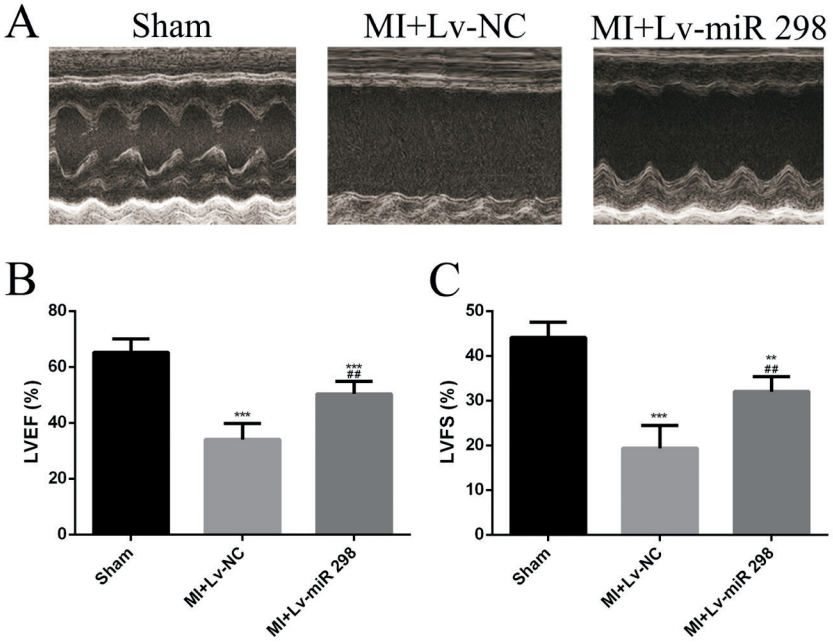

Figure 2. Up-regulation of miR-298 improved the cardiac function after infarction. **A,** Representative transthoracic M-mode echocardiograms from each group. ***B*** and ***C*** were statistical analysis of the data obtained or derived from original echocardiographic records. Data were presented as means ± standard deviations. ($^{**}p<0.01$, $^{***}p<0.001$ compared with Sham group; $^{\#\#}p<0.01$ *vs.* MI+Lv-NC group).

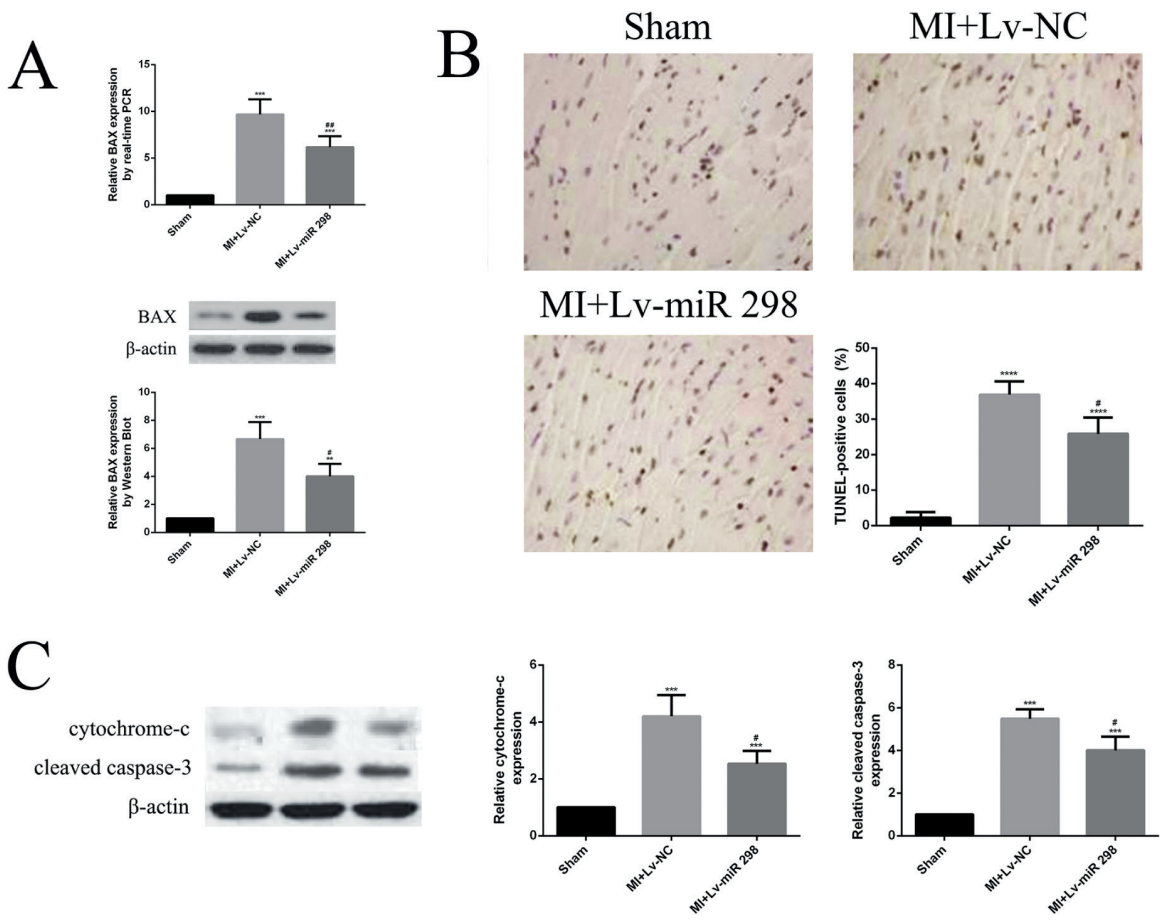

Figure 3. Up-regulation of miR-298inhibit the expression of BAX *in vivo* and reduce the myocardial apoptosis after infarction. ***A,*** Expression of BAX *in vivo* determined by Real-time PCR and Western blot. ***B,*** Images of the myocardial cell stained by TUNEL assay *in vivo*, Photomicrographs were taken at 400× magnification. Apoptotic cardiomyocyte nuclei appear brown stained, histogram shows the percentage of TUNEL-positive cells (brown staining). ***C,*** Protein expression of cytochrome-c and cleaved caspase-3 *in vivo* determined by Western blot. Data were presented as means ± standard deviations. ($^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ compared with Sham group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* MI+Lv-NC group).

ulate the myocardial apoptosis through the target gene BAX (Figure 5).

Discussion

With the improvement of people's living standards and the change of their life styles, CHD represented by acute myocardial infarction has become one of the most main causes of human death in the world. Although the development and application of PCI technology has decreased the mortality of CHD, including that of the myocardial infarction, it remains a challenge in the cardiovascular field that the heart cannot be repaired through the regeneration of the myocar-

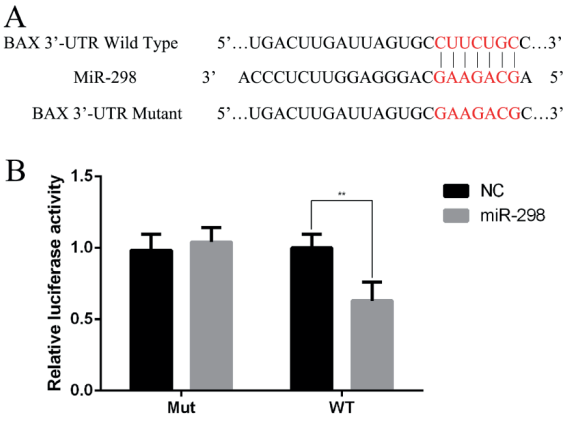

Figure 4. BAX is a direct and functional target of miR-298. ***A,*** Diagram of putative miR-298 binding sites of BAX. ***B,*** Relative activities of luciferase reporters ($^{**}p<0.01$).

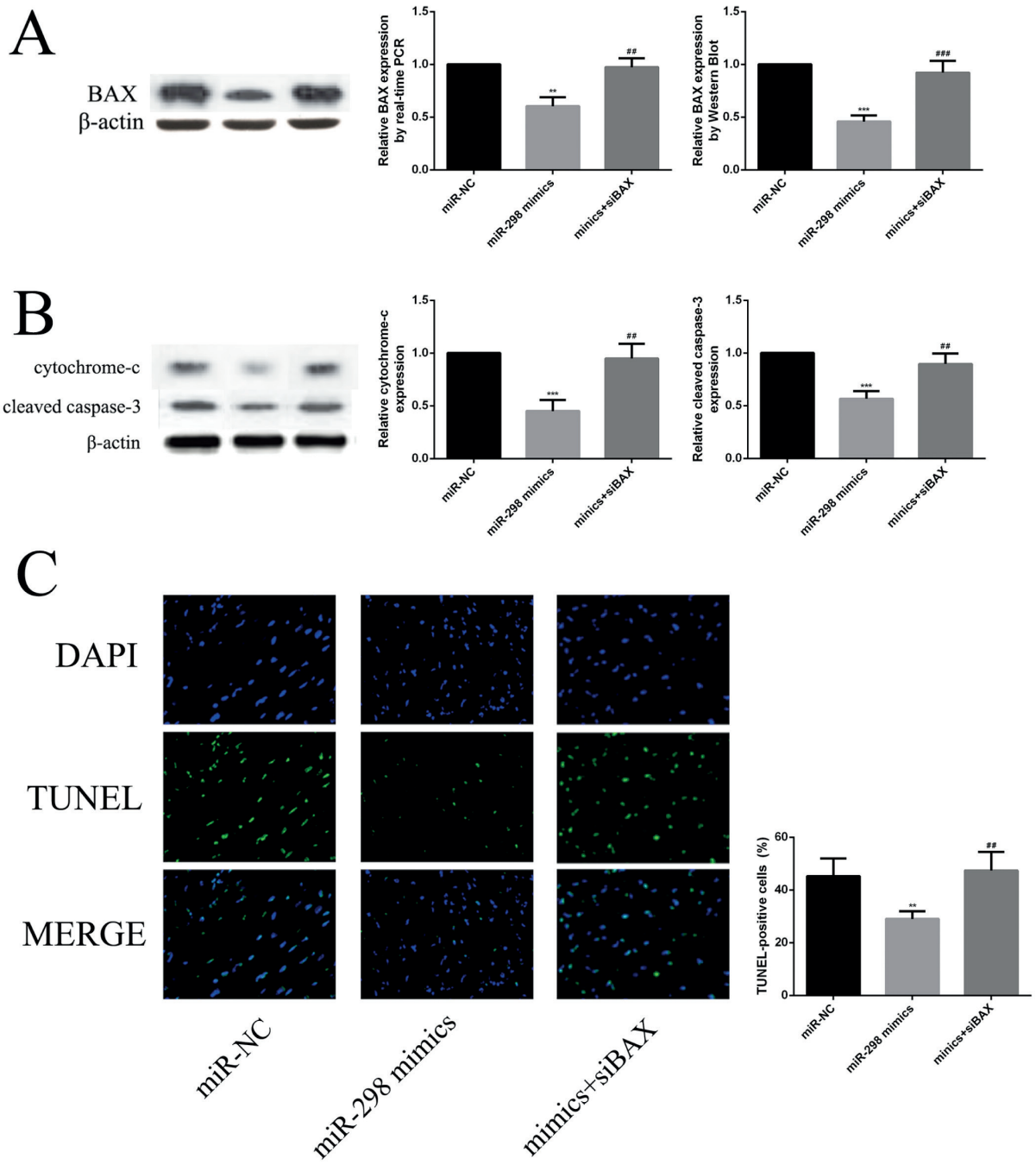

Figure 5. *In vitro* experiment. Up-regulation of miR-298 inhibited the expressions of BAX, reduce the myocardial apoptosis and prevent the protein expression of cytochrome-c and cleaved caspase-3. ***A,*** Expression level of BAX *in vitro*. ***B,*** Protein expression of cytochrome-c and cleaved caspase-3 *in vitro* determined by Western blot. ***C,*** Images of the myocardial cell stained by TUNEL assay *in vitro*, TUNEL-positive cells (green fluorescence) were observed under a fluorescence microscope at 400× magnification. Data were presented as means ± standard deviations. ($^{**}p<0.01$, $^{***}p<0.001$ *vs.* NC group; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* Mimics group).

dial cells after myocardial infarction, which will result in the ventricular remodeling and finally lead to heart failure. Therefore, the maintaining of survival of ischemic myocardial cells is of great importance for the heart functions and long-term prognosis after acute myocardial infarction. Apoptosis is regulated by intracellular apoptosis-modulating proteins, which are divid-

ed into pro-apoptotic proteins and anti-apoptotic proteins. Apoptosis is the result of the loss of balance between these two proteins that are antagonistic to each other. Studies have shown that the expression levels of Bcl-2 associated X protein (BAX) is directly related to the apoptosis regulation. Increased BAX promotes the apoptosis; decreased BAX reduce the apoptosis. It has been proposed that the survival ability of cells after being stimulated by apoptosis depends on the ratio of Bcl-2 to BAX[21,22]. When the BAX expression level is lower, the ratio is increased. The heterodimers of Bcl-2/BAX are formed and the apoptosis is inhibited. When the BAX expression level is higher, the ratio is decreased. The homodimers of Bcl-2/BAX are formed and the apoptosis occurs. Studies have indicated that caspases family is the promoter and executioner of apoptosis for mammals, among which Caspase-3 is the most critical apoptotic protease in the downstream of caspase cascade[20]. BAX worked as a component of the ion channel on the mitochondrial membrane, enables cytochrome-c to pass through the mitochondrial membrane and to further activate Caspase-3, leading to apoptosis[23]. Recent studies have reported that miRNAs are closely involved in the regulation of pathological processes such as angiogenesis, heart development, cardiac hypertrophy, heart failure, and reperfusion injury, and it may be one of the key regulatory factors[24]. The latest researches confirmed the relationship between part of miRNAs and myocardial ischemia injury. MiR-15, miR-140 and miR-320 can promote the apoptosis of myocardial ischemia through corresponding target genes[10-12]; miR-24 and miR-214 can inhibit the apoptosis of myocardial cells[13,14], so as to protect the ischemic myocardium. Thus revealing the regulation rules of miRNAs on apoptosis in myocardial ischemic injury is conducive to our further understanding of the mechanism of myocardial ischemic injury and to looking for new intervention strategies. In this work, the expression of miR-298 in myocardial tissues of rats after myocardial infarction was analyzed, and it was found that the expression level of miR-298 was significantly decreased in myocardial tissues of rats after myocardial infarction. After the intervention in miR-298 expression in myocardial infarction rats with lentivirus, the cardiac color ultrasound, TUNEL assay, qT-PCR and Western blotting were performed, and it was found that miR-298 could inhibit the expressions of BAX in the myocardial infarction border zone, improve the cardiac function after myocardial infarction and reduce the apoptosis level. To study the molecular mechanism of abnormally high expression of miR-298 in myocardial tissues after myocardial infarction, it was found first through bioinformatics that BAX was a regulatory target of miR-298. Then, myocardial cells of neonatal rats were extracted and cultured *in vitro*, an OGD model was performed to mimicking the ischemic condition and luciferase reporter gene assay showed that the lentivirus transfected with miR-298 significantly reduced the fluorescence expression of pGL3-Basic vector with WT-BAX, but had no effect on the fluorescence expression of pGL3-Basic vector with Mut-BAX, indicating that BAX is a target gene of miR-298. The transfection with miR-298 mimics and BAX siRNA confirmed that miR-298 could down-regulate the cytochrome-c and cleaved caspase-3 protein expression and reduce the myocardial apoptosis through the target gene BAX.

Conclusions

Molecular targeted therapy is the direction of medical development and has made a great breakthrough in anti-tumor therapy. In our study, we found that MiR-298 can improve the myocardial apoptosis through the target gene BAX, which may be the theoretical basis for the molecular targeted therapy in the myocardial ischemic injury.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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