MiR-1908 improves cardiac fibrosis after myocardial infarction by targeting TGF-β1

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Abstract. – OBJECTIVE: To investigate the role and mechanism of micro ribonucleic acid (miR)-1908 in myocardial fibrosis after myocardial infarction.

MATERIALS AND METHODS: In *in-vivo* experiments, the rat model of myocardial infarction was established, and miR-1908 was up-regulated by lentivirus with miR-1908 overexpression. Cardiac function of rats was detected by echocardiography. Transforming growth factor-β1 (TGF-β1) and Smad2/3 expressions in infarction border zone were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. Masson staining was used to detect the fibrosis, thus studying the role of miR-1908 in the myocardial fibrosis model. In *in-vitro* experiments, myocardial fibroblasts were isolated and cultured. Oxygen glucose deprivation (OGD) model was established to mimicking the ischemic condition; the relationship between miR-1908 and TGF-β1 was verified using luciferase reporter vector, lentivirus and small-interfering RNA (siRNA) in TGF-β1.

RESULTS: *In-vivo* experiments showed that the miR-1908 expression was down-regulated at 4 weeks after myocardial infarction. The up-regulation of miR-1908 significantly improved the cardiac function, reduced the myocardial fibrosis, and inhibited the expressions of TGF-β1 and Smad2/3. *In-vitro* experiments revealed that TGF-β1 was a target gene of miR-1908 and miR-1908 could inhibit the Smad2/3 expression through TGF-β1.

CONCLUSIONS: MiR-1908 can improve the myocardial fibrosis through the target gene TGF-β1.

Key Words:
    miR-1908, TGF-β1, Myocardial infarction, Fibrosis.

Introduction

Ischemic heart disease is the most common type of cardiovascular disease. Progress continues to be made in thrombolysis, endovascular stenting and coronary artery bypass grafting, so the mortality rate of acute myocardial infarction (AMI) has been decreased year by year. However, during the recovery stage of AMI, cardiac fibroblasts (CFB), due to the over-secretion of extracellular matrixes, such as collagen, fibronectin, elastin, fibrillin, and other proteins, can reduce the ventricular compliance, and obstruct the normal conduction of electrocardiogram, inducing heart failure, malignant arrhythmia or sudden cardiac death[1]. Therefore, alleviating the myocardial fibrosis is an important research direction in improving the prognosis of myocardial infarction.

Micro ribonucleic acid (miRNA) is an evolutionarily highly-conserved non-coding RNA with a length of about 20-25 nucleotides, which promotes the mRNA degradation, suppresses the mRNA translation and isolates the mRNA target genes through identifying the 3'-untranslated region (UTR) of target mRNA via 5'-end base, thereby regulating the cell differentiation, apoptosis, proliferation, and development[2-4]. There is always a lack of biological indexes for the early diagnosis of myocardial fibrosis. Recent studies on miRNA have shown that miRNA, as an upstream regulator that may be involved in the regulation of myocardial fibrosis, attracts more and more attention. The process of myocardial fibrosis has been proved to be associated with a series of cellular signaling pathways and some recently discovered miRNAs[5]. MiR-1908 is located in the first intron

of the human chromosome 11 FADS1 gene, which belongs to the intragenic miRNA. Researches[6-8] have shown that miR-1908 plays a variety of biological roles in the body, and gets involved in the regulation of cell proliferation and differentiation; besides, its expression disorder is closely related to a variety of tumors, and it plays an important role in tumor occurrence, invasion, and metastasis. Xie et al[9] confirmed that MiR-1908 could promote scar formation post-burn wound healing by suppressing Ski-mediated inflammation and fibroblast proliferation. Therefore, the study on miR-1908 is of great significance in revealing the regulatory mechanism of gene expression and disease prevention and treatment. We detected the expression of miR-1908 in myocardial fibrosis after myocardial infarction. Moreover, its role and mechanism in myocardial fibrosis 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. Rats were divided into 4 groups: sham-operation group (n=10), myocardial infarction group (MI group, n=10), miR-1908 group (MI + Lv-miR 1908 group, n=10), and blank lentivirus control group (MI + Lv-NC group, n=10). 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-1908 overexpression was given to miR-1908 group, and the blank lentivirus (GenePharma) was given to blank lentivirus control group[10]. 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. Rats were fed until the 4th week after operation for further investigation. This investigation was approved by the Animal Ethics Committee of Shandong 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 by the cardiac M-mode ultrasound images of parasternal left long-axis section using a small animal ultrasonic apparatus; 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, washed with normal saline, fixed with 4% formaldehyde solution and embedded in paraffin for Masson and hematoxylin-eosin (HE) staining. The distribution and morphology of myocardial cells and collagen were observed, and the collagen volume fraction (CVF) was detected.

qRT-PCR

Total RNA was obtained by TRIzol Reagent in accordance with the manufacturer's protocol. SYBR green qPCR assay was used to measure the level of TGF-β1, Smad2/3 expression and endogenous controlled by glyceraldheyde 3-phosphate dehydrogenase (GAPDH). TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) were used to measure the level of miR-1908 expression normalized to miRNA U6.

Western Blotting

Myocardial tissues in the infarction border zone and 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 being homogenized and centrifuged. The protein level was determined using bicinchoninic acid (BCA) method. According to the level, 20 μg protein were 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-TGF-β1 and anti-Smad2/3 antibodies (Abcam, Cambridge, MA, USA) for incubation at 4°C overnight. After washing, the horseradish peroxidase (HRP)-labeled secondary antibody was added for incubation at room temperature for 1 h, followed by color development via chemiluminescence instrument and analysis via Image-J software. β-actin was used as the internal reference.

Cell Culture

Sprague-Dawley (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 tis-

sues were removed. The ventricular tissues were cut into pieces (1 mm³) 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. Cardiac fibroblasts were obtained using the differential adhesion method, and continued to be cultured in an incubator with 5% CO_2 at 37°C, followed by passage once after 1-2 d. Cells in the 2nd-4th generations were used for experiments.

Cell Transfection and Treatment

Cardiac fibroblasts were pre-cultured in a 24-well plate for 24 h. After that, three group were established to study the potential relevance between miR-1908 and cardiac fibroblast cell: NC group (negative control), miR-1908 mimics (cardiac fibroblast cell transfected by miR-144 mimics) and mimics + TGF-β1 (cardiac fibroblast cell transfected by miR-1908 mimics and siTGF-β1). All the products were 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 mimic the ischemic condition[11]. OGD was performed by placing plates containing cardiac fibroblasts in DMEM without medium in an anaerobic chamber (BD Anaerobe Pouch System, San Jose, CA, USA). 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 TGF-β1 is the target gene of miR-1908. The binding sequence of miR-1908 at the 3'-end of TGF-β1 was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA), and the mutated TGF-β1 (Mut-type) and non-mutant TGF-β1 (WT-type) were connected with the pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA). PGL3-basic vector with mutant TGF-β1 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 TGF-β1 according to steps in the luciferase reporter gene assay kit. Next, the luciferase activity was detected in a multi-function microplate reader.

Statistical Analysis

All results are presented as the means ± standard deviation (SD). The statistical analyses were performed using both GraphPad Prism 5.01 (GraphPad 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 for comparison between two groups. $p < 0.05$ was considered statistically significant.

Results

MiR-1908 was Downregulated in Myocardium Border Zone After Infarction

Compared with that in sham-operation group, the expression of miR-1908 in the myocardial infarction border zone in myocardial infarction group at 4 weeks was decreased. However, treated with miR-1908 lentivirus could increase the expression of miR-1908, demonstrating that the lentivirus with miR-1908 overexpression can up-regulate the expression of miR-1908 in myocardium (Figure 1).
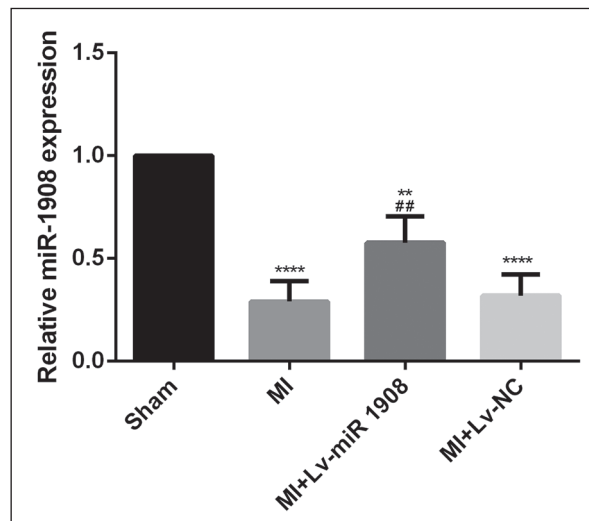

Figure 1. The expressions of miR-144 in myocardium border zone after infarction (**$p < 0.01$, ****$p < 0.0001$ compared with Sham-operation group, ##$p < 0.01$ *vs.* MI group). The up-regulation of miR-1908 significantly improved the cardiac function, reduced the myocardial fibrosis and inhibited the expressions of TGF-β1 and Smad2/3. *In-vitro* experiments revealed that TGF-β1 was a target gene of miR-1908 and miR-1908 could inhibit the Smad2/3 expression through TGF-β1.

miR-1908 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-1908 group were significantly higher than those in blank lentivirus control group and myocardial infarction group. In the M-mode ultrasound images, the left ventricular anterior wall myocardial systole in sham-operation group was good; the myocardial systolic function in miR-1908 group was worse than that in sham-operation group, but better than those in blank lentivirus control group and myocardial infarction group (Figure 2).

miR-1908 Reduced the Myocardial Fibrosis After Infarction

In Masson and HE images, sham-operation group had the least blue collagens, and cells were distributed most orderly with the same nuclear size. The lentivirus control group and myocardial infarction group had the most collagen, and cells were distributed most disorderly with the largest differences in nuclear accumulation and size. MiR-1908 group had moderate collagen, and cells were arranged in a disorderly manner compared with that in sham-operation group, but orderly compared with that in lentivirus control group; the nucleus size and shape were basically the same. CVF in myocardial infarction group was significantly higher than that in sham-operation group, but CVF in miR-1908 group was significantly lower than those in blank lentivirus control group and myocardial infarction group, indicating that miR-1908 improves the myocardial fibrosis after myocardial infarction (Figure 3).

miR-1908 Inhibited the Expressions of TGF-Ð1 and Smad2/3 After Infarction

The mRNA levels of TGF-β1 and Smad2/3 in the myocardial infarction border zone in myocardial infarction group at 4 weeks were higher than those in sham-operation group, but the mRNA levels of TGF-β1 and Smad2/3 in miR-1908 group were significantly lower than those in blank len-
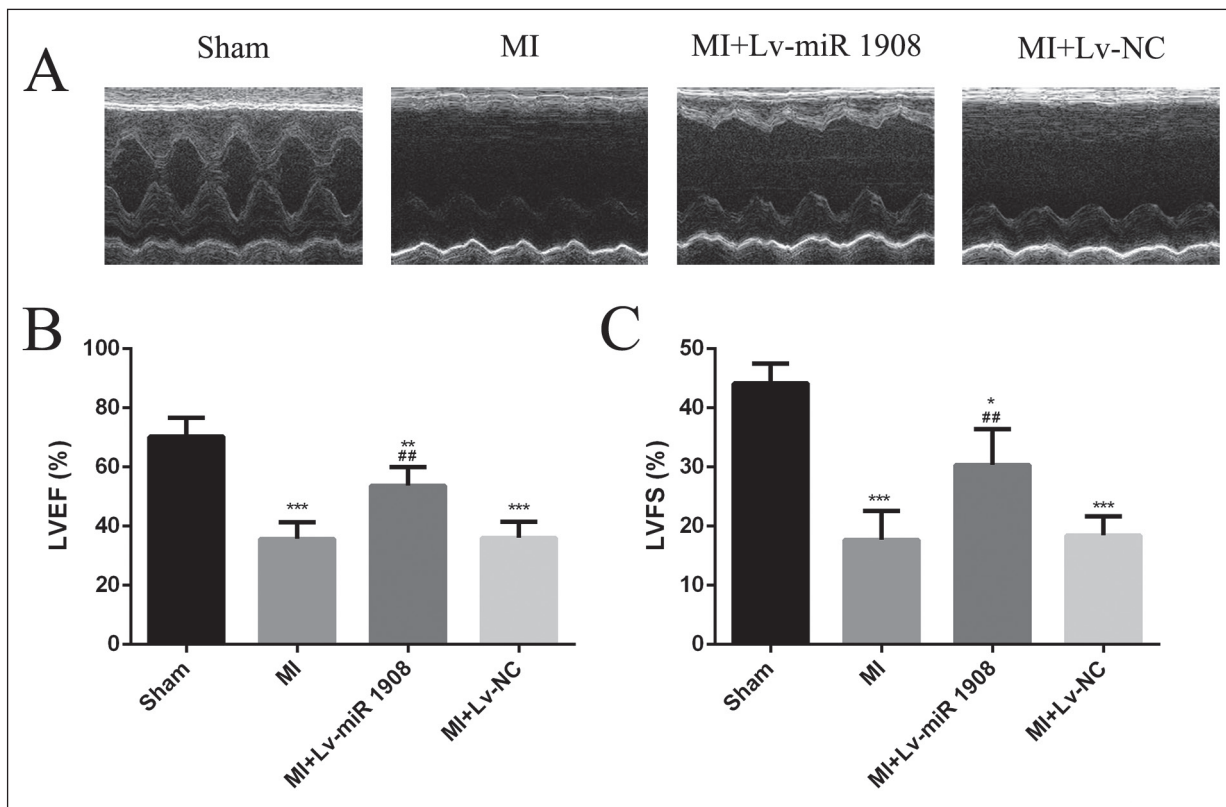

Figure 2. Up-regulation of miR-1908 improved the cardiac function after infarction. **A,** Representative transthoracic M-mode echocardiograms from each group. **B,-C,** were statistical analysis of the data obtained or derived from original echocardiographic records. Data were presented as means ± standard deviations. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with Sham-operation group, #$p < 0.05$, ##$p < 0.01$ vs. MI group).
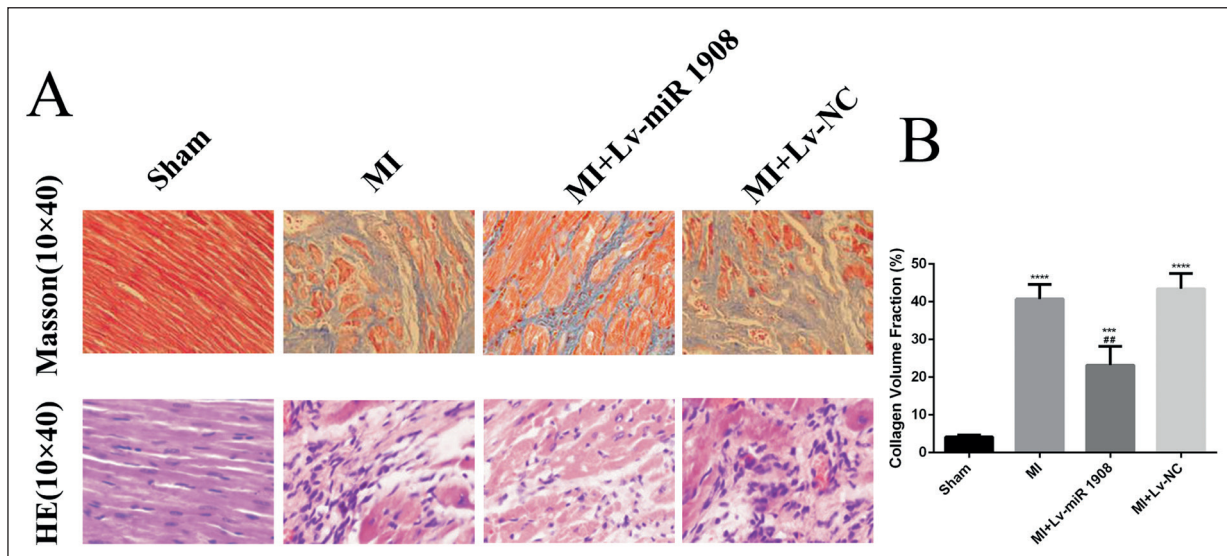
Figure 3. Up-regulation of miR-1908 reduced the myocardial fibrosis. ***A,*** Images of the myocardium and interstitial fibrosis stained with Masson-Trichrome and images of the myocardium stained with hematoxylin-eosin (HE) after infarction (400×). ***B,*** Collagen volume fraction determined by Masson staining. (***$p < 0.001$ compared with Sham-operation group, $^{\#\#}p < 0.01$ *vs.* MI group).

tivirus control group and myocardial infarction group. Similar results to PCR were obtained by Western blotting, indicating that miR-1908 inhibits the expressions of TGF-β1 and Smad2/3 in the myocardial infarction border zone (Figure 4).

TGF-β1 Was a Target Gene of miR-1908

To research potential target of miR-1908, we checked it in three publicly available algorithms, TargetScan, miRDB, and microRNA to elucidate the putative and possible targets of miR-1908. Finally, we found the TGF-β1 was checked as supposed target of miR-1908 (Figure 5 A). In *in-vitro* experiments, the luciferase reporter gene assay showed that the lentivirus transfected with miR-1908 significantly reduced the fluorescence expression of pGL3-basic vector with WT-TGF-β1, but did not reduce the fluorescence expression of pGL3-basic vector with Mut-TGF-β1, indicating that TGF-β1 is a target gene of miR-1908 (Figure 5 B). The transfection with miR-1908 mimics could reduce the mRNA and protein levels of TGF-β1 and Smad2/3 in myocardial fibroblasts after treated with OGD. After the TGF-β1 siRNA was increased, the mRNA and protein levels of Smad2/3 in myocardial fibroblasts were also increased, suggesting that miR-1908 can down-regulate the expression of Smad2/3 and improve the myocardial fibrosis through the target gene TGF-β1 (Figure 6).

Discussion

We analyzed the expression of miR-1908 in myocardial tissues of rats after myocardial infarction and we found that the expression level of miR-1908 was significantly decreased in myocardial tissues of rats after myocardial infarction. After the intervention in miR-1908 expression in myocardial infarction rats with lentivirus, the cardiac color ultrasound, Masson staining, RT-PCR, and Western blotting were performed. It was found that miR-1908 could inhibit the myocardial fibrosis and expressions of TGF-β1 and Smad2/3 in the myocardial infarction border zone, and could improve the cardiac function after myocardial infarction. To analyze the molecular mechanism of abnormally high expression of miR-1908 in myocardial tissues after myocardial infarction, it was found first through bioinformatics that TGF-β1 was a regulatory target of miR-1908. Then, cardiac fibroblasts 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-1908 significantly reduced the fluorescence expression of pGL3-basic vector with WT-TGF-β1, but had no effect on the fluorescence expression of pGL3-basic vector with Mut-TGF-β1, indicat-
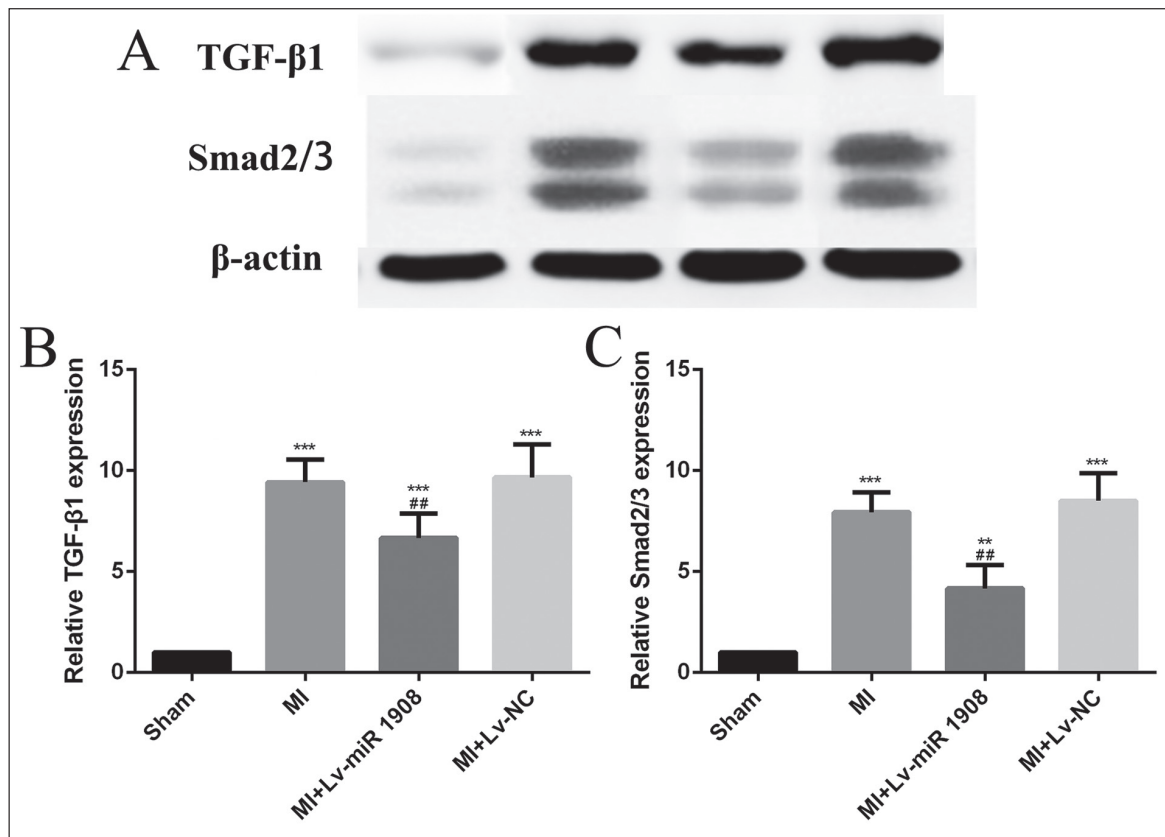
Figure 4. Up-regulation of miR-1908 inhibited the expressions of TGF-β1 and Smad2/3 *in vivo*. **A,** Protein expression of TGF-β1 and Smad2/3 *in vivo* determined by Western blot. **B,** Quantification of TGF-β1 expression. **C,** Quantification of Smad2/3 expression (**$p < 0.01$, ***$p < 0.001$ compared with Sham-operation group, ##$p < 0.01$ *vs.* MI group).

ing that TGF-β1 is a target gene of miR-1908. The transfection with miR-1908 mimics and TGF-β1 siRNA confirmed that miR-1908 could
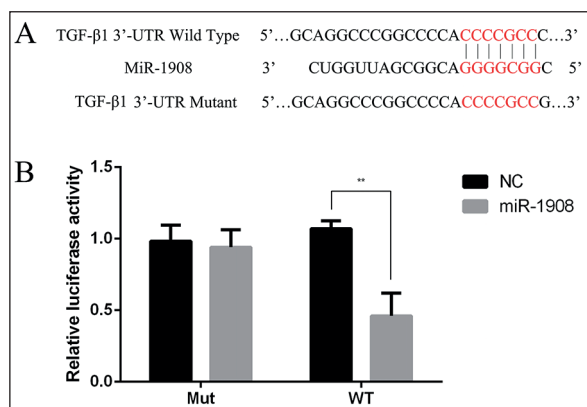

Figure 5. TGF-β1 is a direct and functional target of miR-1908. Cardiac fibroblasts cell were transfection with miR-1908 mimics and inhibitor. **A,** Diagram of putative miR-1908 binding sites of TGF-β1. **B,** Relative activities of luciferase reporters (**$p < 0.01$).

down-regulate the Smad2/3 mRNA expression and improve the myocardial fibrosis through the target gene TGF-β1. Myocardial fibrosis is one of the mechanisms of progression and aggravation of a variety of cardiovascular diseases, which is manifested as myocardial stiffness, decreased compliance, and decreased systolic and diastolic capacities in the terminal stage, eventually resulting in heart failure. It can also lead to cardiac conduction system dysfunction, such as arrhythmia and cardiac arrest. The main pathological changes of cardiac fibrosis are as follows: cardiac fibroblasts migrate and proliferate into the myofibroblasts, and secrete a large number of extracellular matrixes, such as collagen, elastin and laminin, as well as increased collagen deposition, decreased degradation, imbalanced proportion of all types of collagen, and disordered arrangement[12,13]. After MI, severe myocardial ischemia occurs, injury and necrosis of a large number of myocardial cells happen, and myocardial repair also starts. After MI,
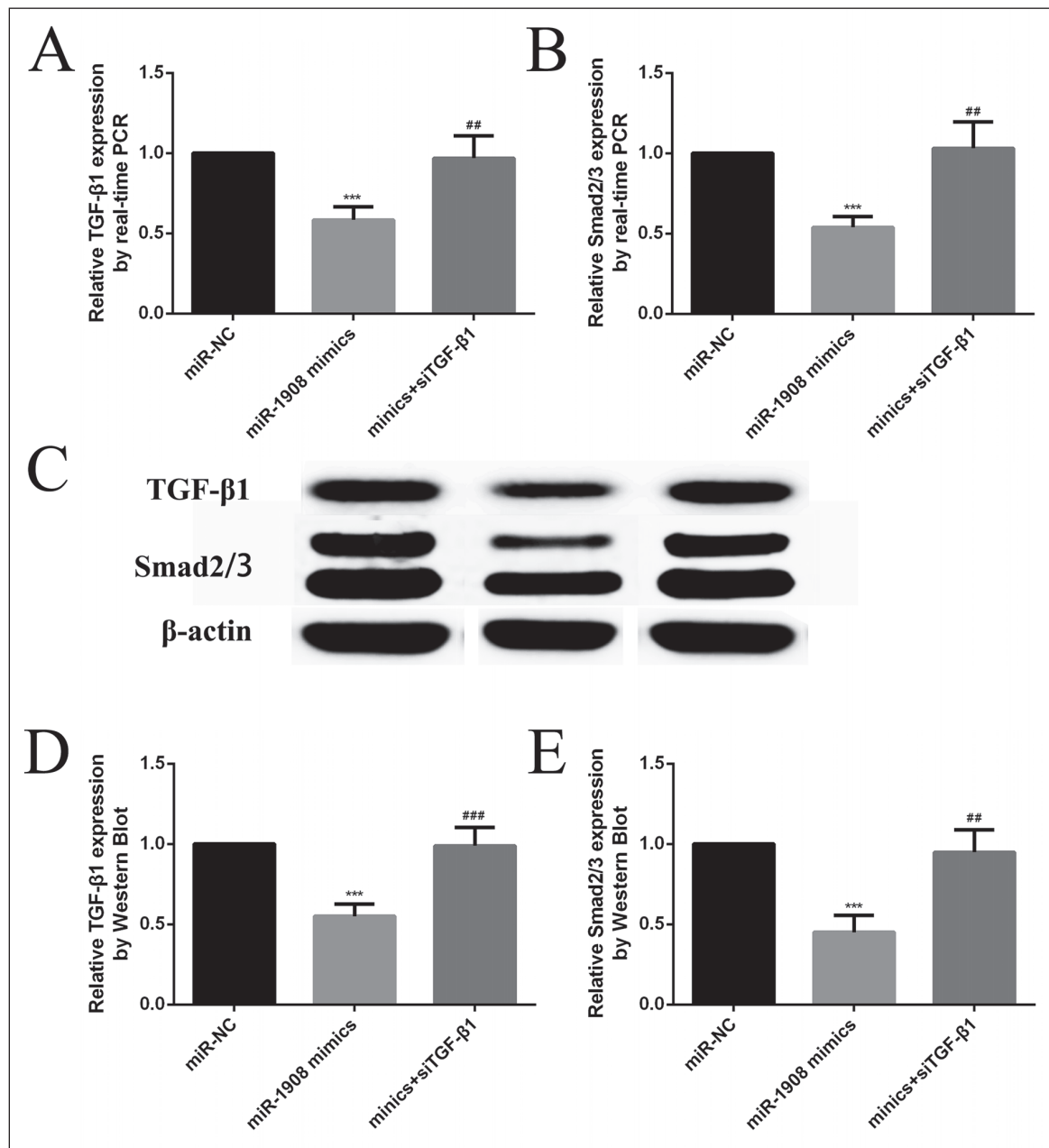
Figure 6. Up-regulation of miR-1908 inhibited the expressions of TGF-β1 and Smad2/3 *in vitro*. **A,-B,** Expression level of TGF-β1 and Smad2/3 *in vitro* determined by Real-time PCR analysis. **C,** Protein expression of TGF-β1 and Smad2/3 *in vitro* determined by Western blot. **D,** Quantification of TGF-β1 expression. **E,** Quantification of Smad2/3 expression (***$p < 0.001$ *vs.* NC group; [#]$p < 0.05$, [##]$p < 0.01$ *vs.* Mimics group).

on one hand, thrombolysis, stent implantation and coronary artery bypass grafting can save part of dying myocardium; on the other hand, it can lead to excessive myocardial fibrosis. The formation of scar tissues further results in coronary artery occlusion and aggravates the myocardial ischemia, thereby causing a vicious circle of ischemia-fibrosis-ischemia[14,15]. The main factors involved in myocardial fibrosis are the renin-angiotensin-aldosterone system[16,17], matrix metalloproteinase, tissue inhibitor 41, vascular endothelial dysfunction[18,19], and TGF-β1[20,21]. Among them, TGF-β1 is the most closely related to the fibrosis, which can regulate the cell growth and differentiation, and promote the cell proliferation, etc. Relevant literature has shown

that TGF-β1 plays an important role in myocardial fibrosis. Smad2/3 is a key intracellular signal transduction protein of TGF-β1-induced fibrosis and specifically transduces TGF-β1[22]. TGF-β1 binds to its receptor and phosphorylates Smad2/3, and then forms the heteromeric complexes with Smad4 in cytoplasm to transfer signals from the cytoplasm to the nucleus. Moreover, it regulates the transcription of downstream target genes, together with other transcriptional supporters and repressors[23]. Because of the decisive role of TGF-β1/Smad signal transduction pathway in the pathogenesis of cardiac fibrosis, the intervention in this signal pathway has become an ideal choice for the prevention and treatment of cardiac fibrosis.

Conclusions

We found that miR-1908 inhibits myocardial fibrosis after myocardial infarction through the target gene TGF-β1, which may provide a new direction for the treatment of myocardial infarction.

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

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