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MicroRNA-29b upregulation improves myocardial fibrosis and cardiac function in myocardial infarction rats through targeting SH2B3

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Abstract. – OBJECTIVE: Myocardial fibrosis (MF) seriously affects normal cardiac function. Meanwhile, MF at post-myocardial infarction (MI) is the leading cause of cardiac dysfunction in patients with acute myocardial infarction (AMI). Therefore, the aim of this study was to investigate the potential effect of microRNA-29b on MF and cardiac function after MI in rats.

MATERIALS AND METHODS: *In vivo* MI model was constructed by ligation of the left anterior descending coronary artery in Sprague-Dawley rats. Lentivirus overexpressing microRNA-29b was established and transfected to up-regulate microRNA-29b expression in rat myocardial tissues. The effect of microRNA-29b on luciferase activity of SH2B3 3'UTR was detected by the luciferase reporter gene assay. The mRNA levels of microRNA-29b, SH1B3, COL1A1, and α-SMA in the infarct border zone and cardiomyocytes were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Meanwhile, the protein levels of SH1B3, COL1A1, and α-SMA in the MI border zone and cardiomyocytes were determined by Western blot. In addition, cardiac function and MF in MI rats were evaluated by echocardiography, hematoxylin and eosin (HE) and Masson staining, respectively.

RESULTS: MicroRNA-29b expression decreased significantly in the infarct border zone at day 28 after MI ($p<0.05$). In addition, microRNA-29b overexpression in myocardial tissues of MI rats significantly improved impaired cardiac function, reduced collagen volume fraction and down-regulated the expressions of COL1A1 and α-SMA. Subsequent luciferase reporter gene assay verified the binding relation between microRNA-29b and SH2B3. Furthermore, the expressions of COL1A1 and α-SMA were confirmed negatively regulated by SH2B3.

CONCLUSIONS: MicroRNA-29b overexpression alleviates MF and cardiac dysfunction in MI rats through targeting SH2B3.

Key Words:
Acute myocardial infarction (AMI), MicroRNA-29b, Myocardial fibrosis (MF), Cardiac function.

Introduction

Myocardial fibrosis (MF) refers to changes in the cardiac structure due to the mass accumulation of extracellular matrix (ECM). It will eventually lead to a variety of heart diseases such as cardiac hypertrophy, heart failure, myocardial infarction, arrhythmia, and inflammatory cardiomyopathy[1,2]. MF seriously affects normal cardiac function, which is also a key influencing factor in malignant arrhythmia after MI. Prevention of fibrosis occurrence or alleviation of fibrosis progression remarkably improves myocardial function, which is of great significance for improving the prognosis of MI[3,4].

Studies have shown that thousands of microRNAs are expressed in the human heart. About 60-70% of the present biological functions closely related to MF are progressed from heart diseases[5]. MicroRNAs participate in the transcriptional regulation of translation inhibition or mRNA degradation by binding to the 3'UTR of target mRNAs. Meanwhile, they are involved in various biological processes such as cell proliferation, differentiation, and apoptosis[6]. It is believed that microRNAs may serve as key regulators in cardiac fibrotic diseases.

The microRNA-29 family contains three members, namely microRNA-29a, microRNA-29b, and microRNA-29c. The expression level of microRNA-29 in infarct myocardium of mouse is down-regulated. MicroRNA-29 exerts a crucial role in the process of fibrosis after MI by regulating TGF-β pathway. Van Rooij et al[7] have demonstrated that microRNA-29 is lowly expressed in many heart diseases, including MI and aortic atrophy. Collagen, metalloproteinase, elastin, and fibrillin are ECM genes, which are also target genes for microRNA-29. Low expression of microRNA-29

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accelerates the production of ECM, thereby leading to the occurrence of MF[7,8]. An *in vivo* research has demonstrated that blocking the TGF-β/Smad3 pathway can alleviate or even reverse cardiac dysfunction and MF results from microRNA-29b deficiency. Myocardial fibroblasts are the most important cells for synthesis and secretion of myocardial ECM proteins. They are differentiated into specific myocardial fibroblasts after physicochemical or biological stimulation. This is manifested as an increased expression of α-SMA, as well as accumulative deposition of ECM proteins, including type I, III, and IV collagen[10,11]. The promotive effect of microRNA-29a on MF development is closely related to myocardial fibroblasts. Furthermore, microRNA-29a has been identified to inhibit the proliferative behaviors of myocardial fibroblasts, eventually inhibiting the occurrence and progression of fibrosis[12].

SH2B3 is a member of the SH2B adaptor family, which affects the sub-localization of molecules and multiple pathways mediated by Janus kinase and receptor tyrosine kinase[13,14]. SH2B3 is involved in the differentiation of hematopoietic cells. Meanwhile, it is closely related to MI, metabolic syndrome, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and other diseases[15]. A previous *in vivo* MI model in mice has shown that the degree of fibrosis is aggravated by SH2B3 deficiency[16]. Therefore, the biological function of SH2B3 in MI is ascertained. However, the exact role of SH2B3 in MF at post-MI has not been fully elucidated. The aim of this study was to investigate the specific function of microRNA-29b in MF and cardiac dysfunction at post-MI and to further clarify the regulation of microRNA-29b on SH2B3.

Materials and Methods

Experimental Animals

As previously described, the *in vivo* MI model was constructed by ligation of the left anterior descending coronary artery (LAD) in Sprague-Dawley rats weighing 200-250 g with 8-10 weeks old[17]. All rats were randomly assigned into three groups, including sham group, negative control group (NC group) and microRNA-29b overexpression group (microRNA-29b group), with 15 in each group. Rats in NC and microRNA-29b group were administrated with NC lentivirus or lentivirus overexpressing microRNA-29b, respectively. This study was approved by the Animal Ethics Committee of Jinzhou Medical University Animal Center.

Rat Echocardiography

On the 28th day after LAD ligation, rats were intraperitoneally injected with 4% chloral hydrate (0.65 mL/kg). After the righting response disappeared, rat incisors and limbs were fixed so that they could be placed on self-made cardboard in a supine position. M-mode echocardiography was obtained using a small-animal ultrasound probe (model Veno2100) on the long axis of the parastolic left ventricle. Left ventricular end-systolic dimension (LVESD), left ventricular end-diastolic dimension (LVEDD), left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were finally recorded.

Histological Analysis

Myocardial tissue in the infarct border zone was fixed in 4% paraformaldehyde for 24 h, embedded in paraffin and sectioned. After tissue dewaxing and hydration, the sections were stained with hematoxylin and eosin (HE) for morphological evaluation. Meanwhile, Masson's trichrome staining was performed to assess the degree of fibrosis. Each section was observed under a 200× light microscope Collagen volume fraction (CVF) was analyzed by Image-Pro 6.2 software with the following formula: CVF=collagen area/total area.

Cell Culture

H9c2 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 5% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 37°C, 5% CO_2 incubator. The culture medium was replaced based on the condition of cell growth. Cell passage was performed using trypsin up to 80% of confluence.

Luciferase Reporter Gene Assay

Cells were co-transfected with 20 nmol/L mimic control or microRNA-29b and 600 ng SH2B3 3'UTR-pmirGLO reporter plasmid. Luciferase activity at 36 h was determined according to the instructions of relative commercial kit. Relative light units of Firefly (RLU-1) and Renilla (RLU-2) of target genes were recorded.

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

Total RNA in myocardial tissues or cells were extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, the extracted RNA was reverse-transcribed into complementary Deoxyribose Nucleic Acid (cDNA), and qRT-PCR

was performed to amplify the target genes. Specific qRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. The relative expression level of target gene was expressed by the $2^{-\Delta\Delta CT}$ method. U6 was used as an internal reference in the quantitative analysis of microRNA-29b expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference in the quantitative analysis of SH2B3, COL1A1, and α-SMA expression. The experiment was repeated 3 times. Primer sequences used were as follows: microRNA-29b, F: 5'-GTGAATGATAGTGAGGAACC-3', R: 5'-GTCGAACGATCTTGCCACACGGA-3'; SH2B3, F: 5'-GCGTGAATGATAGTGAGGAG-3', R: 5'-AGAACGATTTGCCACACG-3'; COL1A1, F: 5'-ACGATGCACCTGTACGATCA-3', R: 5'-CTTCAACACGCAGGACACT-3'; α-SMA, F: 5'-GAGGGACTGGAACGACAA-3', R: 5'-GTGTAAGCGAACATCCTAATACT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

The total protein was extracted from transfected cells by the radioimmunoprecipitation assay (RIPA) solution (Yeasen, Shanghai, China). Protein samples were separated by electrophoresis on 10% of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Then, the membranes were incubated with primary antibodies of SH2B3, COL1A1m, and α-SMA (1:1000) at 4°C overnight. On the next day, membranes were incubated with horseradish peroxidase (HRP)-labeled IgG (1:5000) for 2 h. Immunoreactive bands were exposed by the enhanced chemiluminescence (ECL) method, and integrated optical density was analyzed by gel imaging analysis system. β-actin was used as an internal reference.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for all statistical analysis, and GraphPadPrism5.0 (La Jolla, CA, USA) was applied for image editing. Quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). Categorical data and measurement data were analyzed by Chi-square test and t-test, respectively. $p<0.05$ was considered statistically significant.

Results

MicroRNA-29b Overexpression Alleviated Cardiac Dysfunction at Post-MI

After 28 days of animal procedures, the expression level of microRNA-29b in the infarct border zone was determined by qRT-PCR. Results showed that compared to sham group, microRNA-29b expression in NC group was significantly lower ($p<0.05$). MicroRNA-29b expression was markedly higher in microRNA-29b group than that of NC group ($p<0.05$, Figure 1A). Cardiac function of rats in each group was measured at day 28 through echocardiography. Rats in sham group and NC group presented the highest and lowest levels of LVEF and LVFS among the three groups, respectively (Figure 1B and 1C). M-mode echocardiography revealed good myocardial contraction of rats in sham group. However, rats in NC group and microRNA-29b group showed a worse myocardial contraction of the left ventricular anterior wall, which was more pronounced in NC group (Figure 1D). These results indicated that microRNA-29b overexpression improved cardiac function at post-MI.

MicroRNA-29b Reduced CVF at Post-MI

HE staining showed orderly cardiomyocytes with uniform nuclear size. Cardiomyocytes in NC group showed derangement, nucleus aggregation, and the most abnormal morphology. By comparison, the microRNA-29b group showed an irregular structure with basically even nuclear size (Figure 2A). Next, the Masson trichrome was performed. Among the three groups, sham group and NC group showed the fewest and the most blue-stained collagen, respectively (Figure 2B). CVF was analyzed based on Masson images using image analysis software. Results indicated that CVF was markedly lower in microRNA-29b group than that of NC group ($p<0.001$, Figure 2C).

Expression Levels of SH2B3, COL1A1, and α-SMA After Overexpression of microRNA-29b

The mRNA and protein levels of SH2B3, COL1A1, and α-SMA in the infarct border zone were determined at day 28 by qRT-PCT and Western blot. SH2B3 expression was significantly higher in microRNA-29b group than that of NC group. However, the expressions of COL1A1 and α-SMA showed the opposite trends ($p<0.05$, Figure 3A-3C). Western blot yielded identical results at protein level (Figure 3). Therefore, we might conclude that microRNA-29b overexpression reduced the myocardial fibrotic level.

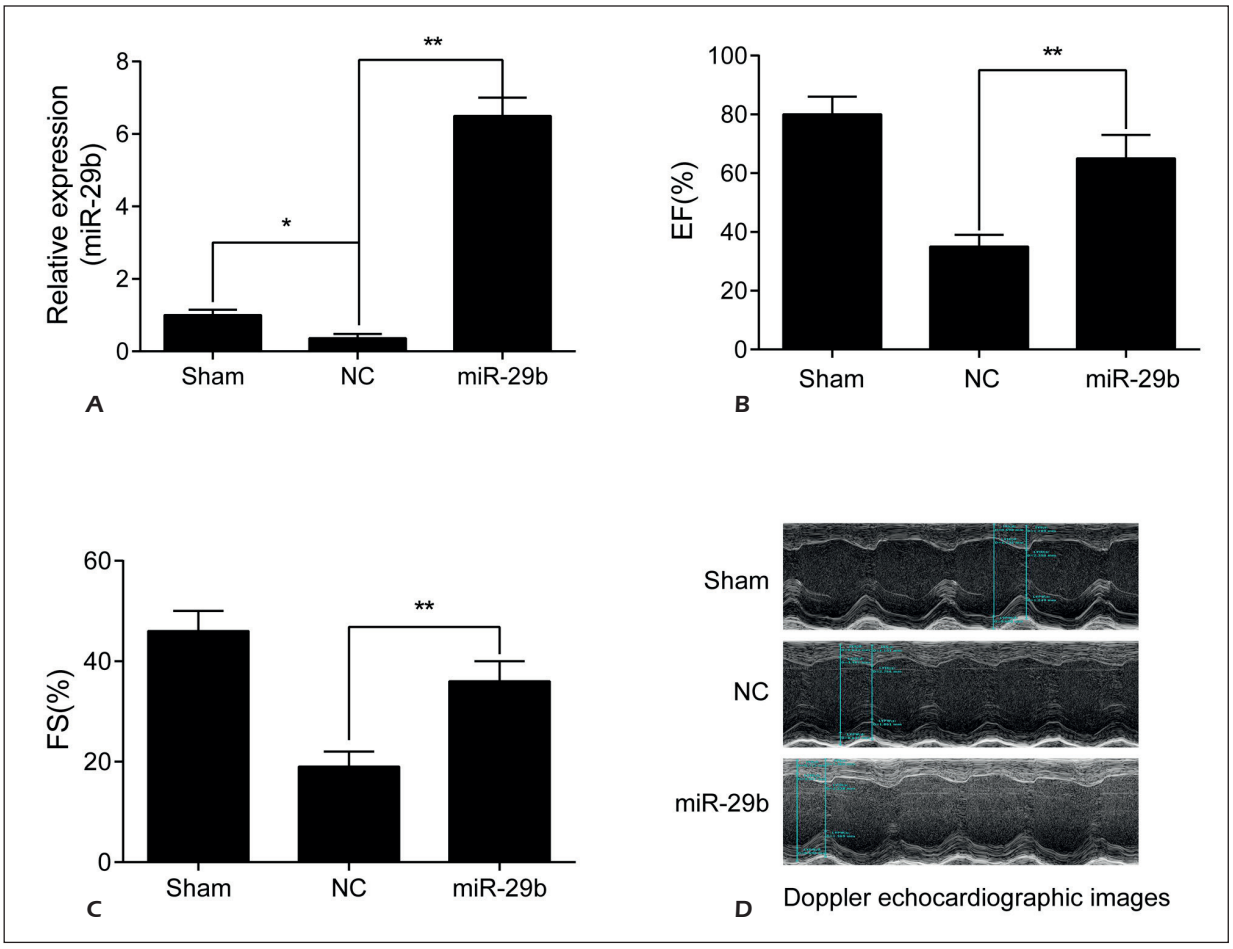

Figure 1. MicroRNA-29b overexpression alleviated cardiac dysfunction at post-MI. **A**, MicroRNA-29b expression in the infarct border zone at day 28. **B**-**C**, LVEF and LVFS in rats of sham group, NC group, and miR-29b group. **D**, M-mode echocardiograph in rats of sham group, NC group, and miR-29b group. *p<0.05 *vs.* Sham group; **p<0.01 *vs.* NC group.

MicroRNA-29b Alleviated MF Through Targeting SH2B3

The SH2B3 luciferase reporter gene vector targeting microRNA-29b site was first constructed. Subsequently, the effect of microRNA-29b on the luciferase activity of SH2B3 3'UTR was detected by luciferase reporter gene assay. Results demonstrated that luciferase activity was significantly inhibited after co-transfection of wild-type vector and microRNA-29b mimics. However, no significant changes were observed in the luciferase activity of mutant group (Figure 4A). To further validate the regulation of SH2B3 on MF, H9c2 cells induced with H_2O_2 were transfected with SH2B3-RNAi. Compared to those transfected with SH2B3-NC, the SH2B3 knockdown significantly decreased SH2B3 expression, whereas increased the expressions of COL1A1 and α-SMA at both mRNA and protein levels (Figure 4B-4E).

Discussion

In recent years, scholars[18-20] have confirmed a number of fibrosis-relative microRNAs. They can regulate the majority of fibrotic-relative molecules and pathways such as TGF-β, MAPK, integrins and EMT pathways. MicroRNA-21, microRNA-29b, and microRNA-133a have been proved to participate in the process of myocardial fibrosis[21]. Previous studies have found that the injection of microRNA-29 inhibitor into the tail vein of mice can remarkably down-regulate expressions of fibrosis-related genes, indicating its close relationship with ECM. Panizo et al[22] have shown that microRNA-29b is capable of regulating MF through negatively mediating the expressions of Collagen I, MMP-2 and CTGF. In our work, we found that microRNA-29b expression decreased significantly in the infarct border zone at day 28 after MI, which was consistent with the previous findings[7].

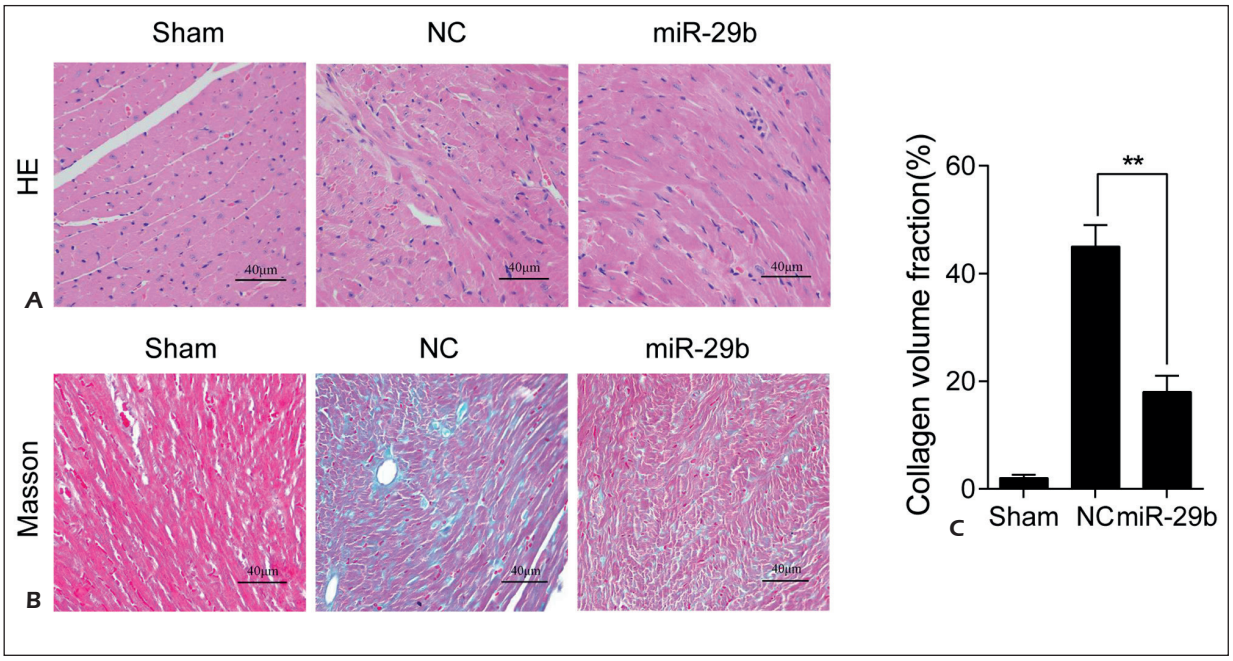

Figure 2. MicroRNA-29b reduced CVF at post-MI. **A-B**, HE and Masson images of sham group, NC group and miR-29b group (magnification 400×). **C**, CVF in sham group, NC group and miR-29b group. **$p<0.01$ *vs.* NC group.

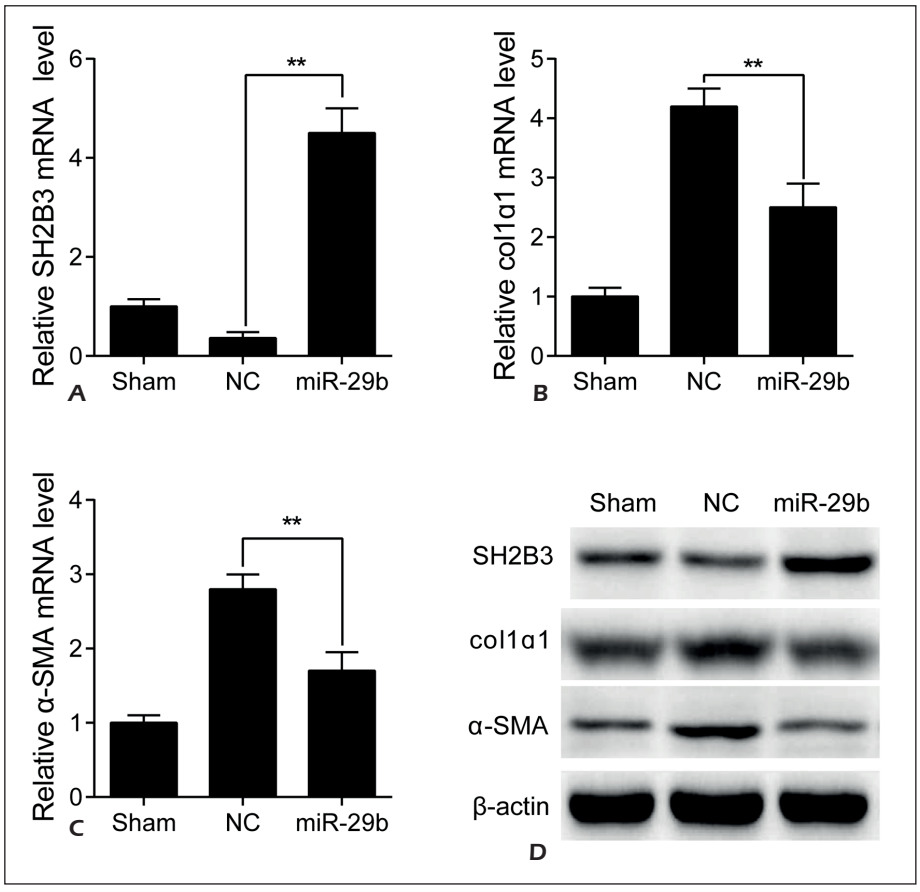

Figure 3. Expression levels of SH2B3, COL1A1, and α-SMA after overexpression of microRNA-29b. **A-C**, mRNA levels of SH2B3, COL1A1, and α-SMA in the infarct border zone of sham group, NC group, and miR-29b group. **D**, Western blot analyses of SH2B3, COL1A1, and α-SMA in sham group, NC group, and miR-29b group. **$p<0.01$ *vs.* NC group.

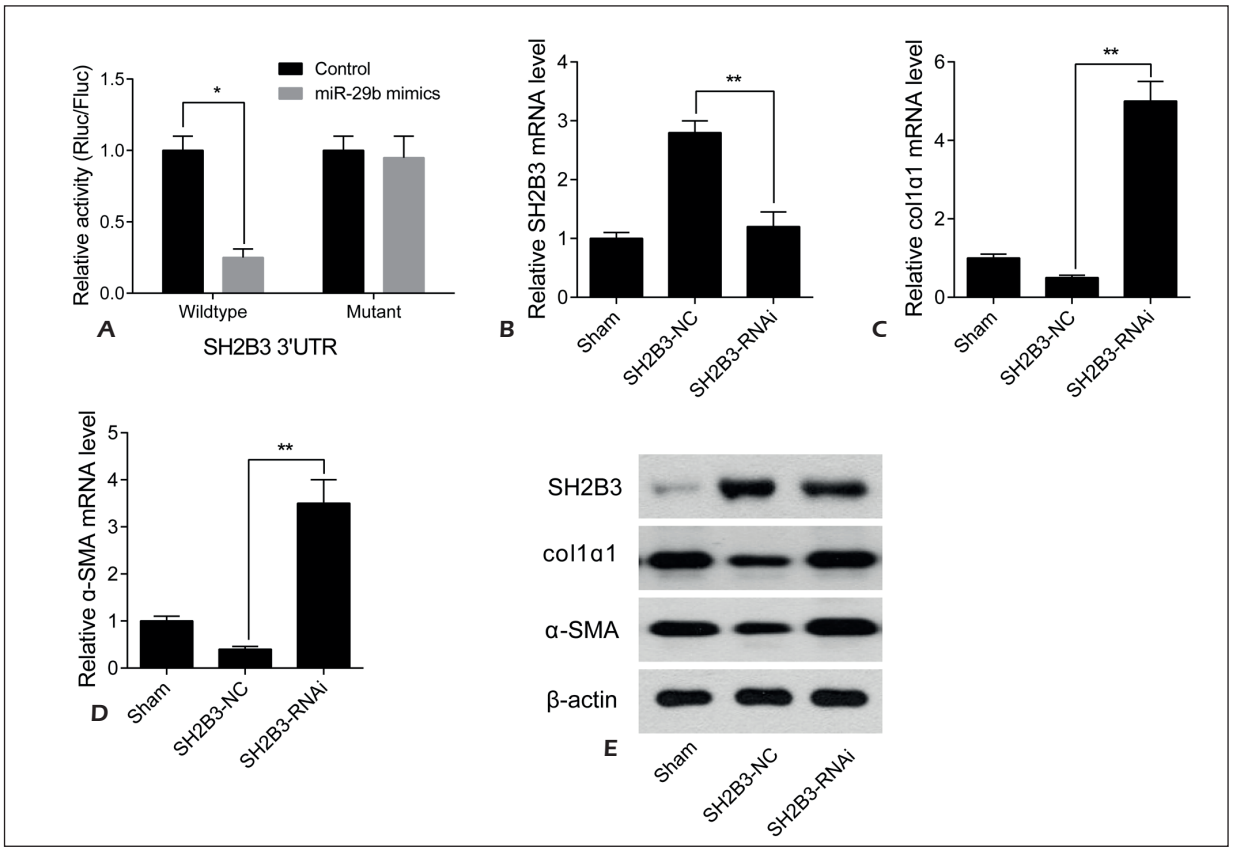

Figure 4. MicroRNA-29b alleviated MF through targeting SH2B3. **A**, Luciferase activity in SH2B3 3'UTR. **B**-**D**, mRNA levels of SH2B3, COL1A1, and α-SMA in H_2O_2-induced H9c2 cells transfected with SH2B3-RNAi or SH2B3-NC. E, Western blot analyses of SH2B3, COL1A1, and α-SMA in H_2O_2-induced H9c2 cells transfected with SH2B3-RNAi or SH2B3-NC. **$p<0.01$ *vs.* SH2B3-RNAi group.

Migration and proliferation of cardiac fibroblasts are the major pathological changes of MF. The transformation from cardiac fibroblasts to myocardial fibroblasts further secrets a large number of ECM, including collagen, elastin, and fibronectin. As a consequence, the imbalance and disordered structure of collagens due to massive deposition and reduced degradation can eventually lead to MF[23,24]. Severely ischemic myocardium after AMI impairs cardiomyocytes and initiates myocardial repair. Thrombolysis, stenting and coronary artery bypass grafting at post-AMI can indeed save part of the necrotic myocardium. However, it can also lead to excessive MF. Scar formation strikingly aggravates coronary occlusion and myocardial dysfunction, resulting in a vicious circle of ischemia-fibrosis-ischemia[25,26]. It has been found that the renin-angiotensin-aldosterone system, matrix metalloproteinase, tissue inhibitory factor, vascular endothelial dysfunction, and transforming growth factor (TGF-β1) are greatly involved in the progression of MF[27-31]. MicroRNAs exert a regulatory effect on MF. For example, microRNA-26a[32] suppresses the expressions of connective tissue growth factor (CTGF) and type I collagen, thus alleviating MF. Furthermore, novel fibrotic-related microRNAs have been searched in recent years.

MF is an advanced pathological manifestation of various cardiovascular diseases, including AMI, hypertension, diabetic cardiomyopathy, and pulmonary hypertension. The main pathological changes are ischemia-induced myocardial fibroblast activation, hyperplasia, and differentiation into myocardial fibroblasts expressing α-SMA. This may further synthesize and secret large amounts of ECM proteins[33,34]. α-SMA is a typical marker for the transformation of cardiac fibroblasts into myocardial fibroblasts. Meanwhile, it is often determined as a clinical index during the process of MF. Current studies have indicated that heart failure is frequently observed at post-MI. The pathogenesis of heart failure includes myocardial remodeling, neuroendocrine activa-

tion, and inflammatory response. Among them, cardiomyocyte hypertrophy, cell death, and extracellular matrix fibrosis are the basic mechanisms of myocardial remodeling[35]. Excessive deposition of ECM alters cardiac mechanical strength and electrical conduction, reduces cardiac compliance and ultimately leads to arrhythmias[36]. Therefore, cardiac function is often monitored during the clinical treatment of MF.

Through echocardiography, HE staining, Masson trichrome, CVF determination and COL1A1, and α-SMA expression determination, we verified that microRNA-29b overexpression could prevent MF, downregulate COL1A1 and α-SMA, and improve cardiac function at post-MI. Further investigations were conducted to elucidate whether SH2B3 was involved in the regulatory effect of microRNA-29b on MF-related gene expressions. By H_2O_2 induction in H9c2 cells transfected with SH2B3-RNAi, both the mRNA and protein levels of COL1A1 and α-SMA were determined. Our results demonstrated that microRNA-29b overexpression could significantly down-regulate the expressions of MF-relative genes by upregulating SH2B3. To sum up, microRNA-29b was capable of alleviating MF and cardiac dysfunction at post-MI through upregulating SH2B3. Our findings might provide a novel direction for the therapeutic therapy of post-MI.

Conclusions

We revealed that microRNA-29b overexpression alleviates MF and cardiac dysfunction in MI rats through targeting SH2B3.

Competing interests

The authors declared that they have no competing interests.

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