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CircRNA 010567 improves myocardial infarction rats through inhibiting TGF-β1

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Abstract. – OBJECTIVE: To observe the intervention effect of circular ribonucleic acid (circRNA) 010567 on myocardial infarction (MI)-induced myocardial fibrosis (MF) in rats, and to explore whether its mechanism of action is related to the regulation on the transforming growth factor-β1 (TGF-β1) signaling pathway.

MATERIALS AND METHODS: The rat model of acute MI was established using ligation of the left anterior descending coronary artery. Model rats were randomly divided into circRNA 010567 siRNA group and Model group, with sham operation group as Control group. The effects of circRNA 010567 on the cardiac function, MF, myocardial apoptosis, mRNA, and protein expression levels of TGF-β1 and Smad3 in heart tissues of MI rats were detected using the small animal ultrasound system, Masson staining, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, reverse transcription-polymerase chain reaction (RT-PCR), and Western blotting, respectively.

RESULTS: Compared with Control group, Model group had significantly decreased cardiac function, significantly lower left ventricular ejection fraction (LVEF), and left ventricular fractional shortening (LVFS), markedly increased left ventricular end-diastolic diameter (LVDd), and left ventricular end-systolic diameter (LVDs), severe MF, as well as a significantly higher apoptosis rate of myocardial cells, and evidently increased mRNA and protein levels of TGF-β1 and Smad3 in heart tissues. Compared with Model group, circRNA 010567 siRNA group had evidently improved cardiac function, significantly higher LVEF and LVFS, markedly decreased LVDd and LVDs, alleviated MF, a significantly lower apoptosis rate of myocardial cells, and evidently decreased mRNA and protein levels of TGF-β1 and Smad3 in heart tissues.

CONCLUSIONS: CircRNA 010567 siRNA can improve the cardiac function, alleviate the MF, and inhibit the myocardial apoptosis, thereby further suppressing MI-induced MF, whose mechanism may be related to the inhibition on the TGF-β1 signaling pathway.

Key Words:
Myocardial infarction, CircRNA, Myocardial fibrosis, TGF-β1.

Introduction

Myocardial infarction (MI) is a common and frequently-occurring disease in coronary heart diseases. Also, it is the most serious with the highest mortality rate. It is the common pathological change in many cardiovascular diseases when developing to a certain stage, characterized by a high mortality rate[1-3]. After MI, ventricular remodeling and cardiac dysfunction will be caused, and myocardial fibrosis (MF) is one of the important manifestations of ventricular remodeling[4]. In the chronic phase, MF is mainly manifested as the increased myocardial apoptosis and decline in neovascularization, thereby aggravating the occurrence and development of MF[5,6]. Therefore, in the treatment of MF anti-myocardial apoptosis has become an important therapeutic regimen to reduce ventricular remodeling and improve myocardial function.

Researchers[7-9] have found that transforming growth factor-β1 (TGF-β1) is the most important trigger in the fibrosis of organs or tissues, and it is involved in many physiological and pathological processes, such as cell growth, proliferation, differentiation, and apoptosis. Wang et al[10] found that MF will be caused after the expressions of TGF-β1 and Smad3 increased significantly in

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heart tissues of rats, indicating that the occurrence of MF is closely associated with the activation of the TGF-β1 signaling pathway.

In recent years, circular ribonucleic acid (circRNA) has been a research hotspot in the biological field. It is a special type of non-coding RNA without 5'-end poly(A) tail and 3'-end cap[11]. CircRNA, characterized by high conservation, tissue specificity, and time sequence, is not degraded by the exonuclease *in vivo*, but can interact with RNA-binding proteins to regulate gene expression[12]. It has been proved that circRNA plays an important regulatory role in cardiovascular diseases. Zhou et al[13] found that circRNA 010567 is significantly up-regulated in MF, but its mechanism of action remains unclear. Therefore, in the present work the rat model of MI was established using ligation of the left anterior descending coronary artery to explore whether circRNA 010567 can alleviate MF by regulating the activity of the TGF-β1 signaling pathway.

Materials and Methods

Reagents

CircRNA 010567 siRNA was purchased from Guangzhou Geneseed Biotechnology Co., Ltd. (Guangzhou, China); one-step terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining kit from Shanghai Beyotime Biotechnology Co., Ltd. (Shanghai, China); Masson staining kit from Nanjing SBJ Biotechnology Co., Ltd. (Nanjing, China); RNA extraction kit from Omega (Doraville, GA, USA); first-strand complementary deoxyribose nucleic acid (cDNA) synthesis kit from Biomiga (San Diego, CA, USA); TGF-β1 and Smad3 primers from Thermo Fisher Scientific (Waltham, MA, USA); TGF-β1, Smad3, and β-actin primary antibodies from Cell Signaling Technology (Danvers, MA, USA); horseradish peroxidase (HRP)-labeled secondary antibodies from Beijing Bioss Biotechnology Co., Ltd. (Beijing, China), and diaminobenzidine (DAB) HRP developing solution from Suzhou Everbright Biotechnology Co., Ltd. (Suzhou, China)

Instruments

Color Doppler ultrasound system was purchased from Siemens (Berlin, Germany), polymerase chain reaction (PCR) instrument and electrophoresis apparatus from Bio-Rad (Hercules, CA, USA), gel imager from Thermo Fisher Scientific (Waltham, MA, USA), inverted microscope from Olympus (Tokyo, Japan), pipette from Eppendorf (EP; Hamburg, Germany), and tissue microtome from Leica (Wetzlar, Germany).

Rats

This research was approved by the Animal Ethics Committee of Lanzhou University Animal Center. A total of 30 specific pathogen-free male Sprague-Dawley rats aged 8 weeks old and weighing (200±20) g were purchased from Lanzhou University. They were fed with ordinary rat feeds and had free access to food and water under 12/12 h light/dark cycle. The rats were divided into Control group, Model group, and circRNA 010567 siRNA group. The rats in circRNA 010567 siRNA group were injected with siRNA solution *via* the caudal vein (siRNA sequence: 5'-CAACAGACGCCAUAAGCAAUU-3'), while those in Control group and Model group were injected with an equal volume of solvent *via* the caudal vein.

Detection of Effect of CircRNA 010567 on Cardiac Function of MI Rats Using Small Animal Ultrasound System

Rat model of MI was established using ligation of the left anterior descending coronary artery. After anesthesia with pentobarbital sodium (30 mg/kg) and tracheal intubation, the small animal ventilator was connected, the chest was cut open from the left 3rd rib, and the inferior margin of left auricle was ligated at about 1-2 mm, followed by injection of 0.1 mL of lidocaine immediately. Then, the muscle layer and skin were sutured, and the rats should be kept warm. At 4 weeks after modeling, the left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS), significantly increased left ventricular end-diastolic diameter (LVDd) and left ventricular end-systolic diameter (LVDs) were detected using the small animal ultrasound system.

Detection of Effect of CircRNA 010567 on MF in MI Rats via Masson Staining

Masson staining is one of the methods used to detect the degree of tissue fibrosis. The sections were stained with iron hematoxylin for 5 min, Masson staining solution, and Ponceau staining solution for 5 min. Then, the sections were washed with a phosphomolybdic acid solution for 1 min, directly stained with aniline blue staining solution for 1 min, transparentized with xylene for 2 min, and sealed with neutral resins.

Detection of Effect of CircRNA 010567 on Myocardial Apoptosis in MI Rats Via TUNEL Staining

The myocardial apoptosis in rats was determined using TUNEL staining. 50 μL of TUNEL assay buffer was dropwise added onto the sections, followed by incubation for 60 min in a dark place. Next, the sections were washed with phosphate-buffered saline (PBS) for 3 times, added with fluorescence quencher and sealed, followed by observation of staining under a microscope.

Detection of Effect of CircRNA 010567 on mRNA Expressions of TGF-β1 and Smad3 in Heart Tissues in MI Rats Through RT-PCR

The total RNA was extracted from heart tissues using 1 mL of TRIzol lysis buffer (Invitrogen, Carlsbad, CA, USA), and synthesized into cDNA according to the instructions. Then, PCR amplification was performed using 1 μL of total RNA, 1 μL of 10 × reaction buffer, 1 μL of DNase I, and nuclease-free water till the total volume of 10 μL, as well as 1 μL of forward and reverse primers of TGF-β1 and Smad3, for a total of 40 cycles. The primers are shown in Table I.

Detection of Effect of CircRNA 010567 on Protein Expressions of TGF-β1 and Smad3 in Heart Tissues in MI Rats Through Western Blotting

The total protein was extracted from myocardial tissues using radioimmunoprecipitation assay (RIPA) lysis buffer (Yeasen, Shanghai, China). The protein concentration was determined using the bicinchoninic acid (BCA) method (Abcam, Cambridge, MA, USA), and the standard curves were plotted. Then, the proteins were added with an appropriate amount of 5 ×sodium dodecyl sulphate (SDS) loading buffer, separated *via* 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a membrane using the wet method, sealed with 5% skim milk powder solution for 2 h, and incubated with TGF-β1 (1:500), Smad3 (1:1000), and β-actin (1:1000) primary antibodies at 4°C overnight. Then, the proteins were washed with Tris-Buffered-Saline and Tween-20 (TBST) for 3 times, and incubated again with HRP-labeled secondary antibodies for 60 min. DAB developing solution was added for color development and the optical density of bands was analyzed using the ImageJ software (NIH, Bethesda, MD, USA).

Statistical Analysis

The data were analyzed using Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA). All data were consistent with the homogeneity of variance and normal distribution, and they were expressed as mean ± standard deviation. The *t*-test was used for analyzing measurement data. Differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ suggested a statistically significant difference.

Results

CircRNA 010567 SiRNA Could Improve Cardiac Function of MI Rats

The cardiac function of rats was detected using the small animal ultrasound system. As shown in Table II, compared with Control group, Model group had a significantly decreased LVEF and LVFS ($^{*}p<0.05$, $^{*}p<0.05$) and a significantly increased LVDd and LVDs ($^{*}p<0.05$, $^{*}p<0.05$). Compared with Model group, circRNA 010567 siRNA group had a significantly increased LVEF and LVFS ($^{\#}p<0.05$, $^{\#}p<0.05$), and a significantly decreased LVDd and LVDs ($^{\#}p<0.05$, $^{\#}p<0.05$).

CircRNA 010567 SiRNA Could Reduce MF in MI Rats

According to the results of Masson staining (Figure 1), there was myocardial cell hypertrophy, and the cells were arranged disorderly with a large number of fibrotic cells in Model group compared with Control group. In cir-

Table I. Primers.

List	F primer	R primer
TGF-β1	CCACCTGCAAGACCATCGAC	CTGGCGAGCCTTAGTTTGGAC
Smad3	TGGACGCAGGTTCTCCAAAC	CCGGCTCGCAGTAGGTAAC
β-actin	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC

Table II. Comparison of cardiac function of rats.

Group	LVEF (%)	LVFS (%)	LVDd (mm)	LVDs (mm)
Control	89.34 ± 3.51	64.39 ± 3.84	6.47 ± 1.12	3.43 ± 0.32
Model	34.29 ± 2.28*	21.52 ± 2.93*	11.83 ± 2.06*	7.48 ± 1.38*
CircRNA 010567 siRNA	56.93 ± 3.46#	37.85 ± 3.05#	9.42 ± 1.77#	5.64 ± 1.87#

Note: *p<0.05: Model group *vs*. Control group, #p<0.05: circRNA 010567 siRNA group *vs*. Model group.

cRNA 010567 siRNA group, the myocardial cells were arranged orderly, and myocardial interstitial fibrosis was alleviated compared with Model group.

CircRNA 010567 SiRNA Could Inhibit Myocardial Apoptosis in MI Rats

The results of TUNEL staining revealed that the number of apoptotic myocardial cells was increased in Model group compared with that in Control group (*p<0.05; Figure 2A), while it declined in circRNA 010567 siRNA group compared with that in Model group (#p<0.05) (Figure 2B).

CircRNA 010567 SiRNA Could Suppress the mRNA Expressions of TGF-β1 and Smad3 in Heart Tissues of MI Rats

According to the results of RT-PCR, Model group had evidently higher mRNA levels of TGF-β1 and Smad3 in heart tissues compared with Control group (*p<0.05, *p<0.05) (Figure 3A), while circRNA 010567 siRNA group had evidently lower mRNA levels of TGF-β1 and Smad3 in heart tissues compared with Model group (#p<0.05, #p<0.05) (Figure 3B).

CircRNA 010567 SiRNA Could Suppress the Protein Expressions of TGF-β1 and Smad3 in Heart Tissues of MI Rats

According to the results of Western blotting, Model group had a significantly higher protein levels of TGF-β1 and Smad3 in heart tissues compared with Control group (*p<0.05, *p<0.05; Figure 4A), while circRNA 010567 siRNA group had a significantly lower protein levels of TGF-β1 and Smad3 in heart tissues compared with Model group (#p<0.05, #p<0.05) (Figure 4B).

Discussion

Coronary heart disease is a scientific task urgently to be solved globally, greatly harming the health of patients. With the increase in population, the number of patients with coronary heart disease has increased by 3 times in the past decade. The morbidity rate of the cardiovascular disease shows a decreasing trend with the development of medicine, but the mortality rate of MI in coronary heart disease still ranks first in human diseases[14]. To improve the quality of life of MI patients, researchers have made constant

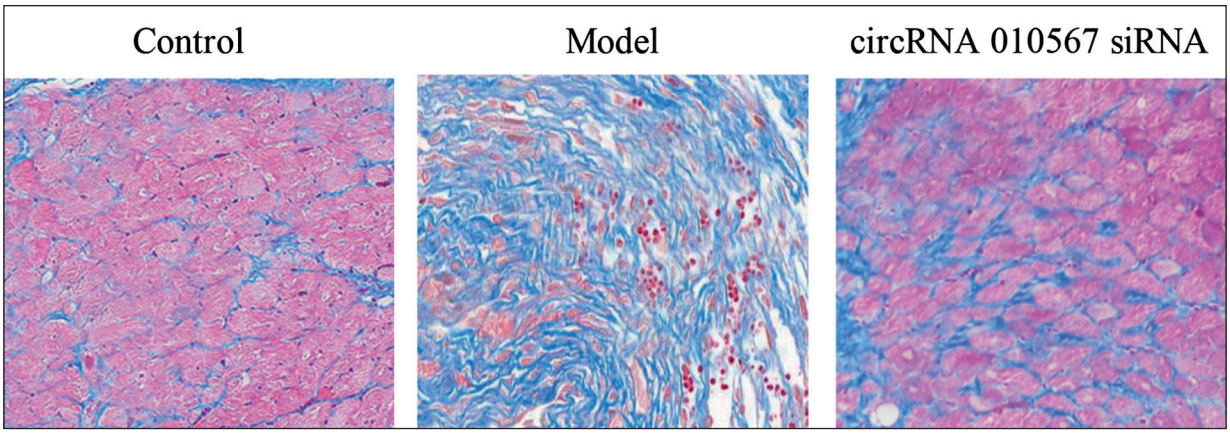

Figure 1. Masson staining of 3 different groups (200×).CircRNA 010567 siRNA significantly reduced MF in MI rats.

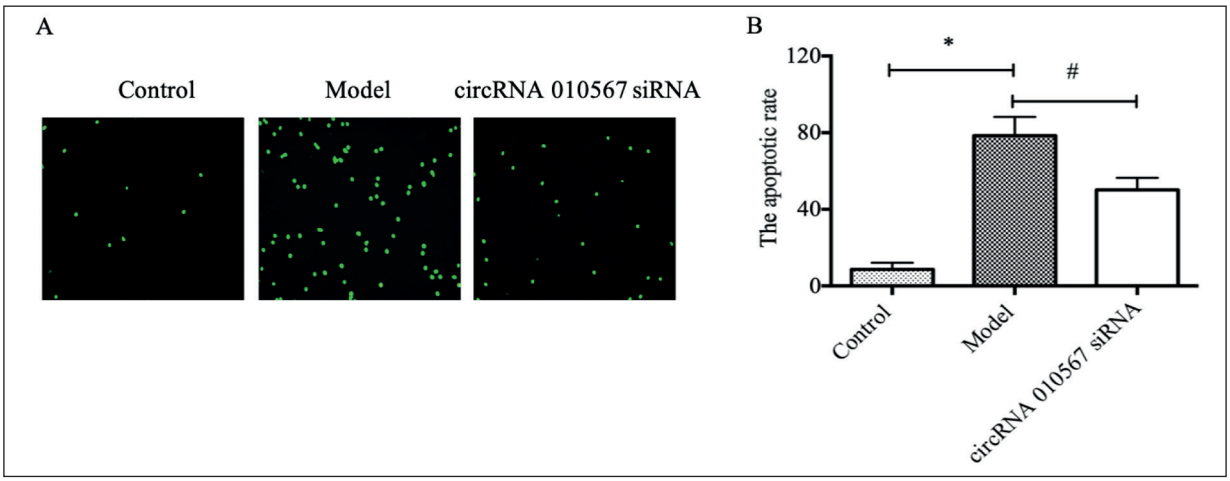

Figure 2. Myocardial apoptosis in each group. **A,** TUNEL staining (20×), **B,** Statistical graph of myocardial apoptosis rate (*p<0.05, #p<0.05).

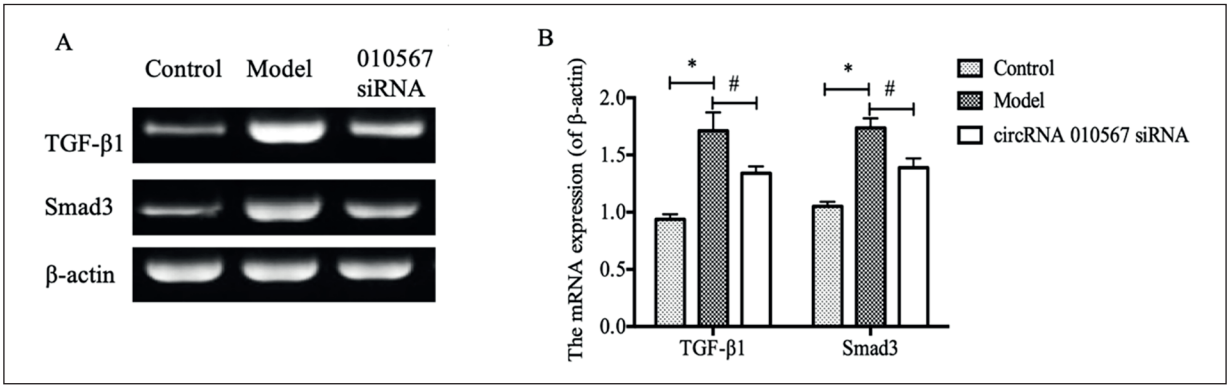

Figure 3. mRNA levels of TGF-β1 and Smad3 in each group detected using RT-PCR. **A,** Band chart, **B,** statistical graph of bands (*p<0.05, #p<0.05).

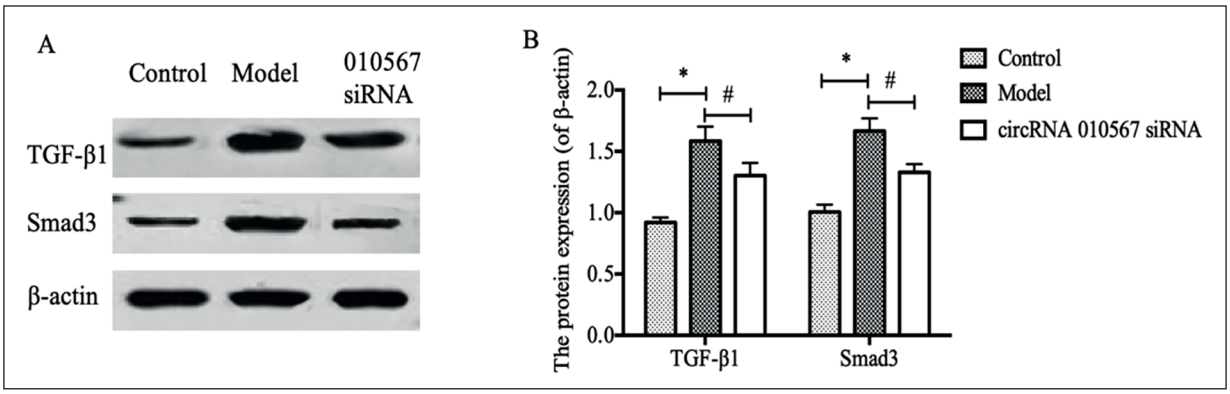

Figure 4. Protein levels of TGF-β1 and Smad3 in each group detected using Western blotting. **A,** Western blotting bands, **B,** statistical graph of bands (*p<0.05, #p<0.05).

efforts. The pathogenesis of fibrotic diseases in organs or tissues is complex, and the related factors include the infection, alcohol, obesity, hypertension, and gene mutation. Fibrotic diseases are a series of complications caused by an imbalance between extracellular matrix production and degradation due to trauma or inflammatory infiltration of organs or tissues, seriously threatening

the lives of patients[15]. As an important factor in cardiac remodeling, MF seriously affects the cardiac function, directly determining the quality of life and health of patients.

In recent years, circRNA, with about 100 nucleotides in length, has become a new research hotspot in the biological field[16]. The biological functions of circRNA include the miRNA sponge, alternative splicing or transcription, which regulates gene expression *in vivo* through non-classical splicing mode[17]. With the deepening of research, reports have demonstrated that circRNA plays an important role in the occurrence and development of cardiovascular diseases. Altesha et al[18] found that the abnormally expressed circRNA is closely related to the occurrence of myocardial ischemia-reperfusion, MI, myocardial failure, cardiomyopathy, and atherosclerosis. Therefore, in this work, circRNA 010567 siRNA was injected *via* caudal vein for interference in the expression of circRNA 010567. We found that the cardiac function of rats in circRNA 010567 siRNA group was significantly improved compared with that in Model group. The results of Masson staining showed that MF was also significantly improved. Then, it was observed that myocardial apoptosis in circRNA 010567 siRNA group was also markedly ameliorated, indicating that circRNA 010567 siRNA can evidently inhibit myocardial apoptosis in MI rats.

Activated TGF-β1 plays a key role in fibrosis of organs or tissues. TGF-β1 widely exists in mammalian cells, with abundant biological activity, which, after stimulation, can bind to receptors on the cell membrane, and transmit the biological signals to the downstream target protein in the cytoplasm, thereby exerting biological functions. Chen et al[19] manifested that miR-1908 improves cardiac fibrosis after myocardial infarction by targeting TGF-beta1. Sun et al[20] found in the study on patients with rheumatic heart disease that the level of TGF-β1 is positively correlated with the occurrence of MF, demonstrating that the selective inhibition on TGF-β1 may be a treatment means for MI. The above research results indicate that TGF-β1 has important significance for the occurrence and development of MF. To explore the mechanism of circRNA 010567 in inhibiting MF, the gene and protein levels of TGF-β1 and Smad3 were detected using RT-PCR and Western blotting. Results revealed that circRNA 010567 siRNA could remarkably reduce the gene and protein levels of TGF-β1 and Smad3, demonstrating that the inhibitory effect of circRNA 010567 siRNA on MF in MI rats is related to the inhibition on TGF-β1 signaling pathway.

Conclusions

We found that circRNA 010567 siRNA can inhibit MF in MI rats. Its regulatory mechanism may be related to the inhibition on the TGF-β1 signaling pathway, which shows that regulating TGF-β1 signaling pathway through interference in the abnormally expressed circRNA has good prospects for application in prevention and in the treatment of MI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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