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Effect of lncRNA GAS5 on rats with acute myocardial infarction through regulating miR-21

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of long non-coding ribonucleic acid (lncRNA) growth arrest specific 5 (GAS5) on acute myocardial infarction (AMI) model rats and to explore its regulatory mechanism.

MATERIALS AND METHODS: The rat model of AMI was established by subcutaneous injection of isoproterenol (ISO). 30 Sprague Dawley (SD) rats were randomly divided into three groups, including Control group, Model group, and lncRNA GAS5 inhibitor [small interfering ribonucleic acid (siRNA) GAS5] group. Hematoxylin and eosin (H&E) staining was used to detect the pathological damage of myocardial tissues in rats of each group. Myocardial cell apoptosis in each group determined *via* terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The levels of matrix metalloproteinase (MMP)-2 and MMP-9 in the serum of rats in each group were examined by enzyme-linked immunosorbent assay (ELISA). Reverse transcription-polymerase chain reaction (RT-PCR) was adopted to measure the expression level of miR-21 in rat myocardial tissues.

RESULTS: Compared with Control group, rats in Model group had significantly poor cardiac function, serious pathological damage of myocardial tissues, as well as increased apoptosis rate of myocardial cells. Meanwhile, the levels of MMP-2 and MMP-9 were significantly elevated in serum of Model group, while miR-21 level was down-regulated. In comparison with Model group, rats in siRNA GAS5 group exhibited significantly improved cardiac function, alleviated pathological damage to myocardial tissues, as well as decreased apoptosis rate of myocardial cells. Furthermore, the levels of MMP-2 and MMP-9 decreased significantly in serum of siRNA GAS5 group, whereas the expression level of miR-21 in myocardial tissues was down-regulated.

CONCLUSIONS: SiRNA GAS5 can enhance the cardiac function of AMI model rats, relieve pathological damage, reduce myocardial cell apoptosis, and inhibit the occurrence of myocardial fibrosis. The possible underlying mechanism may be associated with up-regulation of miR-21.

Key Words:
LncRNA GAS5, MiR-21, Acute myocardial infarction (AMI), Myocardial fibrosis (MF).

Introduction

According to the "Summary of Report on Cardiovascular Disease in China", there are about 290 million patients with cardiovascular diseases in China. This accounts for more than 40% of disease-related resident deaths, which is notably higher than other diseases, including malignant tumors[1]. Meanwhile, the death rate of cardiovascular diseases in rural areas is obviously higher than that of urban areas in China. This has brought heavy economic burdens to the families of rural residents and the society. In cardiovascular diseases, acute myocardial infarction (AMI) is a critical and severe type of irreversible myocardial necrosis caused by acute and persistent ischemia and hypoxia on the basis of coronary atherosclerosis. AMI severely threatens the life quality of patients[2-4]. In clinic, myocardial fibrosis (MF) symptoms occur in most of AMI patients. Currently, how to inhibit MF after AMI has become a research hotspot[5]. MF is mainly pathologically manifested as excessively accumulated extracellular matrix. Repressing such accumulation will be considered as an effective way to inhibit MF development. Numerous reports[6,7] have proved that the mutual regulation of tissue inhibitor of metalloproteinase (TIMP) and matrix metalloproteinase (MMP) is a crucial

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factor in maintaining the balance of extracellular matrix. MMP, a member of the endogenous zinc ion-dependent family, includes MMP-9, MMP-2, MMP-7, and MMP-1. The primary physiological function of MMP is to degrade extracellular matrix. Previous studies have verified that abnormal expression levels of MMP-2 and MMP-9 can result in the occurrence and development of MF. All these findings indicate that drugs suppressing the expressions of MMP-2 and MMP-9 have great potential in the treatment AMI-induced MF[8,9].

Non-coding ribonucleic acids (ncRNAs) are a type of RNAs with no protein-coding function *in vivo*. Meanwhile, they participate in regulating protein expression. It is noticeable that ncRNAs are involved in the occurrence and development of AMI, mainly including long ncRNAs (lncRNAs) and micro RNAs (miRNAs)[10,11]. LncRNAs are long-chain RNAs with more than 200 nucleotides in length, while miRNAs are short-chain RNAs with about 22 nucleotides in length. Although researches on ncRNAs are still in the initial stage, some progress has been made in the interaction between lncRNAs and miRNAs[12]. Growth arrest specific 5 (GAS5) exerts crucial regulatory effects on cell proliferation, apoptosis, and invasion[13]. However, the function of lncRNA GAS5 in AMI has not been fully elucidated. Therefore, the aim of this research was to investigate the regulatory effect of lncRNA GAS5 on AMI-induced MF and to explore its regulatory mechanism by construction of the rat model of AMI through injection of isoproterenol (ISO).

Materials and Methods

Reagents

ISO was purchased from Sigma-Aldrich (St. Louis, MO, USA), chloral hydrate from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), TRIzol and first strand complementary deoxyribonucleic acid (cDNA) synthesis kit from Thermo Fisher Scientific (Waltham, MA, USA), MMP-2, and MMP-9 enzyme-linked immunosorbent assay (ELISA) kits from R&D System (Minneapolis, MN, USA), Hematoxylin and eosin (H&E) staining solution and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) cell apoptosis kit from Beijing Solarbio Life Sciences (Beijing, China), small interfering RNA (siRNA) GAS5 from Guangzhou Ribobio Co., Ltd. (Guangzhou, China), and miR-21 primers from Invitrogen (Carlsbad, CA, USA).

Instruments

Optical microscope was bought from Nikon (Tokyo, Japan), thermostat water bath pot from Tangshan Lichen Science & Technology Co., Ltd. (Tangshan, China), reverse transcription-polymerase chain reaction (RT-PCR) apparatus from Bio-Rad (Hercules, CA, USA), slicer from Leica (Wetzlar, Germany), and spectrophotometer and analytical balance from Mettler Toledo (Columbus, OH, USA).

Animals

A total of 30 clean-grade Sprague Dawley (SD) rats [License No.: SYXK (Jilin) 2018-0014] were purchased from the Changchun University of Traditional Chinese Medicine (Changchun, China). All rats were randomly divided into three groups using a random number table, including Control group, Model group, and lncRNA GAS5 inhibitor (siRNA GAS5) group. The rats were raised in separate cages, with free access to food and water. This research was approved by the Animal Ethics Committee of Guizhou Provincial People's Hospital Animal Center.

Preparation of Rat Models of AMI

Rats in Model group were subcutaneously injected with ISO (150 mg/kg) at a volume ratio of 0.2 mL/100 g for 14 d. Rats in siRNA GAS5 group were given tail vein injection once every 3 d for 15 d. Meanwhile, rats in Control group were injected with an equal volume of normal saline. Left ventricular ejection fraction (LVEF), left ventricular end diastolic diameter (LVEDD), and left ventricular end systolic diameter (LVESD) of rats in each group were detected.

Detection of Pathological Damage to Myocardial Tissues Via H&E Staining

Rat heart tissues were embedded in paraffin, sliced into 8-μm sections, and soaked in xylene for 15 min. Subsequently, the sections were soaked in ethanol solution at gradient concentration from high to low for 1 min, followed by washing with distilled water. After that, the sections were subjected to H&E staining, followed by soaking in ethanol solution at gradient concentration from low to high. After permeabilization in xylene, the sections were sealed with neutral resin added dropwise. Finally, pathological damage was observed under an optical microscope.

Examination of the Apoptosis of Rat Myocardial Cells Via TUNEL Assay

Chromosome DNA fragmentation in apoptosis can be marked with fluorescent staining solution. Myocardial tissue sections were fixed with 4% paraformaldehyde, rinsed with phosphate-buffered saline (PBS) solution, and permeabilized by 0.1% Triton X-100 for 2 min. Thereafter, the sections were added dropwise with 500 μL TUNEL reaction solution, followed by rinsing with PBS solution for 3 times. Anti-quenching agent was added drop-wise for sealing. Finally, staining was observed under an inverted fluorescent microscope.

Determination of the Levels of MMP-2 and MMP-9 in Rat Serum by ELISA

Different concentrations of standard solution were added, and a standard curve was plotted. Then, the sample solution was added. After sealing with plate sealers, the sections were incubated at 37°C for 30 min. Subsequently, the liquid was discarded, and the sections were repeatedly washed with washing solution for 5 times and patted dry on absorbent paper. 50 μL of enzyme reagents were added for 30 min of incubation in the same way, followed by washing with washing solution. Thereafter, the mixed solution (50 μL of A solution + 50 μL of B solution) was added for 15 min of incubation in the dark. Next, 50 μL of stop buffer was added drop-wise to terminate the reaction, and the solution turned yellow at this moment. Finally, absorbance at the wavelength of 450 nm was measured by a micro-plate reader.

Measurement of MiR-21 Level in Rat Myocardial Tissues by RT-PCR

Rat myocardial tissues (50 mg) were added with 1 mL TRIzol lysis buffer and homogenized, followed by an ice bath for 5 min. After centrifugation, the supernatant was transferred to a new Eppendorf (EP) tube. Chloroform was then added for centrifugation. The supernatant was collected and ISO was added for centrifugation again. After discarding the supernatant, diethyl pyrocarbonate (DEPC)-treated water was added to dissolve the precipitate. The resulting solution was total RNA solution. According to the instructions of the first strand complementary deoxyribonucleic acids (cDNAs) synthesis kit, the reaction system was added for incubation at 70°C for 5 min. Next, the samples were incubated with reverse transcription (RT) buffer at 37°C for 60 min. Specific PCR amplification (50 μL in total) was carried out as follows: denaturation at 94°C, annealing at 70°C, and extension at 72°C for 7 min. Primers used in this study were as follows: MiR-21 primer sequences: F: 5'-ACACTC-CAGCTGGGTAGCTTATCAGACTGA-3' and R: 5'-GTGTCGTGGAGTCGGCAATTC-3'. Finally, gel electrophoresis was performed, and changes in bands in each group were observed.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (SPSS, Chicago, IL, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation (SD). The *t*-test was used for analyzing measurement data. Student's *t*-test was applied to compare the difference between the two groups. One-way ANOVA test was used to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Cardiac Function of AMI Model Rats

Compared with Control group, LVEF decreased significantly in Model group, while LVEDD and LVESD increased significantly ($^*p<0.05$) (Table I). In comparison with Model group, siRNA GAS5 group showed remarkably elevated LVEF and reduced LVEDD and LVESD ($^\#p<0.05$). This indicated that siRNA GAS5 could improve the cardiac function of AMI rats.

Table I. Comparison of the cardiac function of the rats in each group.

Group	LVEF (%)	LVEDD (mm)	LVESD (mm)
Control	73.89 ± 6.11	4.25 ± 0.63	2.26 ± 0.15
Model	42.94 ± 4.57*	9.93 ± 0.74**	5.27 ± 0.53*
SiRNA GAS5	62.65 ± 7.24#	7.35 ± 0.82#	4.18 ± 0.36#

Note: $^*p < 0.05$, $^{**}p < 0.01$: Control group *vs*. Model group, $^\#p < 0.05$: Model group *vs*. siRNA GAS5 group.

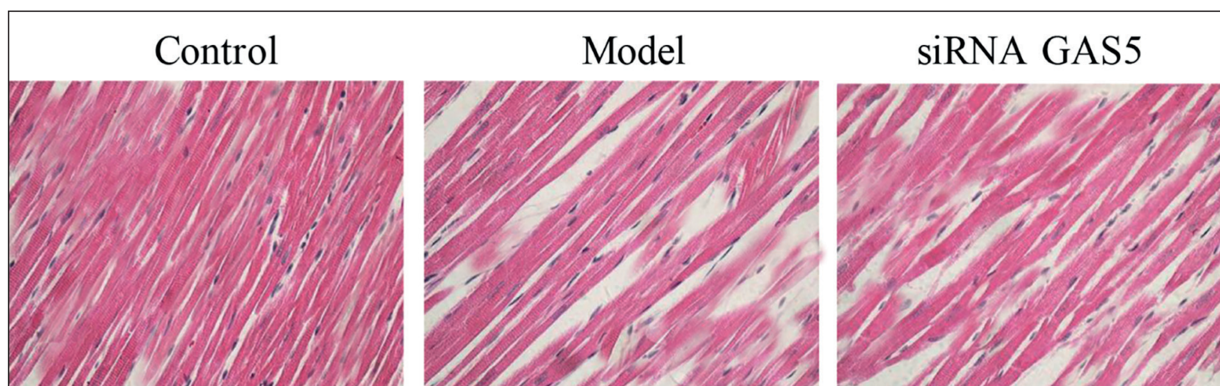

Figure 1. Comparison of pathological morphology of rat myocardial tissues in each group (magnification 20×).

SiRNA GAS5 Was Able to Reduce Myocardial Tissue Injury in Rats

According to H&E staining results (Figure 1), Control group had orderly arranged myocardial fiber cells, with no proliferation of myocardial interstitium. However, Model group had disorderly arranged and irregularly myocardial fiber cells, as well as proliferation in the interstitium. All these findings suggested that siRNA GAS5 could notably reduce myocardial tissue injury in AMI rats.

SiRNA GAS5 Could Suppress the Apoptosis of Myocardial Cells in Rats

TUNEL assay (Figure 2) manifested that the apoptosis rate of myocardial tissue cells in Model group was significantly elevated when compared with Control group ($^{*}p<0.05$). In comparison with Model group, the apoptosis rate of rat myocardial tissue cells in siRNA GAS5 group was significantly reduced ($^{\#}p < 0.05$) (Figure 3). It could be concluded that siRNA GAS5 was capable of inhibiting the apoptosis of rat myocardial tissue cells.

SiRNA GAS5 Could Down-regulate the Levels of MMP-2 and MMP-9 in Rat Serum

As shown in Figure 4-5, ELISA results revealed that compared with Control group, the levels of MMP-2 and MMP-9 in Model group were significantly elevated ($^{*}p<0.05$). In comparison with Model group, siRNA GAS5 group exhibited significantly decreased levels of MMP-2 and MMP-9 in rat serum ($^{\#}p<0.05$). The above findings implied that siRNA GAS5 could reduce the levels of MMP-2 and MMP-9 in the serum of AMI rats, and suppress extracellular matrix accumulation.

SiRNA GAS5 Was Capable of Elevating MiR-21 Level in Rat Myocardial Tissues

RT-PCR results (Figure 6) showed that compared with Control group, miR-21 level decreased significantly in rat myocardial tissues of Model group ($^{*}p<0.05$). In comparison with Model group, siRNA GAS5 group showed significantly up-regulated level of miR-21 in rat myocardial tissues ($^{\#}p<0.05$) (Figure 7). All these results

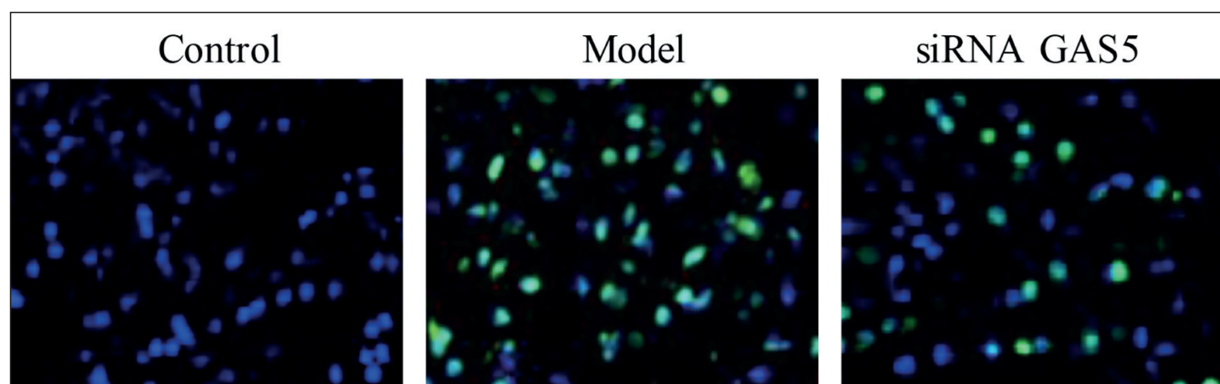

Figure 2. TUNEL staining images (magnification 20×).

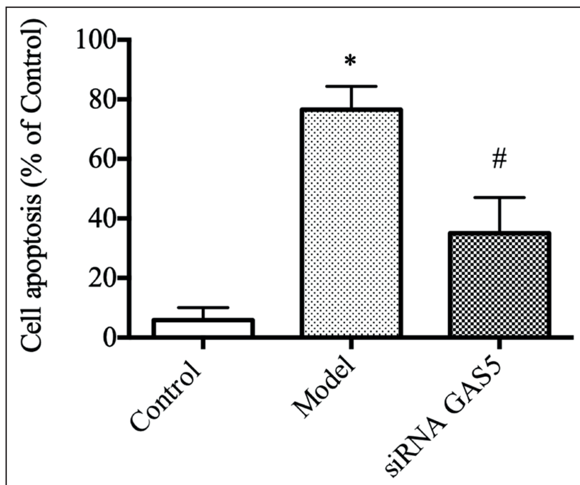

Figure 3. Comparison of the apoptosis rate of myocardial tissue cells (*$p<0.05$, **$p<0.01$: Control group *vs.* Model group, $^{\#}p<0.05$: Model group *vs.* siRNA GAS5 group).

demonstrated that siRNA GAS5 could elevate the expression of miR-21, thereby playing a protective role in AMI rats.

Discussion

AMI is the leading cause of cardiovascular disease-related death in China. AMI-induced MF severely threatens the health and life of patients, which is also a scientific problem urgently to be solved by researchers and medical workers[14]. Pathologically, AMI is primarily manifested as the reduction of coronary artery blood flow. This

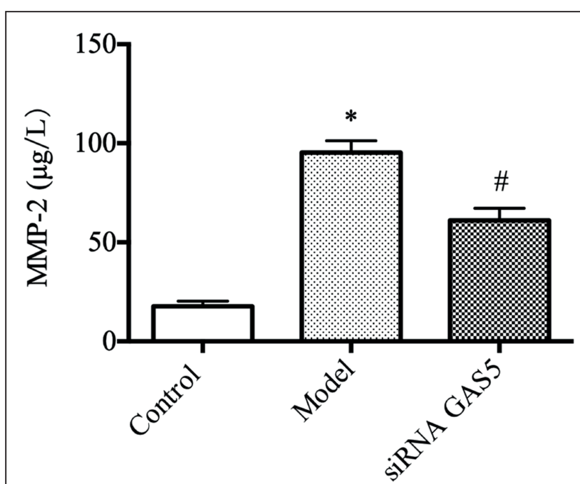

Figure 4. Comparison of MMP-2 level (*$p<0.05$, **$p<0.01$: Control group *vs*. Model group, $^{\#}p<0.05$: Model group *vs*. siRNA GAS5 group).

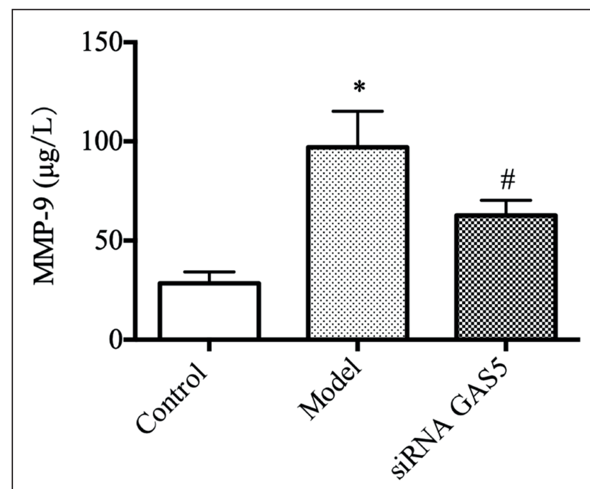

Figure 5. Comparison of MMP-2 level (*$p<0.05$, **$p<0.01$: Control group *vs*. Model group, $^{\#}p<0.05$: Model group *vs*. siRNA GAS5 group).

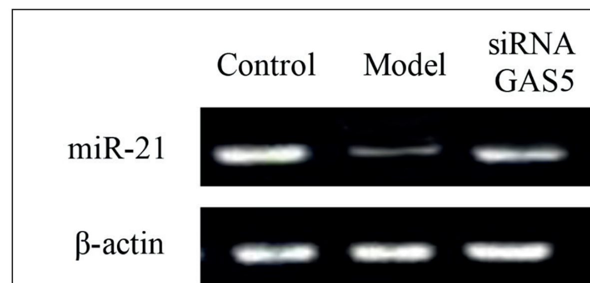

Figure 6. RT-PCR bands.

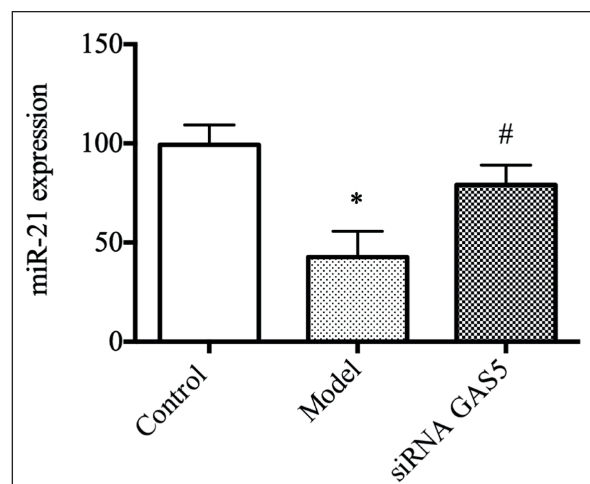

Figure 7. Comparison of miR-21 level (*$p<0.05$, **$p<0.01$: Control group *vs*. Model group, $^{\#}p<0.05$: Model group *vs*. siRNA GAS5 group).

may result in persistent myocardial ischemia or hypoxia, myocardial cell necrosis, and apoptosis. Clinically, the main treatment of AMI includes coronary intervention, thrombolysis or cardiac bypass, with certain therapeutic effects[15]. However, current treatment methods cannot effectively cure AMI-induced MF. Talman and Ruskoaho[16] have revealed that the accumulation of myocardial extracellular matrix is a vital factor leading to MF in the process of AMI. Extracellular matrix is a network structure consisting of glycoproteins, proteoglycans, collagen, and other macromolecular substances. It mainly has the function of heart support and signal transmission. Meanwhile, its synthesis and decomposition are in the dynamic balance of healthy human body. However, the imbalance of MMP and TIMP ratio will lead to the accumulation of cell matrix, as well as the occurrence and development of MF. Han et al[17] and Wang et al[18] have discovered that abnormal MMP-2 and MMP-9 levels are closely associated with MF. If the expressions of MMP-2 and MMP-9 can be suppressed, MF development can be delayed to a certain degree.

LncRNAs are a type of non-coding RNAs with about 200 nucleotides in length. They are located in the nucleus and can regulate about 4-10% of the genome sequences *in vivo*. LncRNAs are involved in gene transcription, transportation, translation, and other processes. Currently, the exploration of their roles in the occurrence of human diseases is still in the initial stage[19]. GAS5 has exerted a pivotal regulatory role in the occurrence and development of different kinds of acute and chronic diseases. Numerous studies have demonstrated that it participates in the regulation of cell biological functions and organ development. Meanwhile, it is the key mitigation of cell proliferation and apoptosis. Scholars have manifested that the abnormal expression of GAS5 is related to the occurrence of a variety of diseases. Ye et al[20] have discovered that the level of GAS5 in atherosclerotic model rats is significantly elevated. This may aggravate the secretion and release of pro-inflammatory factors (IL-6, IL-1β, and TNF-α) and chemokines. All these findings indicate that GAS5 also exerts a key effect on cardiovascular diseases. However, few reports have focused on the regulation of lncRNA GAS5 on AMI rats.

In this study, the rat model of AMI was first successfully established by subcutaneous injection of ISO. Subsequent findings demonstrated that siRNA GAS5 transfection could evidently enhance the cardiac function of AMI rats and reduce the pathological damage to myocardial tissues. TUNEL staining was employed to detect the apoptosis of myocardial cells in rats of each group. It was found that siRNA GAS5 transfection could significantly block myocardial cell apoptosis. Besides, ELISA results demonstrated that siRNA GAS5 was able to significantly down-regulate the levels of MMP-2 and MMP-2 in serum of AMI rats. This implied that siRNA GAS5 could repress the accumulation of extracellular matrix in the myocardium of rats, as well as relieve AMI-induced MF. To further explore the regulatory mechanism of siRNA GAS5 on AMI rats, miR-21 level in rat myocardial tissues was determined *via* RT-PCR. The results illustrated that siRNA GAS5 was capable of up-regulating miR-21 expression level. It could be seen that siRNA GAS5 could notably enhance cardiac function and relieve MF in AMI rats. The possible underlying mechanism might be correlated with the up-regulation of miR-21. Our data provided an experimental basis for the application of lncRNA GAS5 in the treatment of AMI.

Conclusions

We first observed that inhibition of GAS5 can enhance the cardiac function of AMI model rats, relieve pathological damage, reduce myocardial cell apoptosis, and inhibit the occurrence of myocardial fibrosis. The possible underlying mechanism may be associated with the up-regulation of miR-21.

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

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