European Review for Medical and Pharmacological Sciences 2019; 23: 8566-8572

Silence of lncRNA XIST represses myocardial cell apoptosis in rats with acute myocardial infarction through regulating miR-449

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Abstract. – OBJECTIVE: To study the influences of long non-coding ribonucleic acid (lncRNA) X-inactive specific transcript (XIST) on rats with acute myocardial infarction (AMI), and its regulatory mechanism.

MATERIALS AND METHODS: A total of 30 Sprague-Dawley rats were randomly assigned into Sham group, Model group, and lncRNA XIST small interfering RNA (XIST siRNA) group. The AMI rat model was prepared through ligating the left anterior descending coronary artery. The left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), left ventricular systolic diameter (LVDs), and left ventricular diastolic diameter (LVDd) of rats were determined using a color Doppler ultrasound system. Reverse transcription-polymerase chain reaction was performed to measure the expression levels of lncRNA XIST, microRNA (miR)-449, and Notch1 in rat heart tissues in each group. Pathological morphology of rat heart tissues in each group was observed *via* hematoxylin-eosin (HE) staining. Cell apoptosis in rat heart tissues was evaluated through terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

RESULTS: Compared with those in Sham group, rats in Model group had significantly increased LVEDV, LVESV, LVDs, and LVDd. After transfection with lncRNA XIST siRNA, XIST level in rat heart tissues was remarkably declined in XIST siRNA group compared with that in Model group. According to HE staining results, the pathological injuries in rat heart tissues were greatly improved in XIST siRNA group compared with those in Model group. TUNEL staining results revealed that the apoptosis rate of cells in rat heart tissues in XIST siRNA group was markedly lower than that in Model group. Higher level of miR-449 and lower level of Notch1 were observed in rats of XIST siRNA group than those of Model group.

CONCLUSIONS: Knockdown of lncRNA XIST can repress the myocardial cell apoptosis in AMI model rats by downregulating miR-449 level.

Key Words:
Acute myocardial infarction, LncRNA XIST, MiR-449, Apoptosis.

Introduction

The morbidity rate of acute myocardial infarction (AMI) increases year by year due to improvement in living standard, diet habits and gradual deterioration of the environment, and its mortality rate is extremely high[1,2]. It is expected that the number of AMI patients will rise to 25 million by 2020, and the relevant medical cost will increase by two folds in the following 20 years in China. Early identification and detection of AMI, as well as safe and efficacious treatment measures can significantly lower the mortality rate of cardiovascular disease[3,4]. AMI patients have partial acute myocardial necrosis due to persistent and severe cardiac ischemia. Its main clinical manifestations include a series of irreversible pathological changes, such as intense and long-lasting chest pain, arrhythmia, and myocardial injury and necrosis, as well as a high white cell count[5,6]. Certain progress has been made in the treatment of AMI with the improvement in modern medicine, but the curing purpose has not yet been achieved. Therefore, exploring the pathogenesis of AMI is of great significance for effective treatment[7]. Recent studies have found that the aberrant apoptosis of myocardial cells

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plays a crucial role in the development and progression of AMI. Searching for the drugs or schemes for inhibiting myocardial cell apoptosis remains to be solved as an urgent scientific problem.

Long non-coding ribonucleic acids (lncRNAs), a class of highly-conservative endogenous ncRNAs with the length exceeding 200 nt, is ubiquitous in eukaryotic cells and involved in multiple physiological and pathological processes of humans[8]. Guo et al[9] discovered the regulatory function of lncRNAs in AMI, indicating that lncRNAs can serve as the potential therapeutic targets for AMI. Based on the findings in the latest research, X-inactive specific transcript (XIST) is not only implicated in X-chromosome inactivation, but also in the development and progression of numerous diseases by regulating target microRNAs (miRNAs)[10]. Sun et al[11] found that lncRNA XIST plays a vital role in the drug resistance of non-small cell lung cancer (NSCLC) by regulating autophagy, suggesting the potential of XIST as a biomarker in NSCLC. According to the study results of Wei et al[12], the expression level of lncRNA XIST is significantly raised in pancreatic cancer cells. High expression of lncRNA XIST predicts the poor prognosis of patients with pancreatic cancer. Moreover, XIST is identified to affect the proliferation of pancreatic cancer cells by targeting miR-133a. LncRNA XIST can exert an important regulatory effect on several malignancies, but its role and mechanism in AMI remain elusive. The present paper, therefore, aims to explore the influences of lncRNA XIST on AMI rats and its potential mechanism.

Materials and Methods

Reagents

Primers of XIST, β-actin, miR-449 and Notch1 were purchased from Invitrogen (Carlsbad, CA, USA), one-step reverse transcription-polymerase chain reaction (RT-PCR) kit from Promega (Madison, WI, USA), hematoxylin-eosin (HE) staining solution from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China), lncRNA XIST siRNA from Shanghai GenePharma Co., Ltd. (Shanghai, China), and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit from Shanghai Beyotime Biotechnology Co., Ltd. (Shanghai, China).

Instruments

Ultralow-temperature refrigerator was provided by Kuansons Biotechnology (Shanghai, China) Co., Ltd., Nano-200 ultra-micro nucleic acid analyzer by Shanghai Langfu Industrial Co., Ltd. (Shanghai, China), RT-PCR instrument by Bio-Rad, Hercules, CA, USA, microtome and embedding machine by Leica (Wetzlar, Germany), ACUSON Sequoia 512 color Doppler ultrasound system by Siemens (Munich, Germany), and fluorescence inverted microscope by Nikon (Tokyo, Japan).

Animals

Specific pathogen-free male (SPF) Sprague-Dawley rats weighing (220±10) g [Shaanxi Provincial People's Hospital, animal certificate approval No.: SYXK (Shaanxi) 2016-006)] had free access to water and food based on the 12/12 h light-dark schedule. This investigation was approved by the Animal Ethics Committee of Aerospace Center Hospital Animal Center.

Establishment of AMI Rat Model

After fasting for food and water for 12 h preoperatively, the rats were intraperitoneally injected with 10% chloral hydrate for anesthesia. Four limbs and teeth of rats were fixed, and the chest furs of rats were shaved, followed by disinfection. Then, the skin was cut open between the 4^{th}-5^{th} ribs in the left chest. The muscle was stripped using a blunt separator, and the pericardium was cut open to expose the heart. Subsequently, the left anterior descending artery was pinpointed at the junction between the pulmonary cone and left atrial appendage. Following ligation, the apex was observed to be pale, indicating the successful ligation. Residual blood in the thoracic cavity was cleared away. Finally, the incision was sutured layer by layer. Rats can be radiated using baking lamps to increase their temperature intraoperatively and postoperatively, thereby benefiting postoperative recovery.

Administration of LncRNA XIST siRNA Into AMI Rats

To explore the influences of lncRNA XIST on AMI rats, lncRNA XIST siRNA was injected into the rat tail vein at 40 nmol/mL once every 2 days, with lncRNA XIST siRNA-negative control (NC) as the blank control. Target sequences of lncRNA XIST siRNA were listed in Table I. The expression level of XIST in rat heart tissues in each group was determined using RT-PCR.

Table I. LncRNA XIST siRNA and XIST primer sequences.

Gene	Sequence
LncRNA XIST siRNA	GACCUUGUCAUGUGGAUAUTT AUAUCCACAUGACAAGGUCTT
LncRNA XIST siRNA-NC	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT
XIST	CGGGTCTCTTCAAGGACATTTAGCC GCACCAATACAGAGGAATGGAGGG

Detection of Morphopathological Changes in Rat Heart Tissues Via HE Staining

The rat heart tissues were taken, fixed in 4% paraformaldehyde solution overnight, dehydrated using 80% methanol solution and embedded. Then, the embedded tissues were prepared into 5 μm-thick sections, and the sections were soaked in xylene solution for 30 min for de-paraffinization, and in 100%, 90%, 80%, and 70% ethanol successively each for 1 min. Subsequently, the resulting sections were stained with hematoxylin for 3 min, flushed using running water for 5 min, stained with eosin solution for 3 min. Finally, the tissue sections were sealed in neutral resin.

Evaluation of Cell Apoptosis in Rat Heart Tissues Via TUNEL Assay

The rat heart tissue sections were obtained, fixed in 4% paraformaldehyde for 30 min, and immersed in 0.2% Triton X-100 permeabilization buffer for 15 min. Then, each sample was added dropwise with 100 μL of TdT reaction solution and reacted at 37°C in the dark for 1 h. Subsequently, the products were added dropwise with reaction stop solution and rinsed with phosphate-buffered saline (PBS) at room temperature for 5 min × 3 times. Finally, staining was observed under a fluorescence inverted microscope.

Measurement of mRNA Expressions of MiR-449 and Notch1 in Rat Heart Tissues Via RT-PCR

A total of 50 mg of tissues were taken, lysed with 1 mL of TRIzol lysis buffer (Invitrogen, Carlsbad, CA, USA), bathed on ice for 5 min for complete lysis and centrifuged at a low temperature and 12,000 rpm for 10 min. Then, the supernatant was added with 200 μL of chloroform solution, and the mixture was shaken, allowed to stand at room temperature for 15 min, and centrifuged for 15 min. The upper-layer aqueous phase was transferred into new Eppendorf (EP) tubes (Hamburg, Germany), and added with 500 μL of isopropanol for centrifugation. The precipitant was harvested and dissolved using TE buffer. After the concentration of total RNAs was determined using Nano-200 ultra-micro nucleic acid analyzer, PCR was performed according to the instructions, with the primers shown in Table II. Finally, gel electrophoresis was conducted, and the optical density of bands was analyzed using ImageJ software.

Statistical Analysis

Measurement data in each group were presented as mean ± standard deviation. Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was employed for data analysis, and statistical graphs were plotted. Differences between 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). $p<0.05$ represented the statistically significant difference.

Results

The AMI Rat Model Was Prepared Successfully

After the establishment of the AMI rat model, the rats had decreased food intake, mental status decline, and cool limbs, while those in

Table II. Primer sequences of miR-449 and Notch1.

Gene	Sequence
MiR-449	TGGCAGTGTATTGTTAGCTGGT AGGCAGTGTATTGTTAGCTGGC
Notch1	GATGGCCTCAATGGGTACAAG TCGTTGTTGTTGATGTCACAGT
β-actin	GGCTGTATTCCCCTCCATCG CCAGTTGGTAACAATGCCATGT

Table III. LVESV, LVEDV, LVDs, and LVDd of rats (n=30).

List	Sham	Model
LVESV (mm)	0.06 ± 0.09	0.21 ± 0.04*
LVEDV (mm)	0.43 ± 0.05	0.76 ± 0.08*
LVDs (mm)	3.16 ± 0.25	6.47 ± 0.29*
LVDd (mm)	5.61 ± 0.32	8.12 ± 0.22*

Note: *$p < 0.05$: Model group *vs*. Sham group.

Sham group exhibited favorable food intake and mental status. The left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), left ventricular systolic diameter (LVDs), and left ventricular diastolic diameter (LVDd) of rats were measured through small animal echocardiography. It is shown that compared with those in Sham group, rat LVESV, LEDV, LVDs, and LVDd were significantly elevated in Model group (*p<0.05, *p<0.05, *p<0.05, *p<0.05, respectively) (Table III), illustrating that the AMI rat model was established successfully.

The Expression of XIST in the Heart Tissues of AMI Rats Was Effectively Downregulated

The mRNA level of XIST in rat heart tissues was determined using RT-PCR, and the RT-PCR bands were shown in Figure 1A. The analysis results showed that the mRNA level of XIST in rat heart tissues in Model group was evidently higher than that in Sham group (*p<0.05), and compared with that in Model group, its level declined markedly in XIST siRNA group ($^{\#}p$<0.05) (Figure 1B).

Knockdown of LncRNA XIST Could Improve the Pathological Morphology of Heart Tissues in the AMI Model Rats

The rats in Sham group had a ruddy heart with normal function, and orderly arranged myocardial cells with clear structure and no adhesion. In comparison, those in Model group showed weakened function and enlarged volume of the heart, and disorderedly arranged myocardial cells in different sizes with massive inflammatory cell infiltration. After intervention with lncRNA XIST siRNA, the pathological injuries in the rat heart were greatly alleviated (Figure 2).

Knockdown of LncRNA XIST Could Lower the Apoptosis Rate of Cells in Heart Tissues in the AMI Model Rats

TUNEL staining results (Figure 3A) revealed that the apoptosis rate of cells in rat heart tissues was notably elevated in Model group compared with that in Sham group (*p<0.05), while it was evidently lowered in XIST siRNA group compared with that in Model group ($^{\#}p$<0.05) (Figure 3B). It is suggested that knockdown of lncRNA XIST can repress the cardiac cell apoptosis in AMI model rats.

Knockdown of LncRNA XIST Upregulated MiR-44 Level and Downregulated Notch1 Level

According to the statistical results of the RT-PCR bands (Figure 4A), rats in Model group had a lower level of miR-449, but a higher level of Notch1 in heart tissues than Sham group

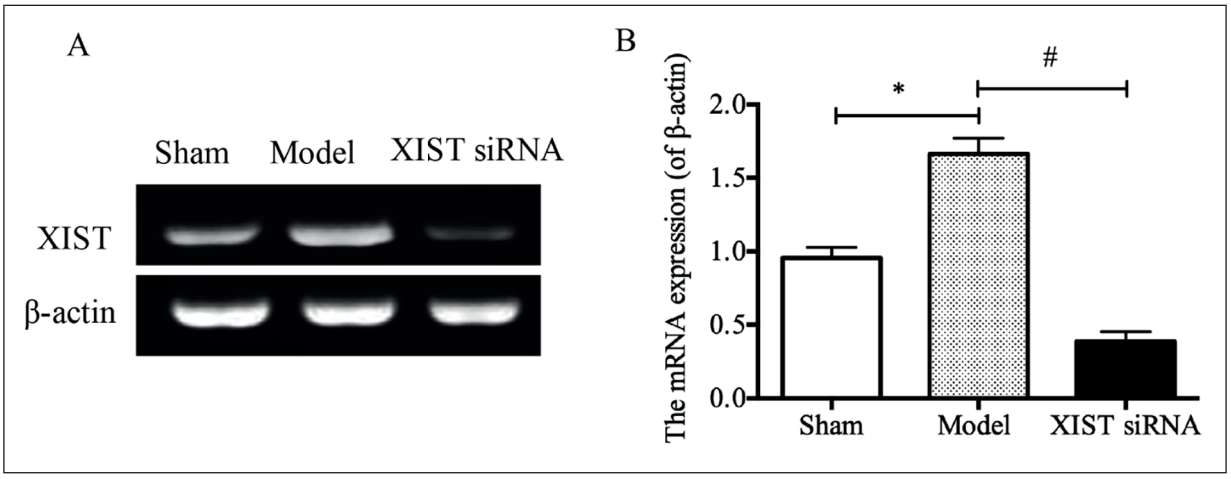

Figure 1. The mRNA level of XIST in rat heart tissues in each group. **A,** RT-PCR bands. **B,** Statistical graph of bands (*p<0.05, $^{\#}p$<0.05).

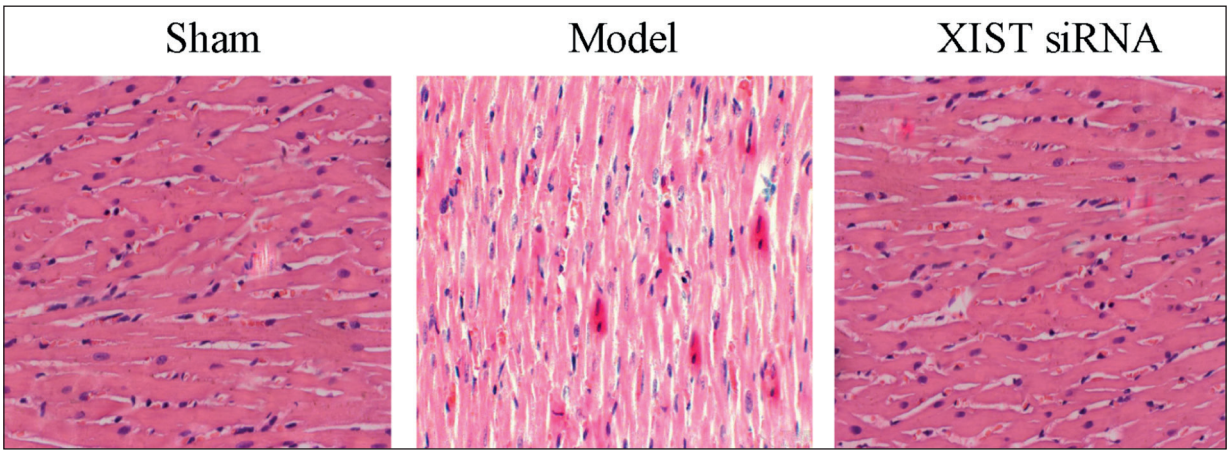

Figure 2. Pathological injuries in rat heart tissues (×20).

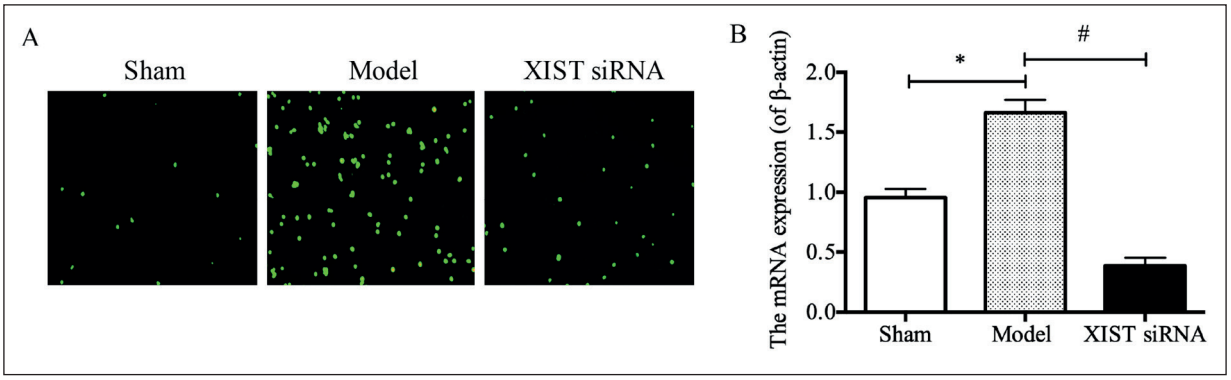

Figure 3. Cell apoptosis in rat heart tissues evaluated *via* TUNEL assay. **A,** TUNEL staining results (×20). **B,** Cell apoptosis rate (*p<0.05, #p<0.05).

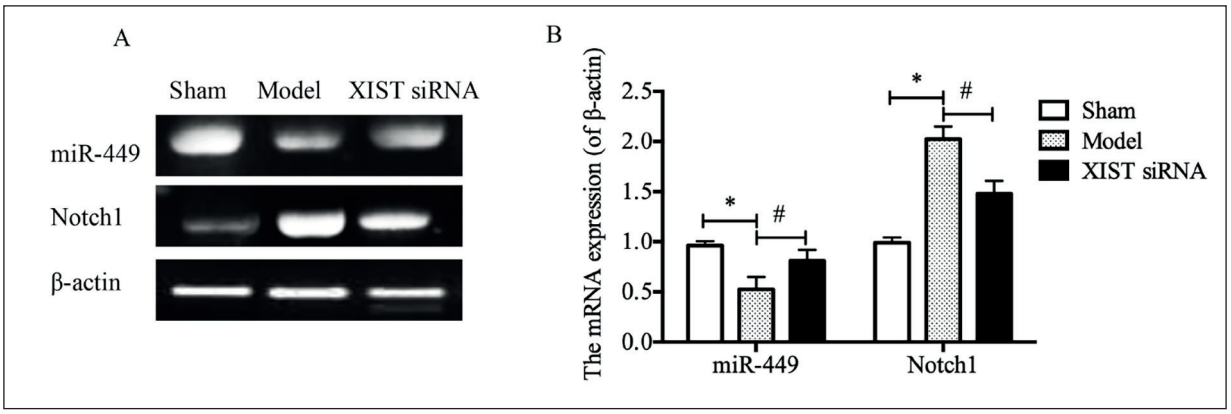

Figure 4. The mRNA levels of miR-449 and Notch1 in rat heart tissues in each group determined *via* RT-PCR. **A,** RT-PCR bands. **B,** The statistical graph of bands (*p<0.05, #p<0.05).

(*p<0.05, *p<0.05). Rats in XIST siRNA group had a higher level of miR-449, but a lower level of Notch than Model group ($^{\#}p$<0.05, $^{\#}p$<0.05) (Figure 4B).

Discussion

The morbidity rate of AMI in 30-80-year-old Chinese people is 70% in males and 25% in females, respectively. Despite the decline in the mortality rate of AMI in the past decade, 80% of AMI patients die because of the absence of in time treatment within 1 h of onset. AMI poses a huge physical and mental pain to patients and heavy economic burden on their family members[13]. Currently, the main clinical treatment measures for AMI include surgery, intervention, and medications, but these treatment strategies can only prolong the disease, instead of curing it[14]. Therefore, understanding the pathogenesis of AMI has important implications for its treatment.

Previous research demonstrated that protein-coding genes are considered as the majority of human genomes. However, the roles of non-coding RNAs in the prevention and treatment of diseases have received growing attention in recent years[15]. LncRNAs, a class of RNA fragments with the length exceeding 200 nt, is extensively distributed in cells and tissues and possesses tissue specificity. Li et al[16] found that knockdown of lncRNA Mirt1 can alleviate AMI in mice by inactivating the NF-κB signaling pathway. Additionally, Li et al[17] discovered through the *in vitro* experiment that over-expression of lncRNA Gm2691 can inhibit the myocardial cell apoptosis in newly born rats receiving anaerobic-anoxic processing. LncRNA Gm2691 is able to ameliorate the cardiac function of AMI model rats and raise the survival rate of myocardial cells according to the results of the *in vivo* experiment. Another study revealed that lncRNAs exert a protective effect against AMI. Yu et al[18] proposed that lncRNA H19 is able to attenuate AMI through inhibiting myocardial cell apoptosis by regulating miR-29 level. The above study results corroborated that the aberrant apoptosis of myocardial cells plays a crucial role in the development and progression of AMI.

Thus, the AMI rat model was prepared by ligating the anterior descending coronary artery[19], and the cardiac function of rats in Sham group and Model group was detected using the small animal ultrasound system in this study. According to the results (Table III), Model group had significantly worse LVESV, LVEDV, LVDs, and LVDd than those in Sham group, suggesting the declined cardiac function in rats of Model group. Moreover, downregulated XIST in rats of XIST siRNA group suggested the successful intervention in XIST expression. Subsequently, HE staining was performed to detect the pathological injuries in rat heart tissues in each group, and it was found that the pathological injuries in the heart tissues of AMI rats were substantially relieved after knockdown of lncRNA XIST. Furthermore, the apoptosis in rat heart tissues in each group was evaluated *via* TUNEL staining, and according to the results, the apoptosis rate of cells in rat heart tissues was significantly raised in Model group in comparison with that in Sham group, and it was evidently lowered after silence of lncRNA XIST. The mRNA levels of miR-449 and its downstream Notch1 in rat heart tissues were also determined *via* RT-PCR. The results manifested that miR-449 was upregulated and Notch1 was downregulated in rat heart tissues in XIST siRNA group compared with those in Model group. Likewise, Liu et al[20] demonstrated that the level of Notch1 rises in the heart tissues of AMI model rats, and myocardial hypertrophy and fibrosis in rats can be prevented through the regulation of the Notch signaling pathway. The results of the present study indicated that knockdown of lncRNA XIST can repress the myocardial cell apoptosis to alleviate the cardiac pathological injuries in the AMI model rats and improve the cardiac function through downregulating miR-449 level.

Conclusions

In summary, these results showed that knockdown of lncRNA XIST is able to inhibit myocardial cell apoptosis and relieve the pathological injuries in AMI model rats by downregulating miR-449, thereby enhancing cardiac function. LncRNA XIST is likely to be a potential therapeutic target for AMI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

Medical Research Projects of China Aerospace Science and Industry Corporation (No: 2017-LCYL-003).

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