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LncRNA-P21 suppresses apoptosis of myocardial cells in rats with acute myocardial infarction *via* regulating Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the expression of long non-coding ribonucleic acid (lncRNA)-p21 in rats with acute myocardial infarction (AMI) and its influences on the viability and apoptosis of myocardial cells.

MATERIALS AND METHODS: Sprague-Dawley rats were utilized to establish the AMI model. Myocardial tissues were extracted, and myocardial cells were isolated. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression of lncRNA-p21. Subsequently, myocardial cells of AMI rats were cultured and transfected with lncRNA-p21 or small interfering (si)-lncRNA-p21. 48 h later, cell proliferation was determined using Cell Counting Kit-8 (CCK-8). Caspase-3 kit was applied to examine the changes in Caspase-3 after myocardial cell transfection. Moreover, Western blotting assay was performed to measure the protein expressions of apoptosis-associated indexes [B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax)] and key members of the Wnt signaling pathway (Wnt-5a and β-catenin).

RESULTS: The expression of lncRNA-p21 decreased significantly in myocardial tissues ($p<0.01$). However, the mRNA expression of inflammatory factors was significantly upregulated in AMI group compared with those in Sham group ($p<0.01$). Compared with Vehicle group, LncRNA-p21 group exhibited remarkably enhanced myocardial cell viability ($p<0.05$). However, Si-lncRNA-p21 group weakened myocardial cell viability ($p<0.05$). The expression of Caspase-3 in myocardial cells was reduced in LncRNA-p21 group ($p<0.05$), but remarkably rose in Si-lncRNA-p21 group ($p<0.05$) in comparison with Vehicle group. In addition, the protein expressions of Wnt-5a and β-catenin in myocardial cells were significantly higher in LncRNA-p21 group ($p<0.05$), whereas were lower in Si-lncRNA-p21 group ($p<0.05$) than those in Vehicle group.

CONCLUSIONS: LncRNA-p21 was lowly expressed in myocardial tissues of AMI rats. Furthermore, it affected the proliferation, apoptosis and inflammation level of myocardial cells in AMI rats by activating the Wnt/β-catenin signaling pathway.

Key Words:
Myocardial infarction, LncRNA-p21, Proliferation, Apoptosis, Wnt/β-catenin signaling pathway.

Introduction

Acute myocardial infarction (AMI) is one of the most common causes of death worldwide[1], in which coronary thrombosis is the major precipitating factor[2]. Over the past few decades, there has been a significant short-term improvement in the survival of MI. However, the degree of left ventricular (LV) dysfunction is associated with the development of heart failure and long-term death rate. Poor LV myocardial remodeling after MI will even lead to LV wall thinning, LV dilatation and systolic dysfunction. Myocardial remodeling is considered as an important cause of congestive heart failure[3-5]. Existing drugs and therapeutic methods can only slow down the progression of disease at most. Therefore, there is an urgent need to find and develop alternative treatment strategies due to the deficiency of available drugs, so as to prevent myocardial remodeling following MI and the progression into heart failure.

Numerous kinds of ribonucleic acids (RNAs) have been transcribed from human genomes. However, protein-coding sequences only accounts for a very small proportion of total transcripts, and the remaining transcripts are non-coding RNA (ncRNA) sequences. Based on the size, ncRNAs can be classified into small ncRNAs with a length of less than 200 nt, represented by micro RNAs (miRNAs), and long ncRNAs (lncRNAs) with over 200 nt in length[6]. LncRNAs can serve as

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main regulators of gene expressions in various biological functions and disease development, including malignant tumors[7]. LcRNAs act as ceRNAs to capture the response elements of target miRNAs and affect their post-transcriptional regulation level[8,9]. They have been confirmed to exert extensive regulatory effects on AMI. LncRNA XIST inhibits hypoxia-induced myocardial cell apoptosis *via* mediating miR-150-5p/B-cell lymphoma-2 (Bcl-2)-associated X protein (Bax) in AMI[10]. LncRNA NEAT1 promotes the apoptosis and suppresses the proliferation of myocardial cells by regulating miR-129-5p[11]. LncRNA-p21, as a synergetic repressor of p53[12], activates p21 to stimulate the expression of its target gene PRC2, thus influencing the epigenetic regulation of cells[13]. In the case of prostate cancer, lncRNA-p21 has been identified as a novel biomarker. It alters the anti-androgen-induced neuroendocrine differentiation of prostate cancer *via* modulating the EZH2/STAT3 signaling pathway[14,15]. As for osteosarcoma (OS), lncRNA-p21 inhibits the proliferation of OS cells through the miR-130b/PTEN/AKT signaling pathway[16]. However, few studies have elucidated the role of lncRNA-p21 in the pathogenesis of AMI.

The pathological process of MI injury includes three major phases: inflammatory response, granulation tissue formation and fibrosis. Wnt signal transduction is triggered in the pathological process of MI injury. According to the quantitative analysis of Wnt protein expression, Wnt-2, Wnt-4, Wnt-10b and Wnt-11 are vigorously upregulated at 5 d after MI[17]. This demonstrates that the persistent activation of Wnt is related to the pathological stages following MI, including inflammation, angiogenesis and fibrosis. Wnt-5a, a subtype of Wnt proteins, is specifically expressed in myocardial cells[18]. It can also facilitate the release of interleukin-1 (IL-1), IL-6 and IL-8 from mononuclear cells, illustrating the pro-inflammatory effects of the Wnt signaling[19]. Though the Wnt signaling pathway plays a vital role in AMI, there is still no literature report about the regulation of AMI progression by lncRNA-p21 by acting on the Wnt signaling pathway. In this research, it was found that lncRNA-p21 expression significantly decreased in AMI rats. Overexpression of lncRNA-p21 was accompanied with changes in the Wnt signaling pathway components. Furthermore, the influences of lncRNA-p21 on AMI rats were verified using subsequent molecular biology experiments. Our findings explained to some extent that the upregulation of lncRNA-p21 expression in AMI rats could enhance the proliferation and invasion and attenuate the apoptosis of cancer cells, thus affecting the progression and prognosis of AMI. It was proposed in this research that lncRNA-p21 was expected to become a potential target of early AMI diagnosis.

Materials and Methods

Preparation of AMI Model in Rats

A total of 20 male Sprague-Dawley rats weighing about 250 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All rats were randomly divided into two groups, including: AMI group and Sham group. The model of AMI was successfully established by ligating the left anterior descending coronary artery. The rats were anesthetized by isoflurane, and the chest was shaved and cleaned using alcohol. Next, the thoracic cavity was cut opened through a left parasternal incision, and the heart was exposed from the left third and fourth intercostal spaces. Afterwards, the pericardium was opened, and the coronary artery was ligated by sutures. In Sham group, the thoracic cavity was sutured after opening, without ligation of the coronary artery. All the rats were sacrificed by euthanasia at 6 h after treatments, so as to obtain cardiac tissues. Finally, total RNAs were extracted and myocardial cells were isolated. This study was approved by the Animal Ethics Committee of Tengzhou Central People's Hospital Animal Center.

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

Total RNAs were extracted from tissues or cells according to the instructions of total RNA kit (Tiangen Biotech, Beijing, China). The concentration and purity of extracted RNAs were measured at 260/280 nm using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, extracted RNAs were synthesized into cDNAs by M-MLV reverse transcriptase. The primers were synthesized by GenScript (Piscataway, NJ, USA). SYBR-Green reagent (Solarbio, Beijing, China) was applied to quantify total RNAs on Exicycler 96 instrument (Bioneer, Daejeon, South Korea). The relative expressions of lncRNA-p21 and genes were calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were shown in Table I.

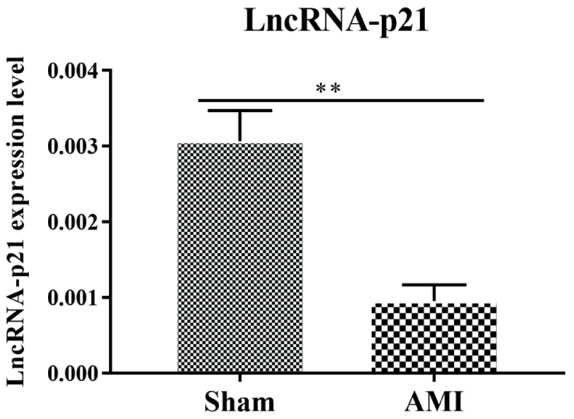

Figure 1. LncRNA-p21 expression decreased significantly in cardiac tissues of AMI rats. Note: The expression of lncRNA-p21 was down-regulated compared with that in Sham group (**p<0.01).

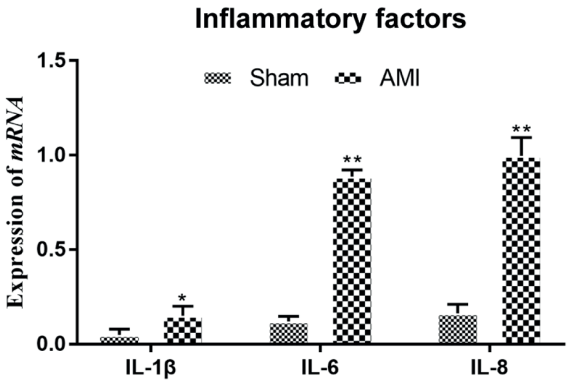

Figure 2. Expression levels of inflammatory factors were up-regulated in cardiac tissues of AMI rats. Note: *p<0.05 & **p<0.01 *vs.* Sham group.

Cell Culture and Transfection

Myocardial cells of AMI rats were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 UI/ml penicillin and 100 μg/ mL streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified incubator under 5% CO_2 at 37°C. Subsequently, myocardial cells were seeded into 24-well plates, with untreated myocardial cells as normal controls. Additionally, myocardial cells were transfected with empty vehicle (Vehicle group), lncRNA-p21 overexpression vehicle (LncRNA-p21 group), lncRNA-p21 silencing vehicle [small interfering (Si)-lncRNA-p21 group] and Dickkopf (DKK; inhibitor of the Wnt signaling pathway, DKK group), respectively. All the vehicles and inhibitor were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). At 24 and 48 h after transfection, the cells were collected to extract total RNAs and proteins for subsequent analyses.

Determination of Cell Proliferation Via Cell Counting Kit-8 (CCK-8) Assay

After 24 h of continuous transfection, the cells were harvested and inoculated into 96-well plates. Next, 10 μL of CCK-8 assay solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added into each well at 12, 24 and 48 h, respectively, followed by incubation for 2 h in the dark. Optical density at 450 nm (OD_{450}) was measured using a MultiSkan FC micro-plate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA), and cell growth curves were finally plotted.

Detection of Caspase-3 Activity

The cells in each group were utilized to detect the activity of Caspase-3 in accordance with the kit instructions, and OD_{450} was determined by a micro-plate reader.

Table I. Primer sequences for qRT-PCR.

Index	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
LncRNA-p21	CCCGGGCTTGTCTTTTGTT	GAGTGGGTGGCTCACTCTTCTC
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-8	CAAGGCTGGTCCATGCTCC	TGCTATCACTTCCTTTCTGTTGC
Wnt-5a	CAACTGGCAGGACTTTCTCAA	CATCTCCGATGCCGGAACT
β-catenin	ATGGAGCCGGACAGAAAAGC	CTTGCCACTCAGGGAAGGA

Data recorded from the first case of appearance of SARS-COV-2 in San Francisco, from March 20, 2020 to Sept 16, 2020. The values are presented in Mean and SEM.

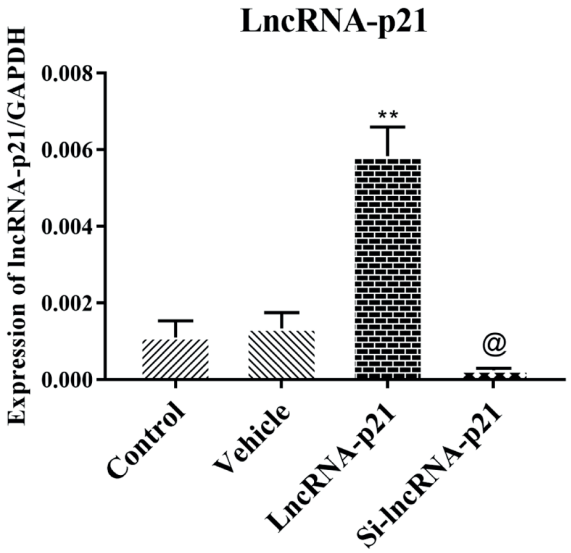

Figure 3. Expression of lncRNA-p21 in myocardial cells after transfection. Note: $^{**}p<0.01$: The difference was statistically significant compared with that in Vehicle group, and $^{@}p<0.05$: the difference was statistically significant compared with that in Vehicle group.

Western Blotting

After washing with PBS, 200 μL of lysis buffer was added into each well of cells. After sufficient shaking, the cells were scrapped and subjected to ultrasonication and centrifugation, so as to obtain total protein samples. Protein concentration was measured by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA), which was maintained uniform at a constant volume. Subsequently, extracted proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Next, the membranes were incubated with primary antibodies of anti-Bax (Abcam, Cambridge, MA, USA, diluted at 1:2000), anti-Bcl-2 (Abcam, Cambridge, MA, USA, diluted at 1:2000), anti-Wnt-5a (Abcam, Cambridge, MA, USA, diluted at 1:1000), and anti-β-catenin (Abcam, Cambridge, MA, USA, diluted at 1:2500) at 4°C overnight. On the next day, the membranes were incubated with horse radish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (CST, Danvers, MA, USA, diluted at 1:2000) at room temperature for 1 h. Immuno-reactive bands were finally exposed using enhanced chemiluminescence (ECL) Plus Western blotting reagent.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was employed for statistical analysis. Numerical variables were expressed as mean ± standard deviation. Differences between two groups were analyzed by using the Student's t-test. $p<0.05$ was considered statistically significant.

Results

LncRNA-p21 Expression Was Downregulated in Cardiac Tissues of AMI Rats

QRT-PCR analysis found that the expression of lncRNA-p21 in cardiac tissues was significantly

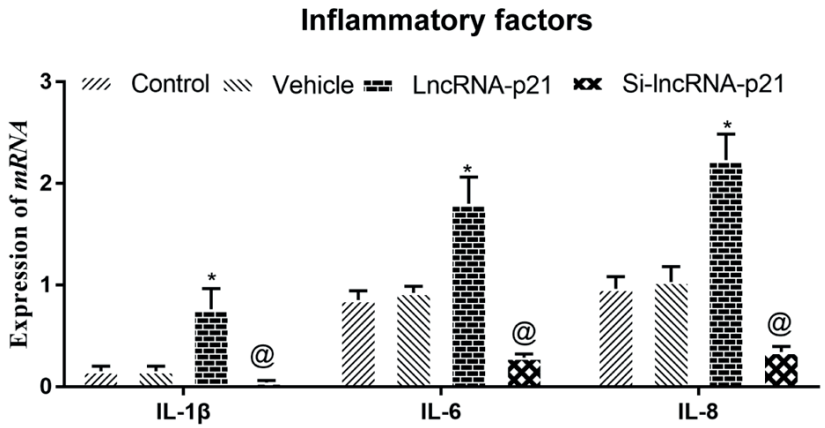

Figure 4. Expression of inflammatory factors in myocardial cells after transfection. Note: $^{*}p<0.05$: The difference was statistically significant compared with that in Vehicle group, and $^{@}p<0.05$: the difference was statistically significant compared with that in Vehicle group.

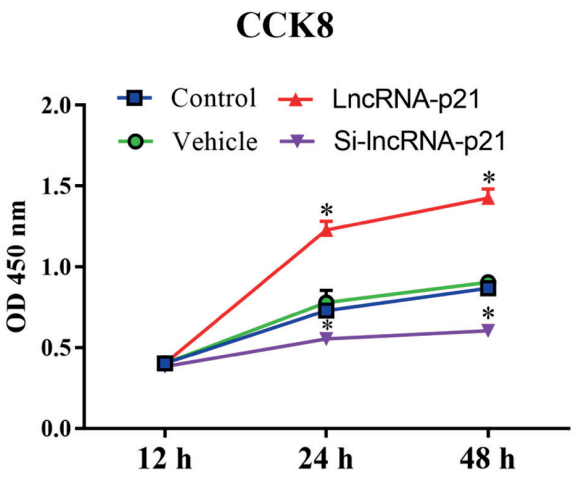

Figure 5. LncRNA-p21 expression influenced myocardial cell proliferation of AMI rats. Note: *p<0.05: The difference was statistically significant compared with that in Vehicle group.

downregulated in AMI group compared with that in Sham group (p<0.01) (Figure 1).

Inflammation Level Was Elevated in Cardiac Tissues of AMI Rats

QRT-PCR results manifested that the messenger RNA (mRNA) expressions of inflammatory factors IL-1β, IL-6, and TNF-α in cardiac tissues were significantly upregulated in AMI group in comparison with those in the Sham group (p<0.01) (Figure 2).

Myocardial Cell Transfection Affected LncRNA-p21 Expression in AMI Rats

The expression of lncRNA-p21 in myocardial cells after transfection was detected *via* qRT-PCR. The results indicated that the expression of lncRNA-p21 remarkably increased in LncRNA-p21 group (p<0.01), but notably decreased in Si-lncRNA-p21 group (p<0.05) compared with Vehicle group (Figure 3).

LncRNA-p21 Positively Regulated Inflammation Level in Myocardial Cells of AMI Rats

In order to identify the expression of lncRNA-p21 in transfected myocardial cells, the mRNA expressions of inflammatory factors in transfected myocardial cells were determined by qRT-PCR. It was shown that the mRNA expressions of IL-1β, IL-6 and IL-8 were evidently higher in LncRNA-p21 group (p<0.05), but lower in Si-lncRNA-p21 group (p<0.05) than those in Vehicle group (Figure 4).

LncRNA-p21 Expression Influenced Myocardial Cell Proliferation of AMI Rats

The changes in myocardial cell proliferation were examined using CCK-8 after transfection. The results manifested that OD_{450} values at 24 and 48 h were markedly upregulated in LncRNA-p21 group (p<0.05), whereas were reduced distinctly in Si-lncRNA-p21 group (p<0.05). These findings suggested that lncRNA-p21 overexpression promoted the proliferation of myocardial cells (Figure 5).

LncRNA-p21 Expression Influenced Myocardial Cell Apoptosis of AMI Rats

The expression of Caspase-3 was measured to observe the changes in myocardial cell apoptosis after transfection. Compared with Vehicle group, LncRNA-p21 group had evidently weakened activity of Caspase-3 in myocardial cells (p<0.05, Figure 6A). However, Si-lncRNA-p21 group

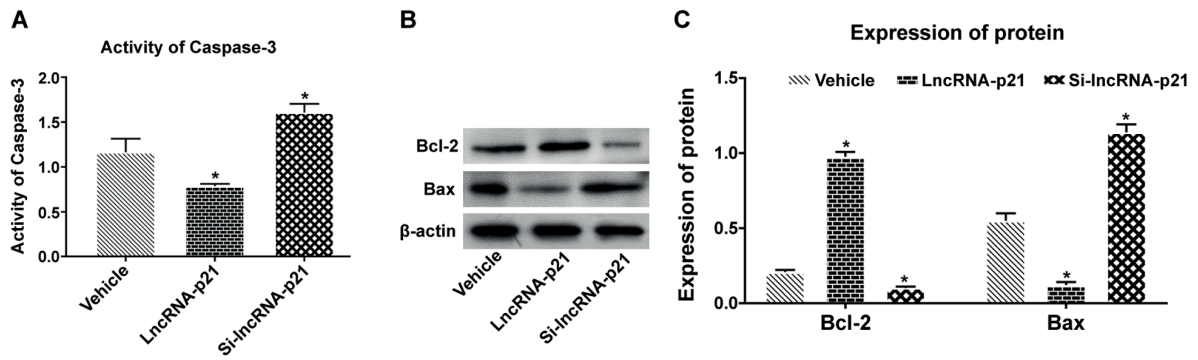

Figure 6. Comprehensive impact of lncRNA-p21 expression on apoptosis-associated indexes in myocardial cells of AMI rats. Note: *p<0.05: The difference was statistically significant compared with that in Vehicle group.

exhibited strengthened activity of Caspase-3 (p<0.05, Figure 6A). Subsequent Western Blotting results demonstrated that the protein level of Bax was lower, while Bcl-2 was higher in myocardial cells of LncRNA-p21 group than those in Vehicle group (p<0.05, Figure 6B and 6C). However, opposite trends were observed in Si-lncRNA-p21 group (p<0.05, Figure 6B and 6C), implying that lncRNA-p21 overexpression repressed myocardial cell apoptosis.

LncRNA-p21 Positively Regulated the Wnt Signaling Pathway

To determine the variations in the Wnt signaling pathway after transfection in myocardial cells of AMI rats, the levels of Wnt-5a and β-catenin were tested. According to the results, the mRNA expressions of Wnt-5a and β-catenin in myocardial cells were remarkably higher in LncRNA-p21 group (p<0.05, Figure 7A), but were lower in Si-lncRNA-p21 group (p<0.05, Figure 7A) than Vehicle group. Subsequent results indicated that LncRNA-p21 group displayed significantly higher protein expressions of Wnt-5a and β-catenin in myocardial cells (p<0.05, Figure 7B and 7C). However, Si-lncRNA-p21 group exhibited significantly lower expressions of Wnt-5a and β-catenin (p<0.05, Figure 7B and 7C) than Vehicle group. All these findings illustrated that lncRNA-p21 overexpression activated the Wnt signaling pathway.

Discussion

In this research, lncRNA-p21 was lowly expressed in myocardial tissues of AMI rats. Overexpression of lncRNA-p21 activated the Wnt/β-catenin signaling pathway to up-regulate the inflammation level and viability of myocardial cells in AMI rats, as well as inhibit cell apoptosis at the same time. It can be seen that lncRNA-p21 can indicate the inflammation level in AMI to some extent, serving as an early diagnostic marker for AMI. Inflammatory response plays a crucial role in the pathological process of AMI. Previous studies have denoted that lncRNA-p21 is lowly expressed in patients with coronary heart disease, but its mechanism of action remains unclear. The results in this research showed that lncRNA-p21 was able to promote the proliferation and suppress the apoptosis of myocardial cells of AMI rats.

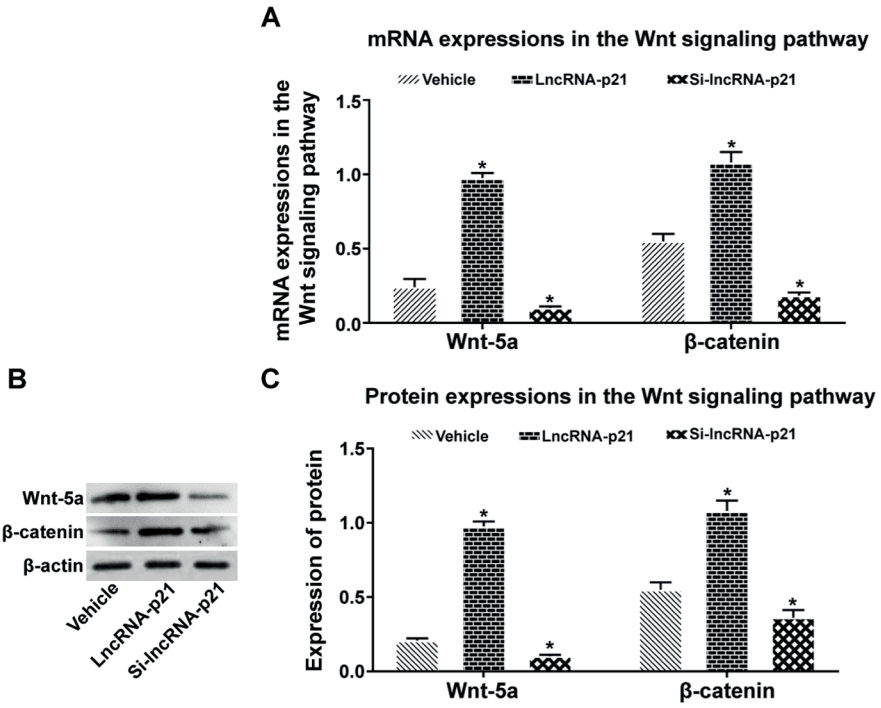

Figure 7. Overexpressed lncRNA-p21 activated the Wnt/β-catenin signaling pathway. Note: *p<0.05 *vs.* Vehicle group.

LncRNAs serve as crucial players in cell proliferation, differentiation, migration, apoptosis, and immune responses by affecting the expressions of deoxyribonucleic acid (DNA), RNA and protein as well as their interactions[17,20,21]. LncRNA-p21 is located at about 15 kb upstream of cell cycle regulatory gene p21/CDKN1A, with a length of nearly 3.0 kb. It acts as a novel regulator of cell proliferation, apoptosis and DNA damage response. Meanwhile, lncRNA-p21 plays an important role in human development and evolution, which also participates in the occurrence and development of tumors[22,23]. Numerous studies have reported that lncRNA-p21 is correlated with the progression of skin tumor, prostate cancer and chronic lymphocytic leukemia[14,24,25]. LncRNA-p21 has also been observed to be involved in the process of atherosclerosis[26]. LncRNA-p21 has prominently downregulated expression in patients with coronary heart disease. It can bind to p53 to restrain MDM2 and regulate the proliferation and apoptosis of vascular smooth muscle cells. The findings elucidate that lncRNA-p21 is a promising new therapeutic target of cardiovascular diseases. The Wnt/β-catenin signaling pathway exerts pivotal effects in normal life processes and disease progression, becoming an emerging therapeutic method and target of various diseases[27]. LncRNA-p21 targets the Wnt/β-catenin signaling pathway to interfere in the occurrence of multiple diseases. Notably, lncRNA-p21 represses the Wnt/β-catenin signaling pathway by competitive binding to miR-17-5p in hepatocytes[28]. Nevertheless, the exact role of lncRNA-p21 in AMI has not been clarified yet. Therefore, whether lncRNAs were implicated in the pathogenesis of AMI *via* the Wnt/β-catenin signaling pathway was explored in this research.

Given the limitations of animal experiments, the role of lncRNA-p21 as a diagnostic criterion of AMI needs to be further verified by clinical samples. In addition, lncRNA-p21 is capable of working and making its regulatory mechanisms complex by controlling more miRNAs or target genes. Hence, two or more human myocardial cell lines and clinical samples of AMI should be used in more in-depth experiments to explore the biological effects of lncRNA-p21 in AMI patients, so as to comprehensively understand the mechanism of lncRNA-p21 in AMI.

Conclusions

In summary, lncRNA-p21 is significantly downregulated in AMI rats. Overexpression of lncRNA-p21 facilitates the proliferation and suppresses the apoptosis of impaired myocardial cells by activating the Wnt/β-catenin signaling pathway. LncRNA-p21 can be taken as an inducer of myocardial cell recovery after injury. Therefore, interfering in the expression of lncRNA-p21 in AMI patients in some degrees and by certain means may partly alleviate the progression of AMI. The novelty of this study was that lncRNA-p21 may become a target of early diagnosis and treatment of AMI. All our findings provide definite references for the prevention and treatment of the disease.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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