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LncRNA ROR facilitates myocardial fibrosis in rats with viral myocarditis through regulating C-Myc expression

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Abstract. – OBJECTIVE: The aim of this study was to explore the effect of long non-coding ribonucleic acid (lncRNA) ROR on myocardial fibrosis in rats with viral myocarditis by regulating c-myc expression.

MATERIALS AND METHODS: A total of 120 healthy male rats were randomly divided into three groups, including: blank control group (group N, healthy rats, n=40), model control group (group M, viral myocarditis, n=40), and group P (lncRNA ROR + viral myocarditis, n=40). At 5, 10, and 15 d after modeling, the rats were sacrificed. Meanwhile, the left ventricular mass index (LVMI), the left ventricular weight/tibial length (LVW/TL) and the heart mass index (HMI) were measured, respectively. The myocardial tissues of rats were observed *via* staining. The protein and mRNA expressions of the transforming growth factor-β (TGF-β) and c-myc in myocardial tissues were detected *via* Western blotting and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), respectively. Moreover, the level of the serum interleukin-6 (IL-6) was detected *via* enzyme-linked immunosorbent assay (ELISA).

RESULTS: Compared with group N, HMI, LVMI, and LVW/TL in group M significantly increased at each time point ($p<0.01$). Meanwhile, they were remarkably elevated at each time point in group P when compared with those in group M ($p<0.01$). According to the results of hematoxylin-eosin staining (HE) staining, the myocardial fibers were normal and orderly arranged in group N. Meanwhile, no inflammatory cells were observed in group N. In group M, the proliferation of a large number of inflammatory cells was observed, and the myocardial cells were disorderly arranged and became enlarged. In group P, the myocardial fibrosis was significantly severer than that of the other two groups ($p<0.05$). The results of the Masson staining revealed that there was significantly less accumulation of collagen fibers in myocardium in group N. There was a little accumulation of collagen fibers in the myocardium and fibrosis declined in group M. The myocardial fibrosis in group P remarkably increased when compared with group M ($p<0.05$). According to the results of the Western blotting, the protein expressions of c-myc and TGF-β were significantly up-regulated in group P, compared with those in group M and N ($p<0.05$). However, they were remarkably higher in group M than in group N ($p<0.05$). QRT-PCR showed that the relative messenger ribonucleic acid (mRNA) expressions of c-myc and TGF-β were remarkably up-regulated in group P when compared with those in group M and N, showing statistically significant differences ($p<0.05$). Moreover, they were significantly higher in group M than those in group N ($p<0.05$). Besides, the expression level of serum IL-6 remained highest in group P ($p<0.01$).

CONCLUSIONS: LncRNA ROR up-regulates the expression of c-myc and increases the level of serum IL-6, thereby facilitating the proliferation and differentiation of cardiac fibroblasts.

Key Words:
LncRNA ROR, Cardiac fibroblasts, C-myc, TGF-β, IL-6.

Introduction

Currently, the most significant features of the viral myocarditis (VMC) are the structural changes in myocardial fibers. This can lead to pathological reactions, such as myocardial remodeling, myocardial ischemia, pressure load, and volume load[1]. The main pathology of VMC in chronic phase is the occurrence and development of fibrosis in the myocardium, commonly known as myocardial fibrosis[2]. During the development of

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myocardial fibrosis, the collagen fibers accumulate excessively in normal myocardial tissues. Ultimately, this increases the original concentration in myocardial tissues and promotes the differentiation of collagens. This is also one of the major causes of the disease[3]. The key to VMC treatment is to regulate the activity of cardiac interstitial collagenase, to modulate the proliferation and apoptosis of myocardial fibroblasts, and to inhibit the increase of extracellular matrix by controlling cytokines and other related signals, thereby effectively improving myocardial fibrosis[4].

Long non-coding ribonucleic acids (lncRNAs) are generally considered as a kind of long-chain RNAs without protein-encoding function. They have been confirmed to be closely related to human heart diseases. Current investigations[5,6] have found that lncRNAs are involved in body growth, development, reproduction, aging, apoptosis, and other changes. The c-myc signaling pathway is involved in cell proliferation, growth, and differentiation, as well as gene expression. Studies have demonstrated that the c-myc signaling pathway participates in the occurrence and development of myocardial fibrosis. During this process, it is involved in inflammatory response and imbalance of cytokines, closely associated with the heart[7]. Transforming the growth factor-β (TGF-β), which regulates the c-myc signaling pathway, may induce changes in myocardial cells, activate myocardial inflammatory factors, and promote the proliferation of myocardial cells. Myocardial fibrosis affects the hardness of heart wall, reduces the adaptability, and even leads to abnormalities in cardiac contraction and relaxation[8,9]. However, the mechanism of lncRNA ROR in regulating the c-myc signaling pathway and the occurrence and development of myocardial fibrosis has not been fully elucidated. In this study, the VMC model was first established in rats. The aim of this study was to explore the role of lncRNA ROR in the occurrence and development of myocardial fibrosis through the c-myc signaling pathway. Our findings might help to provide references for the prevention and treatment of VMC.

Materials and Methods

Laboratory Animals

A total of 120 healthy male rats weighing 150-210 g were purchased from the Animal Experimental Center of Harbin Medical University. All rats were adaptively fed for 2 weeks and used for subsequent experiments. This study was approved by the Animal Ethics Committee of Qiqihar Medical University Animal Center.

Main Reagents and Instruments

The main reagents were: lncRNA ROR reagent (AlphaPharm Pty Limited, San Antonio, TX, USA), isoprenaline (ISO; Sigma, Louis, MO, USA), interleukin-6 (IL-6) kit (Novus, Littleton, CO, USA), goat anti-rabbit β-actin antibody (Abcam, Cambridge, MA, USA), ultra-speed refrigerated centrifuge (Hunan Xiangyi Laboratory Instrument Development Co., Ltd., Xiangyi, China), ELX-800 microplate reader (BioTek, Biotek Winooski, VT, USA), electrophoresis apparatus, transfer tank and gel imaging system (Beijing Liuyi Instrument Factory, Beijing, China).

Modeling and Drug Administration

The rats were randomly divided into three groups, including: blank control group (group N, healthy rats, n=40), model control group (group M, VMC, n=40), and group P (lncRNA ROR + VMC, n=40). They were given free access to food and water. The rats in group N were subcutaneously injected with 0.9% sodium chloride solution. The myocardial fibrosis model was established *via* long-term persistent induction using ISO in rats of group M and P. The specific procedure was as follows: ISO solution was injected into subcutaneous tissues on the back once a day (10 $mg \cdot kg^{-1}$ at 1 d, 5 $mg \cdot kg^{-1}$ at 2 d and 3 $mg \cdot kg^{-1}$ at 3 d). Subsequently, 5 μL of lncRNA ROR siRNA lentivirus was intrathecally injected into the rats of group P since the 2th d for 7 d.

Determination of Cardiac Indexes

After anesthesia *via* intraperitoneal injection of chloral hydrate, the rats were sacrificed. Subsequently, the heart was immediately taken out. The blood clots were washed away with pre-cooled 0.9% sodium chloride solution, and the tissues around the heart and the heart vessels were removed. Next, the heart was sucked dry with clean filter paper, and the heart weight (HW) was accurately measured. The excess tissues were then cut off, and the left ventricle was cleaned. The left ventricular weight (LVW) and the tibial length (TL) were calculated. Finally, the heart mass index (HMI; HMI=HW/BW), the left ventricular mass index (LVMI; LVMI=LVW/BW), and LVM/TL were calculated in each group, respectively.

Pathological Examination of Myocardial Tissues

Left ventricular apical tissues were first taken and fixed with 10% formaldehyde solution. Then, the tissues were dehydrated with gradient alcohol, embedded in paraffin and prepared into 4 μm-thick sections. Subsequently, the hematoxylin-eosin (HE) staining and the Masson staining were performed. The myocardial cells (arrangement, morphology, and inflammatory infiltration) and the collagen fibrosis were finally observed under a microscope (×200).

Detection of Protein Expressions of C-myc and TGF-β in Myocardial Cells Via Western Blotting

The protein expressions of TGF-β and c-myc in left ventricular myocardial cells were determined as follows. The myocardial tissues were first washed with Tris-Buffered Saline Tween 20 (TBST) twice and lysed with lysis buffer, followed by centrifugation at 11,000 rpm for 12 min (R=210 mm). The supernatant was collected, and the protein samples were obtained. Subsequently, the total proteins were separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) at 4°C overnight and transferred onto membranes. After sealing, the membranes were incubated with primary antibodies overnight. On the next day, the membranes were incubated with corresponding secondary antibody at room temperature for 1.5 h. After washing, color development, and gray scanning, the optical density of target band was determined using the gel image processing system (GelAnalzer software). The protein expression level was expressed as the ratio of the gray value of the target band to that of the internal reference β-actin.

Detection of mRNA Expressions of C-myc and TGF-β in Myocardial Cells Via qRT-PCR

25 mg of myocardial tissues were taken into an Eppendorf (EP) tube. After digestion with trypsin, the tissues were cooled and washed with Phosphate-Buffer Saline (PBS) twice. 2 mL of lncRNA ROR vector reagent was added to the tissues to extract total RNA. Subsequently, the extracted total RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA), followed by qRT-PCR amplification with lncRNA ROR vector as the template. The specific PCR conditions were as follows: 95°C for 6 min, 95°C for 28 s, 85°C for 30 s, and 75°C for 4 min, for a total of 45 cycles. β-actin was used as an internal reference. The primer sequences used in this study were shown in Table I.

Determination of Serum IL-6 Level

The levels of serum IL-6 in group N, M, and P were detected *via* enzyme-linked immunosorbent assay (ELISA; Novus, Littleton, CO, USA). After anesthesia *via* intraperitoneal injection of chloral hydrate, the abdominal aortic blood was collected. After incubation for 1 h, the samples were centrifuged at 3,000 rpm for 15 min. The serum was collected and stored at -22°C, followed by detection of IL-6 level.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 19.0 (IBM Corp., Armonk, NY, USA) software was used for all statistical analysis. The *t*-test was performed to compare the differences in cardiac indexes, pathology of myocardial tissues, and protein and mRNA expressions of c-myc and TGF-β in group N, M, and P. The pairwise comparison was achieved using analysis of variance (ANOVA), followed by the post-hoc test (Least Significant Difference). The measurement data were expressed as ($\bar{x} \pm s$). $p<0.05$ was considered statistically significant.

Table I. Primer sequences.

Primer	Gene	Sequence
c-myc	Forward	5'-TGATGACCGAGTTACTTGGAG-3'
	Reverse	5'-GGCTGGTGCTGTCTTTGC-3'
TGF-β	Forward	5'-CCCGCATCCCAGGACCTCTCT-3'
	Reverse	5'-CGGGGGACTGGCGAGCCTTAG-3'
β-actin	Forward	5'-ATATCGCTGCGCTGGTCGTC-3'
	Reverse	5'-AGGAGTCCTTCTGACCCATTC-3'

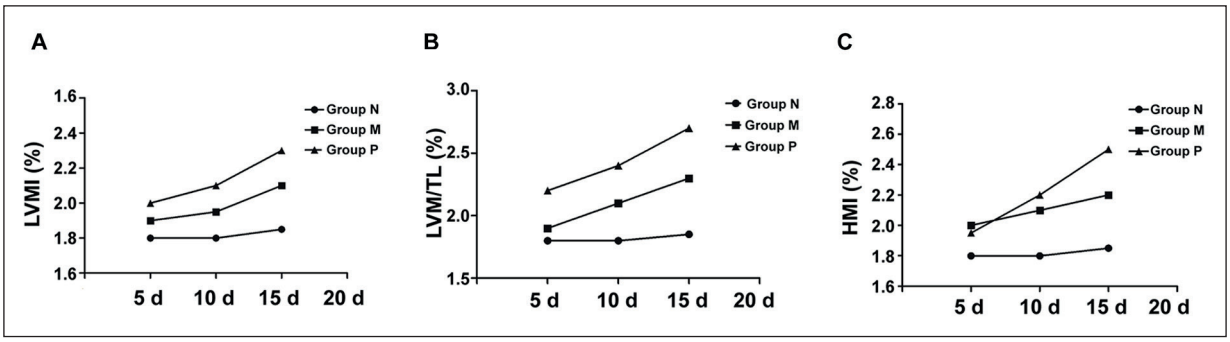

Figure 1. Changes in HMI, LVMI, and LVM/TL in the three groups ($\bar{x} \pm s$, n = 12). Note: At 5 d, HMI, LVMI, and LVM/TL significantly increased in group M and P compared with those in group N ($p<0.05$). HMI significantly increased in group M and P compared with that in group N. However, it was remarkably lower in group P than that in group M. At 15 d, HMI, LVMI, and LVM/TL reached the peak in the three groups, showing statistically significant differences ($p<0.05$).

Results

Cardiac Indexes

The results showed that at 5 d, HMI, LVMI, and LVM/TL gradually increased in group M. Meanwhile, they were also elevated in group P, which was significantly higher than those in group N and M. This showed a degree of increase similar to that in group M. At 10-15 d, HMI, LVMI, and LVW/TL remarkably increased in group M when compared with those in group N. Meanwhile, they were also significantly elevated in group P when compared with group N and M ($p<0.05$). Compared with group M, the above indexes remarkably increased in group P ($p<0.05$) (Figure 1).

Pathological Examination of Myocardial Tissues

According to HE staining, the myocardial fibers were normal and orderly arranged in group N. Meanwhile, no inflammatory cells were observed. In group M, the proliferation of a large number of inflammatory cells was observed. Moreover, the myocardial cells were arranged disorderly and became enlarged. In group P, myocardial fibrosis was significantly severer than that of the other two groups ($p<0.05$) (Figure 2).

Masson staining revealed that there was less accumulation of collagen fibers in the myocardium in group N. There was a little accumulation of collagen fibers in the myocardium in group M, and fibrosis declined. In addition,

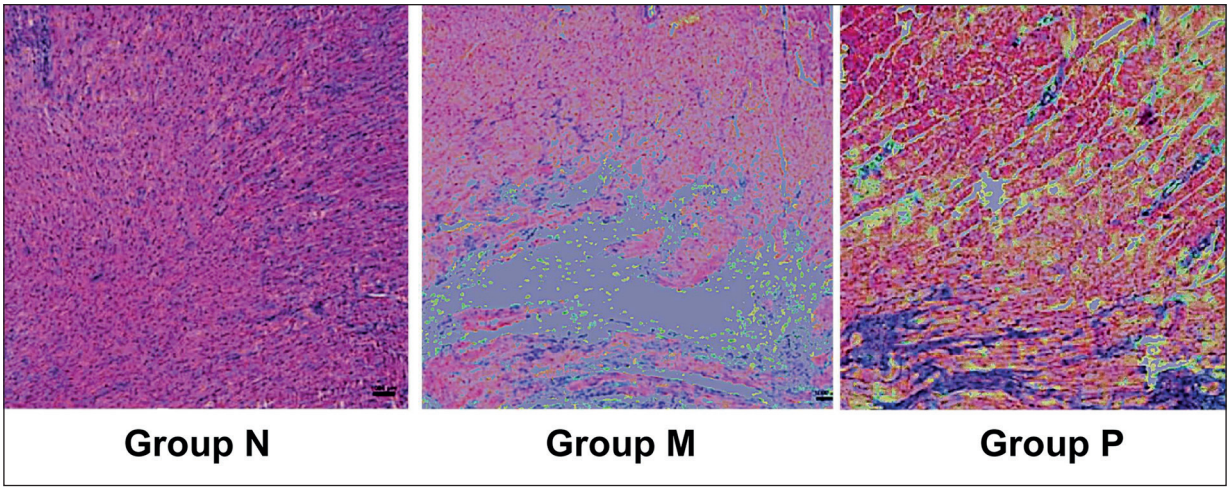

Figure 2. Comparison of pathological features of myocardial tissues in each group *via* HE staining (×200).

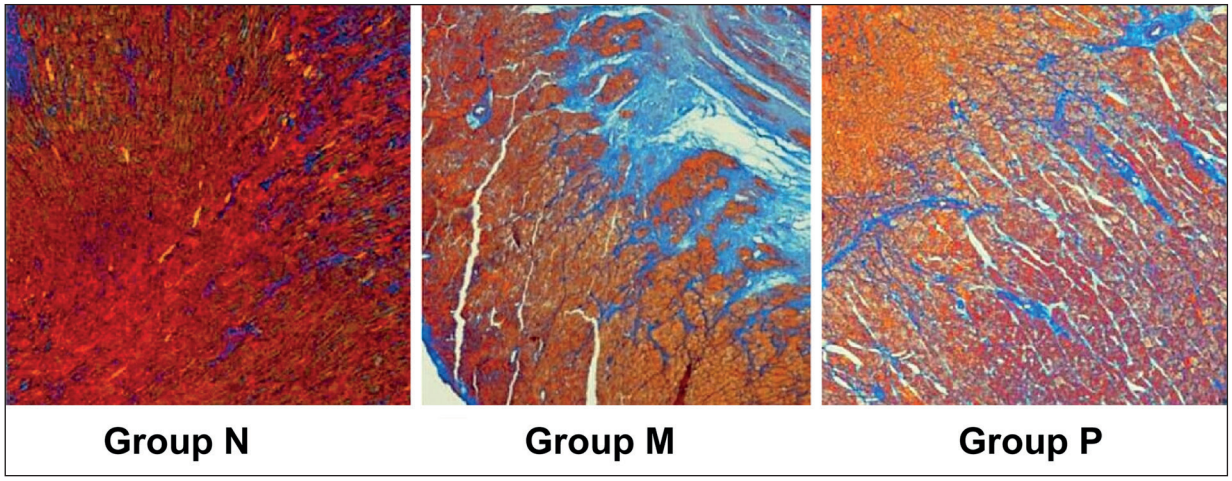

Figure 3. Comparison of pathological features of myocardial tissues in each group *via* Masson staining (×200).

the myocardial fibrosis in group P significantly increased when compared with that in group M (p<0.05) (Figure 3).

Western Blotting Results

According to the results of the Western blotting, the relative protein expressions of c-myc and TGF-β were significantly up-regulated in group M when compared with those in group N (p<0.05). Moreover, they were remarkably up-regulated in group P than those in group M, displaying statistically significant differences (p<0.05) (Figure 4).

MRNA Expressions of C-myc and TGF-β Via qRT-PCR

QRT-PCR results showed that the relative mRNA expressions of c-myc and TGF-β were significantly up-regulated in group P compared with those in group M and N, showing statistically significant differences (p<0.05). Furthermore, they were remarkably higher in group M than group N (p<0.05) (Figure 5).

Serum IL-6 Expression Level

The expression level of serum IL-6 was detected at each time point in the three groups. The results demonstrated that it reached highest levels in group P (p<0.01) (Table II).

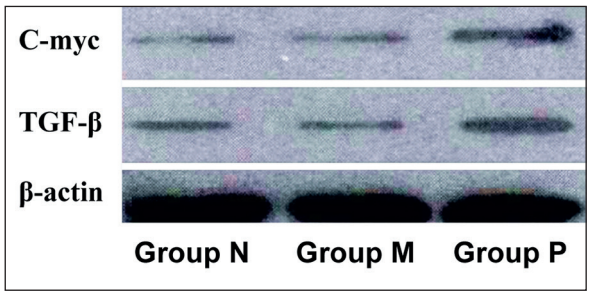

Figure 4. Protein expressions of c-myc and TGF-β detected *via* Western blotting.

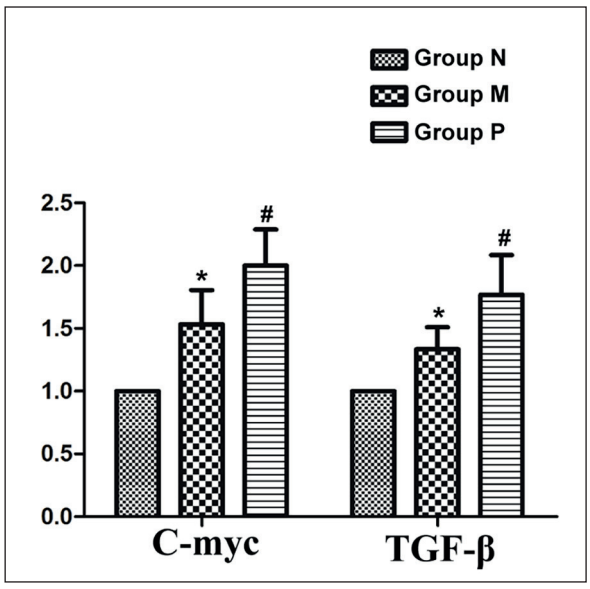

Figure 5. Relative mRNA expressions of c-myc and TGF-β detected *via* qRT-PCR. Note: *p<0.05 *vs.* group N, #p<0.05 *vs.* group M.

Table II. Serum IL-6 expression level in the three groups ($pg \cdot mL^{-1}$, $\bar{x} \pm s$, n = 12).

Group	5 d	10 d	15 d
Group N	57.72 ± 5	58.26 ± 5	57.88 ± 5
Group M	65.92 ± 5*[1]	66.08 ± 5*[1]	66.54 ± 4*[1]
Group P	85.25 ± 6*[2]	86.57 ± 6*[2]	91.90 ± 6*[2]
F	4.37	5.52	6.85
p	< 0.01	< 0.01	< 0.01

Note: *[1]$p < 0.05$ *vs*. group N, *[2]$p < 0.05$ *vs*. group M.

Discussion

VMC refers to the inflammation of myocardial cells due to virus infection in the human body. It has diverse clinical manifestations, including arrhythmia, cardiac enlargement, cardiac insufficiency, even shock, and sudden death[10]. According to the related literature, the myocardial fibrosis is the main pathological change of VMC in the chronic phase. During the development of myocardial fibrosis, the collagen fibers are excessively accumulated in normal myocardial tissues. This may increase the original concentration in myocardial tissues and promotes the differentiation of collagens, also known as one of the major causes of the disease[11]. Previous evidence showed that that lncRNAs with no protein-coding function may affect the genome components[12,13]. Moreover, the targeted regulation of the protein expression by short-chain or long-chain ncRNAs has become a research hotspot in recent years.

In the present study, the VMC model was first established in rats. According to HE staining and Masson staining, the myocardial fibers were normal and orderly arranged in group N. Meanwhile, no inflammatory cells were observed. In group M and P, the proliferation of a large number of inflammatory cells was observed. The myocardial cells were disorderly arranged and became enlarged, and myocardial fibrosis gradually increased. The above findings indicated that the main cause of myocardial hypertrophy, arrhythmia (especially atrial fibrillation), and cardiac insufficiency was the continuous deposition of myocardial fibrinogen. Moreover, cardiac indexes of the left ventricle were determined in group N, M, and P, respectively. It was found that HMI, LVMI and LVW/TL significantly increased in group P when compared with those in group N and M. These findings demonstrated that the damage of myocardial cells could increase the mortality rate of myocarditis patients complicated with heart failure, and hinder myocardial energy supply.

According to the results of the Western blotting, the protein expressions of c-myc and TGF-β were significantly up-regulated in group P, compared with those in group M and N ($p<0.05$). Meanwhile, they were slightly higher in group M than group N ($p<0.05$). QRT-PCR results indicated that the relative mRNA expressions of c-myc and TGF-β were significantly elevated in group P compared with those in group M and N, showing statistically significant differences ($p<0.05$). Moreover, they were remarkably higher in group M than those in group N ($p<0.05$). Transfection of lncRNA ROR demonstrated that the protein expressions of c-myc and TGF-β in myocardial cells were positively correlated with lncRNA ROR. Zhang et al[14] have found that the proliferation of myocardial cells depends on the expression of lncRNA ROR. High-expression lncRNA ROR can also regulate the expression of the related proteins in myocardial cells. LncRNA ROR plays an important role in myocardial tissue. It can induce and activate the expression of the downstream proteins, as well as inhibit the proliferation, growth, and apoptosis of normal myocardial cells[15].

In this study, the serum level of IL-6 was detected. The results found that the level was within the standard value at 5-15 d in group N, and was above the standard value in group M. Meanwhile, the serum level of IL-6 was significantly higher at 5 d in group P than that at 15 d in group M. These results suggested that the increased level of serum IL-6 was often caused by a process of inflammation. It has been found in different studies that IL-6 is the product of monocytes. Its production can be promoted by activating fibroblasts, which is involved in the whole process of inflammation[16]. At the same time, IL-6 is a kind of glycoprotein produced

by various tissue cells. It induces the differentiation of B cells to produce immunoglobulin and promote the growth of the T cells. Moreover, it exerts its function as the mediator of the inflammatory response by inducing the production of acute phase reactive proteins[17]. In addition, IL-6 can lead to immune response by inducing the differentiation of the B cells, macrophages, and natural killer cells, thereby inhibiting viral replication[18]. Excessive production of IL-6 can also destroy the cytokine network and viral clearance, resulting in myocardial damage[19]. Current studies have confirmed that the upregulation of the level of serum IL-6 in model rats will exacerbate myocardial fibrosis, which is consistent with the results in this study.

Conclusions

LncRNA ROR up-regulated the expression of c-myc and increased the level of serum IL-6, thereby facilitating the proliferation and differentiation of cardiac fibroblasts.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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