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HOTAIR promotes myocardial fibrosis through regulating URI1 expression via Wnt pathway

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Abstract. – OBJECTIVE: To investigate whether HOX transcript antisense RNA (HOTAIR) regulates myocardial fibrosis via promoting proliferation of cardiac fibroblasts (CFs) and upregulating the expression levels of fibrotic proteins through activating Wnt signaling pathway.

MATERIALS AND METHODS: The expression level of HOTAIR in Ang II-induced cardiac fibroblasts was detected by quantitative Real-time-polymerase chain reaction (qRT-PCR). Overexpression or knockdown of HOTAIR expression was achieved by lentivirus transfection. The effects of HOTAIR on regulating cell proliferation, migration and apoptosis were measured by cell counting kit-8 (CCK-8) test, transwell assay and flow cytometry, respectively. Western blot and qRT-PCR experiments were performed to detect expressions of fibrosis-related genes and Wnt pathway-related genes. Target gene of HOTAIR was predicted by bioinformatics analysis. Rescue assays were conducted to assess whether HOTAIR could regulate cell proliferation and fibrosis by activating Wnt signaling pathway via URI1.

RESULTS: QRT-PCR results showed that HOTAIR expression in Ang II-induced CF cells was significantly higher than that in the control. HOTAIR overexpression in CF cells can promote cell proliferation and migration, inhibit apoptosis, and promote the expressions of fibrosis-related genes. Western blot results indicated that HOTAIR could upregulate URI1 expression and activate Wnt signaling pathway. In addition, rescue assay demonstrated that overexpression of URI1 reversed the inhibitory effect of HOTAIR knockdown on Wnt pathway.

CONCLUSIONS: Highly expressed HOTAIR promoted proliferation and migration of cardiac fibroblasts. HOTAIR remarkably upregulated fibrosis-related genes in CF cells. The mechanism of HOTAIR in regulating myocardial fibrosis might be related to the activation of Wnt signaling pathway through targeting URI1 expression.

Key Words:
HOTAIR, URI1, Wnt pathway, Myocardial fibrosis.

Introduction

Myocardial fibrosis is caused by abnormally expressed cardiac fibroblasts, excessive accumulation of collagen fibers in the extracellular matrix (ECM) of the heart muscle, increased collagen concentration, or altered collagen composition. These pathological changes are the main manifestations of heart remodeling[1]. The extracellular matrix is mainly composed of type I collagen and type III collagen, accounting for more than 90% of the total amount of myocardial interstitial collagen. Maintenance of proper ratio of myocardial interstitial collagen is important for the integrity of cardiac function and myocardial tissue[2,3]. Myocardial fibrosis increases the stiffness of the heart and decreases ventricular compliance. Meanwhile, the continuous deposition of extracellular matrix seriously affects the normal contraction and diastolic function of the heart, ultimately leading to chronic heart failure, myocardial infarction, malignant arrhythmia or even sudden death. Myocardial fibrosis has become a pathological manifestation and a causative factor of various cardiovascular diseases, and is also an important cause of irreversible and sustained development of ventricular remodeling[4].

Long non-coding RNAs (lncRNAs) consist of 200 to 100,000 nucleotides in length. Due to the lack of an open reading frame, they cannot encode protein, but can participate in regulate target gene expression. Therefore, lncRNAs play an important role not only in the normal development of organisms, but also in various pathophysiological conditions, such as acute myocardial infarction[5,6]. The investigators examined the expression changes of lncRNAs after 4 week myocardial infarction in mice and found that 53 lncRNAs were up-regulated more than 2-fold at the periphery of infarction, and 37 lncRNAs were down-regulated by more than 0.5 times[7]. In addition, overexpres-

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sion of H19 can promote the proliferation of myocardial fibroblasts, thus accelerating myocardial fibrosis[8]. At the same time, H19 was also found to be able to regulate myocardial cell hypertrophy[9]. However, the functions of many other lncRNAs in myocardial fibrosis have not yet been determined, requiring further researches. HOX transcript antisense RNA (HOTAIR) is an lncRNA with 2158 kb that begins to transcribe at the HOXC site. HOTAIR is characterized by differential expression in many digestive system tumors, including colorectal cancers, breast cancer, etc.[10]. Yang et al[11] first discovered that HOTAIR is highly expressed in hepatoma carcinoma cells, which is an independent prognostic factor for predicting the recurrence of liver cancer. Meanwhile, HOTAIR can play a very crucial role in the occurrence and development of many other tumors. For example, HOTAIR inhibits polycomb repressive complex 2 (PRC2) by binding to miR-34a, thereby regulating the process of gastric epithelial mesenization[12]. However, its role in myocardial fibrosis still needs further exploration.

Materials and Methods

Extraction and Culture of Primary Mouse Fibroblasts

The mice were anesthetized and sacrificed. After being disinfected with 75% ethanol, mouse hearts were harvested and washed with normal saline. Heart tissues were fully cut and smashed with a small straight shear. After 4 mL of collagenase were added, the smashed hearts were placed in a water bath at 37°C for 8 min. After centrifugation, the supernatant was discarded. Collected CF cells were cultured in Dulbecco's modified eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin (Gibco, Rockville, MD, USA) in a 5% CO_2 incubator at 37°C. The third-passage CFs were used for experiments. The myocardial fibrosis phenotype was induced by treatment of 0.01 mM Ang II for 24 h. 3-day-old C57BL/6 mice used in this experiment were purchased from the Animal Model Center of Zhengzhou University. All operations in our experiment have obtained approval of First Affiliated Hospital of Zhengzhou University Ethics Committee.

Transfection

One day before transfection, CFs were counted and transferred to a corresponding culture plate until 70-80% of the cell density. The lentivirus solution was diluted in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS at a ratio of 1:1. The original culture medium in the culture plate was removed, and the diluted lentivirus solution was added. Polybrene was added for increasing the transfection efficiency, and a blank control group was set up. Culture medium was replaced 12 h later. The lentivirus used in the experiment was purchased from the Shanghai Gene Pharma Company (Shanghai, China).

Transwell Assay

600 μL of DMEM were added in each well of the 24-well plate, and a transwell chamber with a diameter of 6.5 mm was placed into each well. Cells in different groups were prepared for cell suspension in serum-free DMEM, and then about 8000 cells were added in each chamber. After incubation at 37°C in a constant temperature incubator for 24 hours, the chamber was taken out and cells were fixed for 30 minutes. Giemsa dying assay was performed to evaluate the cell migration under microscope. Cells in 5 randomly selected fields were counted.

Apoptosis

Cells in different groups were digested with trypsin without Ethylene Diamine Tetraacetic Acid (EDTA) (Gibco, Rockville, MD, USA), prepared for cell suspension, and then transferred to a centrifuge tube. After centrifugation, the supernatant was discarded, and 500 μL of Binding Buffer suspension was added to each tube according to the apoptosis kit instructions. 5 μL of Annexin V-APC and 7-AAD were added in succession and mixed in each tube. Cell apoptosis was detected by flow cytometry after 15 minutes at room temperature.

Cell Proliferation

The transfected cells were collected after the trypsin digestion. After cell resuspension, the cell density was adjusted to 5×10^3 cells/ml and seeded in 96-well plates with 100 μL per well. Each group had 5 replicate wells. The culture plate was placed in a 5% CO_2 incubator at 37°C. After cell culture for 24 hours, 10 μL of cell counting kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan) were added in each well and cultured for another 1 h. Microplate reader was used to detect the optical density (OD) value at 450 nm of wavelength. The experiment was repeated for three times.

Total RNA Extraction and Polymerase Chain Reaction (PCR)

The collected cells were washed, lysed with 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and incubated with 0.2 mL of chloroform. After centrifugation, the upper aqueous phase was transferred to a new Eppendorf (EP) tube, and then isopropanol was added to precipitate RNA. After washing with 75% ethanol and dried for 15 minutes, diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China) was used to dissolve the RNA precipitate, followed by preservation in a refrigerator at -80°C. Complementary Deoxyribose Nucleic Acid (cDNA) was obtained from the extracted RNA following the reverse transcription kit instruction. Subsequently, quantitative polymerase chain reaction (qPCR) was performed and 20 μL of the reaction system was arranged in accordance with SYBR Premix Ex Taq kit (TaKaRa, Otsu, Shiga, Japan). The reaction conditions were as follows: 95°C denaturation for 30 s, 95°C for 5 s and 60°C for 30 s, for a total of 40 cycles. The primer sequences were as follows: Collagen I: F: ACGTCCTGGTGAAGTTGGTC, R: CAGGGAAGCCTCTTTCTCCT; Collagen III: F: TGGCCCTGACCCAACTATGAT, R: GCACTTTTTGCCCTTCTTAATGTT; α-SMA: F: ACCTGCAAGACCATCGACATG, R: CGAGCCTTAGTTTGGACAGGAT; HOTAIR: F: GGCGGATGCAAGTTAATAAAAC, R: TACGCCTGAGTGTTCACGAG; URI1: F: GTGGTCACTAACTGCCAAGAG, R: TGAGTCTTTCTCGAAGGGCATTA.

Western Blot Assay

After the cells were collected, radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) protein lysates were used to extract the total proteins of cells in each group. After protein quantitation using bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA), protein sample was electrophoresed on a 10% polyacrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Subsequently, the membrane was blocked by 5% of non-fat milk and then incubated with primary antibody overnight at 4°C. On the next day, the membrane was rinsed in Tris-buffered saline and Tween solution (TBST) (Beyotime, Shanghai, China), and incubated with secondary antibody. At last, membrane were developed by chemiluminescence, and glyceraldheyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The ratio of the gray values of the target protein/reference was considered as the relative expression of each protein.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA), GraphPad (Version X; La Jolla, CA, USA) and other related statistical software were used for data analysis. All data were expressed as mean ± standard deviation. The *t*-test was used to compare the statistical differences between two groups, and $p < 0.05$ was considered statistically significant.

Results

Highly Expressed HOTAIR Promoted the Expression of Fibrosis-Related Genes

HOTAIR expression in Ang II-induced fibrosis model of cardiomyocyte fibroblasts (CFs) was detected by PCR. It is found that HOTAIR expression was significantly increased in the treated group than that of controls (Figure 1A). By lentivirus transfection, intracellular HOTAIR expression was significantly increased (Figure 1B). Overexpression of HOTAIR promoted the proliferation of CFs (Figure 1C). At the same time, mRNA and protein levels of fibrosis-related genes, including Collagen I, Collagen III, and α-SMA were significantly elevated after HOTAIR overexpression (Figure 1D, 1E). Overexpression of HOTAIR could also inhibit cell apoptosis (Figure 1F) while promote cell migration (Figure 1G). The results suggested that HOTAIR may be involved in the process of myocardial fibrosis.

Lowly Expressed HOTAIR Inhibited the Expression of Fibrosis-Related Genes

Intracellular HOTAIR expression was remarkably downregulated after lentivirus transfection (Figure 2A). HOTAIR knockdown significantly inhibited the cell proliferation (Figure 2B), but promoted the apoptotic capacity of CFs (Figure 2C). The mRNA and protein levels of fibrosis-related genes, such as Collagen I, Collagen III, and α-SMA were markedly reduced after HOTAIR knockdown (Figure 2D, 2E). At the same time, knockdown of HOTAIR also dramatically inhibited the migratory ability of CFs (Figure 2F).

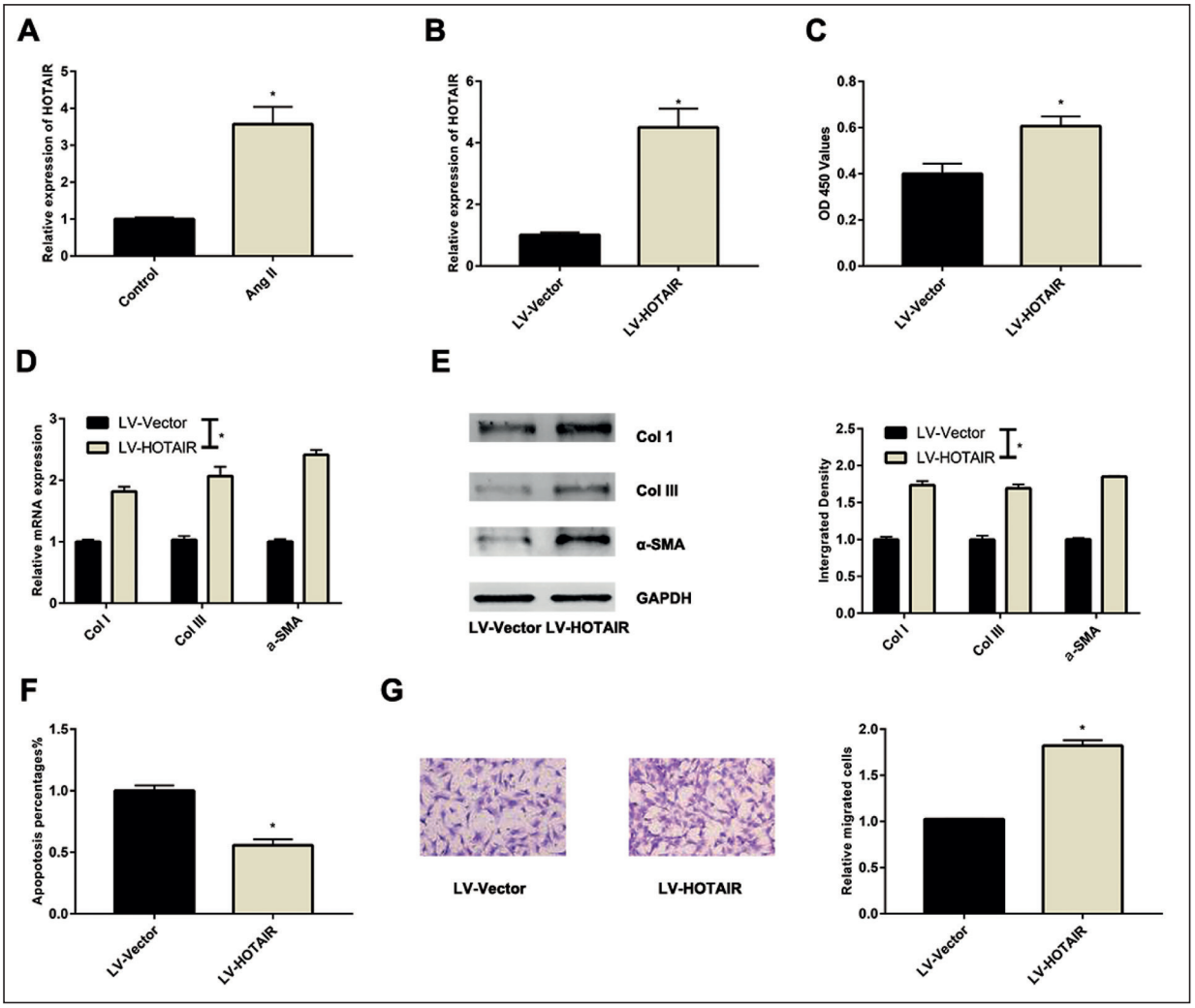

Figure 1. High expression of HOTAIR promoted cell proliferation and fibrotic protein expression. ***A,*** HOTAIR expression was detected in CF cells 24 h after Ang II treatment. ***B,*** Lentiviral transfection significantly increased intracellular HOTAIR expression. ***C,*** High expression of HOTAIR promoted cell proliferation. ***D,*** Highly expressed HOTAIR promoted mRNA expressions of fibrosis-related genes such as Collagen I, Collagen III, and α-SMA. ***E,*** High expression of HOTAIR promoted the expressions of fibrosis-related proteins such as Collagen I, Collagen III, and α-SMA. ***F,*** High expression of HOTAIR inhibited cell apoptosis. ***G,*** High expression of HOTAIR promoted cell migration.

HOTAIR Enhanced URI1 Expression

According to the prediction from Starbase website, URI1 may be the downstream gene of HOTAIR. Both PCR and Western blot confirmed that URI1 expression is positively regulated by HOTAIR (Figure 3A, 3B). Further experiment showed that overexpression of URI1 was capable of promoting cell proliferation (Figure 3C) and migration (Figure 3F). URI1 overexpression also elevated the expressions of fibrosis-related genes (Figure 3D), and inhibited cell apoptosis (Figure 3E). The regulatory effects of URI1 overexpression on CFs functions were as same as those after HOTAIR overexpression, suggesting that HOTAIR may regulate CFs by targeting URI1.

HOTAIR Activated Wnt Pathway

We further explored whether HOTAIR exerts biological functions through the Wnt pathway. Western blot was used to detect the levels of classical molecules in Wnt pathway, such as Axin2, Lef1, β-catenin and p-GSK-3β. It was found that overexpressed HOTAIR significantly activated the Wnt pathway (Figure 4A). Rescue experiment implied that simultaneously overex-

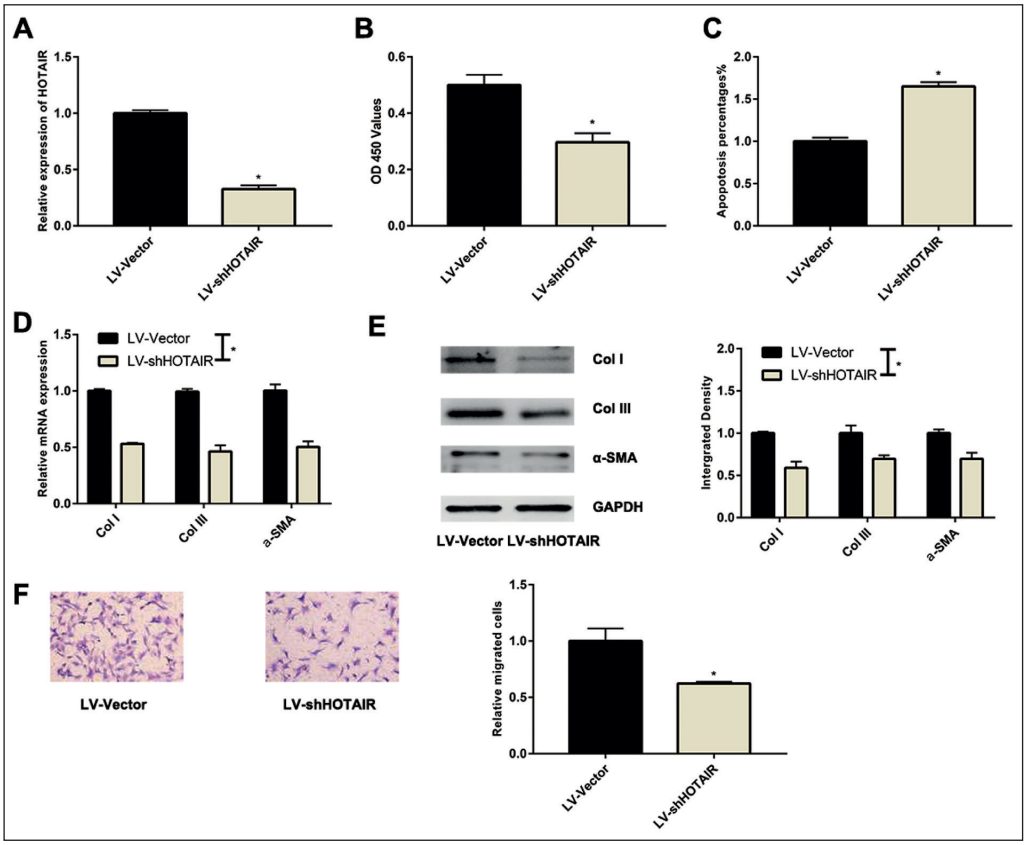

Figure 2. HOTAIR knockdown inhibited cell proliferation and fibrotic protein expression. ***A,*** Lentiviral transfection significantly inhibited intracellular HOTAIR expression. ***B,*** HOTAIR knockdown inhibited cell proliferation. ***C,*** HOTAIR knockdown promoted cell apoptosis. ***D,*** HOTAIR knockdown inhibited mRNA expressions of collagenase-related genes including Collagen I, Collagen III and α-SMA. ***E,*** HOTAIR knockdown inhibited protein expressions of collagenase I, Collagen III, and α-SMA. ***F,*** HOTAIR knockdown inhibited cell migration.

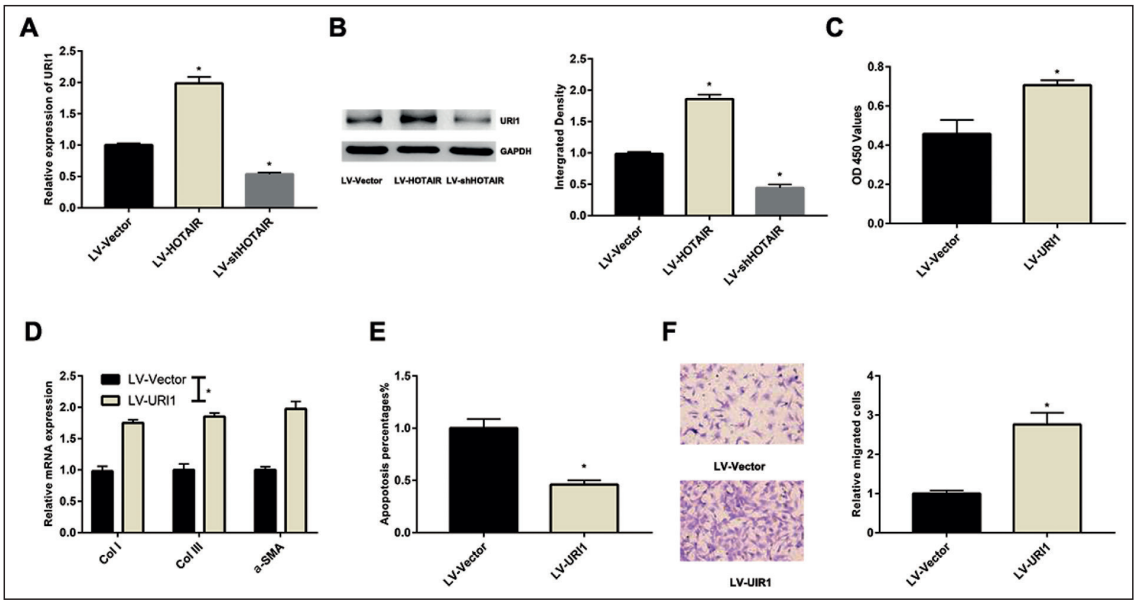

Figure 3. HOTAIR regulated URI1 expression. ***A,*** The mRNA expression of URI1 was detected by PCR after knockdown or overexpression of HOTAIR. ***B,*** Protein expression of URI1 was detected after knockdown or overexpression of HOTAIR. ***C,*** High expression of URI1 promoted cell proliferation. ***D,*** High expression of URI1 promoted mRNA expressions of fibrosis-related genes. ***E,*** High expression of URI1 inhibited cell apoptosis. ***F,*** High expression of URI1 promoted cell migration.

pressing URI1 could partly reverse the inhibitory effect of HOTAIR knockdown on the Wnt pathway (Figure 4B).

Discussion

Researches have shown that MF is a basic lesion of many diseases such as heart failure, myocardial infarction and other cardiovascular diseases, which is also one of the manifestations of myocardial remodeling[13]. The disease development of MF is influenced by many factors, including the immune system, body cytokines, and RAAS system. From the overall view, the imbalance of synthesis, metabolism, and degradation of collagen in the extracellular matrix can promote the development of MF[14, 15]. Cardiac fibroblasts (CFs) are the main cells secreting the extracellular matrix of the myocardium. CFs present a strong proliferative ability but without contractility. CFs are mainly responsible for the generation and precipitation of myocardial interstitium. Under normal circumstances, adult CFs are in a state of equilibrium and inactivation. However, the phenotype of CFs changes under pathological conditions resulted in increased collagen synthesis and secretion[16-18]. Therefore, in order to effectively prevent and treat MF, it is necessary to further explore the possible regulatory mechanisms

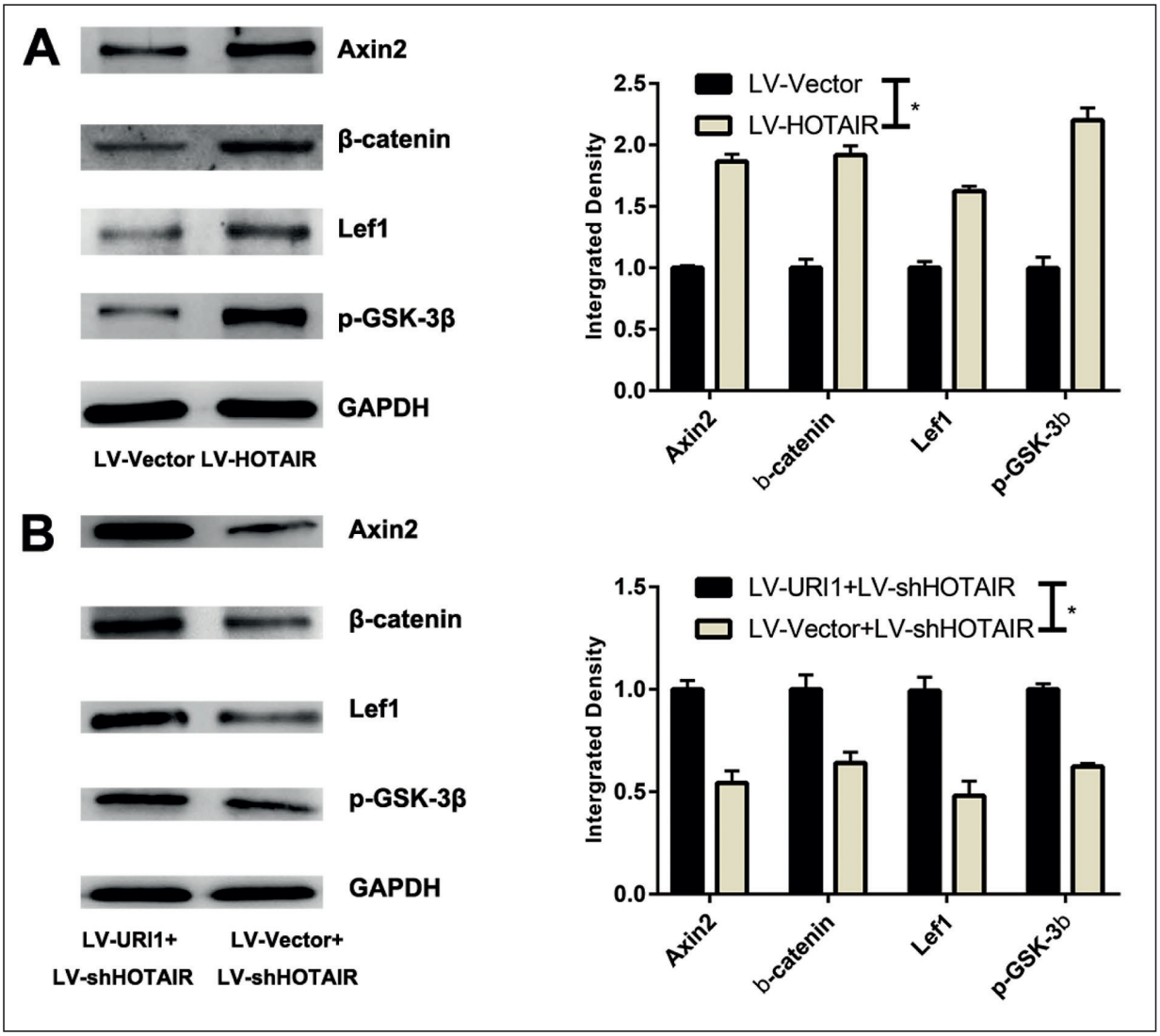

Figure 4. HOTAIR activated the Wnt pathway. ***A,*** Western blot analysis of Wnt pathway related gene expression was shown after HOTAIR overexpression. ***B,*** Wnt pathway-associated gene expression was detected by Western blot after knockdown of HOTAIR and overexpression of URI1.

of phenotypic conversion of CFs, so as to find the targets of myogenic fibroblasts. Wnt signaling pathway is an important pathway that regulates the development of tissues and organs. Wnt pathway is found to participate in cell proliferation, differentiation and metastasis. In recent years, with the deepening of researches, and the role of Wnt pathway in myocardial fibrosis has been well concerned. It was found that in the tissues of human fibrotic diseases, Wnt protein is overexpressed and DKK1 expression is downregulated, indicating that Wnt protein is associated with the occurrence of fibrotic disease[19]. Chen et al[20] found that in the diabetic rat model, angiotensin II can promote myocardial fibrosis, accompanied by the gradual elevation of Wnt expression. Colston et al[21] established the myocardial infarction model in male mice by coronary artery occlusion. Northern method and Western blotting assay confirmed that the expressions of both WISP-1 and adhesin were increased, subsequently leading to activated WISP-1 signaling and cardiac hypertrophy. Finally, fibroblast proliferation and myocardial fibrosis are occurred. Additionally, Wnt signaling pathway is activated in the fibroblasts of infarcted rats, and the downstream signaling molecules including DVL-1 and β-catenin are also increased[22]. Other investigations[23] have also pointed out that overexpression of β-catenin may contribute to the promoted proliferation, differentiation, and metastasis of CFs in young rats, leading to cardiac hypertrophy ultimately. In this study, we found that HOTAIR was highly expressed in the *in vitro*-induced myocardial fibrosis model, leading to increased proliferative and migratory capacity of CFs. Expressions of fibrosis-related genes in CFs were elevated as well. Further investigation of the mechanism suggested that high expression of HOTAIR promoted URI1 expression and activated the Wnt pathway, thereby regulating MF development.

Conclusions

We found that highly expressed HOTAIR can promote the expressions of genes involved in fibroblast fibrosis, and its mechanism may be related to the activation of Wnt signaling pathway.

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

References

1) Porter KE, Turner NA. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol Ther 2009; 123: 255-278.
2) Souders CA, Bowers SL, Baudino TA. Cardiac fibroblast: the renaissance cell. Circ Res 2009; 105: 1164-1176.
3) Ma Y, Iyer RP, Jung M, Czubryt MP, Lindsey ML. Cardiac fibroblast activation post-myocardial infarction: current knowledge gaps. Trends Pharmacol Sci 2017; 38: 448-458.
4) Tallquist MD, Molkentin JD. Redefining the identity of cardiac fibroblasts. Nat Rev Cardiol 2017; 14: 484-491.
5) Kopp F, Mendell JT. Functional classification and experimental dissection of long noncoding RNAs. Cell 2018; 172: 393-407.
6) Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. Nat Rev Genet 2016; 17: 47-62.
7) Zhao N, Yu H, Yu H, Sun M, Zhang Y, Xu M, Gao W. MiRNA-711-SP1-collagen-I pathway is involved in the anti-fibrotic effect of pioglitazone in myocardial infarction. Sci China Life Sci 2013; 56: 431-439.
8) Qu X, Song X, Yuan W, Shu Y, Wang Y, Zhao X, Gao M, Lu R, Luo S, Zhao W, Zhang Y, Sun L, Lu Y. Expression signature of lncRNAs and their potential roles in cardiac fibrosis of post-infarct mice. Biosci Rep 2016; 36(3). pii: e00337.
9) Tao H, Cao W, Yang JJ, Shi KH, Zhou X, Liu LP, Li J. Long noncoding RNA H19 controls DUSP5/ERK1/2 axis in cardiac fibroblast proliferation and fibrosis. Cardiovasc Pathol 2016; 25: 381-389.
10) Jiao ZY, Tian Q, Li N, Wang HB, Li KZ. Plasma long non-coding RNAs (lncRNAs) serve as potential biomarkers for predicting breast cancer. Eur Rev Med Pharmacol Sci 2018; 22: 1994-1999.
11) Yang Z, Zhou L, Wu LM, Lai MC, Xie HY, Zhang F, Zheng SS. Overexpression of long non-coding RNA HOTAIR predicts tumor recurrence in hepatocellular carcinoma patients following liver transplantation. Ann Surg Oncol 2011; 18: 1243-1250.
12) Liu YW, Sun M, Xia R, Zhang EB, Liu XH, Zhang ZH, Xu TP, De W, Liu BR, Wang ZX. LincHOTAIR epigenetically silences miR34a by binding to PRC2 to promote the epithelial-to-mesenchymal transition in human gastric cancer. Cell Death Dis 2015; 6: e1802.
13) Gonzalez A, Schelbert EB, Diez J, Butler J. Myocardial interstitial fibrosis in heart failure: biological and translational perspectives. J Am Coll Cardiol 2018; 71: 1696-1706.

14) Trachtenberg B, Hare J. Response by trachtenberg and hare to letter regarding the article, "inflammatory cardiomyopathic syndromes". Circ Res 2018; 122: e2.

15) Gyongyosi M, Winkler J, Ramos I, Do QT, Firat H, McDonald K, Gonzalez A, Thum T, Diez J, Jaisser F, Pizard A, Zannad F. Myocardial fibrosis: biomedical research from bench to bedside. Eur J Heart Fail 2017; 19: 177-191.

16) van de Schoor FR, Aengevaeren VL, Hopman MT, Oxborough DL, George KP, Thompson PD, Eijsvogels TM. Myocardial fibrosis in athletes. Mayo Clin Proc 2016; 91: 1617-1631.

17) Prabhu SD, Frangogiannis NG. The biological basis for cardiac repair after myocardial infarction: from inflammation to fibrosis. Circ Res 2016; 119: 91-112.

18) Travers JG, Kamal FA, Robbins J, Yutzey KE, Blaxall BC. Cardiac fibrosis: the fibroblast awakens. Circ Res 2016; 118: 1021-1040.

19) Akhmetshina A, Palumbo K, Dees C, Bergmann C, Venalis P, Zerr P, Horn A, Kireva T, Beyer C, Zwerina J, Schneider H, Sadowski A, Riener MO, MacDougald OA, Distler O, Schett G, Distler JH. Activation of canonical Wnt signalling is required for TGF-beta-mediated fibrosis. Nat Commun 2012; 3: 735.

20) Chen JX, Zeng H, Reese J, Aschner JL, Meyrick B. Overexpression of angiopoietin-2 impairs myocardial angiogenesis and exacerbates cardiac fibrosis in the diabetic db/db mouse model. Am J Physiol Heart Circ Physiol 2012; 302: H1003-H1012.

21) Colston JT, de la Rosa SD, Koehler M, Gonzales K, Mestril R, Freeman GL, Bailey SR, Chandrasekar B. Wnt-induced secreted protein-1 is a prohypertrophic and profibrotic growth factor. Am J Physiol Heart Circ Physiol 2007; 293: H1839-H1846.

22) Chen L, Wu Q, Guo F, Xia B, Zuo J. Expression of dishevelled-1 in wound healing after acute myocardial infarction: possible involvement in myofibroblast proliferation and migration. J Cell Mol Med 2004; 8: 257-264.

23) Hahn JY, Cho HJ, Bae JW, Yuk HS, Kim KI, Park KW, Koo BK, Chae IH, Shin CS, Oh BH, Choi YS, Park YB, Kim HS. Beta-catenin overexpression reduces myocardial infarct size through differential effects on cardiomyocytes and cardiac fibroblasts. J Biol Chem 2006; 281: 30979-30989.