EXPERIMENTAL STUDY

LncRNA MHRT Promotes Cardiac Fibrosis via miR-3185 Pathway Following Myocardial Infarction

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Summary

Long-chain noncoding RNA (lncRNA) is a new class of molecular regulators in heart development and disease. However, the role of specific lncRNA in cardiac fibrosis remains to be fully explored. This study aimed to investigate the role and potential mechanism of lncRNA MHRT in myocardial fibrosis after myocardial infarction (MI).

Cardiac fibroblasts (CFs) were isolated from a mouse model of MI. The expression levels of MHRT and miR-3185 in the hearts of MI and CFs mice treated with transforming growth factor beta 1 (TGF-β1) were analyzed by qRT-PCR. The collagen expression was assessed using qRT-PCR and Western blot. Cell proliferation was assessed by performing MTT and EdU assays. The direct interaction between lncRNA and miRNA was analyzed by luciferase assay, RNA-binding protein immunoprecipitation (RIP) assay, and RNA pull-down assay.

The expression levels of MHRT were raised in MI and CFs mice treated with TGF-β1. Overexpression of MHRT promoted collagen production and CF proliferation, while silencing of MHRT showed the opposite effect. MiR-3185 was a target gene of MHRT. In addition, overexpression of MHRT reduced the expression levels of miR-3185, and siMHRT reversed the inhibitory effect of TGF-β1 on the expression of miR-3185. Overexpression of miR-3185 inhibited the upregulation of Col I and Col III induced by TGF-β1.

MHRT promoted cardiac fibrosis after MI through miR-3185 and increased myocardial collagen deposition and promoted myocardial fibrosis.

Key words: TGF-β1-induced, Myocardial fibrosis, Myocardial collagen

A acute myocardial infarction (MI) is acute ischemic necrosis of myocardial tissue. It generally refers to the clinical syndrome of myocardial, injury, and necrosis after the blood supply is greatly reduced or interrupted,1 which is a common critical medical emergency. Although percutaneous coronary intervention, thrombolysis, and emergency coronary artery bypass graft greatly reduce the death of myocardial cells in patients with acute MI and reduce the infarct area of myocardial tissue, ischemic necrosis of myocardial cells still activates the neuroendocrine system to induce abnormal expression of some genes and lead to myocardial remodeling.2,3 Myocardial remodeling is an important pathophysiological mechanism of heart failure in patients with acute MI, and myocardial fibrosis after MI is one of the important links of myocardial remodeling.4,5 The main pathological changes are activation of myocardial fibroblasts (CFs) and proliferation and synthesis of extracellular matrix, resulting in excessive deposition of extracellular matrix and the alteration of collagen components.6 If the ischemia or fibrosis reaction is continuously induced, the extracellular matrix will be excessively deposited, resulting in fibrous scar formation, which will further change the shape and structure of the heart and reduce the compliance of the heart.8,9 Moreover, excessive accumulation of extracellular matrix easily induces arrhythmias and leads to permanent arrhythmia.10 Therefore, preventing excessive myocardial fibrosis, late reactive fibrosis of MI, is an important research direction for interventional treatment of acute MI disease.

Long-chain noncoding RNA (lncRNAs) have been shown to be abnormally expressed in many pathological tissues and play important regulatory roles in various biological processes.11,12 The regulation of lncRNAs has been demonstrated as a potential target in treating human diseases.13,14 Recent studies have shown the involvement of lncRNAs in the development of cardiovascular diseases, including cardiac hypertrophy, cardiac metabolic disease, and heart failure.15,16 LncRNA MHRT is a recently discovered lncRNA that is abnormally expressed in a variety of malignant diseases.17 However, the role of MHRT in myocardial fibrosis after MI remains unclear.

Recently, the regulatory relationship between lncRNAs and miRNAs has been extensively studied.18,19
MiRNAs can intervene in the physiological and pathological processes of cellular signals.20 A large number of miRNAs have been shown to regulate different molecules and signaling pathways of myocardial fibrosis.21,22 For example, miRNA-122 has been demonstrated to inhibit myocardial fibrosis and improve cardiac function.23 MiR-3185 is reported to participate in multiple malignant transformation processes in the cell.24 However, its role and regulatory mechanism in myocardial fibrosis after MI remain unclear. Therefore, this study was carried out to investigate the expression of MHRT and miR-3185 in MI, as well as the effects of MHRT on myocardial fibrosis and cardiac function after MI.

Methods

Experimental animal model of MI: Healthy male C57 BL/6 mice (20-25 g) were purchased from the Animal Center of Sichuan University. All mice had access to food and water freely. All animal experimental procedures were approved by the Laboratory Animal Care and Use Committee of Chengdu Fifth People’s Hospital. MI was induced by suturing the left anterior descending (LAD) artery. Before surgery, mice were fixed in a supine position on a mouse plate and anesthetized by intraperitoneal injection of avertin solution. The neck skin was bluntly separated, and the trachea was intubated and connected to a small animal ventilator. The skin in front of the heart was blunt. The muscles were separated, and the chest wall was cut in the fourth intercostal space. The LAD was sutured to form a MI. Mice in the sham group underwent the same experimental procedure, but there was no LAD constraction. The general conditions of the mice were monitored daily, and mice were sacrificed 4 weeks after MI.

Isolation of cardiac fibroblasts: Under aseptic conditions, the chest was opened to take the heart of SD rats. The atrial large blood vessels and pericardium were removed and placed in PBS buffer. The obtained myocardial tissue was cut into pieces and digested in a water bath containing a mixture of 1 g · L-1 type I collagenase and 1 g · L-1 trypsin. The supernatant suspension was discarded. The second and third digestion were performed with 5 minutes each time, and the supernatant cell suspension was collected. The dead cardiac muscle cells, fibroblasts, and other cells were removed by adhering method, and the adherent living cells were cardiac muscle fibroblasts, which were cultured in DMEM medium containing 10% FBS.

Transfection and TGF-β1 administration: pCMV6-MALAT1, IncRNA MHRT siRNA, miR-3185 mimics, and corresponding negative controls were synthesized by GenePharma Co., Ltd. (Shanghai, China). Mouse cardiac myocytes were plated in 24-well plates and transiently transfected with RNAiMax using Lipofectamine 3000 with Plus Reagent (Thermo Fisher Scientific) to transfect cells. Cells were harvested at 24 hours post-transfection and treated with 20 ng/mL TGF-β1 for 48 hours.

Quantitative reverse transcription-PCR (qRT-PCR): Total RNAs were extracted from cells using TRIzol Reagent (Lianmai, Shanghai, China). After reverse transcription, qRT-PCR was performed using a ViiA™ 7 real-time PCR system (Life Technologies, Grand Island, NY). GAPDH and U6 were used as the internal references. The expression levels of IncRNA and miRNA were calculated using the 2^(-ΔΔCT) method.25 The primer sequences were as follows:

MHRT-Forward: 5’-CCGACTGCACTCCTCATAC-3’
MHRT-Reverse: 5’-GGCTGAAGAGTCCTTGTT-3’
miR-3185-Forward: 5’-ATGGAAGAAGAAGGCGGTCG-3’
miR-3185-Reverse: 5’-ACATGGACAGAAGCGGATG-3’

Col I-Forward: 5’-AGTGTTTGGATGTTGCCAA-3’
Col I-Reverse: 5’-GCAACCCTCATTCCACGAGC-3’
Col III-Forward: 5’-ATCTCTCCAGGCCATTAGT-3’
Col III-Reverse: 5’-ATTCCACCCATTTCCAGGGTG-3’
GAPDH-Forward: 5’-GATTCACCATGCGCAAATTC-3’
GAPDH-Reverse: 5’-CTGGAAGATGGTGATGGGGT-3’

U6-Forward: 5’-CTTCGCTTCCGCAGCACA-3’
U6-Reverse: 5’-AACCGGTTCGAATTTGTGC-3’

Cell viability assay: Cardiac fibroblast proliferation experiments were performed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) cell proliferation and cytotoxicity detection kits. After culturing in 96-well plates, transfected cells were incubated with MTT solution for 4 hours, and 200 μl DMSO was added to each well. Optical density at 570 nm was measured by a microplate reader (BioTek, Winoski, VT, USA).

EdU staining and measurement of collagen content: Cell proliferation was determined using EdU (5-ethyl-2’-deoxyuridine) staining. The cells were exposed to 100 μM of 1x Apollo® reaction mixture for 30 minutes. Next, cells were incubated with 5 μg/mL of DAPI for 30 minutes. EdU in the cells was examined by a fluorescence microscope (Olympus, Tokyo, Japan). The total collagen content was measured using the Sircol Collagen Assay Kit (Biocolor Ltd., Northern Ireland, UK). The absorbance at 540 nm was measured using a microplate reader.

Masson’s trichrome staining: The hearts of mice were dissected and fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin and sectioned into 5-μm-thick slices. The slices were then stained with Masson’s trichrome (Solarbio, Beijing, China). The area occupied by collagen was calculated using Image analysis software (Image-Pro Plus v4.0; Media Cybernetics, Bethesda, MD, USA).

Western blot: Cells were collected after transfection, and total proteins were extracted from cells. Protein concentration was determined using BCA Protein Assay Kit. Protein samples were denatured in boiling water for 10 minutes and then separated using SDS-PAGE gel (10%). Proteins were then transferred to PVDF membranes, which were then incubated with anti-collagen I (1:1,000, Youliante, Wuhan, China), anti-collagen III (1:1,000, Youliante, Wuhan, China), and anti-GAPDH antibodies (1:1,000, Youliante, Wuhan, China) overnight. It was then incubated with 1:5,000 labeled anti-rabbit secondary antibody for 1 hour. Western blot analysis was performed as previously described.26

Luciferase reporter assay: The interaction between
ncRNA MHRT and miR-3185 was predicted using Starbase web server v2.0 (http://starbase.sysu.edu.cn) or target scan v7.2 (http://www.targetscan.org/vert_72/). The pmirGLO luciferase reporter gene vectors containing wild-type (MHRT-wt) or mutant (MHRT-mut) H1919 were transfected into CF cells with Lipofectamine 2000 reagent (Invitrogen), and co-transfected with miR-3185 mimic or control cells. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (E1910; Promega, Beijing, China) 48 hours after transfection.

RNA-binding protein immunoprecipitation (RIP) and pull-down assay: RNA immunoprecipitation was performed using an RNA immunoprecipitation kit. Magnetic beads were combined with anti-Ago2 or IgG (Millipore, Billerica, MA, USA). The immunoprecipitated RNA was analyzed by qRT-PCR. Biotin-labeled negative controls, biotin-labeled miR-3185, and biotin-labeled miR-3185-Mut were used to transfect cells for 48 hours. Cell extracts were combined with M-280 Streptavidin magnetic beads (Invitrogen, Carlsbad, CA, USA) for 4 hours. Co-precipitated RNA was then isolated by lysis buffer containing proteinase K (Invitrogen, Carlsbad, CA, USA) and 10% sodium dodecyl sulfate (SDS). PCR analysis was then performed.

Echocardiographic measurements: The cardiac function of mice was evaluated using an ultrasound machine (Visual Sonics, Toronto, ON, Canada) with a 30 MHz phase-array transducer. The parameters, including left ventricular ejection fraction (EF) and fractional shortening (FS), were calculated from M-mode recording.

Infection of lentivirus carrying siMHRT in vivo: The lentivirus carrying siMHRT plasmids and empty vectors (NC) were constructed by Genechem Co., Ltd. (Shanghai, China). The procedure for intracardiac injection of lentivirus was as previously described. Briefly, mice were anesthetized, a thoracotomy was performed through the fourth intercostal space, and after that, the ascending aorta was clamped. The lentivirus (1.0 × 10⁷ TU/75 μL) was injected into the left ventricular cavity. The aorta was occluded for 10 s after injection. MI model was established 2 weeks after injection.

Statistical analysis: Data were analyzed using SPSS19.0 statistical software. The results were shown as the mean ± standard deviation (mean ± SD). Comparisons among multigroup data were performed using one-way ANOVA. LSD test was used for subsequent analysis. Two-sided P values less than 0.05 indicated significant difference.

Results

Overexpression of MHRT promoted collagen production and proliferation of CFs: The qRT-PCR results showed that compared with the normal group, the expression levels of MHRT in the heart marginal region of the MI group were significantly raised (P < 0.05) (Figure 1A). After treatment with 20 ng/mL of TGF-β1, the expression levels of MHRT in cardiac fibroblasts (CF) were also significantly raised (P < 0.05) (Figure 1B). To evaluate the effect of MHRT on CF, the MHRT overexpression plasmid was transfected into cultured CF. As shown in Figure 1C, compared with the control group, the expression levels of MHRT in the MHRT-transfected group were significantly increased (P < 0.05), but not in the NC group. In addition, the expression levels of collagen I and III in CF in the TGF-β1-induced group were significantly increased (P < 0.05) at both mRNA and protein levels. Compared with the NC group, the expression levels of Col I and Col III in CF with overexpression of MHRT were significantly increased (P < 0.05) (Figure 1D-G). Meanwhile, the content of total collagen in CF with overexpression of MHRT was significantly increased (P < 0.05) (Figure 1H). In addition, MTT and EdU staining assay results showed that compared with the control group, the cell viability and the number of EdU-positive cells in CF in the TGF-β1 induction group were significantly increased (P < 0.05). Compared with the NC group, the cell viability and the number of EdU-positive cells in CF with overexpression of MHRT were also significantly increased (P < 0.05), which was similar to the role that TGF-β1 played (Figure 1I and J). These results showed that overexpression of MHRT activated CF and promoted collagen production.

Silencing of MHRT inhibited TGF-β1-induced CF collagen production and proliferation: To further explore the role of MHRT in cardiac fibrosis, the effect of silencing of MHRT on CFs collagen production was analyzed. As shown in Figure 2A, compared with the control group, the expression levels of MHRT in CF induced by TGF-β1 were significantly increased (P < 0.05), and siMHRT significantly reduced the expression levels of MHRT in CF treated with TGF-β1 (P < 0.05). In addition, siMHRT significantly reduced the expression levels of Col I and Col III in CF treated with TGF-β1 at both mRNA and protein levels (P < 0.05) (Figure 2B-E). Moreover, siMHRT also reduced the total collagen content in CF treated with TGF-β1 (P < 0.05) (Figure 2F). Moreover, MTT and EdU staining assay results showed that siMHRT significantly inhibited CF activity induced by TGF-β1 and the number of EdU-positive cells (P < 0.05) (Figure 2G and H). These results indicated that silencing of MHRT can inhibit TGF-β1-induced CF collagen production and proliferation.

The relationship between the expression of MHRT and miR-3185 in CFs: Starbase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php) was used to predict the interaction between MHRT and miR-3185, and it showed that miR-3185 was a potential target of MHRT (Figure 3A). To verify the interaction between them, the WT-MHRT or mutant (mut)-MHRT luciferase reporter plasmid was used for the luciferase reporter gene assay. The results showed that the luciferase activity of pGL3-REPOR-MHRT-WT was reduced by miR-3185 mimics (P < 0.05), but the luciferase activity of pGL3-REPOR-MHRT-mut did not change significantly (Figure 3B). The results of RIP and RNA pull-down assay further confirmed the direct interaction between MHRT and miR-3185 (Figure 3C and D). In addition, the expression of MHRT and miR-3185 was negatively and significantly correlated in MI mice (P < 0.05) (Figure 3E). These results indicated that MHRT functioned as a competing endogenous RNA (ceRNA) of miR-3185 in MI.

MHRT may exert its biological function through miR-3185: Compared with the control group, the expression
levels of miR-3185 with the overexpression of MHRT were significantly reduced (Figure 4A), while the negative control (NC) group had no such effect. The expression levels of miR-3185 in CF in the TGF-β1-induced group
were significantly reduced ($P < 0.05$), and siMHRT reversed the inhibitory effect of TGF-$\beta$1 on the expression of miR-3185 ($P < 0.05$) (Figure 4B). As shown in Figure 4C, overexpression of miR-3185 downregulated TGF-$\beta$1-induced CF proliferation. Overexpression of miR-3185 inhibited the expression of Col I and Col III induced by TGF-$\beta$1 at both mRNA and protein levels ($P < 0.05$) (Figure 4D-G). These results indicated that MHRT may exert its biological function through miR-3185. The involvement of miR-3185 in the regulation of cardiac fibrosis by MHRT was then evaluated. As shown in Figure 4H, knockdown of miR-3185 reversed siMHRT-induced decreases of CF proliferation. Moreover, MHRT siRNA abrogated TGF-$\beta$1-induced expression of Col I and Col III at both mRNA and protein levels, which was reversed by knockdown of miR-3185 (Figure 4I-L). These data suggested that miR-3185 is a downstream mediator of MHRT in the regulation of cardiac fibrogenesis.

**Knockout of MHRT suppressed cardiac fibrosis in MI mice:** The role of MHRT in cardiac fibrosis was further explored in vivo using lentivirus transfection. The expression levels of MHRT were lower in Lv-siMHRT mice compared with that in age-matched Lv-control mice. The upregulation of MHRT after MI was to a significantly
Figure 3. Relationship between the expression of MHRT and miR-3185 in CFs. A: Putative binding sites for miR-3185 and MHRT 3’-UTR by searching online program Starbase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php). B: MiR-3185 mimics or NC mimics were transfected into CF cells with MHRT 3’-UTR-WT or MHRT 3’-UTR-WT. Luciferase activity in CF cells were detected. C: RIP assay using anti-Ago2 antibody demonstrated that lncRNA MHRT and miR-3185 formed an RNA-induced silencing complex (RISC). D: RNA pull-down assay indicated that lncRNA MHRT could be enriched by biotin-labeled miR-3185 but not biotin-labeled miR-3185 mutant type. E: Relationship between the expression of MHRT and miR-3185 in MI patients. *P < 0.05. All experiments were performed in three independent trials.

smaller extent in Lv-siMHRT mice compared with that in Lv-controls (Figure 5A). The expression levels of miR-3185 were higher in Lv-siMHRT mice than that in Lv-controls (Figure 5B). The echocardiography data showed that Lv-siMHRT improved cardiac function, as reflected by increased EF and FS in MI mice (Figure 5C and D). Collagen deposition was obviously reduced in MI mice with Lv-siMHRT (Figure 5E and F). Consistently, Lv-siMHRT mitigated MI-induced expression of Col I and Col III at both mRNA and protein levels (Figure 5G-J). These results indicated that suppression of MHRT is a negative determinant of MI-driven cardiac fibrosis.

Discussion

Myocardial fibrosis (MF) is the advanced pathophysiological manifestation of various cardiovascular diseases such as acute MI, hypertension, diabetic cardiomyopathy, and pulmonary hypertension.27,28) The main pathological changes included activation of myocardial fibroblasts, proliferation, and transformation into muscle fibroblasts expressing α-smooth muscle actin (α-SMA).29) The extracellular matrix of the heart, including various structural proteins, matrix proteins, and adhesion proteins, which are synthesized and secreted by cells, can not only provide a structural framework for myocardial cells but also participate in various biological signaling pathways and inhibit the propagation of cardiac electrical activity.30,31) Type I collagen (col 1) and Collagen III (Col 3) are the two main structural proteins of the extracellular matrix and are a typical sign of the transformation of CFs into myofibroblasts.32) Therefore, Col1 and Col 3 are often used as the heart measurement index of myofibrosis.33) In this study, Col I and Col III were used as the measurement indicators of MF.

The factors involved in MF are the renin-angiotensin-aldosterone system, matrix metalloproteinases, tissue inhibitors, vascular endothelial dysfunction, and transforming growth factor (TGF-β1).34) Moreover, TGF-β1 plays a key role in the process of MF by upregulating genes of type I and type III collagen, leading to collagen deposition.35) Studies have also shown that TGF-β1 can stimulate CFs to transform into myofibroblasts, induce CFs to synthesize matrix metalloproteinases, and cause ventricular remodeling.36)

To reduce MF after MI, extensive studies have investigated the mechanisms of MF MI.37) LncRNAs can regulate gene expression, sponge miRNAs, and regulate cell transcription and translation.38,39) Studies have shown that lncRNAs play key roles in cardiovascular diseases such as MF and atrial fibrillation.40,41) An increasing number of lncRNAs have been reported to affect the apoptosis of myocardial fibrotic cells.42) For example, it was found that lncRNA-PFL is upregulated in myocardial fibrotic fibro-
MHRT promotes cardiac fibrosis after MI through miR-3185. This study suggests that MHRT may be a potential pathogenic gene for MF after MI.

Conclusion

MHRT promoted cardiac fibrosis after MI through miR-3185. This study suggests that MHRT may be a potential pathogenic gene for MF after MI.
Figure 5. Heterozygous knockdown of MHRT ameliorated MI-induced cardiac fibrosis in vivo. A: MHRT levels in the hearts of Lv-control-sham, Lv-siMHRT-sham, Lv-control-MI, and Lv-siMHRT-MI mice by qRT-PCR. B: MiR-3185 expression in the hearts of WT-sham, MHRT +/−-sham, WT-MI, and MHRT +/−-MI mice by qRT-PCR. C, D: Knockdown of MHRT restored the impaired cardiac function in infarcted hearts, determined by echocardiography. E, F: Representative Masson images of mice hearts from each group and quantification of the total fibrotic area using Image-Pro Plus. Scale bar = 50 μm. G–J: Knockdown of MHRT inhibited MI-induced Col I and Col III expression by Western blot and qRT-PCR analysis. *P < 0.05 versus WT-sham; *P < 0.05. n ≥ 6 in each group.

Disclosure

Conflicts of interest: The authors declare that they have no conflicts of interests.

Ethics approval and consent to participate: All animal experimental protocols were approved by the Laboratory Animal Care and Use Committee of Chengdu Fifth People’s Hospital.

Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions: MJL: literature research, experiment studies, research design, data analysis, and manuscript writing. DKO, ZHL, YL, XHZ and FPZ: data analysis, experiment work, manuscript editing, and clinical studies. All authors read and approved the final manuscript.

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