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The mechanism of myocardial fibrosis is ameliorated by myocardial infarction-associated transcript through the PI3K/Akt signaling pathway to relieve heart failure

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Abstract

Objective: This study aimed to investigate the role of long noncoding RNA (LncRNA) myocardial infarction-associated transcript (MIAT) in a heart failure (HF) model *in vivo* and *in vitro* by regulating the PI3K/Akt signaling pathway.

Methods: We established HF models *in vivo* and *in vitro* and evaluated the collagen content of these models and other factors.

Results: We found that when LncRNA MIAT was silenced, vascular endothelial growth factor, phosphorylated protein kinase B (Akt), and phosphorylated phosphoinositide 3-kinase (PI3K) mRNA and protein levels were significantly downregulated, which suggested that MIAT activated the PI3K/Akt signaling pathway. Akt and PI3K expression was not significantly changed. We also found that when LncRNA MIAT was silenced, collagen expression was significantly downregulated. This finding suggested that MIAT promoted myocardial fibrosis during the development of HF. The levels of inflammatory factors were also significantly reduced with silencing of LncRNA MIAT. This finding suggested that MIAT promoted the expression of inflammatory factors in myocardial fibrosis by activating the PI3K/Akt signaling pathway.

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Conclusion: This study indicates that silencing LncRNA MIAT may improve myocardial fibrosis and alleviate HF through the PI3K/Akt signaling pathway, which may be helpful for patients with HF to obtain a better therapeutic effect.

Keywords
Long noncoding RNA, myocardial infarction-associated transcript, myocardial fibrosis, heart failure, phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), inflammation

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Introduction

Heart failure (HF) affects more than 23 million people worldwide and causes a high rate of morbidity and mortality.[1] HF is an end-stage disease of various cardiovascular diseases and it has similar pathological characteristics.[2] At present, the focus of HF treatment is to prevent and reverse myocardial remodeling, and the treatment of myocardial fibrosis (MF) is the main research aim. Cardiac fibroblasts (CFs) are important cell groups responsible for extracellular matrix homeostasis. After myocardial infarction (MI), CFs transform into MFs and they play an important role in healing fibrosis.[3,4] Therefore, research on MF is important in the field of HF treatment. Long noncoding RNA (LncRNA) is a new type of molecular regulatory factor in cardiac development and disease. LncRNA plays a part in shaping the structure and function of heart and defining the pathogenesis of cardiovascular disease. LncRNA plays an important role in cardiac remodeling, and MI-associated transcript (MIAT) is a powerful and dynamic modifier for cardiac remodeling.[5] In patients with acute MI, coronary heart disease, or angina pectoralis, the level of MIAT is significantly increased.[6–8] A previous study showed that LncRNA MIAT induces cardiac fibrosis and LncRNA MIAT is a type of fibrotic factor, which is used to control cardiac fibrosis and regulate cardiac function in patients with MI.[8] Additionally, activation of the phosphoinositide 3-kinase/protein kinase B (PI3K/AKt) signaling pathway by LncRNA MIAT aggravates atherosclerosis (AS). Activation of the PI3K/Akt signaling pathway by LncRNA MIAT also promotes angiogenesis and the expression of inflammatory factors in mice.[9]

The mechanism of LncRNA MIAT for improving HF by regulating MF has not been reported. Therefore, this study aimed to investigate this mechanism through the PI3K/Akt signaling pathway to relieve HF. Understanding this mechanism may help patients with HF achieve better treatment results.

Materials and methods

Human CF cultures and grouping

Human CFs (hCFs) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in fibroblast medium (fetal bovine serum, Thermo Fisher Scientific; Waltham, MA, USA) containing 10% fetal bovine serum and 25% CO_2 at 37°C. The medium was changed after 24 to 48 hours and passed once in 48 hours. Cultured hCFs were divided into the

following four groups: negative control (NC) group, NC transfected with MIAT short interfering RNA (siRNA) (NC+si-MIAT) group, NC transfected with scramble siRNA (NC+Scramble) group, and NC induced by transforming growth factor (TGF)-β1 group. Cultured hCFs were treated with TGF-β1 (Sigma Aldrich, St Louis, MO, USA) at 20 ng/mL for 24 hours.

After transfection and induction at 24, 48, and 72 hours in the NC+Scramble, NC+si-MIAT, and TGF-β1 groups, the three groups were treated with TGF-β1 (20 ng/mL) for another 48 hours and then processed for downstream experiments. Cell proliferation activity was measured by the cholecystokinin-8 assay (Beyotime Institute of Biotechnology, Haimen, Jiangsu Province, China) in each group. Cell proliferation activity = (optical density$_{\text{experimental group}}$ − optical density$_{\text{blank control group}}$)/(optical density$_{\text{control group}}$ − optical density$_{\text{blank control group}}$).

Establishment of the HF model in rats and grouping

Healthy male C57BL/6 rats (20–25 g) were fed under an ambient temperature of 25°C±2°C and a 12/12-hour of light/dark environment. All rats were used for subsequent experiments in accordance with the Laboratory Animal Management Regulations and Animal Ethical Requirements before modeling. The animal experimental protocol (approval no. 2019-013) was approved by the Ethics Committee of the Inner Mongolia People's Hospital. Rats were sacrificed and anesthetized by intraperitoneal injection of pentobarbital sodium solution (1%) at a dose of 50 mg/kg. We reduced the number of rats that were used and reduced their suffering as much as possible.

The rats were divided into the four following groups: NC (NC group), HF model (HF group), HF model transfected with MIAT siRNA (HF+si-MIAT group), and HF model transfected with scramble siRNA (HF+Sham group). There were five rats in each group. All of the rats were designed and purchased from Invitrogen (Shanghai, China). The construct was inserted into the lentivirus vector plasmid PHy-LV-KD5.1 (Invitrogen, Shanghai, China). The recombinant lentiviral vector carrying siRNA MIAT was successfully constructed by homologous recombination.

The plasmid (70 μL, 108 TU/mL) carrying si-MIAT or siRNA was injected into the left ventricular chamber and was forced into the coronary arteries when the aortic artery was temporarily occluded for 15 s. Rats in the HF+Sham group were administered 70 μL of Dulbecco's modified Eagle medium as controls.

Measurement of collagen content

The total collagen content was detected by the Sircol Collagen Assay kit (Biocolor Ltd., Northern Ireland, UK) as previously described.[10] The absorbance of the sample at 540 nm was determined by an enzyme-linked immunosorbent assay (ELISA). Using a linear calibration curve, the collagen content was converted into protein units and normalized to the total protein content of each sample.

Real-time reverse transcription polymerase chain reaction

RNA isolation from tissues and cells was performed with the Trizol standard protocol (Invitrogen Inc., Carlsbad, CA, USA), and quantified using the Nano-Drop 8000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). For each sample, RNA was converted to cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLVRT;

Table 1. Primer sequences.

Gene	Primer
VEGF	F: 5′-GCTTCGGCAGCACATATACTAAAAT-3′ R: 5′-CGCTTCACGAATTTGCGTGTCAT-3′
PI3K	F: 5′-GAAAGCCGAGAACCTATTGCGAG-3′ R: 5′-GTTTGACTTCGCCATCTACCAC-3′
Akt	F: 5′-CGCACCGGTATGACGACGTGGCTATTGTGAA-3′ R: 5′-CCGGAATTCTCAGGAAGTGCCGCTGG-3′
TNF-α	F: 5′-CCGATGGGTTGTACCTTGTC-3′ R: 5′-GGGCTGGGTAGAGAATGGAT-3′
IL-1β	F: 5′-TGTGATGAAAGACGGCACAC-3′ R: 5′-CTTCTTCTTTGGGTATTGTTTGG-3′
IL-6	F: 5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′ R: 5′-TCTGACCACAGTGAGGAATGTCCAC-3′
Col I	F: 5′-GCTCCTCTTAGGGGCCACT-3′ R: 5′-CCACGTCTCACCATTGGGG-3′
Col III	F: 5′-ACGTAGATGAATTGGGATGCAG-3′ R: 5′-GGGTTGGGGCAGTCTAGTG-3′
GAPDH	F: 5′-AAGAAGGTGGTGAAGCAGGC-3′ R: 5′-TCCACCACCCTGTTGCTGTA-3′

F, forward; R, reverse; VEGF, vascular endothelial growth factor; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; TNF-α, tumor necrosis factor-α; IL, interleukin; Col, collagen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Promega, Madison, WI, USA). The transcript levels were detected by SYBR Green I incorporation methods on the ABI 7500 fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA). The sequences of primer pairs were synthesized by RiboBio (Guangzhou, China) and are listed in Table 1.

ELISA assay

Levels of interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, collagen (Col) I, and Col III were measured with commercial ELISA kits (Mlbio, Shanghai, China) according to the manufacturer's protocol.

Statistical analysis

All statistical analyses were performed using IBM SPSS 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 7.0 (GraphPad Prism Software Inc., San Diego, CA, USA). Differences between two groups and multiple groups were analyzed using the t-test and one-way analysis of variance, respectively. A p value <0.05 was considered as statistically significant.

Results

Silencing of MIAT inhibits TGF-β1-induced collagen production and proliferation in hCFs

To determine the role of MIAT in CFs, the effects of LncRNA MIAT knockdown on collagen production in hCFs were studied. Cultured hCFs were transfected with MIAT siRNA or scramble siRNA for 48 hours, and TGF-β1 was used to induce hCFs. In the NC+si-MIAT group, cell proliferation activity was significantly lower compared with that in the TGF-β1 group ($p<0.05$, Figure 1a). This finding suggested that MIAT enhanced TGF-β1-induced proliferation of hCFs. LncRNA MIAT knockdown significantly inhibited TGF-β1-induced upregulation of Col I and Col III mRNA (Figure 1i, j) and protein (Figure 1k, l) levels (all $p<0.05$). MIAT mRNA levels in the NC+Scramble and NC+si-MIAT groups were significantly higher than those in the NC group (both $p<0.05$, Figure 1p).

MIAT activates the PI3K/Akt signaling pathway

The activation state of the PI3K/Akt signaling pathway was determined by real-time reverse transcription-polymerase chain

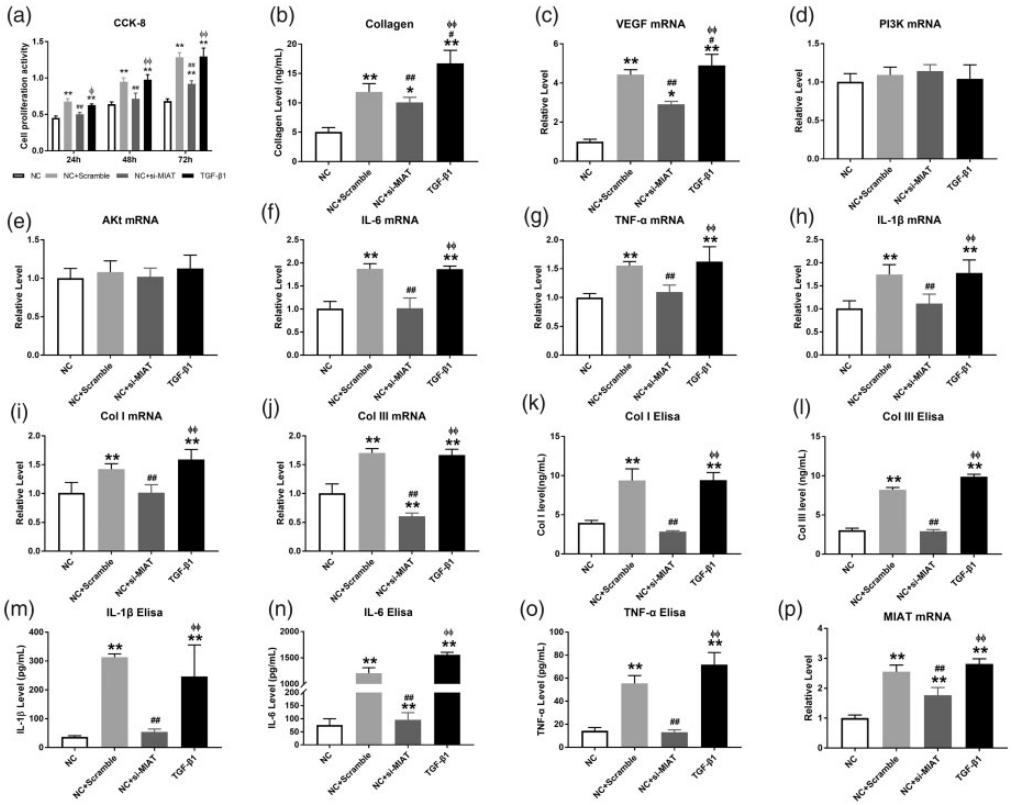

Figure 1. (a) Cell proliferation activity as measured by the CCK-8 assay in each group. (b) Measurement of collagen content in each group. (c–p) Expression levels of mRNA and protein for various factors *in vitro* with measurement by real-time reverse transcription polymerase chain reaction and ELISA assays.
*$p<0.05$ versus the NC group; **$p<0.01$ versus the NC group.
#$p<0.05$ versus the NC+Scramble group; ##$p<0.01$ versus the NC+Scramble group.
$\phi$$p<0.05$ versus the NC+si-MIAT group; $\phi\phi$$p<0.01$ versus the NC+si-MIAT group.
NC, negative control; siMIAT, short interfering RNA myocardial infarction-associated transcript; TGF-ß, transforming growth factor-ß; CCK-8, cholecystokinin-8; VEGF, vascular endothelial growth factor; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; IL, interleukin; TNF-α, tumor necrosis factor-α; Col, collagen; ELISA, enzyme-linked immunosorbent assay; MIAT, myocardial infarction-associated transcript.

reaction (RT-PCR) and western blotting in *in vitro* and *in vivo* experiments. In *in vitro* experiments, PI3K mRNA levels (Figure 1d) and Akt mRNA levels (Figure 1e) in the NC+si-MIAT group were not significantly different compared with those in the NC+Scramble group. However, vascular endothelial growth factor (VEGF) mRNA levels were significantly lower in the NC+si-MIAT group than in the NC+Scramble group (Figure 1c). We also found the same results at the protein expression level for VEGF, PI3K, and Akt (Figure 2). Protein expression levels of phosphorylated (p)-PI3K and p-Akt were significantly lower in the NC+si-MIAT group than in the NC+Scramble group (both $p<0.05$, Figure 2). In *in vivo* experiments, we found that VEGF (Figure 3a),

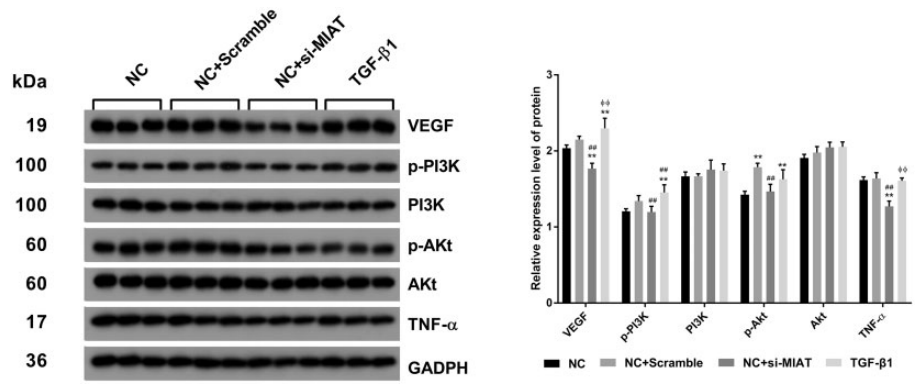

Figure 2. Silencing MIAT inhibits the PI3K/Akt signaling pathway *in vitro*.
**p<0.01 versus the NC group; ##p<0.01 versus the NC+Scramble group.
$\phi\phi$p<0.01 versus the NC+si-MIAT group.
NC, negative control; siMIAT, short interfering RNA myocardial infarction-associated transcript; TGF-ß1, transforming growth factor-ß1; VEGF, vascular endothelial growth factor; p-PI3K, phosphorylated phosphoinositide 3-kinase; PI3K, phosphoinositide 3-kinase; p-Akt, phosphorylated protein kinase B; Akt, protein kinase B; TNF-α, tumor necrosis factor-α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PIK3 (Figure 3b), and Akt (Figure 3c) mRNA expression levels in the HF and HF+Sham groups were significantly upregulated compared with the NC group (all p<0.05). VEGF (p<0.05, Figure 3a) and Akt (p<0.05, Figure 3c), but not PIK3, mRNA expression levels were significantly downregulated in the HF+si-MIAT group compared with the HF group. We found the same results at the protein expression level (Figure 4a). These results suggested that MIAT activated the PI3K/Akt signaling pathway.

MIAT regulates MF through the PI3K/Akt signaling pathway

In *in vivo* experiments, we found that Col I (Figure 3g) and Col II (Figure 3h) mRNA expression levels were significantly upregulated in the HF and HF+Sham groups compared with the NC group (all p<0.05). Col I (Figure 3i) and Col II (Figure 3j) protein expression levels were significantly downregulated in the HF+si-MIAT group compared with the HF group. These results suggested that MIAT promoted MF in rats with HF by activating the PI3K/Akt signaling pathway.

MIAT promotes inflammatory factor expression by activating the PI3K/Akt signaling pathway

In *in vitro* experiments, IL-1β, IL-6, and TNF-α mRNA (Figure 1f–h) and protein (Figure 1m–o) expression levels were significantly downregulated in the NC+si-MIAT group compared with the NC+Scramble group (all p<0.05). In *in vivo* experiments, IL-1β, IL-6, and TNF-α mRNA (Figure 3d–f) and protein (Figure 3k, l, n) expression levels were significantly higher in the HF and HF+Sham groups compared with the NC group (all p<0.05). There were no significant differences in IL-1β, IL-6, or TNF-α expression between the NC and HF+si-MIAT groups. These results suggested that MIAT promoted inflammatory factor expression in MF by activating the PI3K/Akt signaling pathway.

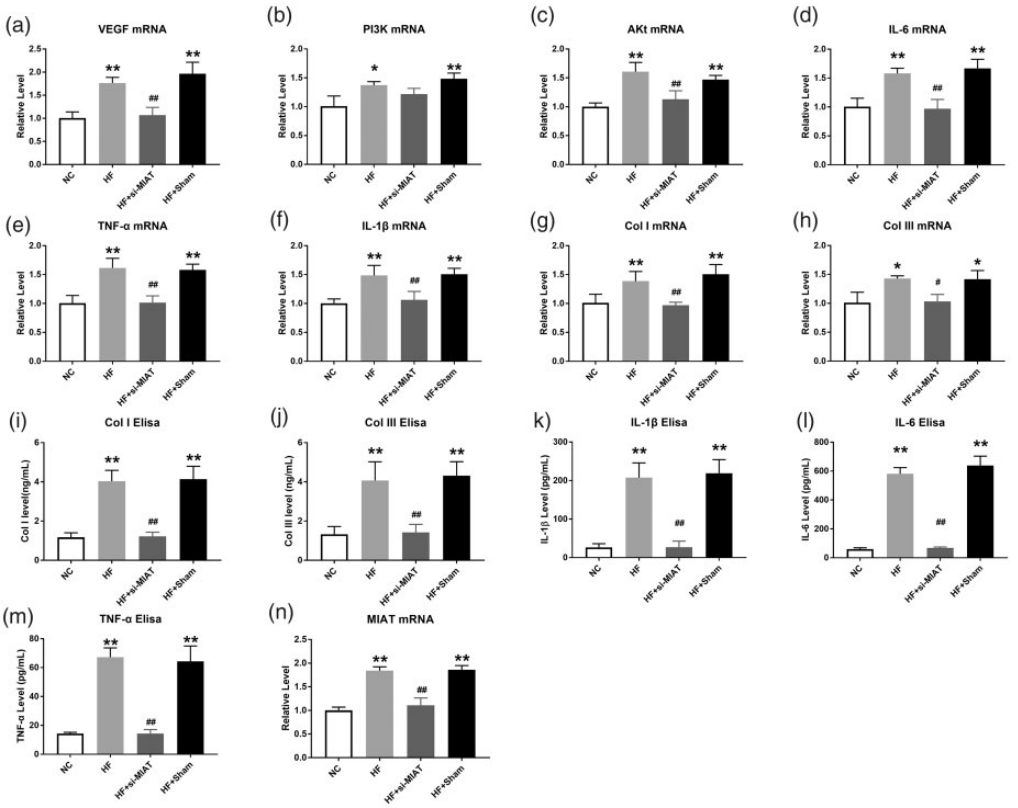

Figure 3. Expression levels of mRNA and protein for various factors *in vivo* with measurement by real-time reverse transcription polymerase chain reaction and ELISA assays.
*$p<0.05$ versus the NC group; **$p<0.01$ versus the NC group.
#$p<0.05$ versus the HF group; ##$p<0.01$ versus the HF group.
NC, negative control; HF, heart failure; siMIAT, short interfering RNA myocardial infarction-associated transcript; VEGF, vascular endothelial growth factor; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; IL, interleukin; TNF-α, tumor necrosis factor-α; Col, collagen; ELISA, enzyme-linked immunosorbent assay; MIAT, myocardial infarction-associated transcript.

Discussion

Cardiomyocyte apoptosis leads to blocked arterial flow to the heart, damage to cardiac muscle, and eventually heart failure and sudden cardiac death.[11] LncRNA is a cell response molecule of bioactive polyphenols that is used in the treatment of many diseases. LncRNA MIAT is expressed in the heart and fetal brain tissue.[12] HF is a common complication of MI, leading to high mortality worldwide.[13–15] Recent studies have shown that upregulation of LncRNA MIAT aggravates the development of AS[16] and ischemic stroke,[17] and the mechanism of developing AS is related to the activation of the PI3K/Akt signaling pathway. Activation of the PI3K/Akt signaling pathway can reduce the level of reactive oxygen species and lipid deposition, thus inhibiting plaque formation and reversing the progression of AS.[18] The role of MIAT in HF through the PI3K/Akt signaling pathway has not been reported. Therefore, there is an urgent

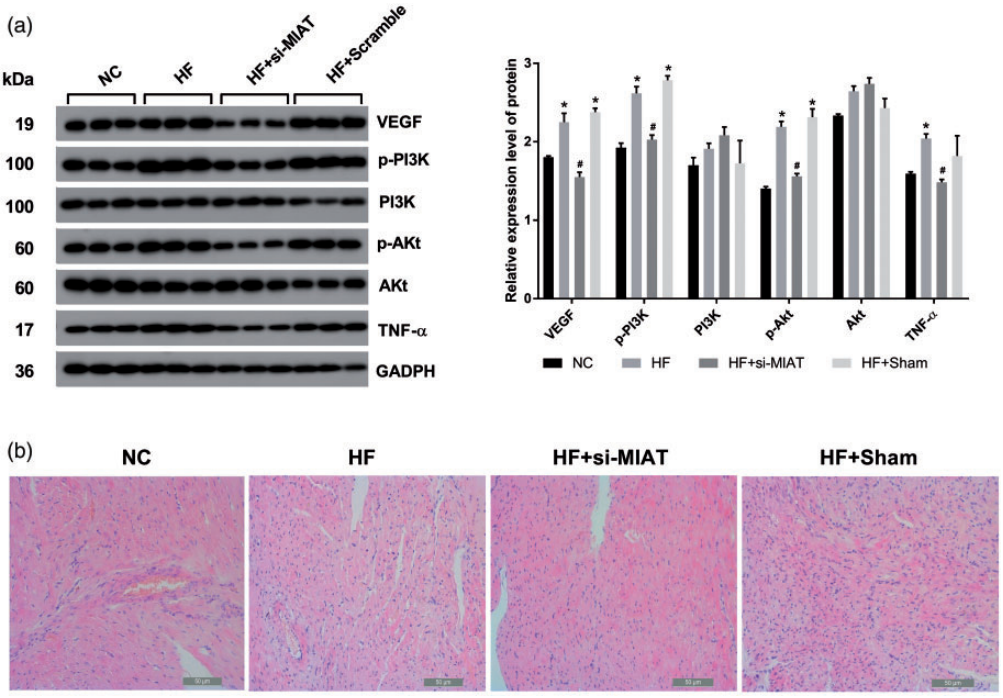

Figure 4. (a) Silencing MIAT inhibits the PI3K/Akt signaling pathway (protein expression). (b) Hematoxylin and eosin staining in *in vivo* experiments.
*$p<0.05$ versus the NC group; #$p<0.05$ versus the HF group.
NC, negative control; HF, heart failure; siMIAT, short interfering RNA myocardial infarction-associated transcript; VEGF, vascular endothelial growth factor; p-PI3K, phosphorylated phosphoinositide 3-kinase; PI3K, phosphoinositide 3-kinase; p-Akt, phosphorylated protein kinase B; Akt, protein kinase B; TNF-α, tumor necrosis factor-α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

need to identify effective therapeutic targets to reduce the incidence of HF. In the present study, we examined the role of LncRNA MIAT in an HF model through regulation of the PI3K/Akt signaling pathway. Our study suggested that silencing MIAT reduced the occurrence of HF by activating the PI3K/Akt signaling pathway.

The Akt family is involved in a variety of cellular processes, including promoting cell survival, glucose metabolism, and cellular protein synthesis in cardiomyocytes.[19] Generally, Akt contains three isoforms of Akt1, Akt2, and Akt3. Short-term Akt1 activation promotes cardiac growth,[20] whereas long-term Akt1 activation induces pathological hypertrophy[21] because Akt1 overexpression leads to HF.[22] A previous study also showed that MIAT activated the PI3K/Akt signaling pathway.[9] Our study is consistent with this previous study because we found that p-PI3K and p-Akt expression was significantly decreased after silencing MIAT as shown by real-time RT-PCR and WB analysis. Interestingly, MIAT functions as an miR-150-5p sponge and affects the inhibitory effect of VEGF, which promotes the migration of inflammatory cells to the intima and induces pathological angiogenesis.[17] Our study showed that MIAT knockdown inhibited VEGF expression by real-time RT-PCR and WB

analysis. TGF-β is the major fiber-derived growth factor in the MF process.[23] TGF-β signaling plays an important role in inflammation and immune regulation.[24,25] TGF-β1/Smad2/3 signaling contributes to MF.[26] We found that MIAT silencing inhibited the production and proliferation of TGF-β1-induced MF as shown by collagen expression. MIAT promotes angiogenesis and expression of inflammatory factors (IL-1β, IL-6, and TNF-α) in mice with AS by activating the PI3K/Akt signaling pathway.[9] TNF-α is a major cytokine implicated in the progression of AS.[22] In this study, silencing MIAT suppressed inflammatory factor expression by activating the PI3K/Akt signaling pathway. We also found that MIAT knockdown significantly inhibited the proliferation of MF in the HF rat model. Additionally, MIAT knockdown attenuated Col I and Col III levels in the HF model. MIAT may have regulated CF through the PI3K/Akt signaling pathway. Taken together, our results suggest that MF is ameliorated by MIAT through the PI3K/Akt signaling pathway and this relieves HF.

There are some limitations to the present study. Conservation of the MIAT sequence is low between rats and humans, and LncRNAs are generally poorly conserved among species.[27] Therefore, the clinical significance of this study remains unclear. Furthermore, except for the PI3K/Akt pathway, we cannot exclude the involvement of other mechanisms in regulating LncRNA MIAT in HF. Another limitation of this study is that we cannot rule out involvement of other mechanisms of regulation of cardiac fibrosis by MIAT other than the PI3K/Akt pathway.

This study indicates that silencing MIAT ameliorates MF by the PI3K/Akt signaling pathway to relieve heart failure. However, further research is required to support our results. Our findings suggest that LncRNA MIAT is a molecular target for intervention in HF in humans, but further study is required to verify the potential mechanism for LncRNA MIAT in treating HF.

Author contributions

Conception and design of the research: Xingsheng Zhao and Yu Ren; acquisition of data: Hongkun Ren; analysis and interpretation of data: Huan Chen and Chun Ying; statistical analysis: Yun Wu and Xi Liu; drafting the manuscript: Yu Ren; obtaining funding and revision of manuscript for important intellectual content: Xingsheng Zhao.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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