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LncRNA HOTAIR aggravates myocardial ischemia-reperfusion injury by sponging microRNA-126 to upregulate SRSF1

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Abstract. – OBJECTIVE: Acute myocardial infarction (AMI) is a severe fatal disease throughout the world. Myocardial IR limits the recovery of impaired cardiac function in AMI patients. This study aims to elucidate the role of long non-coding RNA (lncRNA) HOTAIR in myocardial ischemia-reperfusion (IR) and the underlying mechanism, thus providing a novel therapy for AMI.

MATERIALS AND METHODS: Myocardial IR model in mice was firstly constructed by LAD. Plasma levels of LDH, CK-MB, HOTAIR, and microRNA-126 in mice were detected. Subsequently, *in vitro* HR model was constructed in H9c2 cells. Regulatory effects of HOTAIR on proliferative ability, LDH release, and Caspase-3 activity in H_2O_2-induced H9c2 cells were determined. Relative levels of inflammatory factors in *in vitro* HR model were measured by enzyme-linked immunosorbent assay (ELISA). The regulatory loop HOTAIR/microRNA-126/SRSF1 was finally verified by Dual-Luciferase reporter assay.

RESULTS: LDH and CK-MB were significantly released in mice with myocardial IR. HOTAIR was upregulated, while microRNA-126 was downregulated in IR mice and H_2O_2-induced H9c2 cells. Overexpression of HOTAIR stimulated proliferative ability, LDH release, and Caspase-3 activity in H_2O_2-induced H9c2 cells. Besides, overexpression of microRNA-126 inhibited the release of inflammatory factors in H9c2 cells undergoing HR induction. The regulatory loop HOTAIR/microRNA-126/SRSF1 was identified to influence IR development.

CONCLUSIONS: HOTAIR aggravates myocardial IR by competitively binding SRSF1 with microRNA-126.

Key Words:
Myocardial IR, HOTAIR, MicroRNA-126, SRSF1.

Introduction

Acute myocardial infarction (AMI) is a heart disease with an extremely high mortality[1]. Myocardial ischemia-reperfusion (IR) could reduce the range of myocardial necrosis, which is the major therapy for AMI[2]. Contradictorily, IR aggravates cardiac dysfunction and structure damage following blood flow restore, leading to secondary damages[3,4]. It is reported that 40-50% of AMI cases suffer myocardium necrosis because of IR injury[5-7]. Hence, prevention and treatment of IR are of significance.

Long non-coding RNAs (LncRNAs) are non-coding transcripts with over 200 nucleotides. They exert diverse functions by interacting with DNAs, miRNAs, mRNAs, and proteins[8]. LncRNAs are extensively involved in cell metabolism, cell differentiation, and tumor progression[9]. So far, potential influences of lncRNAs in ischemic injuries have been identified[10]. Knockdown of lncRNA KCNQ1QT1 protects myocardial IR following AMI through the MAPK/NF-κB pathway[11]. By regulating the release of antioxidants, lncRNA ROR participates in HR-induced myocardial injury[12].

MicroRNAs (miRNAs) are endogenous, single-strand RNAs that exert a post-transcriptional regulation on gene expressions. They participate in almost every aspect of cell behavior[13-15]. Several miRNAs have been discovered to be critical in cardiac diseases[16-18]. As the vital regulation in myocardial IR, miRNAs may be potential therapeutic targets of AMI[19].

In this paper, *in vivo* myocardial IR model in mice was established by LAD. Meanwhile, *in vitro* HR model in H9c2 cells was constructed. Regulatory effects of HOTAIR on myocardial IR were specifically clarified.

Materials and Methods

In Vivo Myocardial IR Model in Mice

Mice were administrated with Xylazin/Ketamine solution and intubated. After exposure of

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the left chest cavity, the left anterior descending coronary artery (LAD) was ligated using an 8-0 prolene suture. A steady elevation of ST segment indicated the successful induction of myocardial ischemia. Thirty minutes later, reperfusion was conducted for 6, 12, and 24 h, respectively. Mice were sacrificed by isoflurane anesthesia at the end of experiments.

Cell Culture and In Vitro HR Model

H9c2 cells were provided by Cell Bank (Shanghai, China). Cells 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 Ul/mL penicillin, and 100 μg/mL streptomycin. For inducing hypoxia/reoxygenation (HR) injury, cells were incubated with $Na_2S_2O_4$ for 0, 2, 4, and 8 h, followed by reoxygenation at 12 h.

Ethical Statement

Mice were provided by Experimental Animal Center (Shanghai, China). This research was approved and supervised by Ethic Committee, Shanxi Cardiovascular Hospital. All experimental protocols followed the guidelines for animal experimentation.

Lentivirus Transfection

LV-NC, LV-HOTAIR, and LV-shHOTAIR were purchased from Genechem (Shanghai, China). Lentivirus screening was conducted by puromycin. In addition, microRNA-126 mimics and inhibitor, as well as negative controls were transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Determination of LDH and CK-MB

Relative levels of LDH and CK-MB in mouse plasma or cell supernatant were detected by commercial kits (Invitrogen, Carlsbad, CA, USA).

Cell Counting Kit-8 (CCK-8)

Cells were inoculated in a 96-well plate. 100 μL of 10% CCK-8 was applied in each well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

5-Ethynyl-2′-Deoxyuridine (EdU)

Cells were inoculated in a 96-well plate, incubated with 50 μM EdU (Sigma-Aldrich, St. Louis, MO, USA), and dyed with Apollo and DAPI (Ribobio, Guangzhou, China) in the dark. Images of EdU-positive cells were captured and counted.

Caspase-3 Activity Determination

Cells were lysed and incubated in enzyme-linked immunosorbent assay (ELISA) buffer (Cell Signaling Technology, Danvers, MA, USA) in the dark, followed by determination of Caspase-3 activity in a microplate reader.

Dual-Luciferase Reporter Assay

Wild-type and mutant-type vectors were constructed by GenePharma (Shanghai, China), according to the binding sequences in the 3′-untranslated region (3′-UTR). Cells were co-transfected with vectors and microRNA-126 mimics/NC using Lipofectamine 3000, followed by determination of Luciferase activity (Promega, Madison, WI, USA).

ELISA

The antibody was diluted to 10 μg/mL with a carbonate coating buffer, and 0.1 mL of diluted antibody was added to each well of a polystyrene plate at 4°C. On the other day, the solution was replaced by 0.1 mL of sample, incubated at 37°C for 1 h, and washed. Subsequently, enzyme-labeled antibodies were applied for 1 h incubation. After washing, the substrate solution was applied and finally, H_2SO_4 was added to terminate the reaction 30 minutes later. The absorbance at 450 nm was measured on an ELISA detector.

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

TRIzol method (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA. Through reverse transcription of RNA, the extracted cDNA was used for PCR detection using the DBI Bestar SybrGreen qRT-PCR Master Mix (DBI Bioscience, Shanghai, China) on Stratagene Mx3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference. HOTAIR Forward (5′-3′): CAGTGGGGAACTCTGACTCG; Reverse (5′-3′): GTGCCTGGTGCTCTCTTACC; microRNA-126 Forward (5′-3′): GGGGTCGTACCGTGAGT; Reverse (5′-3′): CAGTGCGTGTCGTGGAGT; SRSF1 Forward (5′-3′): CCGCAGGGAACAACGATTG; Reverse (5′-3′): GCCGTATTTGTAGAACACGTCCT; GAPDH Forward (5′-3′):

CTCCTCCACCTTTGACGCTG; Reverse (5′-3′): TCCTCTTGTGCTCTTGCTGG; U6 Forward (5′-3′): GCTGAGGTGACGGTCTCAAA; Reverse (5′-3′): GCCTCCCAGTTTCATGGACA.

Western Blot

Cells were lysed for isolating cellular protein and electrophoresed. The protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for indicated time. Primary SRSF1 antibody used was bought from Proteintech (Chicago, IL, USA). Band exposure and analyses were finally conducted. Immuno-reactive bands were visualized by enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Piscataway, NJ, USA). The gray value was analyzed using Image J software (Version 1.38; National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were expressed as mean ± SD (standard deviation). The *t*-test was conducted to compare differences. $p<0.05$ indicated the significant difference.

Results

Dynamic Expressions of HOTAIR and miR-126 in IR Mice

After constructing myocardial IR model in mice, the relative levels of LDH and CK-MB in mice were determined. With the prolongation of reperfusion, releases of LDH and CK-MB in IR mice gradually increased, which achieved the peak at 12 h, and remarkably decreased at 24 h (Figure 1A, 1B). In addition, HOTAIR was gradually upregulated at 6 and 12 h following reperfusion, and it was suddenly reduced at

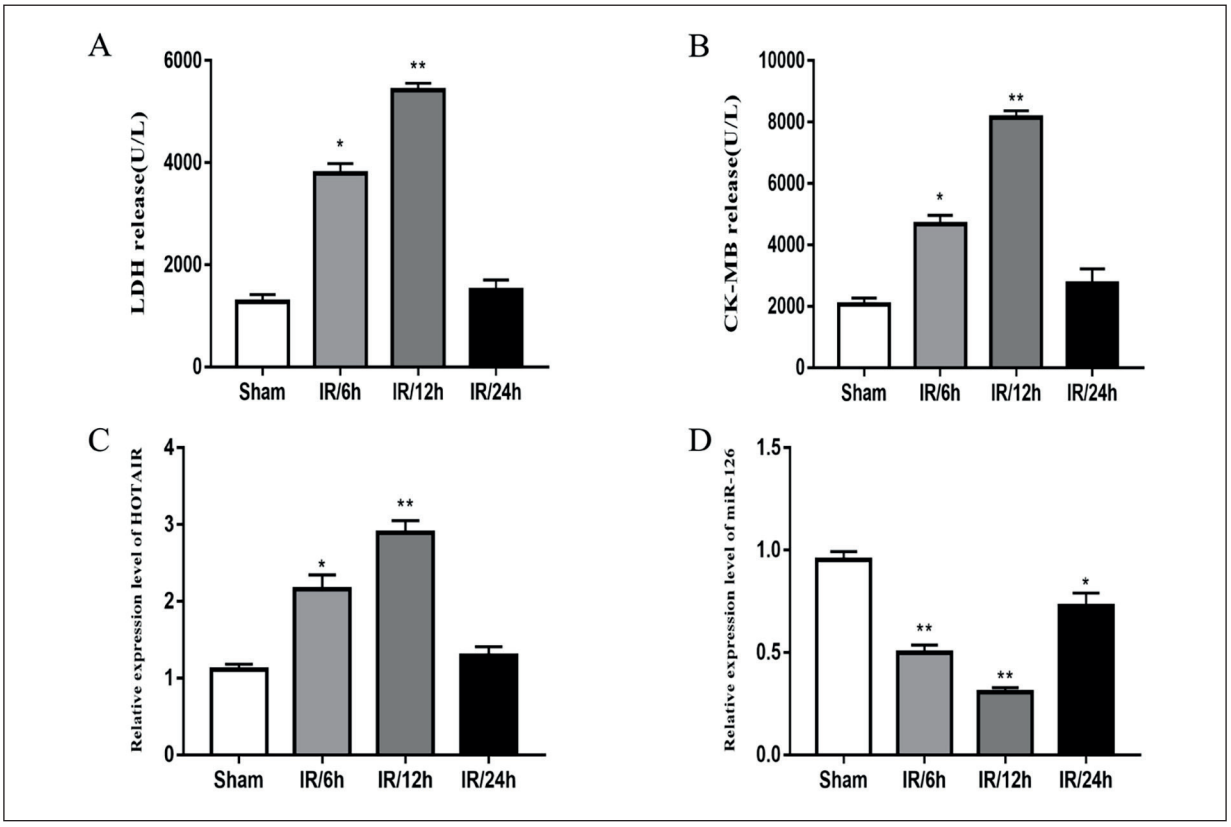

Figure 1. Dynamic expressions of HOTAIR and miR-126 in IR mice. After induction of ischemia in mice, reperfusion was conducted at 6, 12 and 24 h. Mice were assigned into sham group, IR/6h group, IR/12h group and IR/24h group, with 6 in each group. LDH release (**A**), CK-MB release (**B**), HOTAIR level (**C**) and microRNA-126 level (**D**) in mice of each group were determined. *$p<0.05$, **$p<0.01$; Data were present as the mean ± SD.

24 h (Figure 1C). Conversely, microRNA-126 was time-dependently downregulated at 6 and 12 h of IR, which was markedly upregulated at 24 h following IR (Figure 1D). Dynamically expressed HOTAIR and microRNA-126 were believed to be involved in the development of myocardial IR.

Dynamic Expressions of HOTAIR and miR-126 in HR-Induced Cardiomyocytes

H9c2 cells were exposed to 0, 0.25, 0.5 or 1 mmol/L H_2O_2 for 24 h, and HOTAIR level was gradually upregulated at 0.25 and 0.5 mmol/L H_2O_2 induction. However, its level was reduced following 1 mmol/L H_2O_2 treatment (Figure 2A). Moreover, HOTAIR level was time-dependently upregulated following 0.5 mmol/L H_2O_2 induction for 0, 6, 12 and 24 h, respectively (Figure 2B). MicroRNA-126 was downregulated after H_2O_2 induction in H9c2 cells. The most pronounced downregulation trend was observed at 0.5 mmol/L H_2O_2 treatment for 24 h (Figure 2C, 2D). Subsequently, *in vitro* HR model was established by $Na_2S_2O_4$ induction for 0, 2, 4 and 8 h, followed by reoxygenation at 12 h. Similarly, HOTAIR was upregulated to the peak at 4 h, and microRNA-126 was downregulated to the lowest level at 4 h as well (Figure 2E, 2F).

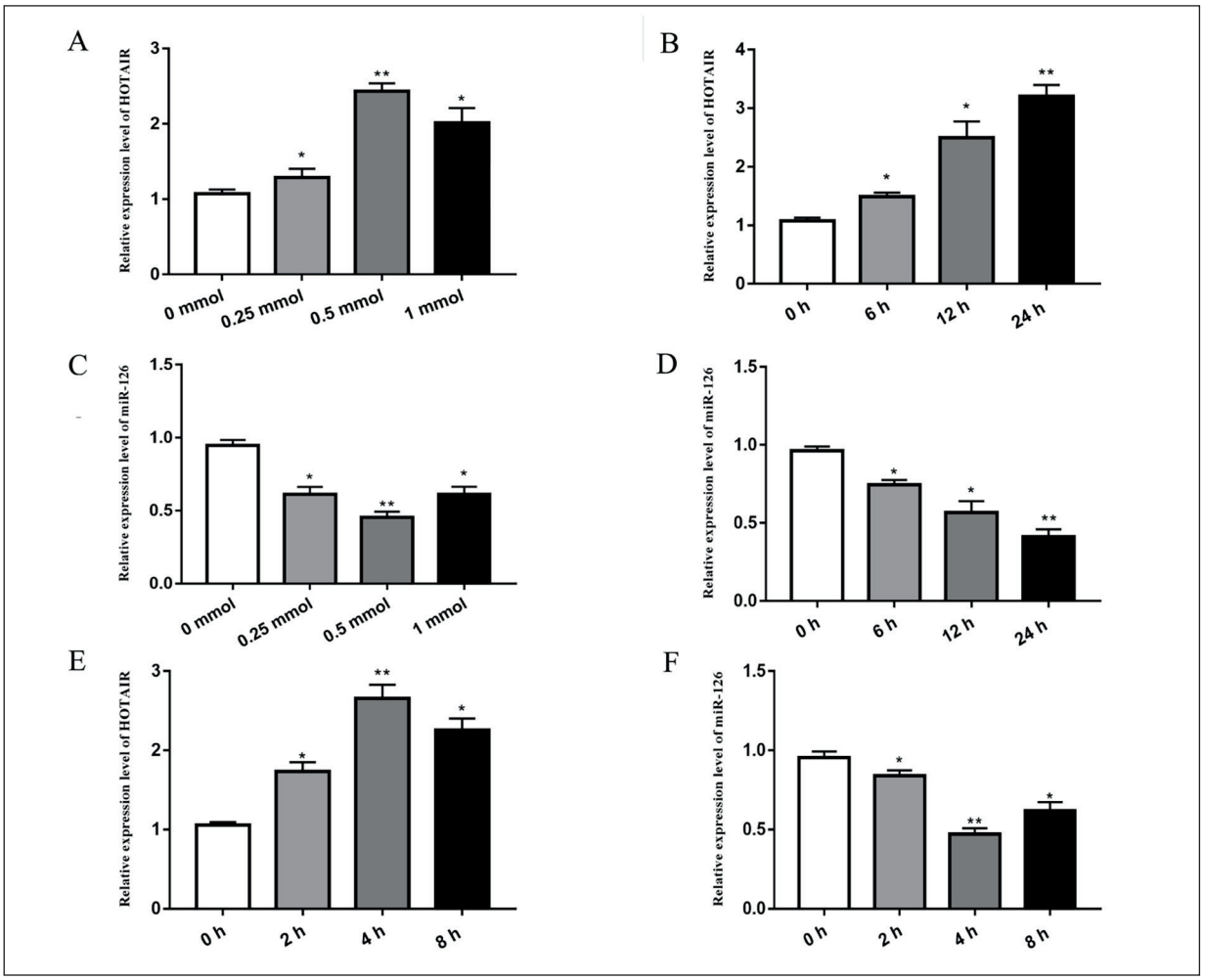

Figure 2. Dynamic expressions of HOTAIR and miR-126 in HR-induced cardiomyocytes. **A,** HOTAIR level in H9c2 cells induced with 0, 0.25, 0.5 or 1 mmol/L H_2O_2 for 24 h. **B,** HOTAIR level in H9c2 cells induced with 0.5 mmol/L H_2O_2 for 0, 6, 12 and 24 h. **C,** MicroRNA-126 level in H9c2 cells induced with 0, 0.25, 0.5 or 1 mmol/L H_2O_2 for 24 h. **D,** MicroRNA-126 level in H9c2 cells induced with 0.5 mmol/L H_2O_2 for 0, 6, 12 and 24 h. **E,** HOTAIR level in H9c2 cells induced with $Na_2S_2O_4$ for 0, 2, 4 and 8 h, followed by reoxygenation at 12 h. **F,** MicroRNA-126 level in H9c2 cells induced with $Na_2S_2O_4$ for 0, 2, 4 and 8 h, followed by reoxygenation at 12 h. *p<0.05, **p<0.01.

HOTAIR Inhibited Viability and Stimulated LDH Release and Caspase-3 Activity in H_2O_2-Induced H9c2 Cells

Transfection efficacy of LV-HOTAIR and LV-shHOTAIR was tested in H9c2 cells (Figure 3A). Interestingly, microRNA-126 level was negatively regulated by HOTAIR (Figure 3B). H_2O_2 induction markedly reduced viability and EdU-positive ratio in H9c2 cells, and the reduced trends were partially improved by knockdown of HOTAIR (Figure 3C, 3D). Notably, transfection of LV-HOTAIR further aggravated the decreased viability and EdU-positive ratio in H_2O_2-induced H9c2 cells. Conversely, LDH release and Caspase-3 activity were stimulated by H_2O_2 induction, and they were reversed by knockdown of HOTAIR (Figure 3E, 3F). As a result, HOTAIR aggravated H_2O_2-induced injury in cardiomyocytes.

HOTAIR Could Sponge MicroRNA-126

Through bioinformatics prediction, the binding sequences were found in the promoter regions of HOTAIR and microRNA-126 (Figure 4A). Declined Luciferase activity after co-transfection of HOTAIR WT and microRNA-126 mimics further indicated the binding between

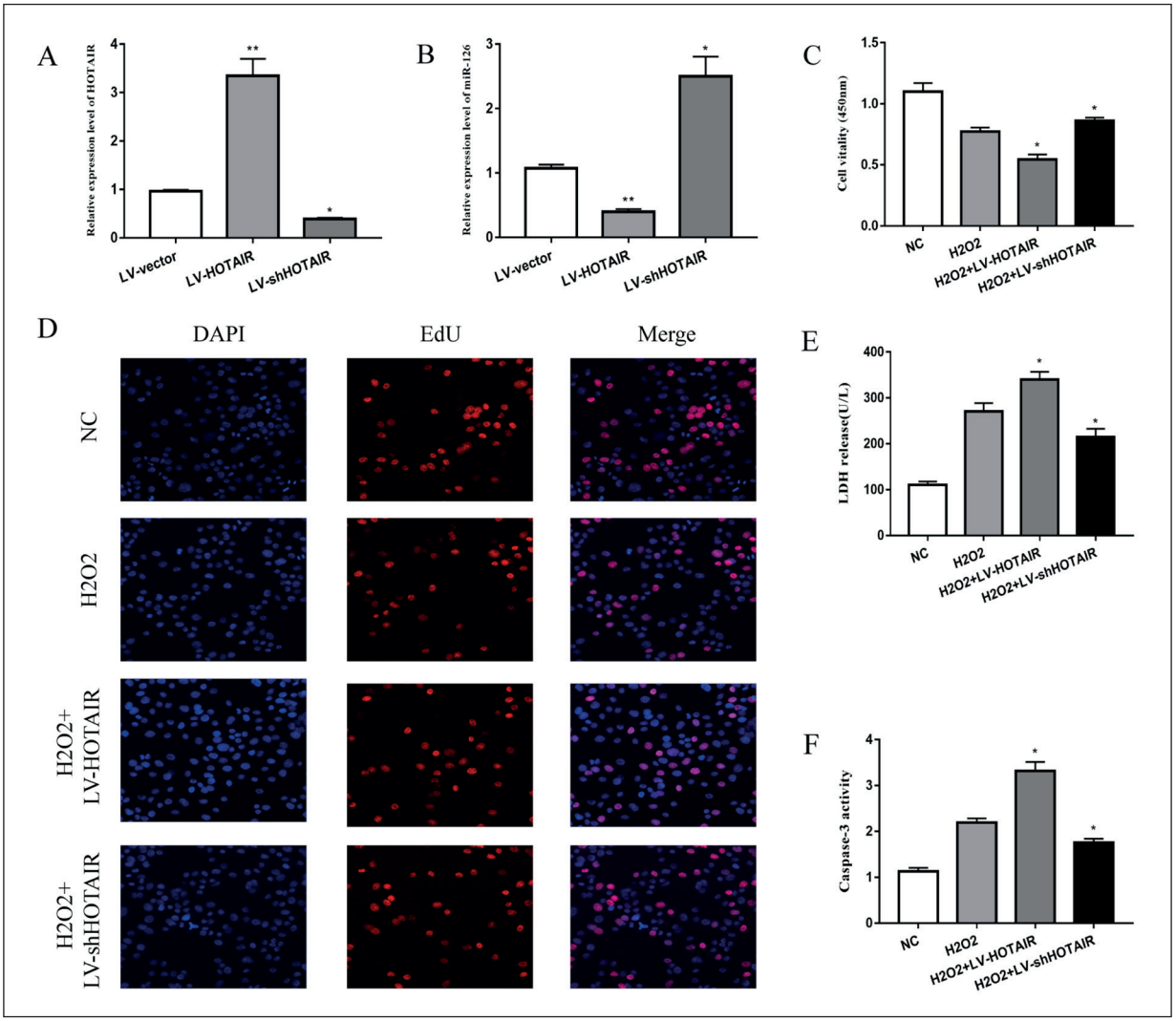

Figure 3. HOTAIR inhibited viability and stimulated LDH release and Caspase-3 activity in H_2O_2-induced H9c2 cells. **A,** Transfection efficacy of LV-HOTAIR and LV-shHOTAIR in H9c2 cells. **B,** MicroRNA-126 level in H9c2 cells transfected with LV-vector, LV-HOTAIR or LV-shHOTAIR. H9c2 cells were treated with blank control, or 0.5 mmol/L H_2O_2 for 24 h, followed by transfection of LV-HOTAIR or LV-shHOTAIR. Cell viability (**C**), EdU-positive ratio (**D**) (magnification: 400×), LDH release (**E**) and Caspase-3 activity (**F**) in each group. *p<0.05, **p<0.01.

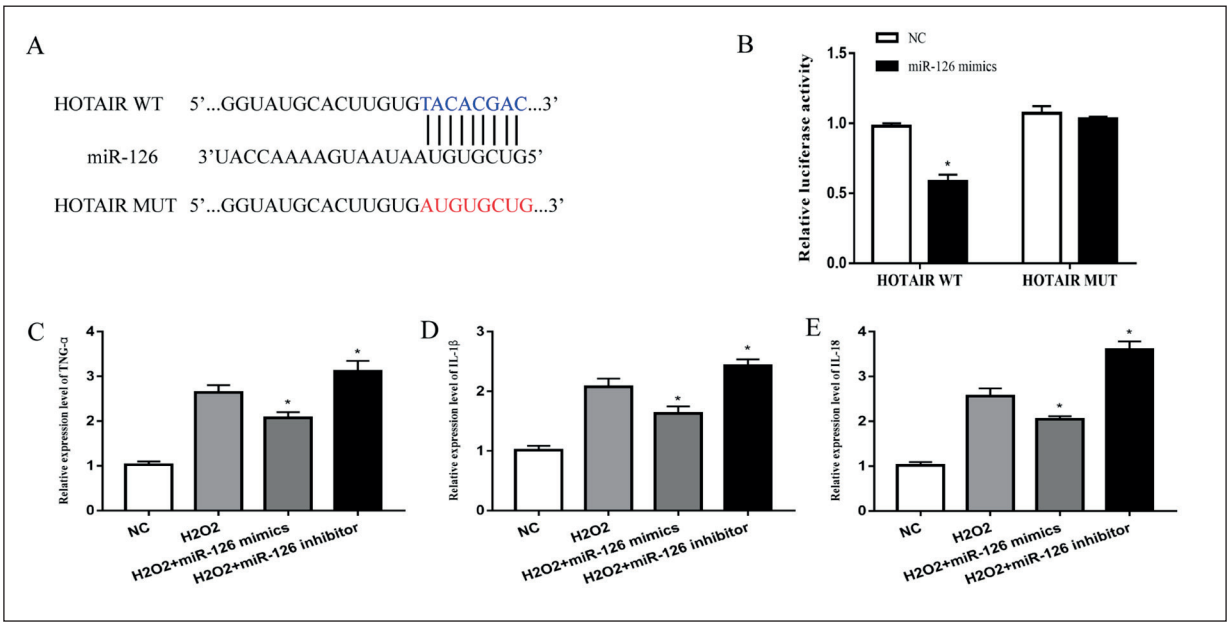

Figure 4. HOTAIR could sponge microRNA-126. **A,** Binding sequences between HOTAIR and microRNA-126. **B,** Luciferase activity after co-transfection of HOTAIR WT/HOTAIR MUT and NC/microRNA-126 mimics. H9c2 cells were treated with blank control, or 0.5 mmol/L H_2O_2 for 24 h, followed by transfection of microRNA-126 mimics or microRNA-126 inhibitor. Contents of TNF-α (**C**), IL-1β (**D**) and IL-18 (**E**) were detected by ELISA. *p<0.05.

HOTAIR and microRNA-126 (Figure 4B). It is shown that contents of TNF-α (Figure 4C), IL-1β (Figure 4D), and IL-18 (Figure 4E) were markedly elevated following H_2O_2 induction. Their increased levels were reduced by overexpression of microRNA-126. Therefore, microRNA-126 exerted a protective effect on inflammatory response in H_2O_2-induced cardiomyocytes.

SRSF1 was the Target Gene of MicroRNA-126

In a similar way, SRSF1 was verified to be the target gene binding microRNA-126 (Figure 5A, 5B). Interestingly, both mRNA and protein levels of SRSF1 were downregulated in H9c2 cells overexpressing microRNA-126 (Figure 5C, 5D). Based on the above results, HOTAIR sponged microRNA-126 to upregulate SRSF1.

Discussion

Researches on myocardial IR have been well concerned in the cardiovascular field[20,21]. Myocardial IR is considered as the therapeutic approach for AMI[22]. During the blood flow reperfusion, however, myocardium suffers pathological lesions to a certain extent. Effective prevention and alleviation of myocardial IR should be well explored.

LncRNAs are non-coding RNAs over 200 nucleotides long[23]. They are extensively involved in disease progression through regulations on epigenetics, cell cycle progression, reprogramming of pluripotent stem cells, etc.[24,25]. Serving as a ceRNA, lncRNA could reduce the regulatory effect of sponged miRNA on its downstream genes. LncRNA HOTAIR is transcribed from HOXC locus, with 2158 nucleotides long. Previous studies[26-28] have demonstrated the oncogenic role of HOTAIR, which is utilized as a potential tumor marker. In recent years, the biological role of HOTAIR in the cardiovascular system has been identified. Zhang et al[29] found that the interaction of miR-519d-3p and HOTAIR can protect MI and hypoxia-induced cardiomyocytes apoptosis, providing the potential therapeutic target for MI treatment. HOTAIR is downregulated in mice with cardiac hypertrophy or end stage of ischemic heart failure, suggesting its potential impact on cardiac functions[30,31].

MiRNAs are able to regulate protein expressions by recognizing target mRNAs to inhibit their translation[32,33]. A growing number of studies have proposed the fundamental effects of miRNAs on cardiovascular diseases[34]. MicroR-

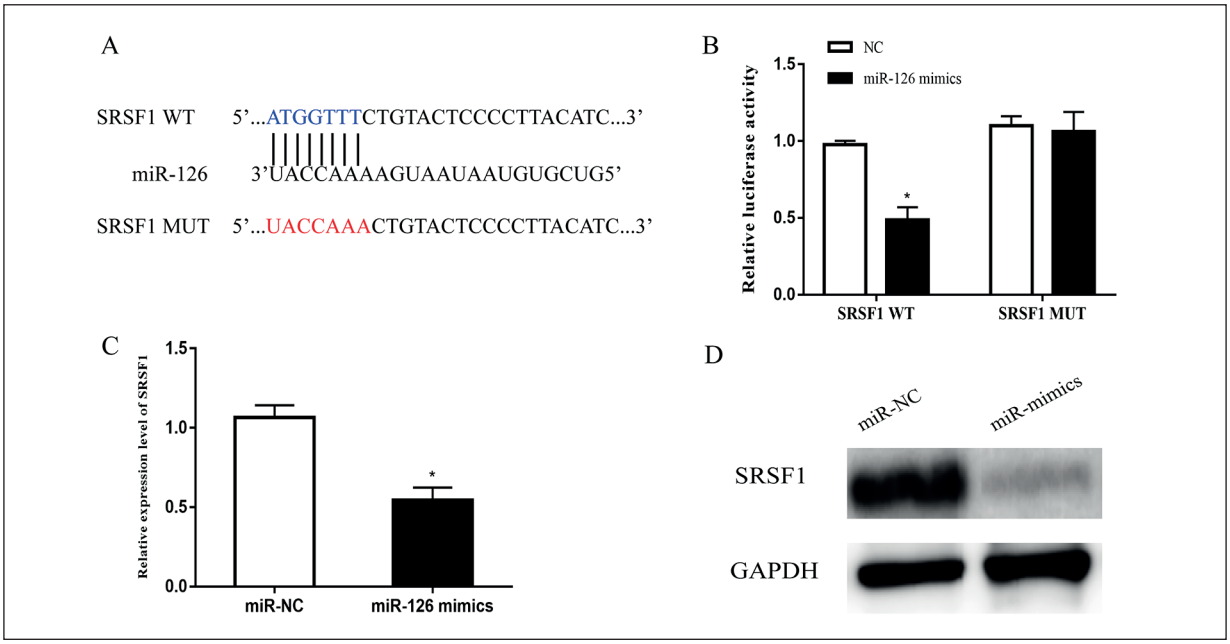

Figure 5. SRSF1 was the target gene of microRNA-126. **A,** Binding sequences between microRNA-126 and SRSF1. **B,** Luciferase activity after co-transfection of SRSF1 WT/SRSF1 MUT and NC/microRNA-126 mimics. **C, D,** The mRNA (**C**) and protein level (**D**) of SRSF1 in H9c2 cells transfected with miR-NC or microRNA-126 mimics. *p<0.05.

NA-126 is abundantly expressed in cardiovascular endothelial cells and aims to inhibit angiogenesis[35]. In this paper, a negative regulatory effect of microRNA-126 on myocardial IR has been demonstrated. SRSF1 is a vital member in the SR protein family, which is capable of regulating splicing regulation, protein translation, RNA transportation, cell invasiveness, and senescence[36-40].

Our findings uncovered that no matter in the *in vivo* mouse IR model or *in vitro* HR model in H9c2 cells, HOTAIR was upregulated and microRNA-126 was downregulated. Overexpression of HOTAIR remarkably suppressed viability, and increased LDH release and Caspase-3 activity in H_2O_2-induced H9c2 cells. Besides, overexpression of microRNA-126 inhibited the release of inflammatory factors in H9c2 cells undergoing HR induction. Through bioinformatics analyses, a regulatory loop HOTAIR/microRNA-126/SRSF1 was responsible for influencing the development of myocardial IR.

Conclusions

In summary, HOTAIR aggravates myocardial IR by competitively binding SRSF1 with microRNA-126. The regulatory loop HOTAIR/microRNA-126/SRSF1 may be novel therapeutic targets for myocardial IR.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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