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LncRNA HOXA-AS2 accelerates the proliferation and migration and inhibits the apoptosis of vascular smooth muscle cells by absorbing miRNA-877-3p

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Abstract. – OBJECTIVE: The aim of this study was to clarify the role of long non-coding RNA (lncRNA) HOXA-AS2 in influencing the proliferative, migratory and apoptotic abilities of human aortic vascular smooth muscle cells (HA-VSMCs) by absorbing microRNA-877-3p (miRNA-877-3p).

MATERIALS AND METHODS: HOXA-AS2 level in HA-VSMCs treated with different doses of oxidized low-density lipoprotein (ox-LDL) and for different time points was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). After transfection of si-HOXA-AS2 in HA-VSMCs undergoing ox-LDL treatment, the viability, apoptotic rate and migration of cells were detected, respectively. Meanwhile, the subcellular distribution of HOXA-AS2 was analyzed. The Dual-Luciferase reporter gene assay was applied to verify the binding relationship between HOXA-AS2 and miRNA-877-3p. MiRNA-877-3p level in HA-VSMCs treated with different doses of ox-LDL was determined as well. Furthermore, the regulatory effects of HOXA-AS2/miRNA-877-3p axis on cellular behaviors of HA-VSMCs were determined.

RESULTS: HOXA-AS2 expression was upregulated by ox-LDL treatment in a time- and dose-dependent manner. After being treated with 100 mg/L ox-LDL for 48 h, the proliferative and migratory abilities of HA-VSMCs were significantly enhanced, while apoptosis was inhibited. Conversely, these changes were reversed by transfection of si-HOXA-AS2. HOXA-AS2 was mainly distributed in the nuclear fraction. Dual-Luciferase reporter gene assay confirmed the direct binding relationship between HOXA-AS2 and miRNA-877-3p. Moreover, miRNA-877-3p was markedly downregulated after transfection of si-HOXA-AS2. MiRNA-877-3p expression decreased gradually with an increased dose of ox-LDL. In addition, knockdown of miRNA-877-3p could reverse the regulatory effects of HOXA-AS2 on proliferative, migratory and apoptotic abilities of HA-VSMCs.

CONCLUSIONS: HOXA-AS2 is upregulated after HA-VSMCs injury, which accelerates the proliferative and migratory abilities, and inhibits the apoptosis of vascular smooth muscle cells by absorbing miRNA-877-3p.

Key Words:
HOXA-AS2, MiRNA-877-3p, HA-VSMCs.

Introduction

Atherosclerosis is a chronic disease that results from myocardial infarction and ischemic stroke. Lipid deposition in blood vessel walls, plaque rupture and thrombosis are the major pathological factors of atherosclerosis[1,2]. Current studies[3,4] have indicated that high levels of lipid, cholesterol and low-density lipoprotein (LDL) are risk factors for atherosclerosis in both human and an-

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imals. During the progression of atherosclerosis, changes in proliferative and apoptotic abilities of vascular smooth muscle cells (VSMCs) are the major initiation factors[5-7]. Nevertheless, the potential mechanisms underlying the proliferative and apoptosis of VSMCs in the progression of atherosclerosis remain to be uncovered.

Long non-coding RNAs (lncRNAs) are a kind of RNAs with 200-100,000 nt in length. Previous studies[8,9] have demonstrated that lncRNAs are responsible for transcriptional and post-transcriptional regulations, serving vital functions in pathological progress. Although lncRNAs do not encode proteins, they can be microRNA (miRNA) sponges to target downstream genes[10]. Differentially expressed lncRNAs in different tissues are important regulators in cell metabolism, proliferation, apoptosis, etc.[11,12]. Scholars[13-15] have identified many functional lncRNAs involved in the progression of cardiovascular diseases.

In this paper, an *in vitro* atherosclerosis model was successfully constructed by ox-LDL treatment in human aortic vascular smooth muscle cells (HA-VSMCs). The aim of this work was to uncover the role of lncRNA HOXA-AS2 in regulating cellular behaviors of HA-VSMCs undergoing ox-LDL treatment.

Materials and Methods

Cell Culture and Ox-LDL Treatment

HA-VSMCs were provided by Cell Bank (Shanghai, China). HA-VSMCs were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 μg/mL penicillin and 0.1 mg/mL streptomycin, and maintained in a 37°C, 5% CO_2 incubator. Until 60% of confluence, HA-VSMCs were treated with different doses of ox-LDL (0, 10 and 100 mg/L) for different time points (0, 24 and 48 h), respectively.

Cell Transfection

Until 60% of confluence, the cells were transfected with si-HOXA-AS2, si-NC, miRNA-877-3p mimics, miRNA-877-3p inhibitor or miRNA-877-3p NC according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 6 hours later, the complete medium was replaced. Transfected cells for 24-48 h were harvested for *in vitro* experiments.

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

Total RNA in cells was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The concentration of extracted RNA was qualified by an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). Subsequently, RNA was reverse transcribed into complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). Specific quantitative Real Time-Polymerase Chain Reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were listed in Table I.

Cell Counting Kit-8 (CCK-8) Assay

Cells were first seeded into 96-well plates and cultured overnight. Absorbance (A) at 450 nm at the appointed time points was recorded in accordance with the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Finally, viability curves were plotted.

Table I. Primer sequences.

Gene	Primer sequences
HOXA-AS2	F: 5'-CCCGTAGGAAGAACCGATGA-3' R: 5'-TTTAGGCCTTCGCAGACAGC-3'
miR-877-3p	F: 5'-GGGTGGCAGTGTCAGUGAU-3' R: 5'-CAGTGCGTGTCGTGGAGT-3' R: 5'-AACGCTTCACGAATTTGCGT-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3' R: 5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGT-3' R: 5'-GGGAAGGATCTGTCTCTGACC-3'

Apoptosis Determination

Cells were first washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) twice and digested with ethylenediaminetetraacetic acid (EDTA)-free trypsin. After resuspension, the density of cells was adjusted to 1×10^6 cells/mL. Subsequently, the cells were transferred to a flow cytometry tube, followed by incubation with buffer and 1.25 μL of fluorescein isothiocyanate (FITC) Annexin V/Propidium Iodide (PI) for 15 min in the dark. Apoptosis was determined within 1 hour by flow cytometry (Partec AG, Arlesheim, Switzerland).

Transwell Assay

HA-VSMCs suspended in 500 μL of serum-free medium were inoculated in the upper chamber of transwell inserts placed in a 24-well plate, with 3×10^4 cells per insert. Meanwhile, 100 μL of complete medium was applied to the lower chamber. After 48 h of incubation, invasive cells were fixed with 4% paraformaldehyde and dyed with crystal violet. The number of penetrating cells was counted under a microscope (Leica, Wetzlar, Germany). 10 fields were randomly selected for each sample.

Determination of Subcellular Distribution

Cytoplasmic and nuclear RNAs were extracted using the PARIS kit (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal references for nucleus and cytoplasm, respectively.

Dual-Luciferase Reporter Gene Assay

HA-VSMCs were inoculated in 24-well plates at a density of 3×10^5 cells per well. Subsequently, they were co-transfected with miRNA-877-3p mimics/NC and HOXA-AS2-WT/HOXA-AS2-MT using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 24 hours later, co-transfected cells were harvested. Luciferase activity was finally determined by a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Experimental data were expressed as mean ± standard deviation. Intergroup differences were analyzed by *t*-test. $p<0.05$ was considered statistically significant.

Results

HOXA-AS2 Was Upregulated in HA-VSMCs With Ox-LDL Treatment

HA-VSMCs were first treated with different doses of ox-LDL to construct the atherosclerotic hyperlipidemia model *in vitro*. QRT-PCR results showed that HOXA-AS2 was dose-dependently upregulated in HA-VSMCs after treatment with 0, 10 and 100 mg/L ox-LDL for 48 h (Figure 1A). Besides, HOXA-AS2 expression was time-dependently upregulated after 100 mg/L ox-LDL treatment for 0, 24 and 48 h (Figure 1B) as well. Transfection of si-HOXA-AS2 remarkably downregulated HOXA-AS2 level in HA-VSMCs, suggesting satisfactory transfection efficacy (Figure 1C). After treatment of 100 mg/L ox-LDL for 48 h, the viability and migration of HA-VSMCs were significantly elevated. However, these indexes were remarkably reduced after transfection of si-HOXA-AS2 (Figure 1D, 1F). Conversely, ox-LDL treatment attenuated the apoptotic rate in HA-VSMCs, and silence of HOXA-AS2 reversed this trend (Figure 1E). Collectively, HOXA-AS2 was upregulated in ox-LDL-treated HA-VSMCs. Silence of HOXA-AS2 attenuated the proliferative and migratory abilities, whereas induced apoptosis of HA-VSMCs.

HOXA-AS2 Sponged MiRNA-877-3p

Subcellular distribution analysis indicated a significantly higher abundance of HOXA-AS2 in nuclear fraction than that of the cytoplasmic part (Figure 2A). Bioinformatics predicted that a potential binding sequencing was identified between HOXA-AS2 and miRNA-877-3p (Figure 2B). Dual-Luciferase reporter gene assay revealed that a marked reduction in the Luciferase activity was observed in cells co-transfected with HOXA-AS2-WT and miRNA-877-3p mimics. The above results confirmed the binding relationship between HOXA-AS2 and miRNA-877-3p (Figure 2C). Besides, transfection of si-HOXA-AS2 remarkably upregulated miRNA-877-3p expression in HA-VSMCs (Figure 2D). With the treatment of increased doses of ox-LDL, miRNA-877-3p level in HA-VSMCs downregulated gradually (Figure 2E). All these findings suggested that HOXA-AS2 could sponge miRNA-877-3p and negatively regulate its level.

HOXA-AS2 Regulated HA-VSMCs by Sponging MiRNA-877-3p

To investigate the role of HOXA-AS2/miRNA-877-3p in regulating ox-LDL-treated HA-VSMCs,

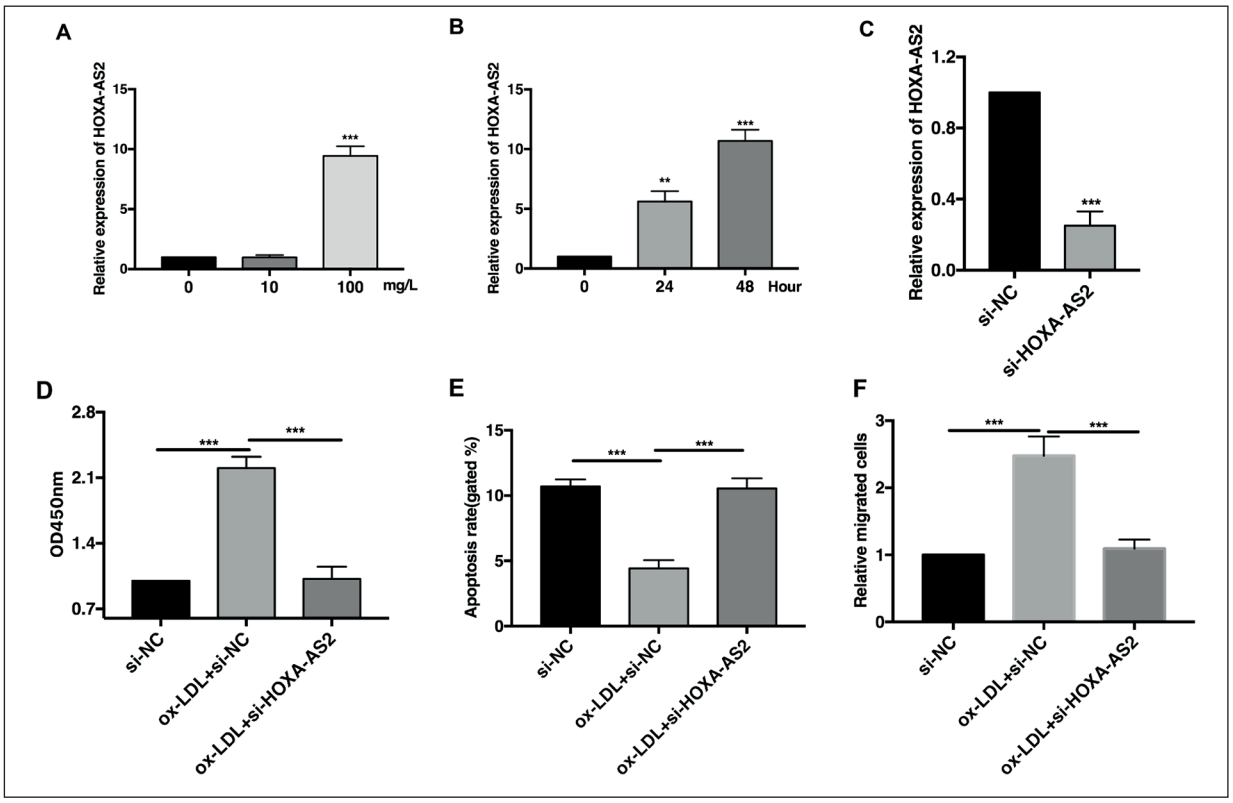

Figure 1. HOXA-AS2 was upregulated in HA-VSMCs with ox-LDL treatment. **A,** Relative level of HOXA-AS2 in HA-VSMCs treated with 0, 10 and 100 mg/L ox-LDL for 48 h. **B,** Relative level of HOXA-AS2 in HA-VSMCs treated with 100 mg/L ox-LDL for 0, 24 and 48 h. **C,** Transfection efficacy of si-HOXA-AS2 in HA-VSMCs. **D,** CCK-8 assay showed the viability of HA-VSMCs transfected with si-NC, ox-LDL induction + si-NC and ox-LDL induction + si-HOXA-AS2. **E,** Apoptotic rate of HA-VSMCs transfected with si-NC, ox-LDL induction + si-NC and ox-LDL induction + si-HOXA-AS2. **F,** Migration ability of HA-VSMCs transfected with si-NC, ox-LDL induction + si-NC and ox-LDL induction + si-HOXA-AS2.

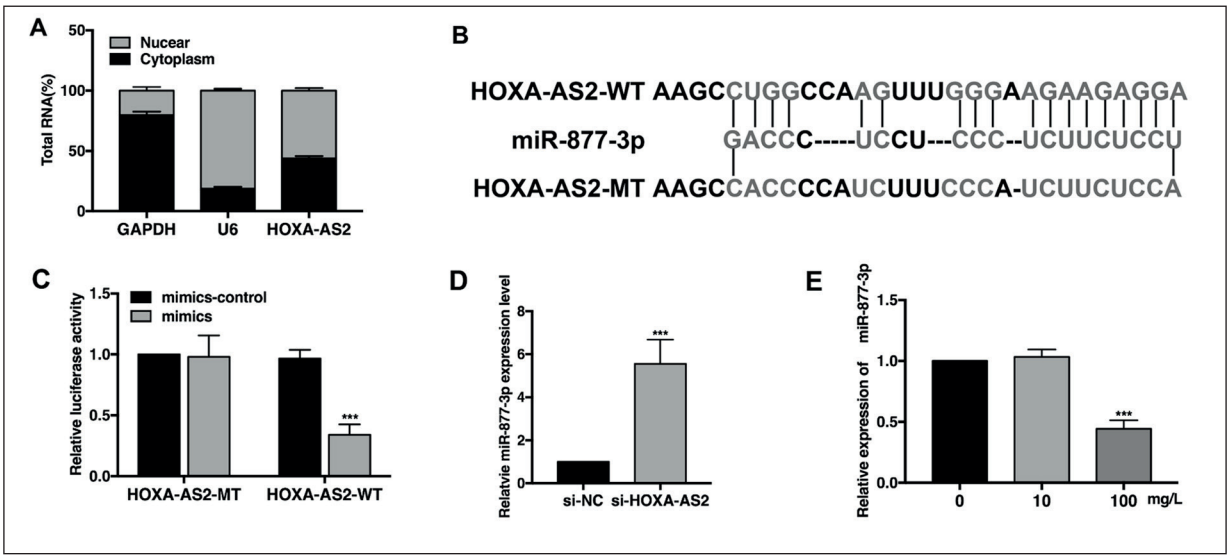

Figure 2. HOXA-AS2 sponged miR-877-3p. **A,** Subcellular distribution of HOXA-AS2 in nuclear and cytoplasmic fractions of HA-VSMCs. GAPDH and U6 were used as internal references for cytoplasm and nucleus, respectively. **B,** Potential binding sequences between HOXA-AS2 and miR-877-3p. **C,** Luciferase activity in cells co-transfected with miR-877-3p mimics/NC and HOXA-AS2-WT/HOXA-AS2-MT. **D,** Relative level of miR-877-3p in HA-VSMCs transfected with si-NC or si-HOXA-AS2. **E,** Relative level of miR-877-3p in HA-VSMCs treated with 0, 10 and 100 mg/L ox-LDL for 48 h.

we constructed miRNA-877-3p inhibitor. Transfection of miRNA-877-3p inhibitor remarkably downregulated miRNA-877-3p expression in HA-VSMCs (Figure 3A). Meanwhile, transfection of si-HOXA-AS2 significantly suppressed the viability and migration of HA-VSMCs undergoing ox-LDL treatment. These results were partially reversed by miRNA-877-3p knockdown (Figure 3B, 3D). Elevated apoptotic rate of ox-LDL-treated HA-VSMCs transfected with si-HOXA-AS2 was further reduced by co-transfection of miRNA-877-3p inhibitor (Figure 3C). Our results indicated that HOXA-AS2 regulated cellular behaviors of HA-VSMCs by sponging miRNA-877-3p.

Discussion

VSMCs proliferate at a very low rate in blood vessels, exhibiting an extremely low activity of protein synthesis and unique contractile capacity[16]. However, in response to the vascular injury,

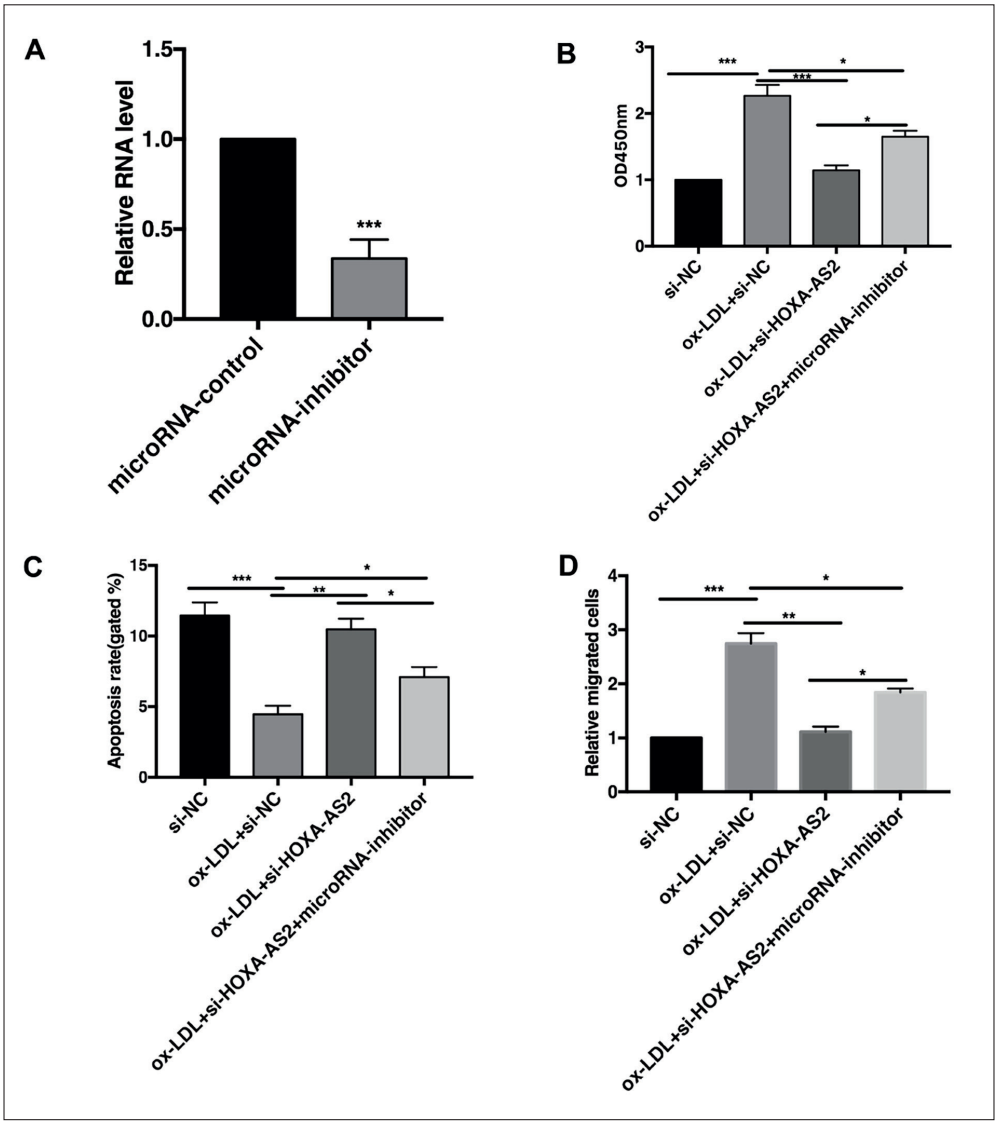

Figure 3. HOXA-AS2 regulated HA-VSMCs by sponging miR-877-3p. **A,** Transfection efficacy of miR-877-3p inhibitor in HA-VSMCs. **B,** CCK-8 assay showed the viability of HA-VSMCs transfected with si-NC, ox-LDL induction + si-NC, ox-LDL induction + si-HOXA-AS2 and ox-LDL induction + si-HOXA-AS2 + miR-877-3p inhibitor. **C,** Apoptotic rate of HA-VSMCs transfected with si-NC, ox-LDL induction + si-NC, ox-LDL induction + si-HOXA-AS2 and ox-LDL induction + si-HOXA-AS2 + miR-877-3p inhibitor. **D,** Migration ability of HA-VSMCs transfected with si-NC, ox-LDL induction + si-NC, ox-LDL induction + si-HOXA-AS2 and ox-LDL induction + si-HOXA-AS2 + miR-877-3p inhibitor.

VSMCs can migrate from tunica media to the intima of blood vessels and proliferate massively. This may eventually form a new intima and lead to arteriosclerosis[17,18]. In this study, we found that HOXA-AS2 was upregulated by ox-LDL treatment in a time- and dose-dependent manner. Silence of HOXA-AS2 significantly inhibited proliferative and migratory abilities, whereas induced apoptosis of HA-VSMCs. The results indicated that HOXA-AS2 exerted a vital role in HA-VSMCs injury.

LncRNA HOXA-AS2 is a 1048-bp lncRNA between HOXA3 and HOXA4 genes in the HOXA cluster[19]. Generally, it is believed[20,21] that lncRNAs participate in multiple cellular and biological pathways by interacting with DNAs, proteins and RNAs. Meanwhile, lncRNAs can competitively bind miRNAs and thereafter suppress their functions[22,23]. MiRNAs are single-stranded, non-coding, small-molecule RNAs with about 18-22 nucleotides in length. They can regulate gene expressions and their biological effects by completely or incompletely complementary to target mRNAs at the post-transcriptional level[24-26]. So far, over 2000 miRNAs have been discovered to participate in the occurrence and progression of human diseases[27,28]. Furthermore, it has been found[29] that upregulation of miRNA-877-3p can inhibit the proliferative rate of bladder cancer cells.

Our study detected the binding relationship between miRNA-877-3p and HOXA-AS2. More importantly, knockdown of miRNA-877-3p could reverse the regulatory effects of HOXA-AS2 on the proliferative, migratory and apoptotic abilities of HA-VSCMs. Hence, it was concluded that HOXA-AS2 sponged miRNA-877-3p to influence HA-VSMCs behaviors.

Conclusions

We found that HOXA-AS2 is upregulated after HA-VSMCs injury, which accelerates the proliferative and migratory abilities, and inhibits apoptosis of HA-VSMCs by absorbing miRNA-877-3p. All our findings suggest that HOXA-AS2 may be a promising target for atherosclerosis treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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