European Review for Medical and Pharmacological Sciences 2020; 24: 3302-3314

LncRNA FOXC2-AS1 regulated proliferation and apoptosis of vascular smooth muscle cell through targeting miR-1253/FOXF1 axis in atherosclerosis

Y.-Q. WANG[1], Z.-M. XU[2], X.-L. WANG[2], J.-K. ZHENG[1], Q. DU[1], J.-X. YANG[1], H.-C. ZHANG[3]

[1]Department of Cardiology, Affiliated Hospital of North Sichuan Medical College, Nanchong, Sichuan Province, China
[2]Department of Pharmacy, North Sichuan Medical College, Nanchong, Sichuan Province, China
[3]Department of Neurology, Dazhou Central Hospital, Dazhou, Sichuan Province, China

Abstract. – OBJECTIVE: Atherosclerosis (AS) is the most dangerous factor for human death, which is responsible for coronary heart disease. Growing evidence has showed that long non-coding RNAs (lncRNAs) are involved in the development of AS. In this study, we mainly aimed at investigating the roles of FOXC2-AS1 in AS patients.

PATIENTS AND METHODS: RT-PCR was performed to detect the expressions of FOXC2-AS1 and miR-1253 in serum samples of AS patients (n=35) and healthy volunteer (n=35). The correlation between FOXC2-AS1 and miR-1253 was further analyzed. Human vascular smooth muscle cells (VSMCs) were respectively treated with ox-LDL, IL-6, CRP, TNF-α and IL-8 to explore the affecting factors. P-FOXC2-AS1 was constructed and transfected into VSMCs. Cell proliferation abilities were measured by CCK-8 assay. Cell apoptotic rates were measured by flow cytometry (FACS) analysis. Western blot (WB) was performed to detect protein levels of FOXF1, Bcl-2, Bax and Cleaved Caspase3. Finally, luciferase gene reporter assay was performed to prove the relationships between FOXC2-AS1 and miR-1253, miR-1253 and FOXF1.

RESULTS: We found that FOXC2-AS1 was significantly upregulated in AS patients, which could be induced by ox-LDL and IL-6 in VSMCs. MiR-1253 was decreased in AS patients, which was negatively correlated with FOXC2-AS1. Furthermore, FOXC2-AS1 overexpression promoted proliferation and inhibited apoptosis in VSMCs. Luciferase gene reporter assay showed that FOXC2-AS1 could bind to miR-1253 in VSMCs and 293 cells. Moreover, miR-1253 overexpression inhibited proliferation and promoted apoptosis of VSMCs. Luciferase reporter assay proved that miR-1253 could target at FOXF1 in VSMCs and 293 cells, which was reported to be associated with cell proliferation and apoptosis in some cancers. Additionally, miR-1253 mimic or GSK343, a FOXF1 inhibitor, was respectively transfected into VSMCs with p-FOXC2-AS1. Results showed that the promoted cell proliferation and inhibited cell apoptosis were reversed as well, confirming that FOXC2-AS1 promoted cell proliferation and inhibited apoptosis via miR-1253/FOXF1 signaling axis in AS patients.

CONCLUSIONS: According to the results, we found that FOXC2-AS1 was upregulated in AS patients; furthermore, FOXC2-AS1 overexpression promoted cell proliferation and inhibited cell apoptosis via targeting miR-1253/FOXF1 signaling axis. Our results elucidated a potential mechanism underlying the role of FOXC2-AS1, which might be used as a promising marker and a potential target for AS patients.

Key Words:
LncRNA FOXC2-AS1, MiR-1253, FOXF1, Cell proliferation, Atherosclerosis.

Introduction

Atherosclerosis (AS) is the leading cause of death in the world[1-3], being the main dangerous factor and mainly responsible for coronary artery disease (CAD)[3-5]. It has been reported that the interactions of different chemokines and various vascular cells and non-vascular cells lead to the formation of atherosclerotic lesion. These cells included vascular smooth muscle cells (VSMCs), mononuclear inflammatory cells (MNCs), endothelial cells (ECs), etc[6-9]. As an important component of blood vessels, the proliferation and migration of VSMCs lead to the intimal thickening,

Corresponding Author: YuQuan Wang, MD; e-mail: 39770191@qq.com

which played an important role for the development of atherosclerotic lesions[10-14].

Long non-coding RNAs (lncRNAs) are a group of RNAs that are more than 200 nucleotides in length, which are characterized as non-protein coding RNAs; however, they play vital roles and biological functions in various diseases[15-19]. Growing evidence demonstrates that lncRNAs are involved in the formation and development of AS[20,21]. Zhang et al[20] revealed that LINC00305 could promote monocytes inflammation by activating NF-κB pathway in AS; Li et al[21] found that lncRNA CDKN2B-AS1 reduced the inflammatory responses and promoted cholesterol efflux by inhibiting ADAM10 in AS. Wang et al[22] found that lncRNA MEG3 could regulate cell proliferation and apoptosis of VSMCs via targeting with miR-361-5p/ABCA1 axis. Forkhead box protein C2-AS1(-FOXC2-AS1) is a single antisense oligonucleotide RNA transcribed from FOXC2, which has been demonstrated to be upregulated in several cancers, such as osteosarcoma[23], breast cancer[24], prostate cancer[25] and non-small cell lung cancer[26]. FOXC2-AS1 has been reported to be associated with proliferation and correlated with poor prognosis of patients[23-26]. However, whether it is involved in the development in AS remains unknown.

MiRNAs are kinds of RNAs, about 22 nucleotides in length, which are involved in various diseases[27-30]. It has been reported that lncRNAs may contact with miRNA through the mechanism[31,32] of "competing endogenous RNA " (ceRNA), which plays important roles in pathological and biological processes. MiR-1253 was involved in the progression of several cancers[33-35], such as pancreatic ductal adenocarcinoma[33], prostate cancer[25], osteosarcoma[34], non-small-cell lung cancer[35], etc. However, the biological role of miR-1253 in AS is unclear.

In this study, we aimed at exploring the expressions and functions of FOXC2-AS1 in patients with AS. Firstly, we found that FOXC2-AS1 was significantly increased in AS, which might contribute to the development of AS. Therefore, we wanted to investigate the functions and underlying mechanism of FOXC2-AS1 in AS patients.

Patients and Methods

Patient Samples

Serum samples were collected from 35 cases of AS patients and 35 cases of healthy volunteers in our hospital from October 2015 to October 2016. No significant differences had been found in age and sex among patients and healthy volunteers. All serum samples were frozen in liquid nitrogen at -80°C. These patients were excluded if they were suffering with other clinical diseases and all patients and healthy volunteers signed the informed consent. This study was approved by the Ethics Committee of our hospital, and it was in accordance with the principles of the Declaration of Helsinki.

Cell Culture

Human vascular smooth muscle cell line (VSMCs) and human embryonic kidney 293 cell line (HEK 293 cells) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), streptomycin (100 μg/ml) and antibiotics penicillin (100 U/ml). Cells with various treatments were cultured in the incubator with 37°C and 5% CO_2.

Construction of Plasmids and Cell Transfection

The full length of human FOXC2-AS1 and FOXF1 cDNA was respectively synthesized and constructed into plasmids (Invitrogen, Carlsbad, CA, USA), resulted with FOXC2-AS1 overexpression and FOXF1 overexpression in plasmids. The negative control (NC) was also constructed. Cells were pre-incubated to about 50% confluence on a 6-well plate and then p-FOXC2-AS1 or p-FOXF1 and p-NC were respectively transfected into the prepared cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Next, the stable VSMCs with FOXC2-AS1 overexpression and FOXF1 overexpression were constructed. Indicated VSMCs were seeded in 6-well plates (1×10^6/well) until reaching 60%; before mimic transfection, the transfection reagent Lipofectamine 2000, DMEM/F12 and miR-1253 NC or miR-1253 mimic were mixed and incubated for 30 mins. After that, the mixtures were added into prepared VSMCs with complete medium containing 10% FBS. At the indicated time point after transfection, cells were harvested.

Cell Proliferation Assay

The cell proliferation abilities were measured by CCK8 assay; the indicated and treated VSMCs were seeded on 96-well plates (2×10^3/well) and cultured with 100 ul DMEM/F12 for 1 d, 2 d and 3 d for each well. Three replicate wells were set for each group. 10 ul Cell Counting Kit 8 (CCK8, Dojindo Molecu-

lar Technologies, Kumamoto, Japan) was added for each well, which was co-cultured at darkness for another 2 hours in the incubator at 37°C and 5% CO_2. Then the proliferation abilities of indicated VSMCs were measured by CCK8 assay. The absorbance (OD) value was measured at 450 nm with microplate reader (Thermo Fisher, Waltham, MA, USA). The data were collected at the time of 0 d, 1 d, 2 d and 3 d. The whole experiment was repeated for three times.

Flow Cytometric Analysis of the Cell Apoptosis

The indicated VSMCs were treated with trypsin and harvested. Each pellet was stained with FITC-Annexin V (Dojindo) and Propidium iodide (PI), and flow cytometry (FACS) was conducted within 5 mins. The apoptotic cells were obtained using a FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed using the FlowJo software (Tree Star Corp, Ashland, OR, USA).

RNA Extraction and Quantitative Real-Time PCR

Total RNAs of serum samples were extracted by using TRIzol LS (Invitrogen, Carlsbad, CA, USA) and total RNAs of VSMCs were extracted by using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the protocols. Reverse transcription was performed using PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) in accordance to the protocol. PCR primers were synthesized by Gene Pharma (Gene Pharma, Shanghai, China) and primer sequences were listed in Table I. The mRNA expressions were detected using SYBR Premix Ex Taq II (TaKaRa), which were normalized to GAPDH or U6, and $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expressions.

Protein Extraction and Western Blot

Total proteins of VSMCs were extracted by using RIPA lysis buffer (Biyuntian, Shanghai, China) and a BCA kit (Sigma-Aldrich, St. Louis, MO, USA) was used to measure the protein concentrations according to its protocol. 50 ug proteins were added to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); proteins in SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membranes when the proteins were separated. Furthermore, these membranes were blocked at room temperature by 5% non-fat milk for 1 h. Then membranes were incubated with primary antibodies at 4°C overnight. All primary antibodies included Bcl-2 (3869, 1:1000, 23 kDa, CST, Danvers, MA, USA), Bax (2774, 1:1000, 20 kDa, CST, Danvers, MA, USA), Cleaved Caspase-3 (9661, 1:500, 17 kDa, CST, Danvers, MA, USA), FOXF1 (ab168383, 1:1000, 40 kDa, Abcam, Cambridge, MA, USA), GAPDH (5174, 1:5000, 37 kDa, CST, Boston, MA, USA). Subsequently, these membranes were incubated with matched secondary antibodies for another 1 h. Protein bands were detected by Pierce ECL Western blot substrate (Sigma-Aldrich, St. Louis, MO, USA) with ECL detection system (Thermo Fisher Scientific, Waltham, MA, USA).

Luciferase Assay

The potential binding sequences, including wt-FOXC2-AS1, mut-FOXC2-AS1, wt-FOXF1 and mut-FOXF1, were synthesized and constructed into pmiR-GLO (Promega, Madison, WI, USA). VSMCs were seeded on 48-well plates for 24 h and pre-incubated to about 40%. Then miR-1253 mimic and miR-NC were respectively co-transfected into indicated VSMCs for 24 h. Plasmids were mixed with Lipofectamine 2000 and DMEM medium at room temperature for 20 mins, which were then added into VSMCs for another 24 h. Finally, VSMCs were lysed and luciferase activities of firefly and Renilla were measured using dual-luciferase reporter assay (Promega, Madison, WI, USA) according to the protocol. Data were normalized to the Renilla luciferase gene and the relative activities of luciferase were analyzed.

Table I. Primer sequences for RT-PCR.

Genes	Primer sequences
FOXC2-AS1	Forward: 5'-TTCATCGGCTGCGTATTCG-3' Reverse: 5'-TTGCCTTCTAGTCGCCTCC-3'
miR-1253	Forward: 5'-GCTGTAACAGCGGCGGAACT -3' Reverse: 5'- ATCCGCAGGAGTGTCCGAGG-3'
GAPDH	Forward: 5'-GGAGTCCACTGGTGTCTTCA-3' Forward: 5'-GGGAACTGAGCAATTGGTGG-3'
U6	Forward: 5'- CGCTTCGGCAGCACATATACT -3' Forward: 5'- CGCTTCACGAATTTGCGTGTC-3'

Statistical Analysis

Data were expressed as the mean±SD, which were analyzed by SPSS 19.0 (SPSS Inc., Armonk, NY, USA) and images were graphed by GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Data were evaluated using Student's *t*-test or one-way ANOVA with Tukey's post-hoc test. Correlations were analyzed using Pearson's correlation analysis. If p value <0.05, it was considered statistically significant.

Results

FOXC2-AS1 Was Increased in AS Patients and Was Induced by ox-LDL and IL-6 in VSMCs

To investigate the roles of FOXC2-AS1 in patients with AS, firstly, we used qRT-PCR to detect the expressions of FOXC2-AS1 in human serum samples from AS patients (n=35) and healthy volunteers (n=35). Results showed that FOXC2-AS1 was significantly increased for 2.2 folds in AS patients, compared to healthy volunteers (Figure 1A) (p<0.001). The VSMCs played critical roles in the development and progression of AS; in order to evaluate the factors that affect the expression of FOXC2-AS1 in VSMCs, ox-LDL and five normal inflammatory factors were respectively treated into VSMCs. Results revealed that the FOXC2-AS1 was induced by treatments of ox-LDL and IL-6 (Figure 1B-C) (p<0.001), while no significant differences had been found with CRP, TNF-α and IL-8 (Figure 1D-F) (p>0.05). These results indicated that FOXC2-AS1 was increased in AS patients and it was induced by ox-LDL and IL-6 in VSMCs, which suggested that it might play some roles in AS patients.

FOXC2-AS1 Overexpression Promoted Cell Proliferation and Inhibited Apoptosis of VSMCs

To explore the underlying roles of FOXC2-AS1 in AS patients, the p-FOXC2-AS1 was synthesized, resulted with FOXC2-AS1 overexpression. After p-FOXC2-AS1 or p-NC was respectively transfected into VSMCs, the expression of FOXC2-AS1 was significantly increased in p-FOXC2-AS1 (Figure 2A) (p<0.001). CCK8 assay was performed

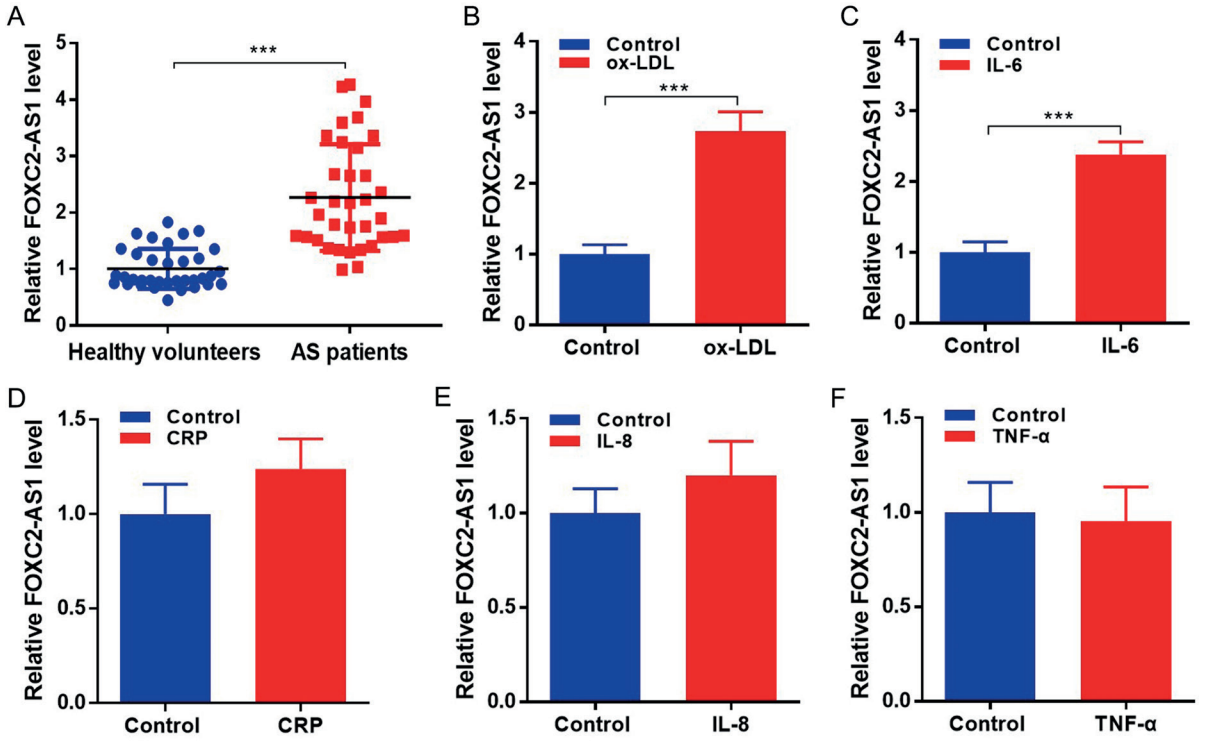

Figure 1. FOXC2-AS1 was increased in AS patients and was induced by ox-LDL and IL-6 in VSMCs. **(A)** The expressions of FOXC2-AS1 in serum samples of AS patients (n=35) and healthy volunteers (n=35) were detected by RT-PCR. **(B-F)** Ox-LDL, IL-6, CRP, TNF-α and IL-8 were respectively treated into VSMCs and then the expressions of FOXC2-AS1 were detected by RT-PCR. Data are shown as mean ± SD based on at least three independent experiments, ***p<0.001.

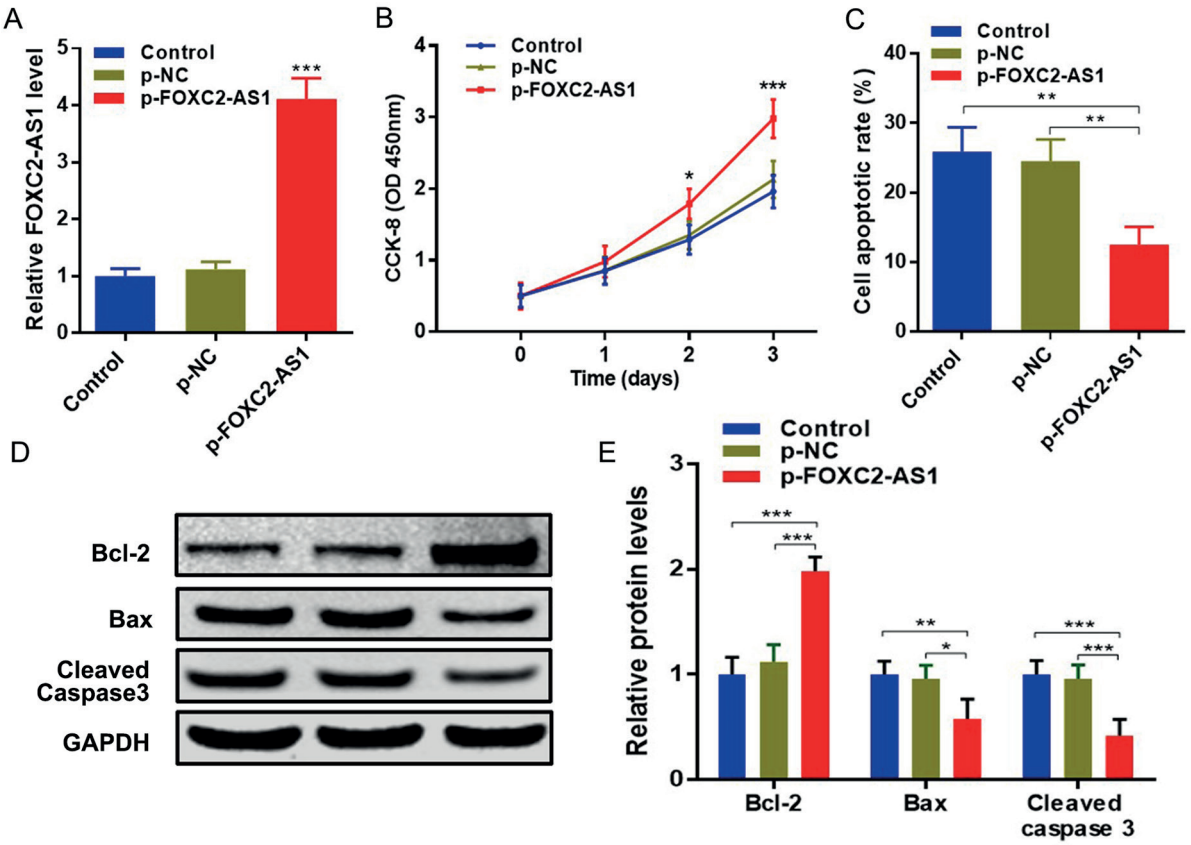

Figure 2. FOXC2-AS1 overexpression promoted cell proliferation and inhibited apoptosis of VSMCs. **(A)** The expressions of FOXC2-AS1 were detected by RT-PCR in VSMCs with blank control, p-NC and p-FOXC2-AS1. **(B)** The proliferation abilities of VSMCs were measured by CCK8 assay. **(C)** FACS was used to measure the apoptotic rates of VSMCs. **(D-E)** The protein levels of apoptotic and anti-apoptotic genes were detected by WB. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05, **p<0.01, ***p<0.001.

to evaluate the proliferation abilities of VSMCs; results showed that FOXC2-AS1 overexpression improved the cell proliferation ability, compared with the blank control and p-NC group (Figure 2B) (p<0.05). Furthermore, flow cytometry (FACS) results showed that apoptotic rate in p-FOXC2-AS1 group was much lower than the other two groups (Figure 2C) (p<0.01). Moreover, the protein level of anti-apoptotic gene Bcl-2 was increased, while apoptotic genes of Bax and cleaved caspase-3 were significantly decreased in p-FOXC2-AS1, compared to the other two groups (Figure 2D-E) (p<0.05). Collectively, these results indicated that FOXC2-AS1 overexpression promoted cell proliferation and inhibited apoptosis of VSMCs.

FOXC2-AS1 Could Directly Bind with miR-1253 in VSMCs and 293 Cells

To further explore the underlying mechanisms of FOXC2-AS1 that promoted cell proliferation and inhibited apoptosis in VSMCs, we used starBase v2.0 database to analyze the targets and miR-1253 was identified as a potential targeting miRNA. Then we detected the expressions of miR-1253 in AS patients and healthy volunteers. Results showed that miR-1253 was significantly decreased in AS patients (n=35) (Figure 3A) (p<0.001). Furthermore, we found that miR-1253 was negatively correlated with FOXC2-AS1 in AS patients (Figure 3B) (p<0.01), but not in healthy volunteers (Figure 3C) (p>0.05). Moreover, expressions of miR-1253 in VSMCs were repressed following with the treatments of ox-LDL and IL-6 (Figure 3D-E) (p<0.01). In addition, expressions of miR-1253 in VSMCs transfected with p-FOXC2-AS1 were significantly repressed following with FOXC2-AS1 overexpression (Figure 3F) (p<0.01). These results suggested that FOXC2-AS1 was negatively interacted with miR-1253, which was predicted as a potential target of FOXC2-AS1. To confirm that FOXC2-AS1 could directly bind with miR-1253, the wild type and mutant sequences

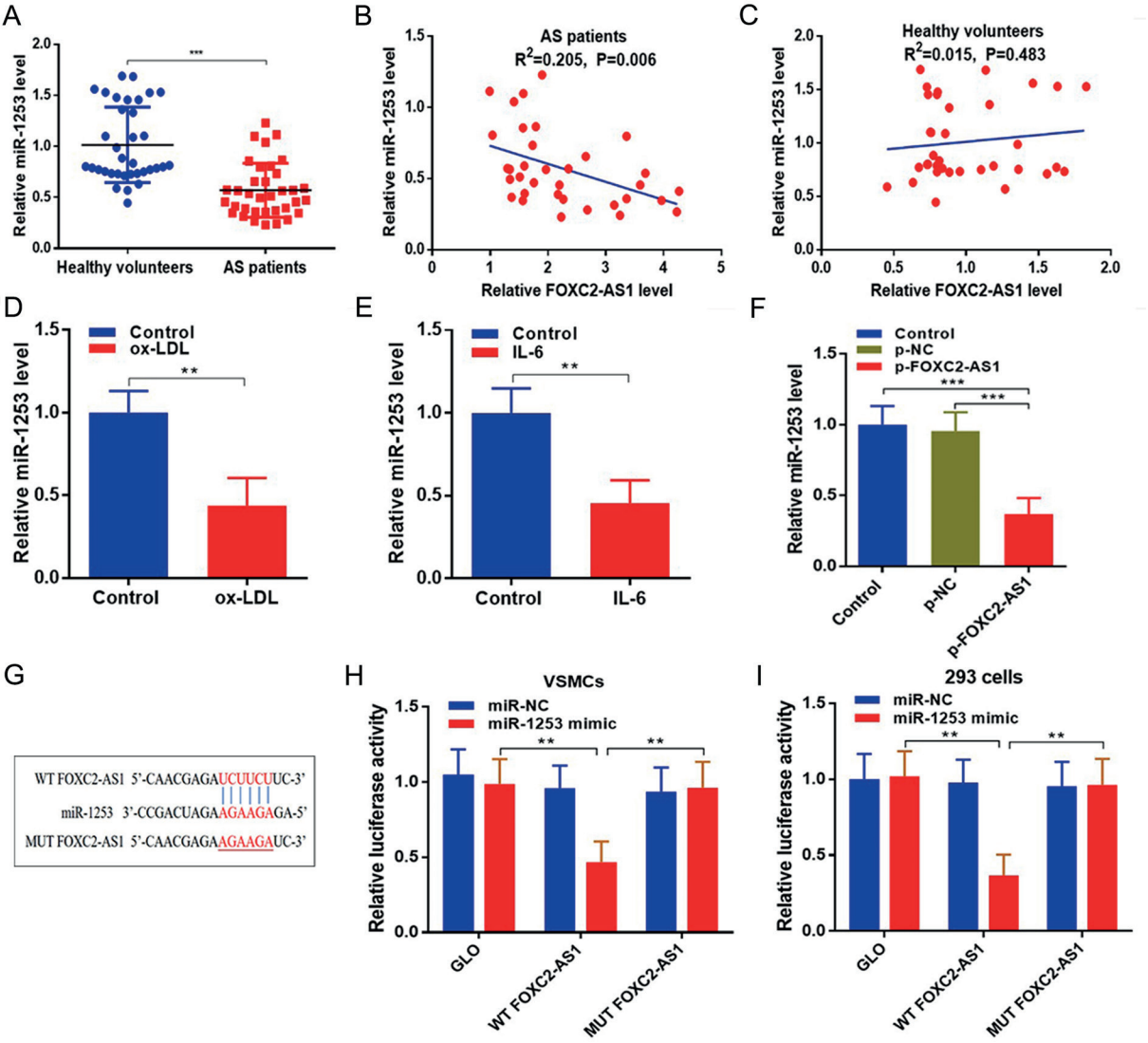

Figure 3. FOXC2-AS1 could directly bind with miR-1253 in VSMCs and 293 cells. **(A)** The expressions of miR-1253 were detected by RT-PCR in AS patients (n=35) and healthy volunteers (n=35). **(B, C)** The correlations between FOXC2-AS1 and miR-1253 were analyzed in AS patients and healthy volunteers. **(D-F)** The miR-1253 expressions were detected by RT-PCR in VSMCs treated with ox-LDL or IL-6 or transfected with p-FOXC2-AS1 or p-NC. **(G)** Wild type and mutant potential binding sequences between FOXC2-AS1 and miR-1253 were constructed into GLO plasmid. **(H, I)** Luciferase reporter assay was performed in VSMCs and 293 cells. Data are shown as mean ± SD based on at least three independent experiments, **p<0.01, ***p<0.001.

were synthesized and constructed in GLO plasmid (Figure 3G); next, the luciferase gene reporter assay was performed. Results showed that the relative luciferase activity in VSMCs co-transfected with WT-FOXC2-AS1 and miR-1253 mimic was significantly repressed compared with that in cells transfected with miR-NC. Furthermore, it was reversed in cells co-transfected with MUT-FOXC2-AS1 (Figure 3H) (p<0.01). Additionally, similar results had been found in 293 cells (Figure 3I) (p<0.01). These results demonstrated that FOXC2-AS1 could directly bind with miR-1253 in VSMCs, which might function as a ceRNA to regulate the development of AS patients.

MiR-1253 Inhibited Proliferation and Promoted Apoptosis in VSMCs

To further investigate the underlying roles of miR-1253 in AS patients, miR-1253 mimic or miR-NC was respectively transfected into VSMCs, resulted with miR-1253 overexpression in comparison with other two groups in VSMCs (Figure

4A) (p<0.001). The cell proliferation ability was significantly repressed and cell apoptotic rate was improved in VSMCs transfected with miR-1253 mimic (Figure 4B-C) (p<0.01). Furthermore, the protein level of Bcl-2 was decreased, while levels of apoptotic genes Bax and Cleaved Caspase3 were increased following with miR-1253 overexpression (Figure 4D-E) (p<0.01). Collectively, these results revealed that miR-1253 overexpression inhibited cell proliferation and promoted apoptosis in VSMCs. MiRNAs had been found to target at the 3′-UTR of target genes and regulate biological functions; however, the detailed mechanism of miR-1253 in AS remained unclear.

MiR-1253 Targeted at Binding with FOXF1 in VSMCs

To further explore the mechanism that miR-1253 inhibited cell proliferation and promoted apoptosis in VSMCs, target genes of miR-1253 were predicted by using TargetScan database. FOXF1 was predicted as a target gene of miR-1253, which was reported to act as an oncogene to promote cell proliferation and malignancy in some cancers[36-38]. We found that the protein level of FOXF1 was upregulated in p-FOXC2-AS1 group, compared with blank control and p-NC (Figure 5A-B) (p<0.01). Furthermore, the protein level of FOXF1 was significantly repressed in miR-1253 mimic group, compared with blank control and miR-NC (Figure 5C-D) (p<0.01). These results indicated that FOXF1 was positively interacted with FOXC2-AS1, while it was negatively interacted with miR-1253, which was also predicted as a target of miR-1253. To confirm that miR-1253 could directly bind with FOXF1, the wild type and mutant sequences of FOXF1 were synthesized and constructed in GLO plasmids (Figure 5E); then, the luciferase gene reporter assay was performed. Results revealed that the relative luciferase activity in VSMCs co-transfected with WT-FOXF1 and miR-1253 mimic was repressed, while it was reversed in cells co-transfected with MUT-FOXF1 and miR-1253 mimic (Figure 5F)

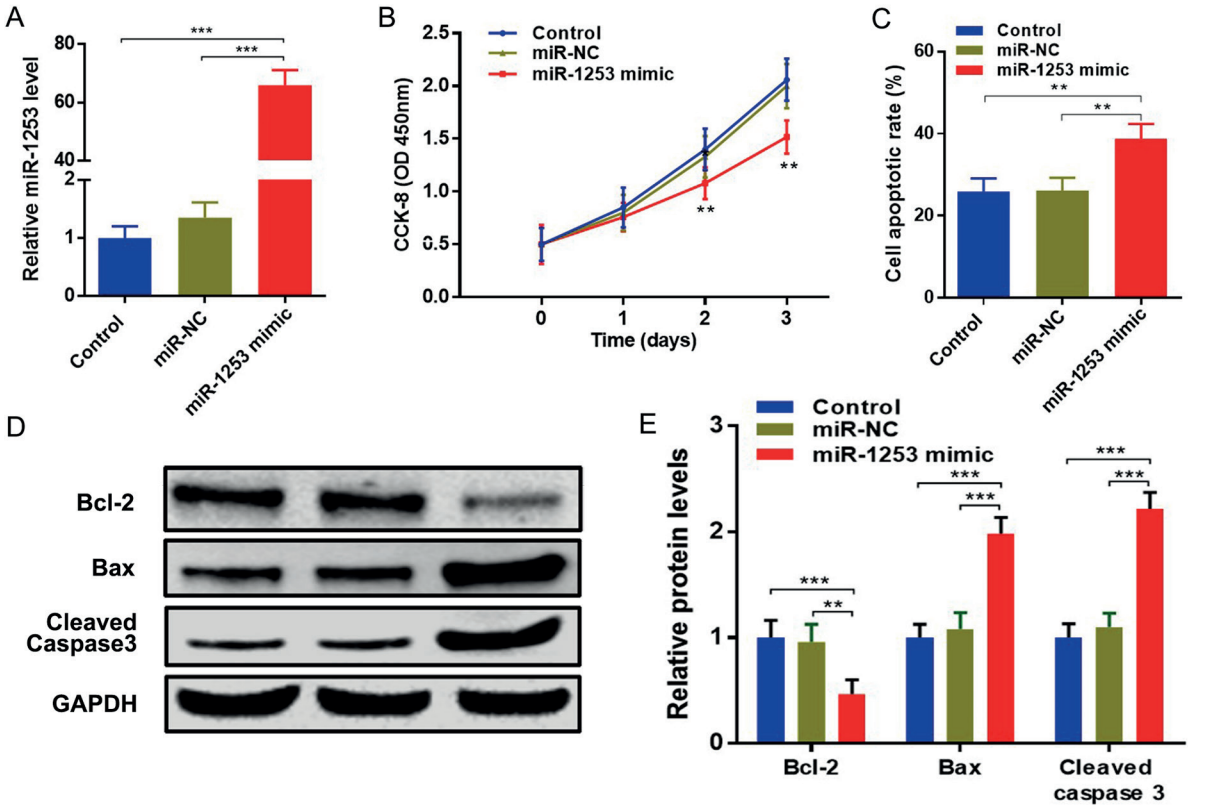

Figure 4. MiR-1253 inhibited proliferation and promoted apoptosis in VSMCs. **(A)** The expression of miR-1253 was detected by RT-PCR after miR-1253 mimic or miR-NC transfecting into VSMCs. **(B)** The proliferation abilities of VSMCs were measured by CCK8 assay. **(C)** The apoptotic rates of VSMCs were measured by FACS. **(D-E)** The protein levels of Bcl-2, Bax and Cleaved Caspase3 were detected by WB. Data are shown as mean ± SD based on at least three independent experiments, **p<0.01, ***p<0.001.

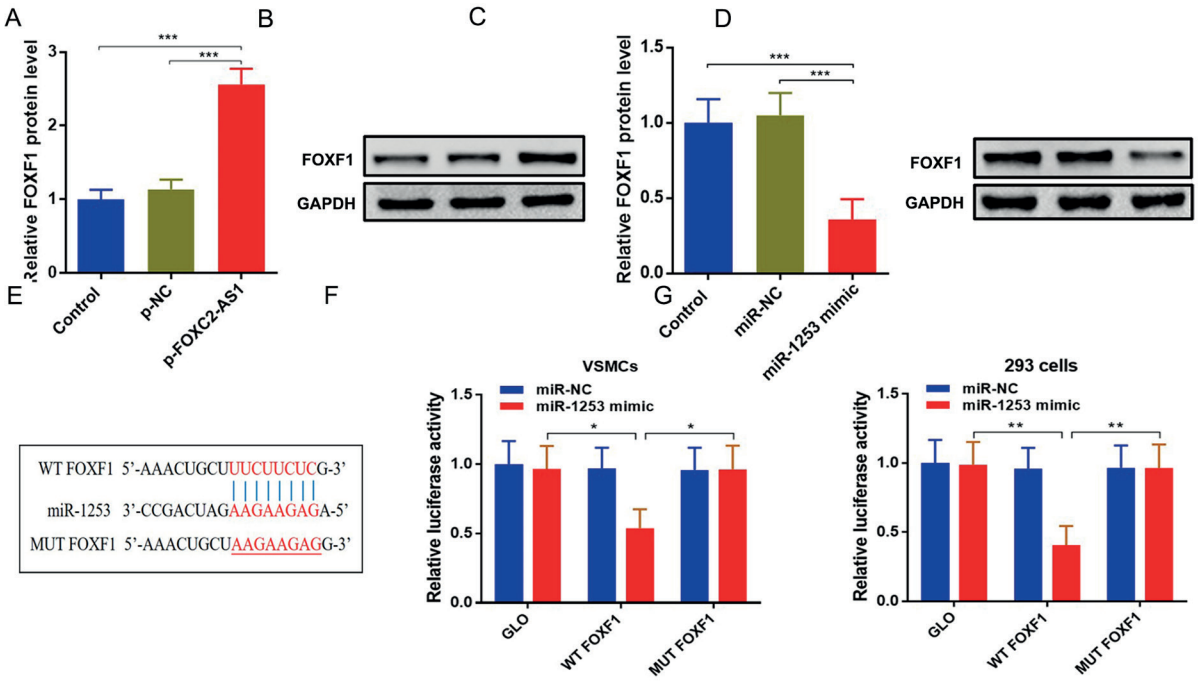

Figure 5. MiR-1253 targeted at binding with FOXF1 in VSMCs. **(A-B)** The protein levels of FOXF1 were detected by WB in VSMCs with blank control, p-NC and p-FOXC2-AS1. **(C-D)** The protein levels of FOXF1 were detected by WB in VSMCs with blank control, miR-NC and miR-1253 mimic. **(E)** Wild type and mutant potential binding sequences between FOXF1 and miR-1253 were constructed into GLO plasmid. **(F,-G)** Luciferase reporter assay was performed in VSMCs and 293 cells. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05, **p<0.01, ***p<0.001.

(p<0.01). Additionally, similar results had been found in 293 cells (Figure 5G) (p<0.01). These data indicated that miR-1253 could directly target at binding with FOXF1 in VSMCs.

MiR-1253/FOXF1 Axis Contributed to Regulate Cell Proliferation and Apoptosis in VSMCs

To confirm that miR-1253/FOXF1 axis contributed to regulate cell proliferation and apoptosis in VSMCs, p-FOXF1 was synthesized and constructed into the plasmid, resulted with FOXF1 overexpression. The p-FOXF1 or p-NC was respectively transfected into VSMCs, and then miR-1253 mimic and miR-NC were respectively transfected into these cells. Results showed that FOXF1 was significantly increased following with p-FOXF1 transfection (Figure 6A) (p<0.01), while it was repressed following with miR-1253 mimic transfection into p-FOXF1 cells (Figure 6A) (p<0.001). Furthermore, CCK8 assay showed that FOXF1 overexpression promoted cell proliferation, while it was repressed following with miR-1253 overexpression (Figure 6B) (p<0.001). Moreover, FACS assay showed that apoptotic rate was decreased following with FOXF1 overexpression, while it was increased following with miR-1253 overexpression (Figure 6C) (p<0.01). Besides, the protein levels of FOXF1 and Bcl-2 were increased, protein levels of Bax and Cleaved Caspase3 were decreased following with FOXF1 overexpression, while these protein levels were reversed following with miR-1253 overexpression (Figure 6D-E) (p<0.01). Collectively, these results indicated that miR-1253/FOXF1 axis contributed to regulate cell proliferation and apoptosis in VSMCs. In summary, these results demonstrated that FOXC2-AS1 could bind to miR-1253, which then targeted at binding with FOXF1 in VSMCs and regulated the cell proliferation and development of AS.

FOXC2-AS1 Promoted Proliferation and Inhibited Apoptosis via of miR-1253/FOXF1 in VSMCs

To further show the above assumption, we transfected miR-1253 mimic or miR-NC into VSMCs with p-FOXC2-AS1 or p-NC. Results

detected that FOXC2-AS1 was upregulated and miR-1253 was decreased following with FOXC2-AS1 overexpression, while FOXC2-AS1 was repressed and miR-1253 was increased following with miR-1253 mimic transfection (Figure 7A) (p<0.01). Furthermore, CCK8 assays revealed that cell proliferation ability was increased following with FOXC2-AS1 overexpression, while it was repressed following with miR-1253 mimic transfection (Figure 7B) (p<0.05). Moreover, FACS assay showed that the apoptotic rate was decreased following with FOXC2-AS1 overexpression, while it was increased following with miR-1253 overexpression (Figure 7C) (p<0.01). In addition, WB results showed that protein levels of FOXF1 and Bcl-2 were increased, levels of Bax and Cleaved Caspase3 were decreased following with FOXC2-AS1 overexpression, while they were reversed after miR-1253 mimic transfection (Figure 7D-E) (p<0.01). Collectively, our study disclosed that FOXC2-AS1 was upregulated in AS samples, which might sponge with miR-1253, and the repressed miR-1253 could increase the FOXF1 expression, thereby promoting proliferation of VSMCs and regulating the development of AS.

Discussion

LncRNAs participated in the progression in various diseases and studies had proved that some lncRNAs are involved in different biological functions of AS[20,21]. Therefore, it is important to making a better understanding of lncRNAs, which may provide special markers and therapeutic targets for AS. In this study, we found that FOXC2-AS1 was significantly increased in AS patients, which was induced by ox-LDL and IL-6 in VSMCs. These data indicated that FOXC2-AS1 might play some roles in the development of AS. However, its functions in AS remained unclear.

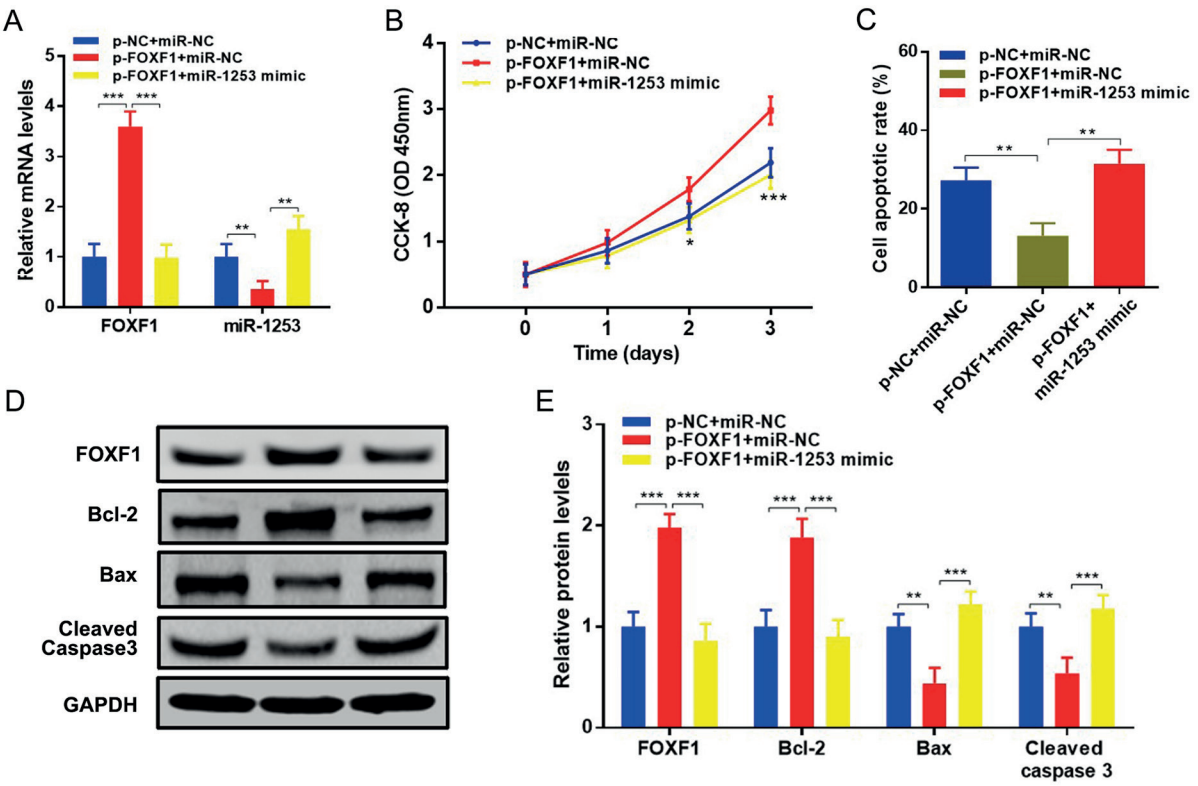

Figure 6. MiR-1253/FOXF1 axis contributed to regulate cell proliferation and apoptosis in VSMCs. **(A)** The expressions of FOXF1 and miR-1253 were detected by RT-PCR in VSMCs. **(B)** CCK8 assay was used to measure the proliferation abilities of VSMCs. **(C)** FACS was used to measure the apoptotic rates of VSMCs. **(D-E)** The protein levels of FOXF1, Bcl-2, Bax and Cleaved Caspase3 were detected by WB. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05, **p<0.01, ***p<0.001.

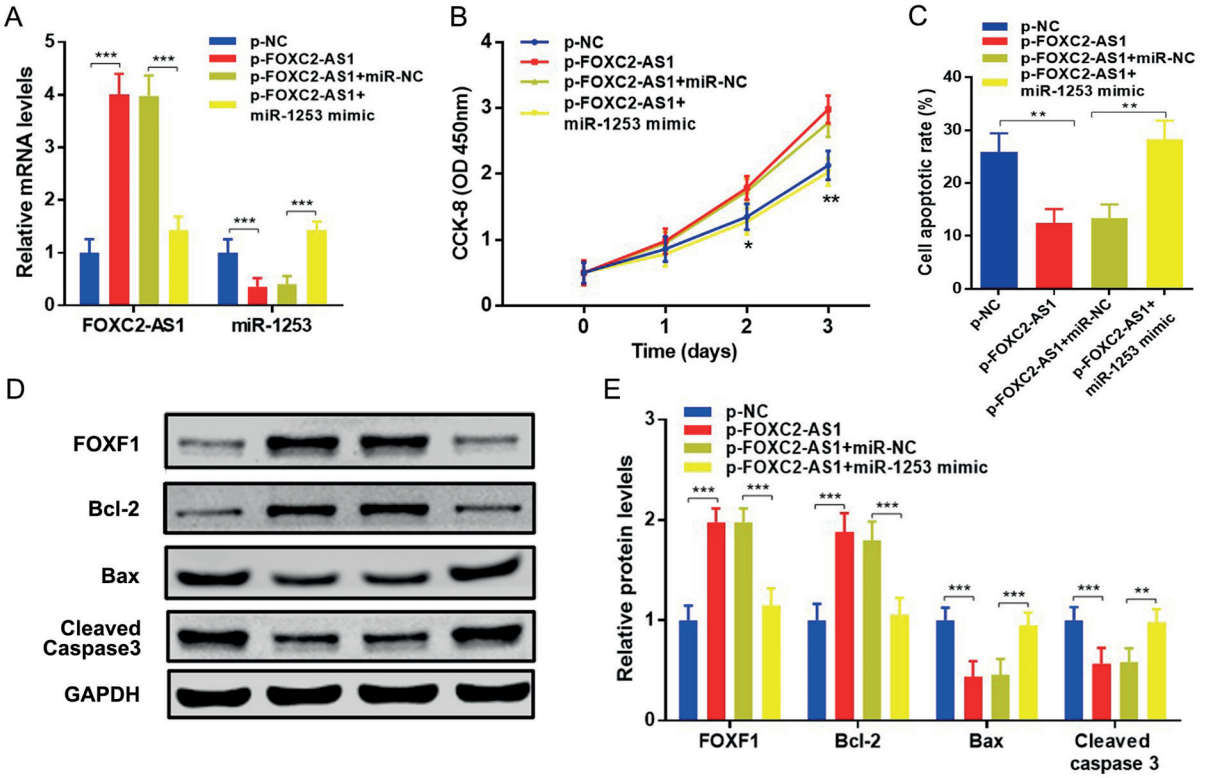

Figure 7. FOXC2-AS1 promoted proliferation and inhibited apoptosis via of miR-1253/FOXF1 in VSMCs. **(A)** The expressions of FOXC2-AS1 and miR-1253 were detected by RT-PCR in VSMCs co-transfected with p-NC or p-FOXC2-AS1 and miR-NC or miR-1253 mimic. **(B)** CCK8 assay was used to measure the proliferation abilities of VSMCs. **(C)** FACS was used to measure the apoptotic rates of VSMCs. **(D-E)** The protein levels of FOXF1, Bcl-2, Bax and Cleaved Caspase3 were detected by WB. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05, **p<0.01, ***p<0.001.

Then p-FOXC2-AS1 was synthesized and transfected into VSMCs, the proliferation abilities of VSMCs were increased and cell apoptotic rate was inhibited following with FOXC2-AS1 overexpression.

LncRNAs might act as a "sponge" molecule to interact with miRNAs[31,32], which had been reported to regulate the formation and progression of AS through binding to 3'-untranslated regions (UTR) of target genes, thereby regulating the progression of AS. Then, we used starBase v2.0 database to analyze the targets of FOXC2-AS1 and miR-1253 was identified as a potential target. We found that miR-1253 was decreased in AS patients, which was negatively correlated with FOXC2-AS1 in AS patients. Furthermore, miR-1253 was repressed following with FOXC2-AS1 overexpression. These results suggested that FOXC2-AS1 was negatively interacted with miR-1253, which was predicted as a potential target of FOXC2-AS1. Moreover, luciferase gene reporter assay confirmed that FOXC2-AS1 could directly bind with miR-1253 in VSMCs and 293 cells. However, the detailed roles of miR-1253 in patients with AS remained unknown.

Forkhead box F1 (FOXF1) is a kind of transcription factor that has been regarded as an oncogene, which plays important roles in cell proliferation and development of cancers[36-38]. It was reported that FOXF1 could promote prostate cancer progression by stimulating the mitogen-activated protein kinase ERK5 expression[36]. Kun-Peng et al[37] found that FOXF1 could promote migration and invasion of osteosarcoma[38]. Recent study showed that miR-1253 downregulation could modulate cell proliferation and invasion by targeting at FOXF1 in osteosarcoma. To further investigate the roles of miR-1253 in AS, miR-1253 mimic was transfected into VSMCs. We found that the proliferation abilities of VSMCs were inhibited and cell apoptotic rate was improved following with miR-1253 overexpression. Furthermore, Targescan database was used, which showed that FOXF1 might contain the 3'-untrans-

lated regions (3'-UTR) that could bind with miR-1253. Therefore, luciferase gene reporter assay was performed, confirming that miR-1253 could directly bind to FOXF1 in VSMCs and 293 cells.

To confirm that miR-1253/FOXF1 axis contributed to regulate cell proliferation and apoptosis in VSMCs, p-FOXF1 or p-NC was respectively transfected into VSMCs. Then, miR-1253 mimic and miR-NC were respectively transfected into these cells. Results showed that FOXF1 was significantly increased following with p-FOXF1 transfection, while it was repressed following with miR-1253 mimic transfection into p-FOXF1 cells. CCK8 assay and FACS assay showed that FOXF1 overexpression promoted cell proliferation and inhibited cell apoptosis, while it was reversed following with miR-1253 overexpression. Besides, the protein levels of FOXF1 and Bcl-2 were increased, and the protein levels of Bax and Cleaved Caspase3 were decreased following with FOXF1 overexpression, while these protein levels were reversed following with miR-1253 overexpression. These results indicated that miR-1253/FOXF1 axis contributed to regulate cell proliferation and apoptosis in VSMCs. Collectively, results demonstrated that FOXC2-AS1 could bind to miR-1253, which then targeted at binding with FOXF1 in VSMCs and regulated the cell proliferation and development of AS.

To detect our assumption, miR-1253 mimic and miR-NC were respectively transfected into VSMCs with p-FOXC2-AS1. We found that FOXC2-AS1 was increased and miR-1253 was repressed following with overexpression of FOXC2-AS1, while they were reversed following with miR-1253 mimic transfection. Furthermore, CCK8 assay and FACS assay showed that FOXC2-AS1 overexpression promoted cell proliferation and inhibited cell apoptosis, while it was reversed following with miR-1253 overexpression. Finally, the protein levels of FOXF1 and Bcl-2 were increased, protein levels of Bax and Cleaved Caspase3 were decreased following with FOXC2-AS1 overexpression, while these protein levels were reversed following with miR-1253 overexpression. These results indicated that FOXC2-AS1 promoted proliferation and inhibited apoptosis via of miR-1253/FOXF1 in AS patients. Collectively, our study suggested that FOXC2-AS1 was upregulated in AS samples, which might sponge with miR-1253, and the repressed miR-1253 could increase the FOXF1 expression, thereby promoting proliferation of VSMCs and regulating the development of AS.

Conclusions

We found that FOXC2-AS1 was upregulated in AS patients. Furthermore, FOXC2-AS1 overexpression promoted cell proliferation and inhibited cell apoptosis via targeting miR-1253/FOXF1 signaling axis. Our results elucidated a potential mechanism underlying the role of FOXC2-AS1, which might be used as a promising marker and a potential target for AS patients.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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