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ORIGINAL ARTICLE

HOXA cluster antisense RNA 2 elevates KIAA1522 expression through microRNA-520d-3p and insulin like growth factor 2 mRNA binding protein 3 to promote the growth of vascular smooth muscle cells in thoracic aortic aneurysm

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Abstract

Aims Recently, long non-coding RNAs (lncRNAs) have been revealed to mediate smooth muscle dysfunction in thoracic aortic aneurysm (TAA). LncRNA HOXA-AS2 has been proposed to engage in the regulation of diverse diseases. However, its function in TAA remains unknown. This study aimed to reveal the role and mechanism of HOXA-AS2 in VSMCs which were implicated in TAA formation.

Methods and results RT-qPCR or western blot was performed to detect RNA or protein expression levels. The role of HOXA-AS2 in VSMCs was explored by functional assays. The relationship among HOXA-AS2/miR-520d-3p/KIAA1522/IGF2BP3 was analysed via mechanism assays. HOXA-AS2 was detected to have significantly high expression in TAA tissues and function as an oncogene to promote proliferation of VSMCs, while inhibiting cell apoptosis (Figure 1, $^{**}P < 0.01$). HOXA-AS2 was unveiled to bind with miR-520d-3p (Figure 2, $^{*}P < 0.05$, $^{**}P < 0.01$) and further up-regulate KIAA1522 to facilitate the growth of VSMCs (Figure 3–4, $^{*}P < 0.05$, $^{**}P < 0.01$). HOXA-AS2 was also found to recruit IGF2BP3 to stabilize KIAA1522 mRNA (Figure 5, $^{**}P < 0.01$). All data were displayed as mean ± standard deviation.

Conclusions HOXA-AS2 up-regulates KIAA1522 through targeting miR-520d-3p/IGF2BP3 to drive VSMC growth in TAA.

Keywords HOXA-AS2; miR-520d-3p; KIAA1522; IGF2BP3; TAA

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Introduction

Thoracic aortic aneurysm (TAA) has been diagnosed as localized dilatations of the supra-diaphragmatic aorta induced by impairing and expansion of the arterial wall and commonly accepted as one of the dominating causes of human deaths.[1] After the involvement of ascending aorta, acute aortic insufficiency, aortic rupture, pericardial tamponade, or haemopericardium may give rise to the death of patients. According to the size of the ascending aorta and patient's risk factors, it might be necessary to tightly control the arterial blood pressure or even replace ascending aortic.[2] Genetic factors have exerted critical impact on TAA progression. Published studies have unearthed that approximately 20% of patients with nonsyndromic TAA have the affected first-degree relatives.[3,4] In addition, other factors, such as hypertension, arteriosclerosis, aging as well as diseases of inflammatory and autoimmune influencing the aorta, can also lead to the initiation of TAA.[5] The aortic media majorly consists of vascular smooth muscle cells (VSMCs), the major

source of the proteins of extracellular matrix. VSMCs related to the extracellular matrix to a large extent affect the biomechanical properties of the aortic wall. Additionally, in the aortic wall of TAA patients, an enhanced apoptosis of VSMCs is noted, which is regarded as a significant cause for TAA.[6] The development of TAA has been reported to be caused by multiple factors, including an interaction between genetic factors and other risk factors, such as cellular imbalances and some haemodynamic factors.[2] Recently, progresses have been made to enlarge the knowledge of TAA physiopathology, whereas the molecular mechanism of TAA pathogenesis has not been largely uncovered.

A growing number of studies have indicated that only 1.2% of the genome in mammals has the capacity of encoding proteins.[7,8] The genome is mainly transcribed into long noncoding RNAs (lncRNAs), belonging to noncoding RNAs (ncRNAs) with over 200 nucleotides in length.[9,10] Accumulating research work has revealed that lncRNAs have no or limited capability of protein coding on account of lacking an open reading structure of a given length.[11,12] Recent evidence suggests the involvement of lncRNAs in human diseases (TAA included) and also emphasizes the critical effects of lncRNAs on the malignant behaviours of VSMCs. For instance, lncRNA HIF1A-AS1 has been unveiled to modulate the proliferation and apoptosis of VSMCs in TAA.[13] LncRNA LINC00341 has been uncovered to accelerate the growth of VSMCs via miR-214/FOXO4 feedback loop.[14] LncRNA RNCR3 knockdown has been found to induce the dysfunction of VSMCs and ECs in atherosclerosis.[15] Reviewing published research work, we noticed that HOXA cluster antisense RNA 2 (HOXA-AS2) had been uncovered to play an oncogenic role in facilitating the progression of multiple diseases, including prostate cancer,[16] non-small cell lung cancer,[17] and glioblastoma.[18] Although this novel lncRNA has been revealed to be an important regulator in numerous cancers,[19,20] the possible regulatory function of HOXA-AS2 on the proliferative and apoptotic abilities of VSMCs in TAA remains unclear.

This study was the first attempt to dig into the role of HOXA-AS2 and its underlying molecular mechanism in TAA. We hope that the findings of this study could shed new light on developing novel potential diagnostic and therapeutic agent against TAA.

Methods

Collection of clinical specimens

Thirty-nine patients with TAA and 21 healthy volunteers were recruited in this study for collecting the TAA and normal control tissue samples. The ethic approval from Qingdao Municipal Hospital and the signed informed consents from all subjects were obtained before the study. Instantly, after surgical resection, the fresh samples were preserved at −80°C in liquid nitrogen until RNA isolation. The study was run in strict line with the Declaration of Helsinki.

Cell culture and transfection

In terms of cell culture, 1.5×10^5 human aortic VSMCs (Cascade Biologics, Portland, Oregon, USA) were placed in Medium 231 in the presence of smooth muscle growth supplements (Cascade Biologics) in an incubator (5% CO_2, 37°C). Cells of passage 3–8 were collected for further analysis. For cell transfection, HOXA-AS2-specific shRNAs (sh-HOXA-AS2#1/2), IGF2BP3-specific shRNAs (sh-IGF2BP3#1/2), and corresponding negative control (sh-NC), pcDNA3.1/KIAA1522, pcDNA3.1/HOXA-AS2, and empty pcDNA3.1, as well asmiR-520d-3p mimics/inhibitor and NC mimics/inhibitor, were all designed and procured at GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was utilized for 48 h transfection.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Extraction of total cellular RNAs from 2×10^6 VSMCs was achieved with the use of TRIzol reagent (Invitrogen), and then the single-strand cDNA was synthesized as template. QPCR was carried out by SYBR green Imaster mix (Invitrogen) on Step-One Plus System (Applied Biosystems, Foster City, CA, USA). The RNA level, normalized to GAPDH or U6, was calculated by the comparative delta–delta CT method ($2^{-\Delta\Delta Ct}$). Bio-repeats were run in three times.

Fluorescence in situ hybridization (FISH)

FISH assay was performed to determine the location of miR-520d-3p and HOXA-AS2 in VSMCs. At first, 1.5×10^5 VSMCs were fixed in 4% paraformaldehyde (PFA) at room temperature. Next, 0.5% TritonX-100 was employed to permeabilize the cells for 15 min at 4°C. After being rinsed with PBS, cells were co-cultured with the FISH probes targeted miR-520d-3p or HOXA-AS2. Subsequently, DAPI (Beyotime, Shanghai, China) was applied to stain the cells, and laser scanning confocal microscope was eventually used to observe cells. Bio-repeats were run in three times.

Cell counting kit-8 (CCK-8) assay

After transfection for 48 h, the single cell suspension (3×10^4 cells/mL) was acquired and planted in 96-well culture plates containing 10 μL of CCK-8 (Sigma Aldrich, St. Louis, MI,

USA). The OD value (450 nm) was examined once reagent reacted for 0, 24, 48, 72, and 96 h. Bio-repeats were run in three times.

5-Ethynyl-2′-deoxyuridine (EdU) assay

The EdU kit (Ribobio, Guangzhou, China) was bought for detecting proliferation of VSMCs. Transfected cells (2×10^5) in 96-well plates were subjected to incubation with 100 μL of 50 μM EdU for 3 h, fixation with 4% PFA, and permeabilization with 0.5% Troxin X-100; 100 μL of 1× Apollo® 488 fluorescent liquid was added to the reaction for 30 min. When nuclei were stained with DAPI, cells were then analysed under a fluorescent microscope (Leica, Wetzlar, Germany). Bio-repeats were run in three times.

Flow cytometry analysis for cell apoptosis

Cells (4×10^6) were treated with trypsin, washed in pre-cooled PBS solution, and then incubated with 100 μL of binding buffer for 10 min in the dark. Afterwards, 6 μL of Annexin V-FITC and 10 μL of PI (Sigma Aldrich) were used for treating cells for 20 min. Finally, cell apoptosis was analysed using flow cytometry. Bio-repeats were run in three times.

Terminal-deoxynucleotidyl transferase mediated nick end labelling (TUNEL)

The apoptosis of cultured VSMCs was estimated by in situ cell death detection kit (Minneapolis, MN, USA) as per the established protocol. Cells (2.5×10^5) on coverslips were plated to 6-well plates with 4% PFA. Next, cells were permeabilized with 0.5% Triton X-100 and subsequently cultivated with 50 μL TdT reaction mix. Cell apoptosis was finally observed under fluorescence microscope after DAPI staining. Bio-repeats were run in three times.

Caspase-3 activity

Cells (3×10^3) were involved in this assay. Caspase 3 Activity Assay Kit was bought and utilized in light of the recommendation of supplier (C1115, Beyotime). The cellular protein was prepared in 96-well plates and mixed with reaction buffer as well as caspase substrate for 4 h at 37°C, followed by detection using microplate reader at 405 nm. Bio-repeats were run in three times.

Subcellular fractionation assay

Cells (1×10^7) were used in this assay. The nuclear and cytoplasmic RNAs were separated and purified by use of Cytoplasmic & Nuclear RNA Purification Kit (Norgen, Belmont, CA, USA) based on the user guide. The isolated RNAs (HOXA-AS2, GADPH, and U6) were analysed via RT-qPCR. U6 and GAPDH were regarded as nuclear reference and cytoplasm reference. Bio-repeats were run in three times.

RNA pull-down assay

The protein extracts were prepared from 2×10^7 VSMCs, then treated severally with biotinylated HOXA-AS2 RNAs (HOXA-AS2 biotin probe, HOXA-AS2 no-biotin probe, Bio-HOXA-AS2-Mut and Bio-HOXA-AS2-WT). After cell lysates were incubated with probes overnight, magnetic beads were added to collect RNA-protein complex, followed by RT-qPCR or western blot analysis. Bio-repeats were run in three times.

Luciferase reporter assay

The wild-type (WT) HOXA-AS2 sequences were inserted to the pmirGLO Dual-Luciferase Reporter Vector (Promega, Madison, WI, USA) to construct pmirGLO-HOXA-AS2-WT. The HOXA-AS2 sequences with mutant (Mut) binding sites on miR-520d-3p were sub-cloned into pmirGLO plasmids to generate pmirGLO-HOXA-AS2-Mut. Both vectors were co-transfected into 1×10^4 cells with indicated transfection plasmids for 48 h. The luciferase activity was finally analysed by Dual-Luciferase Reporter Assay System (Promega), with *Renilla reniformis* as internal reference. Bio-repeats were run in three times.

RNA immunoprecipitation (RIP)

VSMCs (1×10^7) were prepared in RIP lysis buffer and then incubated with anti-Ago2 or anti-IGF2BP3 (Millipore, Billerica, MA, USA) following the manufacturer's instructions. IgG antibody acted as the NC. After beads were added for coprecipitation, the recovered RNA was purified and assayed by RT-qPCR. Bio-repeats were run in three times.

Western blot

RIPA buffer (Sangon, Shanghai, China) was applied to extract protein samples from 2×10^6 cells. Afterwards, proteins were isolated on 10% SDS-PAGE gel, followed by the transfer to PVDF membranes. Next, PVDF membranes were sealed with 5% fat-free milk and incubated with primary antibodies including anti-KIAA1522 (ab122203, Abcam,

Cambridge, MA, USA), anti-CSTF2T (ab138486, Abcam), anti-DGCR8 (ab191875, Abcam), anti-FMR1 (TA504290, OriGene, Rockwell, Maryland, USA), anti-HNRNPA2B1 (ab31654, Abcam), anti-HNRNPK (ab23644, Abcam), anti-IGF2BP2 (ab129071, Abcam), anti-IGF2BP3, anti-RBFOX2 (PA5–52268, Invitrogen), anti-SRSF1 (32–4,500, Invitrogen), anti-U2AF2 (PA5–30442, Invitrogen), and anti-GAPDH (ab8245, Abcam). GAPDH functioned as internal reference. The following blotting was conducted using HRP-anti-IgG secondary antibody. At last, ECL detection reagent (Millipore) was applied for signal development. Bio-repeats were run in three times.

mRNA stability assay

The 2×10^6 cultured cells were transfected with plasmids in the single 10 cm culture dish and then seeded in a 6-well plate. Actinomycin D (20 μg/mL; Sigma Aldrich) were added to block mRNA transcription. Three groups were set for detecting mRNA stability, including pcDNA3.1, pcDNA3.1/HOXA-AS2, and pcDNA3.1/HOXA-AS2 + sh-IGF2BP3#1. Cells were reaped at 0, 4, 8, and 12 h for extracting RNA, and RT-qPCR was applied to evaluate mRNA stability. Bio-repeats were run in three times.

Statistical analyses

All experiments included three biological repeats. Data analysis was processed by Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA). Experimental results were presented as the mean ± standard deviation (SD). Comparisons between groups were achieved by Student's *t*-test, one-way or two-way analysis of variance (ANOVA) backed by Dunnett's or Tukey's tests, with $P < 0.05$ as the cut-off value of statistically significant.

Results

HOXA cluster antisense RNA 2 knockdown restrains proliferation while promoting apoptosis of vascular smooth muscle cells

Before studying the underlying biological function of HOXA-AS2 in TAA, we detected the expression of HOXA-AS2 in samples from TAA patients and healthy donors via RT-qPCR. The result showed a marked up-regulation of HOXA-AS2 in TAA specimens (*Figure 1A*). After the expression of HOXA-AS2 in VSMCs was significantly cut down by sh-HOXA-AS2#1/2 (*Figure 1B*), CCK-8 and EdU assays were carried out with the more efficient sh-HOXA-AS2#1. The result represented that knockdown of HOXA-AS2 resulted in an observably attenuation of VSMC proliferation (*Figure 1C,D*). Moreover, flow cytometry and TUNEL analyses uncovered that the decrease of HOXA-AS2 contributed to a significant increase of apoptosis capability of VSMCs (*Figure 1E,F*). Apart from that, the elevated caspase-3 activity induced by HOXA-AS2 depletion suggested that the apoptosis ability of VSMCs could be enhanced by reducing HOXA-AS2 expression (*Figure 1G*). In sum, HOXA-AS2 displays a high expression level in TAA tissues and HOXA-AS2 knockdown suppresses VSMCs cell proliferation while facilitating cell apoptosis.

HOXA cluster antisense RNA 2 binds with microRNA-520d-3p in vascular smooth muscle cells

To fathom out the potential mechanism of HOXA-AS2 in TAA, we carried out subcellular fractionation assay and discovered that HOXA-AS2 was mainly located in cytoplasm of VSMCs (*Figure 2A*). Hence, we conjectured that HOXA-AS2 might function as a competing endogenous RNA (ceRNA) to sponge miRNA in VSMCs. Through utilizing starBase (http://starbase.sysu.edu.cn/) and DIANA tools (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted), 16 miRNAs predicted to have binding capacity with HOXA-AS2 were obtained and displayed in *Figure 2B*. Subsequently, RNA pull-down assays were employed to screen out the specific miRNA that could bind with HOXA-AS2 in VSMCs. The result delineated a conspicuous enrichment of miR-520d-3p in HOXA-AS2 biotin probe group (*Figure 2C*). Thus, miR-520d-3p was selected to involve in this study. Next, FISH assay was operated to determine the localization of miR-520d-3p and HOXA-AS2 in VSMCs. It turned out that miR-520d-3p and HOXA-AS2 mainly co-existed in the cytoplasm of VSMCs (Supporting Information, *Figure S1A*). After searching on starBase, a binding site between HOXA-AS2 and miR-520d-3p was predicted (*Figure 2D*). Afterwards, the efficiency of miR-520d-3p overexpression in VSMCs was analysed via RT-qPCR, which turned out to be satisfactory (*Figure 2E*). In addition, data from luciferase reporter assay implied that increased expression of miR-520d-3p weakened the luciferase activity of pmirGLO-HOXA-AS2-WT while exerting no significant effect on the luciferase activity of pmirGLO-HOXA-AS2-Mut (*Figure 2F*). Taken together, HOXA-AS2 binds to miR-520d-3p in VSMCs.

KIAA1522 acts as a downstream target of miR-520d-3p in VSMCs

To delve into the ceRNA mechanism of HOXA-AS2 in TAA, we further employed starBase to find the candidate mRNAs that

Figure 1 HOXA-AS2 is highly expressed in TAA tissues and HOXA-AS2 knockdown inhibits VSMCs proliferation whereas promotes cell apoptosis. (A) HOXA-AS2 expression in specimens of TAA and healthy donors was detected via RT-qPCR. (B) The efficacy of HOXA-AS2 knockdown in VSMCs was tested through RT-qPCR. (C, D) The viability and proliferation of VSMCs upon HOXA-AS2 depletion was evaluated through CCK-8 and EdU assays. (E–G) Flow cytometry, TUNEL and caspase-3 activity analyses were employed to assess the apoptosis ability of VSMCs transfected with indicated plasmids. $^{**}P < 0.01$.

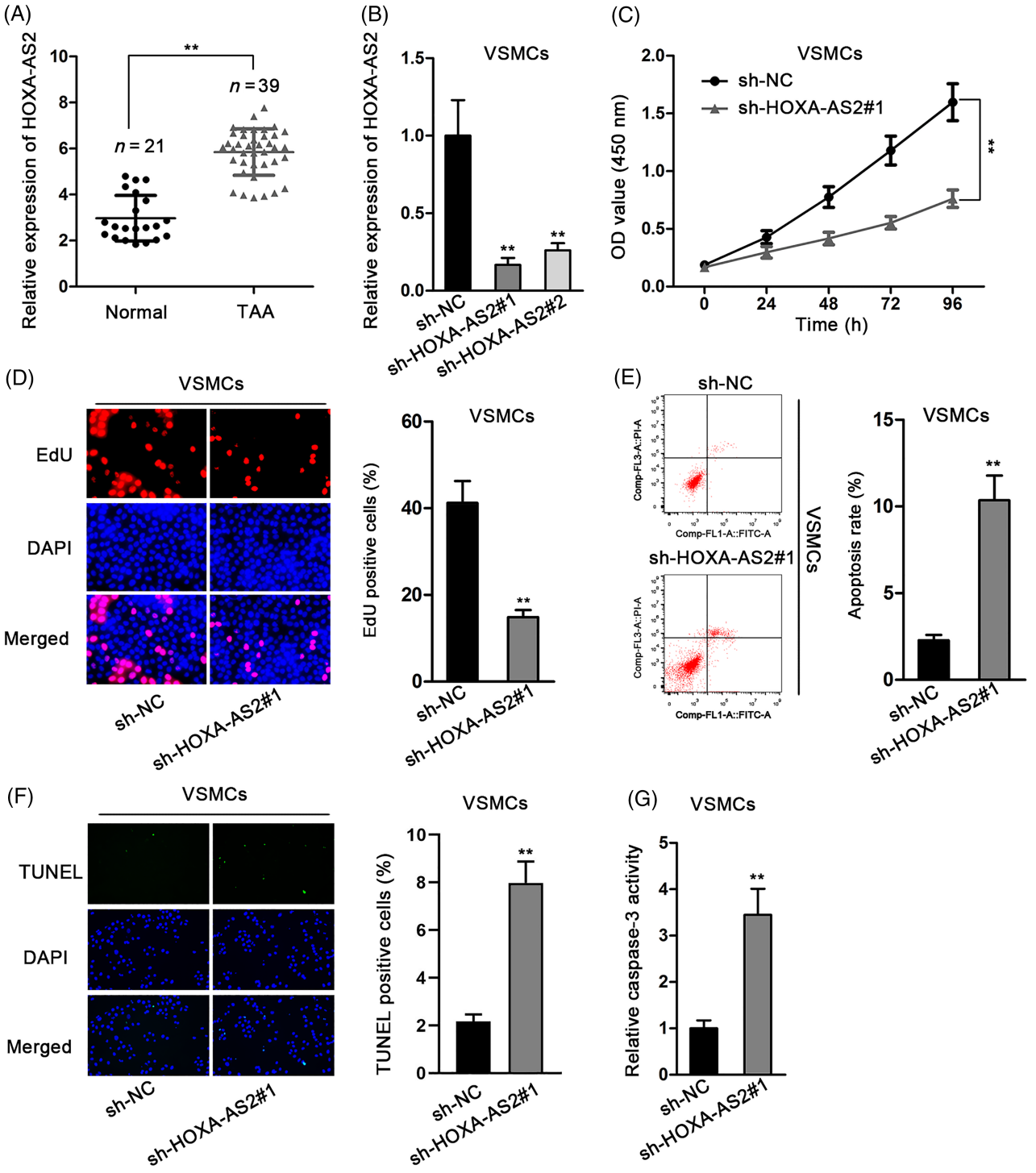

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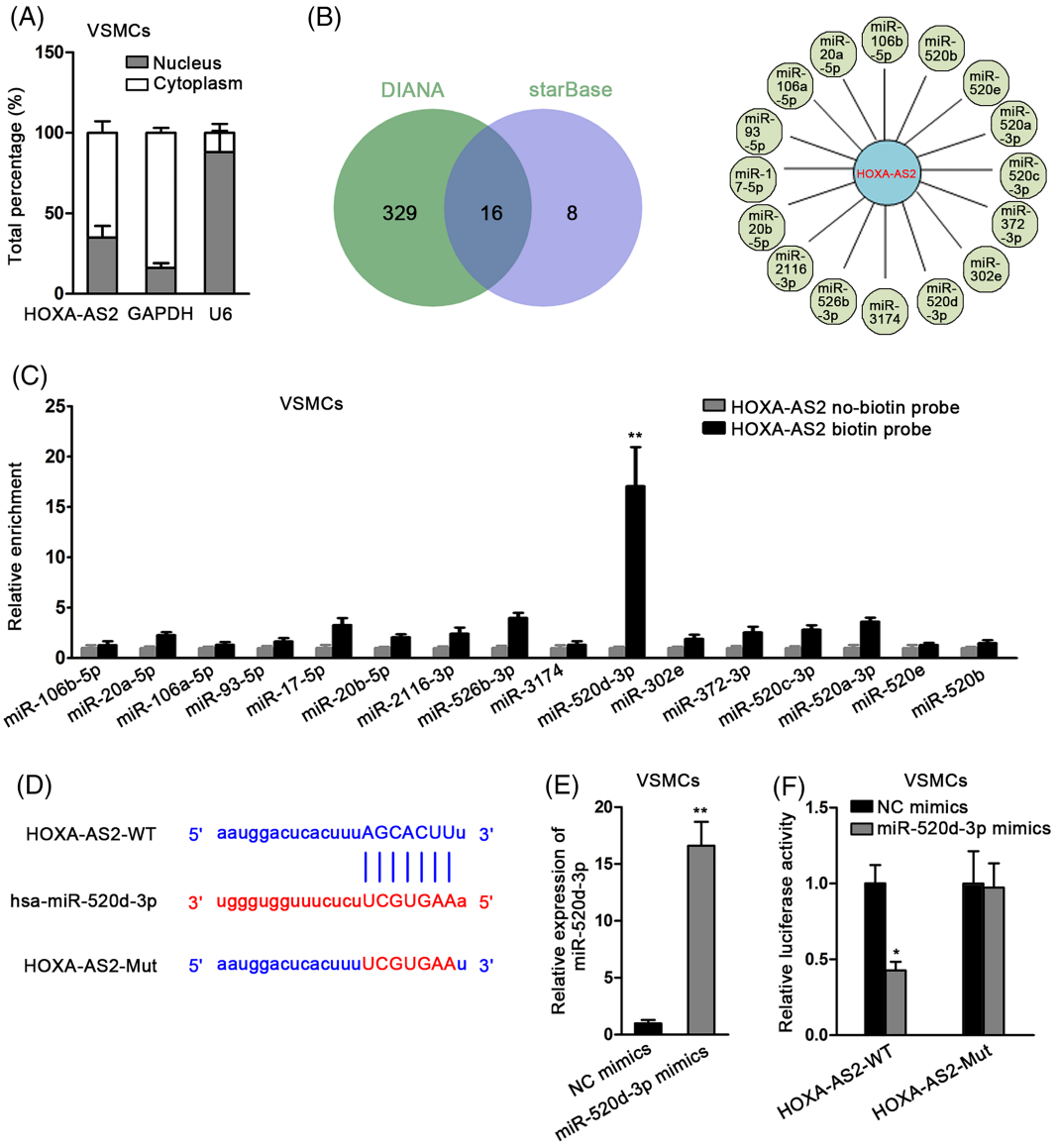
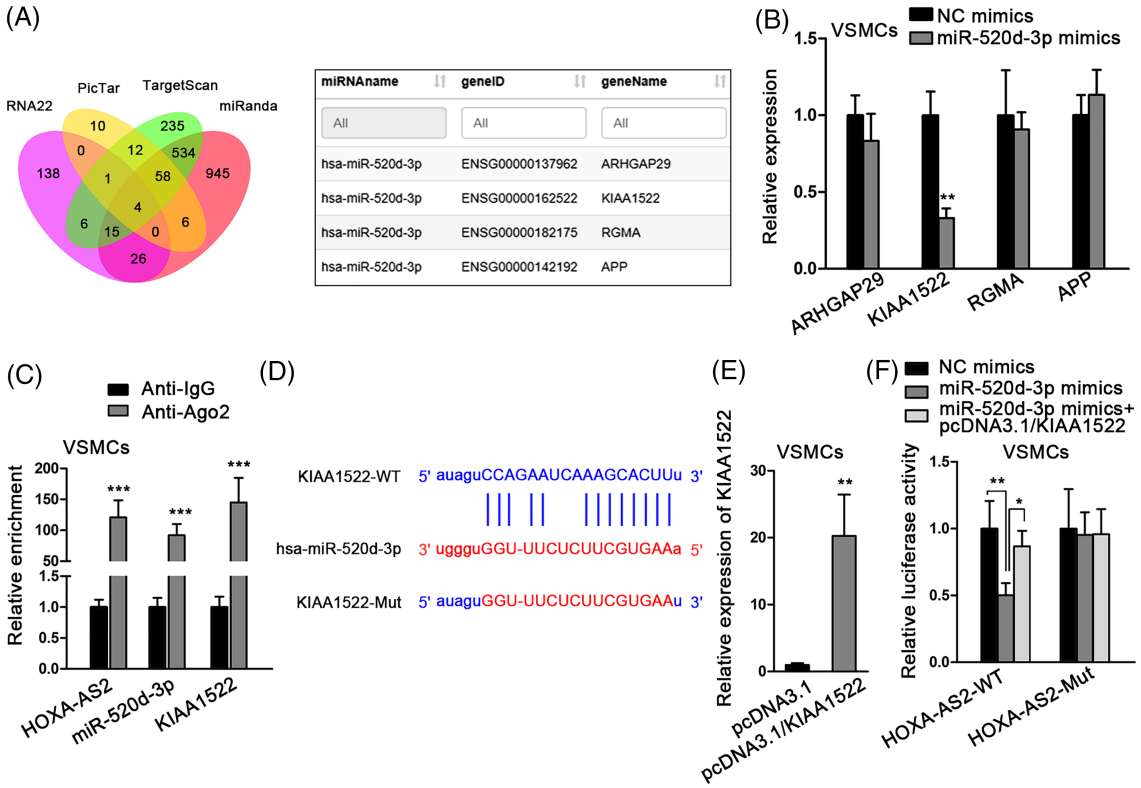
Figure 2 HOXA-AS2 binds with miR-520d-3p in VSMCs. (A) Subcellular fractionation assay was operated to confirm the distribution of HOXA-AS2 in VSMCs. (B) Candidate miRNAs possibly have the binding affinity with HOXA-AS2 were predicted by DIANA and starBase tools. (C) RNA pull-down assays were carried out to testify the binding affinity between HOXA-AS2 and those miRNAs. (D) Binding sites between HOXA-AS2 and miR-520d-3p were predicted through starBase. (E) The efficacy of miR-520d-3p overexpression in VSMCs was examined through RT-qPCR. (F) The interaction between HOXA-AS2 and miR-520d-3p was certified by luciferase reporter assay. $*P < 0.05$, $**P < 0.01$.



might bind with miR-520d-3p. Based on the data of four databases (miRanda, RNA22, PicTar, and TargetScan), Venn diagram was drawn, and four mRNAs having the potential binding affinity with miR-520d-3p were presented in *Figure 3A*. Then, the results of RT-qPCR reflected a significantly decreased expression of KIAA1522 in VSMCs transfected with miR-520d-3p mimics (*Figure 3B*). Aside from that, RIP assay revealed that RNAs (HOXA-AS2, miR-520d-3p and KIAA1522) were noticeably enriched in anti-Ago2 group, which indicated the existence of these RNAs in RNA-induced silencing complex (RISC) (*Figure 3C*). Additionally, a binding site between miR-520d-3p and KIAA1522 was projected through starBase (*Figure 3D*). Moreover, RT-qPCR analysis reflected a notable up-regulation of KIAA1522 in VSMCs transfected with pcDNA3.1/KIAA1522 (*Figure 3E*). More importantly, KIAA1522 overexpression partially countervailed the repressive effect of miR-520d-3p up-regulation on the luciferase activity of pmirGLO-HOXA-AS2-WT. Nonetheless, no conspicuous changes of the luciferase activity of pmirGLO-HOXA-AS2-Mut were noticed among different groups (*Figure 3F*). All in all, KIAA1522 works as a downstream gene of miR-520d-3p in VSMCs.

Figure 3 KIAA1522 is a downstream target of miR-520d-3p in VSMCs. (A) Venn diagram demonstrated the overlaps of bioinformatics prediction, and 4 mRNAs likely to bind with miR-520d-3p were listed. (B) The expression of candidate mRNAs (ARHGAP29, KIAA1522, RGMA, and APP) in VSMCs with or without transfection of miR-520d-3p mimics was quantified by RT-qPCR. (C) RIP analysis was performed to detect the differential enrichment of RNAs (HOXA-AS2, miR-520d-3p, and KIAA1522) in anti-IgG and anti-Ago2 groups. (D) Binding sites between miR-520d-3p and KIAA1522 were projected with the use of starBase. (E) The efficiency of KIAA1522 overexpression in VSMCs was assessed through RT-qPCR analysis. (F) The interaction between RNAs (HOXA-AS2, miR-520d-3p, and KIAA1522) in VSMCs was analysed by luciferase reporter assay. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.



HOXA cluster antisense RNA 2 contributes to vascular smooth muscle cells growth by binding to microRNA-520d-3p and up-regulating KIAA1522 expression

With the intention of testing whether the ceRNA mechanism could regulate VSMCs growth, we conducted functional rescue assays in the following part. First of all, the data collected in RT-qPCR and western blot assays suggested that miR-520d-3p inhibition could only partially recover the impact of HOXA-AS2 knockdown on the expression of KIAA1522 (*Figure 4A*). Later on, cell proliferation assays validated that inhibited expression of miR-520d-3p partly rescued the impact of HOXA-AS2 knockdown on the proliferation of VSMCs (*Figure 4B,C*). Similarly, cell apoptosis assays manifested that reduced miR-520d-3p expression partially countervailed the promoting effect induced by HOXA-AS2 depletion on the apoptosis of VSMCs (*Figure 4D*). Moreover, KIAA1522 up-regulation could nearly completely restore the influence of HOXA-AS2 deficiency on the proliferation of VSMCs (*Figure 4E,F*). In addition, overexpressing KIAA1522 could fully offset the impact of HOXA-AS2 down-regulation on the apoptosis of VSMCs (*Figure 4G–I*). In brief, HOXA-AS2 accelerates the growth of VSMCs by binding to miR-520d-3p and elevating KIAA1522 expression.

HOXA cluster antisense RNA 2 promotes the mRNA stability of KIAA1522 through recruiting insulin like growth factor 2 mRNA binding protein 3 in vascular smooth muscle cells

On the basis of the aforementioned findings, we conjectured that HOXA-AS2 might regulate KIAA1522 expression to drive VSMCs malignant behaviours by another pathway aside from sponging miR-520d-3p. After we overexpressed HOXA-AS2 in VSMCs (*Figure 5A*), the mRNA stability of KIAA1522 was detected in VSMCs treated with Actinomycin D. It turned out HOXA-AS2 elevation enhanced KIAA1522 mRNA stability (*Figure 5B*). Given that HOXA-AS2 post-transcriptionally regu-

Figure 4 HOXA-AS2 contributes to the progression of VSMCs by up-regulating KIAA1522 expression. VSMCs were transfected with different plasmids: sh-NC, sh-HOXA-AS2#1 and sh-HOXA-AS2#1 + miR-520d-3p inhibitor. (A) KIAA1522 expression in transfected cells was detected via RT-qPCR and western blot. (B, C) CCK-8 and EdU assays were carried out to analyse the viability and proliferation of VSMCs transfected with different plasmids. (D) Flow cytometry, TUNEL and caspase-3 activity analyses were implemented to evaluate the apoptosis ability of VSMCs transfected with indicated plasmids. (E, F) CCK-8 and EdU assays were conducted to assess cell proliferation capability in different groups: sh-NC, sh-HOXA-AS2#1 and sh-HOXA-AS2#1 + pcDNA3.1/KIAA1522. (G–I) Cell apoptosis assays were carried out to detect the apoptosis of VSMCs transfected with different plasmids. $^{*}P < 0.05$, $^{**}P < 0.01$.

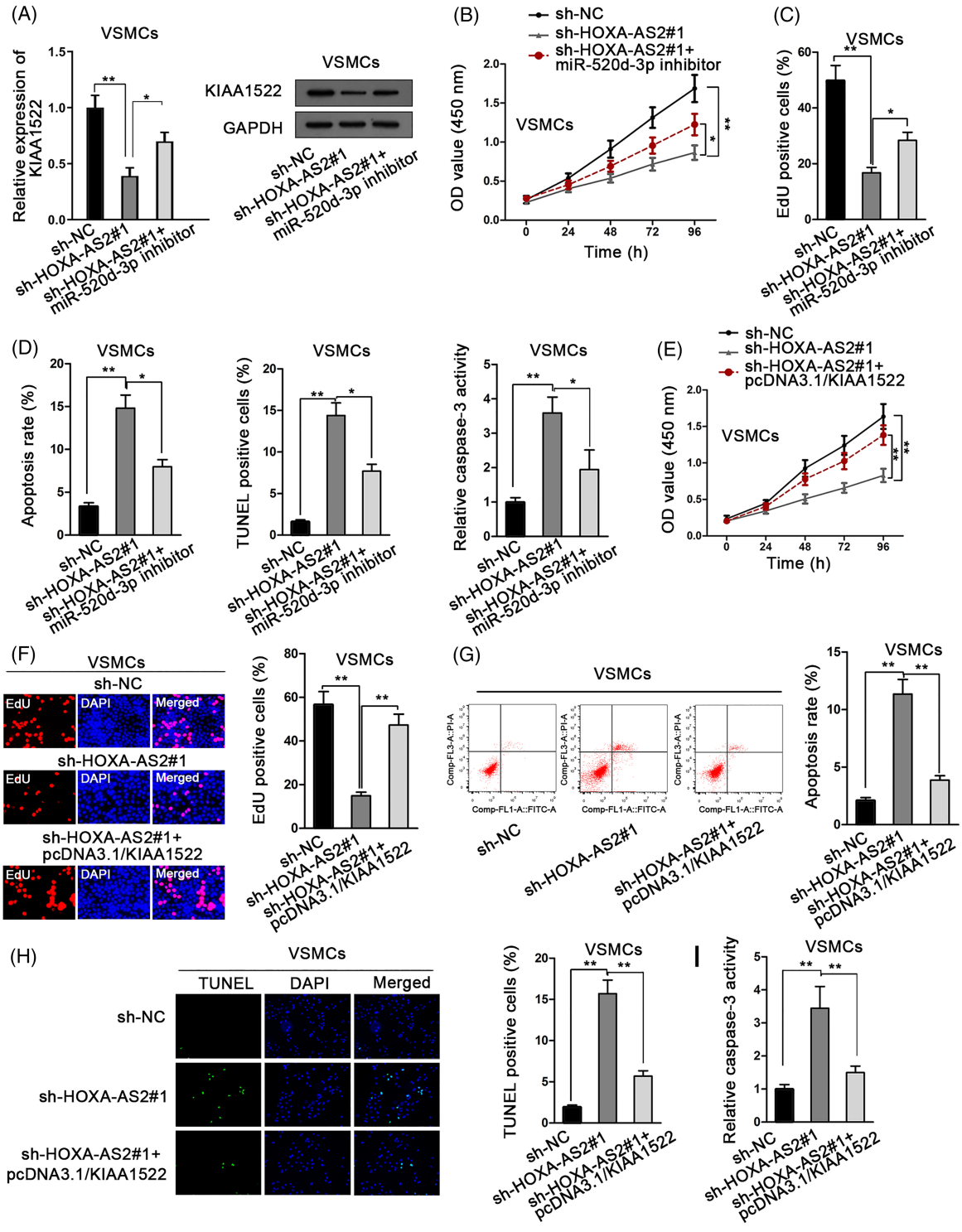


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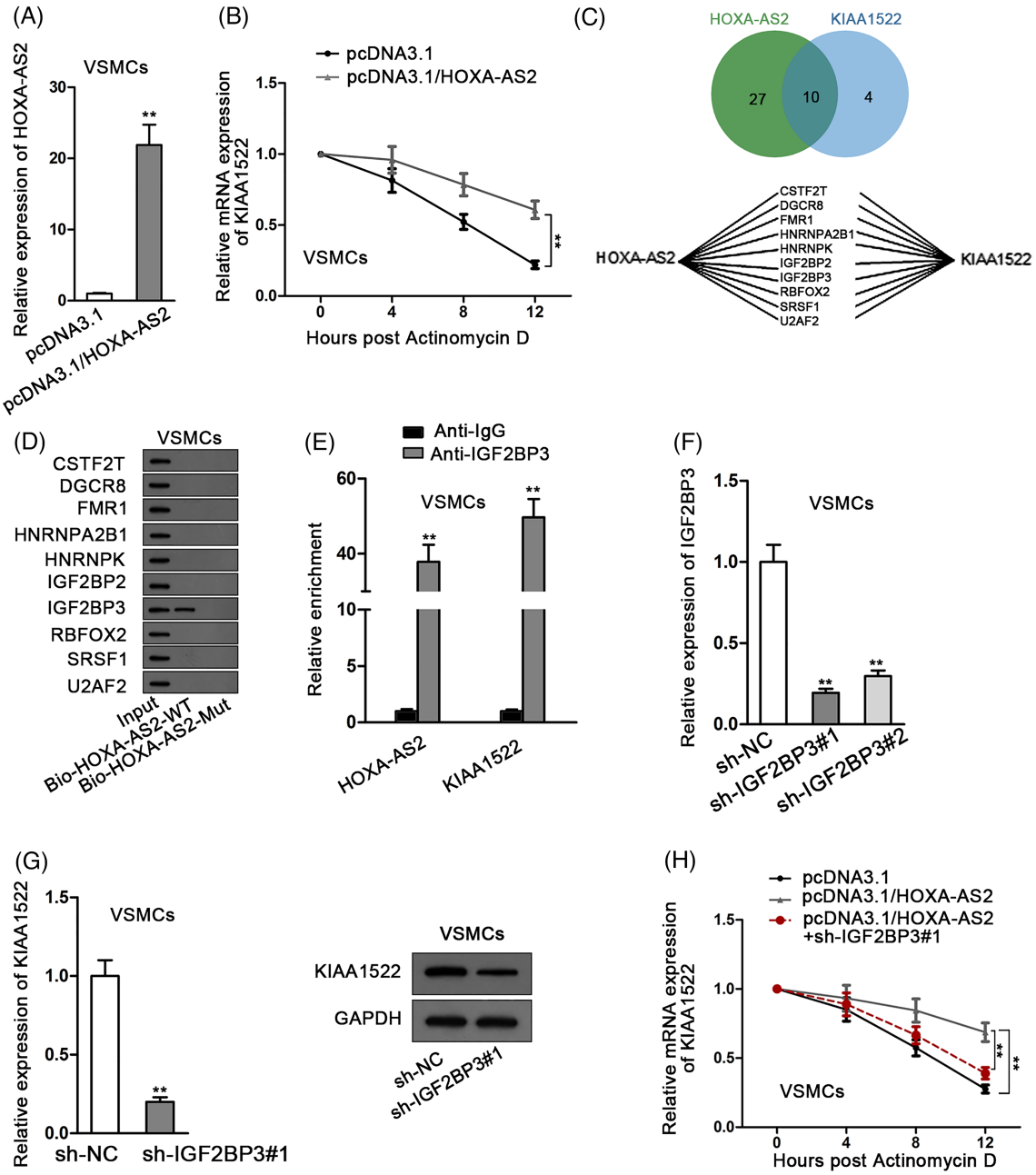
Figure 5 HOXA-AS2 promotes the mRNA stability of KIAA1522 through IGF2BP3 in VSMCs. (A) The efficiency of HOXA-AS2 overexpression was evaluated via RT-qPCR. (B) After treatment with Actinomycin D, RT-qPCR was carried out to evaluate the mRNA stability of KIAA1522 in VSMCs. (C) 10 RNA binding proteins (RBPs) were predicted to bind with HOXA-AS2 and KIAA1522 after searching starBase. (D) RNA pull-down assay was operated to examine the binding affinity between HOXA-AS2 and those candidate RBPs in VSMCs. (E) RIP assay was performed to evaluate the binding affinity between IGF2BP3 and HOXA-AS2 (or KIAA1522) in VSMCs. (F) The efficacy of IGF2BP3 knockdown in VSMCs was evaluated via RT-qPCR. (G) The expression of KIAA1522 in transfected VSMCs was detected via RT-qPCR and western blot. (H) After VSMCs were treated with Actinomycin D, RT-qPCR was carried out to assess the mRNA stability of KIAA1522 in different groups. $^{**}P < 0.01$.



lated KIAA1522 expression, we assumed that there were RNA binding proteins (RBPs) that interacted with HOXA-AS2 to regulate the mRNA stability of KIAA1522 in VSMCs. Subsequently, starBase was utilized, and then 10 candidate RBPs predicted to bind with HOXA-AS2 and KIAA1522 were shown in *Figure 5C*. Later on, RNA pull-down assay revealed that only IGF2BP3 had the strong binding affinity with HOXA-AS2 in VSMCs (*Figure 5D*). Furthermore, RIP assay

confirmed the binding capacity between IGF2BP3 and HOXA-AS2 (or KIAA1522) in VSMCs (*Figure 5E*). After transfecting VSMCs with sh-IGF2BP3#1, sh-IGF2BP3#2 or sh-NC, we observed that the expression of IGF2BP3 was the lowest in sh-IGF2BP3#1-transfected cells (*Figure 5F*). Thus, we chose sh-IGF2BP3#1 to knock down IGF2BP3 in the following assays. As illustrated in *Figure 5G*, KIAA1522 was observably down-regulated by IGF2BP3 reduction in VSMCs. Moreover, after VSMCs were treated with Actinomycin D, knockdown of IGF2BP3 could offset the promoting effect of HOXA-AS2 overexpression on the mRNA stability of KIAA1522 (*Figure 5H*). All these findings uncover that HOXA-AS2 facilitates the mRNA stability of KIAA1522 through recruiting IGF2BP3 in VSMCs.

Discussion

Reports have revealed that dysfunction of VSMCs is implicated with TAA.[6] Increasing investigations have also suggested the significant function that lncRNAs exert on the proliferation and apoptosis of VSMCs in TAA.[13,21] In this research work, HOXA-AS2 in TAA tissues was detected to be highly expressed relative to normal tissues. Moreover, HOXA-AS2 knockdown repressed cell proliferation whereas inducing apoptosis of VSMCs. Based on former literature, HOXA-AS2 could be a vital therapeutic target for diverse vascular disorders.[22] Consistent with this literature, our study indicated HOXA-AS2 might act as a potential biomarker for TAA.

Abundant evidence has revealed that lncRNA can serve as a ceRNA in diverse cancers by sponging miRNA.[23,24] In the present research, through bioinformatics prediction and several molecular mechanism assays, miR-520d-3p, which has been pointed out to function as anti-tumour gene in multiple human diseases (including hepatocellular carcinoma and breast cancer[25,26]), was confirmed to bind with HOXA-AS2 in VSMCs. Moreover, miR-520d-3p inhibition could partially restore the suppressed malignant behaviours of VSMCs induced by HOXA-AS2 depletion. MiR-520d-3p has been suggested to be a potential therapeutic target in diabetic vascular disease.[27] Similar to this study, we uncovered the potential impact of miR-520d-3p on TAA treatment. Moreover, a previous study has reported HOXA-AS2, mainly located in nucleus of HA-VSMCs, contributes to the proliferative and migratory abilities but inhibits the apoptosis of VSMCs via absorbing miRNA-877-3p.[28] Herein, we found HOXA-AS2 mainly distributed in cytoplasm of VSMCs and could promote the malignant behaviours of VSMCs by competitively binding with miR-520d-3p. Comparing the two studies, we discovered that the main distribution of HOXA-AS2 in VSMCs was different in the two studies, but the data difference was not significant. Aside from this discrepancy, both studies unveiled that HOXA-AS2 was able to exert a promoting effect on VSMCs proliferation and an inhibitory effect on apoptosis via binding to miRNA. In this research, we explored the downstream mRNA of HOXA-AS2 and miR-520d-3p which was not discussed in the previous study. To be specific, we subsequently found KIAA1522 was a downstream target of miR-520d-3p in VSMCs. The suppressive effect of HOXA-AS2 depletion on KIAA1522 expression could be partly offset by miR-520d-3p reduction. Furthermore, up-regulated KIAA1522 could completely rescue the influence of HOXA-AS2 knockdown on the proliferation and apoptosis of VSMCs. Previous studies have uncovered that KIAA1522 exerts an oncogenic impact on the proliferation and metastasis of multiple cancers, such as oesophageal cancer[29] and breast cancer.[30] Additionally, KIAA1522 has been identified to be a biomarker for predicting poor survival of non-small cell lung cancer patients.[31] In our study, KIAA1522 was also testified to have a promoting role in malignant processes of VSMCs which might be a possible target for treating TAA.

RBPs have been regarded as a kind of epigenetic regulators in regulating the stability of mRNA by directly binding to mRNAs.[32,33] In this study, IGF2BP3 was confirmed to be the shared RBP of HOXA-AS2 and KIAA1522. IGF2BP3 has been verified as a RBP that participates in the tumorigenesis activity.[34] However, limited knowledge reveals its relation to HOXA-AS2 (or KIAA1522) in VSMCs. A former study has revealed LINC00467 binds with IGF2BP3 to stabilize TRAF5 mRNA in hepatocellular carcinoma. Herein, we drew a similar conclusion that HOXA-AS2 recruited IGF2BP3 to regulate the mRNA stability of KIAA1522 in VSMCs.

In summary, HOXA-AS2 elevates KIAA1522 expression through binding to miR-520d-3p or recruiting IGF2BP3 to regulate the proliferation and apoptosis of VSMCs in TAA. This study did not involve sequencing or microarray analysis in TAA. The expression and distribution of HOXA-AS2 and KIAA1522 were not discussed in TAA and control vessels. TAA mouse models were failed to be constructed to identify the role of HOXA-AS2/miR-520d-3p/IGF2BP3/KIAA1522 in vivo. Despite those limitations, we still hope this finding might provide a new potential direction for the clinical research of TAA treatment.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. (A) The distribution of HOXA-AS2 and miR-520d-3p in VSMCs was determined via FISH assay.

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