



Long non-coding RNA XIST negatively regulates thoracic aortic aneurysm cell proliferation by targeting the miR-193a-5p/KLF7 axis

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ARTICLE INFO

Original paper

Article history:

Received: April 04, 2022

Accepted: July 19, 2022

Published: July 31, 2022

Keywords:

Thoracic aortic aneurysm (TAA), XIST, miR-193a-5p, KLF7.

ABSTRACT

Non-coding RNAs (ncRNAs) are important molecular modulators in diverse pathological processes, influencing the occurrence and progression of carcinomas. Thoracic aortic aneurysm (TAA) is an infrequent disease among aneurysmal diseases and accounts for nearly 3% of diagnosed aneurysms. The functional roles of long ncRNA (lncRNA) XIST and miR-193a-5p and the associated molecular mechanisms are yet to be investigated. In the current study, we discovered that miR-193a-5p was expressed at low levels in the blood of TAA patients. Further, loss-of-function and gain-of-function assays disclosed that miR-193a-5p impacted the proliferation ability of TAA cells. XIST was found to be the most overexpressed lncRNA among predicted lncRNAs binding to miR-193a-5p. The promotive function of XIST in TAA was also explored. Subsequently, KLF7 was proved to be the downstream factor of the XIST/miR-193a-5p axis. Rescue assays testified the whole regulation mechanism of the XIST/miR-193a-5p/KLF7 axis in TAA. MiR-193a-5p was absorbed by XIST for the improvement of KLF7 in TAA. These results concluded that XIST might be engaged in TAA pathogenesis via regulation of the miR-193a-5p/KLF7 axis, supplementing more therapeutic options for TAA treatment.

Doi: <http://dx.doi.org/10.14715/cmb/2022.68.7.31>

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Introduction

Thoracoabdominal aneurysms account just for approximately 3% of diagnosed aneurysms in the USA (1). Thoracic aortic aneurysm (TAA) is rare and life-threatening as patients with this disease are at potential risk for aortic dissection or rupture (2,3). Researchers have made great advances in the treatment of TAA (4-6), whereas we still need to further explore the mechanisms relevant to TAA development.

Noncoding RNAs (ncRNAs), including long noncoding RNAs (lncRNAs) and microRNAs (miRs), are common modulatory molecules in diseases, TAA contained (7-10). For example, human- and smooth muscle cell-abundant lncRNA SMILR boosts cell proliferation by mitotic CENPF mRNA and drives cell cycle progression to affect vascular remodeling (11); miR-203 inhibits VNN1 to suppress septic shock through alleviating lung injury (12); lncRNA HIF1A-AS1 is high in intracranial aneurysms and is involved in the regulating proliferation of vascular smooth muscle cells by TGF- β 1 (13); aneurysm-specific miR-221 and miR-146a are implicated in thoracic and abdominal aortic aneurysms (14). The investigation of regulatory molecules in TAA remains unclear.

Here, miR-193a-5p can repress the cellular activities in

cancers (15-17). In addition, miR-193a-5p has been reported to be downregulated in TAA, but its functional role has never been further probed (18). This evidence provokes us to excavate the possible role of miR-193a-5p in TAA. As for lncRNA X inactive specific transcript (XIST), it has been indicated to be highly expressed in numerous carcinomas (19,20). Interestingly, one research showed that XIST was one up-regulated lncRNA in thoracic aortic dissection (21). Similar to miR-193a-5p, the specific role of XIST has never been investigated in TAA.

Our research purposed to deeply investigate the molecular mechanism in TAA. We validated the low expression of miR-193a-5p in TAA and proved the inhibitory role of miR-193a-5p for TAA proliferation. XIST was found to interact with miR-193a-5p and promote cell proliferation of TAA cells. Thereafter, KLF7, the possible downstream molecule of the XIST/miR-193a-5p axis, was discovered. All in all, XIST boosts TAA via relieving miR-193a-5p-repressed KLF7.

Materials and Methods

Blood specimens

Blood specimens of patients (n=35) with thoracic aortic aneurysm (TAA) were attained from Shandong Provin-

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cial Qianfoshan Hospital. Meanwhile, blood specimens of healthy subjects (n=20) who received routine physical examinations were collected to serve as the control group. Prior to blood extraction, no patients had received chemotherapy or radiotherapy. Blood specimens were frozen at -80°C the moment they were attained. Written informed consent were attained from all participants and experimental procedures have been favored by the Ethics Committee of Shandong Provincial Qianfoshan Hospital. Upon maintaining for 30 min at room temperature, blood specimens underwent centrifugation at room temperature for 20 min at 1,000 x g to collect serum (supernatant).

Cell culture

With a constant airflow of 5%, CO₂, vascular smooth muscle cells (VSMCs) were cultured in a humidified incubator with RPMI-1640 (Invitrogen, USA) adding 10% fetal bovine serum (FBS; Invitrogen, USA) at 37°C.

Cell transfection

For transfection, miR-193a-5p inhibitor, miR-193a-5p mimics and their corresponding NCs (miR-NCs) were constructed by GenePharma (Shanghai, China). Specific shRNAs against XIST (shXIST#1 and shXIST#2) and their corresponding NC (shNC), along with the pcDNA3.1 vector targeting KLF7 and the empty vector, were formed by Genechem (Shanghai, China). Mentioned plasmids were individually transfected into VSMCs via Lipofectamine 3000 (Invitrogen).

qRT-PCR

Whole RNA was separated via TRIzol reagent (Invitrogen) and the PrimeScript reverse-transcription reagent Kit (Thermo Fisher Scientific, Waltham, MA, USA) was utilized for implementing reverse transcription reaction on RNA as per its guide. qRT-PCR was conducted by use of SYBR Green qPCR Master Mixes (Thermo Fisher Scientific) as well as GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). GAPDH and U6 were normalizations and relative gene expressions were detected with the 2^{-ΔΔCt} approach.

Cell Counting Kit-8 (CCK-8) assay

Transfected VSMCs were plated in 96-well plates and sequentially added with 10 μL CCK-8 reagents (Signalway Antibody, College Park, MD, USA) for 1 h of incubation. Following that, absorbance readings were acquired at 450 nm.

EdU staining

EdU staining was carried out as the supplier requested. Upon transfection, VSMCs were incubated for 2 h by 50 μM EdU (RiboBio, Nanjing, China), followed by staining in Apollo (RiboBio) and DAPI (Invitrogen). Eventually, fluorescence microscopy (Nikon, Tokyo, Japan) was applied for the assessment of EdU-positive cells.

Cell apoptosis evaluation

Annexin V-FITC/PI double staining (KeyGen, Nanjing, China) was conducted for cell apoptosis evaluation. The percentage of apoptosis was assayed via flow cytometry with the FACSCalibur machine (BD Biosciences, Franklin Lakes, NJ, USA).

TUNEL assay

Detection of fluorescence of apoptotic cells was undertaken with the fluorometric TUNEL system (Promega, Madison, WI, USA). Transfected VSMCs were plated onto 6-well plates. Following fixation employing formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and permeabilization with 0.5% Triton X-100 (Sigma-Aldrich), cells were treated by 50 μL TdT enzyme buffer. Next, cell nuclei were dyed by DAPI. At last, labeled strand breaks were monitored under a fluorescence microscope.

RNA pull-down

The miR-193a-5p-wt/mut was labeled with biotin through the Pierce RNA 3' End Desthiobiotinylation Kit (Thermo Fisher Scientific). VSMCs cell lysates were incubated with Bio-miR-193a-5p-wt or Bio-miR-193a-5p-mut adding streptavidin-coated magnetic beads (Invitrogen). In the end, the biotin-coupled RNA complex was pulled down and assayed by qRT-PCR.

Luciferase Reporter assay

XIST-WT/Mut or KLF7-WT/Mut was sub-cloned into the pmirGLO dual-luciferase vector (Promega) so as to generate pmirGLO-XIST-WT/Mut or pmirGLO-KLF7-WT/Mut which was then co-transfected with miR-193a-5p mimics or miR-NC into VSMCs. Following co-transfection for 48 h, Dual Luciferase Reporter Assay System (Promega) was applied in line with its protocols.

Western blot

In line with the requirements of RIPA lysis buffer (Beyotime, Shanghai, China), total protein was extracted. Following measurement of protein concentration with a BCA Protein Assay kit (CWBio, Jiangsu, China), proteins in equal amounts were separated by 10% SDS-PAGE (Bio-Rad, Hercules, CA, USA), and sequentially transferred to PVDF (Millipore, Billerica, MA, USA). Upon blocking in 5% nonfat milk, membranes were incubated with primary antibodies, including KLF7 (ab197690, Abcam, Cambridge, USA) and GAPDH (ab9485, Abcam). The secondary antibodies were used, subsequently. Western blot band intensities were quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Differences in groups were assessed by Student's t-test or one-way ANOVA. Data derived from three independently conducted assays were imported into GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA) and shown as mean ± SD. P < 0.05 represented statistical significance. Pearson's correlation analysis was applied in order to analyze the correlation among XIST, miR-193a-5p and KLF7 expressions.

Results

Inhibited or elevated miR-193a-5p negatively or positively modulated the proliferative capacity of VMSCs

This study continued to explore the possible role of miR-193a-5p in the thoracic aortic aneurysm (TAA). The expression of miR-193a-5p in the blood of patients suffering TAA was lower than that in normal individuals (Figure 1A). In vascular smooth muscle cells (VMSCs) from TAA patients, we silenced miR-193a-5p and ove-

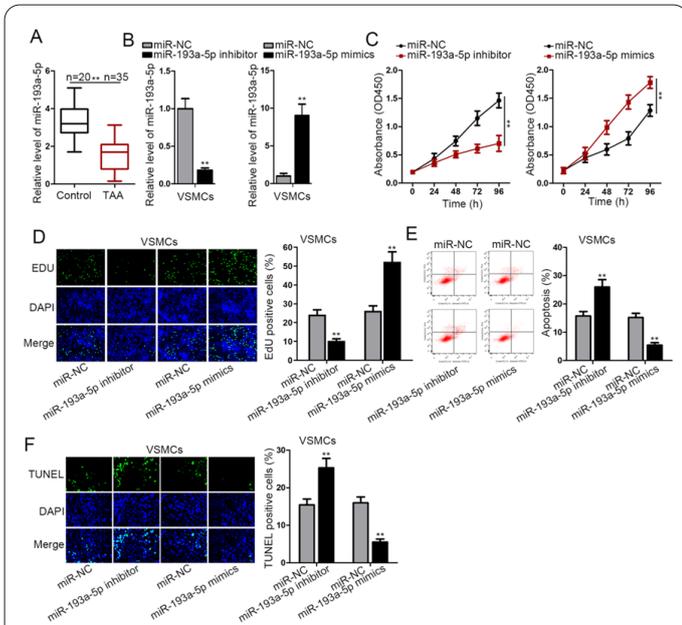


Figure 1. MiR-193a-5p promoted TAA cell proliferation and inhibited cell apoptosis. A. MiR-193a-5p was lowly expressed in the blood of TAA patients. B. MiR-193a-5p was silenced or overexpressed in VSMCs. C-D. CCK-8 and EdU assays demonstrated that VSMCs proliferation was facilitated by upregulating miR-193a-5p, but was hindered by downregulating miR-193a-5p. E-F. Flow cytometry and TUNEL staining assay showed that VSMCs apoptosis was induced through miR-193a-5p inhibitor, but was repressed through miR-193a-5p mimics. ** $P < 0.01$ versus control group.

expressed miR-193a-5p respectively via transfection of miR-193a-5p inhibitor and miR-193a-5p mimics (Figure 1B). Then CCK-8 assay demonstrated that cell viability was significantly repressed by miR-193a-5p inhibition and overtly promoted by miR-193a-5p addition (Figure 1C). The results of the EdU assay showed that cell proliferation was restrained and accelerated separately under the transfection of miR-193a-5p inhibitor and miR-193a-5p mimics (Figure 1D). Flow cytometry analysis and TUNEL assay were utilized to dissect cell apoptosis. The decrease of miR-193a-5p induced more apoptotic cells and TUNEL positive cells, whereas the increase of miR-193a-5p reduced the number of apoptotic cells and TUNEL positive cells (Figure 1E-F). These findings showed that miR-193a-5p in TAA affected the pathological activities of TAA.

MiR-193a-5p was targeted by XIST that also mediated the proliferation ability of TAA cells

Subsequently, we tried to find the related mechanism that miR-193a-5p is involved in. MiRNAs are widely known to be targeted by lncRNAs for the increase of mRNAs (22,23). From starBase, we gained predicted 86 lncRNAs binding to miR-193a-5p. qRT-PCR analysis dissected that lncRNA XIST was the most up-regulated lncRNA in the blood of TAA patients in comparison with that in the control group (Figure 2A). Whether XIST could regulate miR-193a-5p was still unknown. Through qRT-PCR detection, we observed that XIST was significantly elevated in the blood of TAA patients (Figure 2B). Spearman's correlation analysis revealed the negative association between XIST and miR-193a-5p (Figure 2C). After XIST was knocked down by specific shRNAs, miR-193a-5p expression was accordantly augmented (Figure 2D-E). And miR-193a-5p overexpression also inhibited XIST expres-

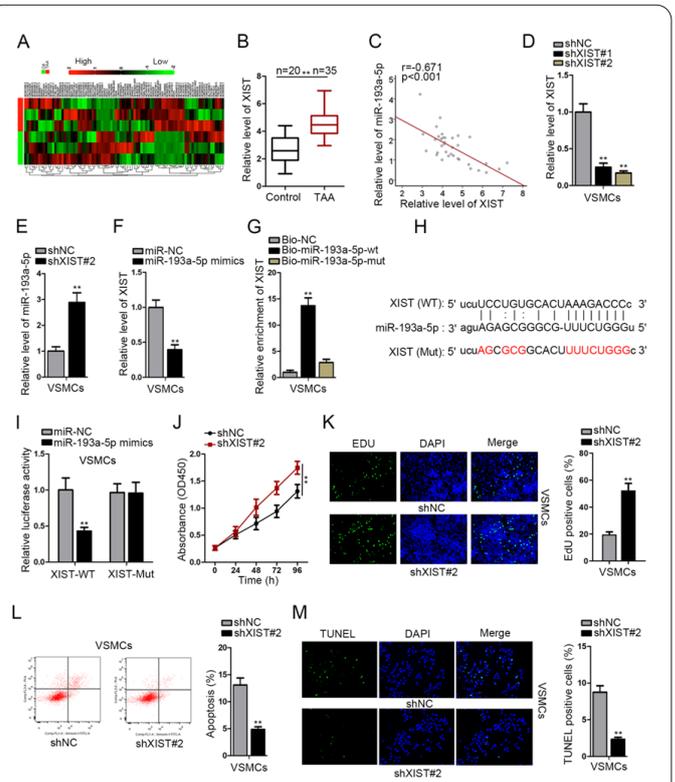


Figure 2. XIST sponged miR-193a-5p and suppressed TAA cell proliferation. A. XIST was the most upregulated lncRNA in the blood from TAA patients, as exhibited in the heatmap. B-C. The high expression of XIST was negatively correlated with miR-193a-5p. D-E. The specific shRNAs to XIST lowered XIST expression but enhanced miR-193a-5p expression in VSMCs. F. XIST expression was lessened via transfecting VSMCs with miR-193a-5p mimics. G. The enrichment of XIST in RNA complex pulled-down by miR-193a-5p was observed in the RNA pull-down experiment. H. The wild-type or mutant binding sites of miR-193a-5p in XIST sequences. I. MiR-193a-5p mimics decreased the luciferase intensity of XIST-WT. J-K. XIST depletion contributed to VSMCs proliferation. L-M. Increased apoptotic VSMCs were attributed to the silenced XIST. ** $P < 0.01$ versus control group.

sion (Figure 2F). Next, the binding affinity between miR-193a-5p and XIST was validated. In the RNA pull-down assay, only the bio-miR-193a-5p-wt probe pulled down a large amount of XIST (Figure 2G). The assumed binding sequences between miR-193a-5p and XIST and the mutated sequences of XIST for miR-193a-5p were displayed in Fig. 2H. It was observed in the luciferase reporter assay that miR-193a-5p mimics merely weakened the luciferase activity of XIST-WT and had no effect on that of XIST-Mut (Figure 2I). To explore whether XIST participated in the regulation of TAA cellular processes, function experiments were carried out. CCK-8 and EdU assays observed that cell proliferation was dramatically enhanced with the knockdown of XIST (Figure 2J-K). Cell apoptosis was also suppressed with the silencing of XIST, as estimated by flow cytometry and TUNEL assay (Figure 2L-M). These data illustrated that XIST targeted miR-193a-5p and modulated cell proliferation and apoptosis in TAA.

KLF7 was the downstream factor of XIST and miR-193a-5p

The downstream genes were discovered by website prediction. 29 mRNAs were selected with the help of microT, miRmap, PicTar and PITA datasets (Figure 3A).

The expression levels of these genes under treatment of miR-193a-5p mimics or shXIST#2 were examined by qRT-PCR and the results represented that these genes showed different expression changes in response to two treatments. Among the down-regulated genes, KLF7, the most affected gene by both miR-193a-5p and XIST, was screened out (Figure 3B-C). In the blood of TAA patients, KLF7 mRNA and protein expression was relatively high (Figure 3D). The negative relationship between KLF7 and miR-193a-5p and the positive relationship between KLF7 and XIST were exposed via Spearman's correlation analysis (Figure 3E-F). These were confirmed by qRT-PCR and western blot assays. The mRNA and protein levels of KLF7 were both lowered by miR-193a-5p promotion or XIST silence (Figure 3G-H). RNA pull-down and luciferase reporter assays were conducted to inquire about the interplay between miR-193a-5p and KLF7. The relative sequences of KLF7-WT, miR-193a-5p and KLF7-Mut were exhibited in Fig. 3J. KLF7 was simply pulled down through biotinylated miR-193a-5p-wt and the luciferase activity of KLF7 was purely impaired by miR-193a-5p mimics (Figure 3I and K). All in all, KLF7 was a target gene of miR-193a-5p.

XIST regulated cell proliferation in TAA via targeting miR-193a-5p/KLF7 pathway

KLF7 was increased for the implement of rescue assays (Figure 4A). In VMSCs, cell viability was firstly accelerated by the down-regulation of XIST but secondly repressed by miR-193a-5p repression or KLF7 overexpression, as examined by the CCK-8 assay (Figure 4B). In the EdU assay, shXIST#2-boosted cell proliferation was restrained with the down-regulation of miR-193a-5p or the addition of KLF7 (Figure 4C). AS for cell apoptosis, according to the results of flow cytometry, we found that XIST inhibition reduced the number of apoptotic cells, which was strengthened by miR-193a-5p obstruction or KLF7 activation (Figure 4D). TUNEL assay indicated that the repressive effect of shXIST#2 on cell apoptosis was counteracted by miR-193a-5p inhibitor or KLF7 (Figure 4E). In summary, miR-193a-5p depletion or KLF7 addition reversed the impact of XIST decrease on the proliferation capacity in TAA.

Discussion

The current paper illustrated that miR-193a-5p participated in the pathogenesis of thoracic aortic aneurysm (TAA) where miR-193a-5p was sponged by X inactive specific transcript (XIST) so that Kruppel-like factor 7 (KLF7) could be protected from degradation.

As increasing reports have elucidated the impact of noncoding RNAs (ncRNAs) on aortic aneurysm (13,14), we determined to do researches on seeking more effective biomarkers for TAA patients. MicroRNA (miR)-193a-5p, which was demonstrated to be under-expressed in thoracic aortic dissection, was figured out from the NCBI website. Whether miR-193a-5p is engaged in the modulation of pathological activities of TAA cells is still obscure. Firstly, the aberrantly low expression of miR-193a-5p in the blood of TAA patients was unveiled. Secondly, the control of miR-193a-5p on cell proliferation and apoptosis was testified by loss-of-function and gain-of-function assays. Herein, the functional role of miR-193a-5p in TAA was

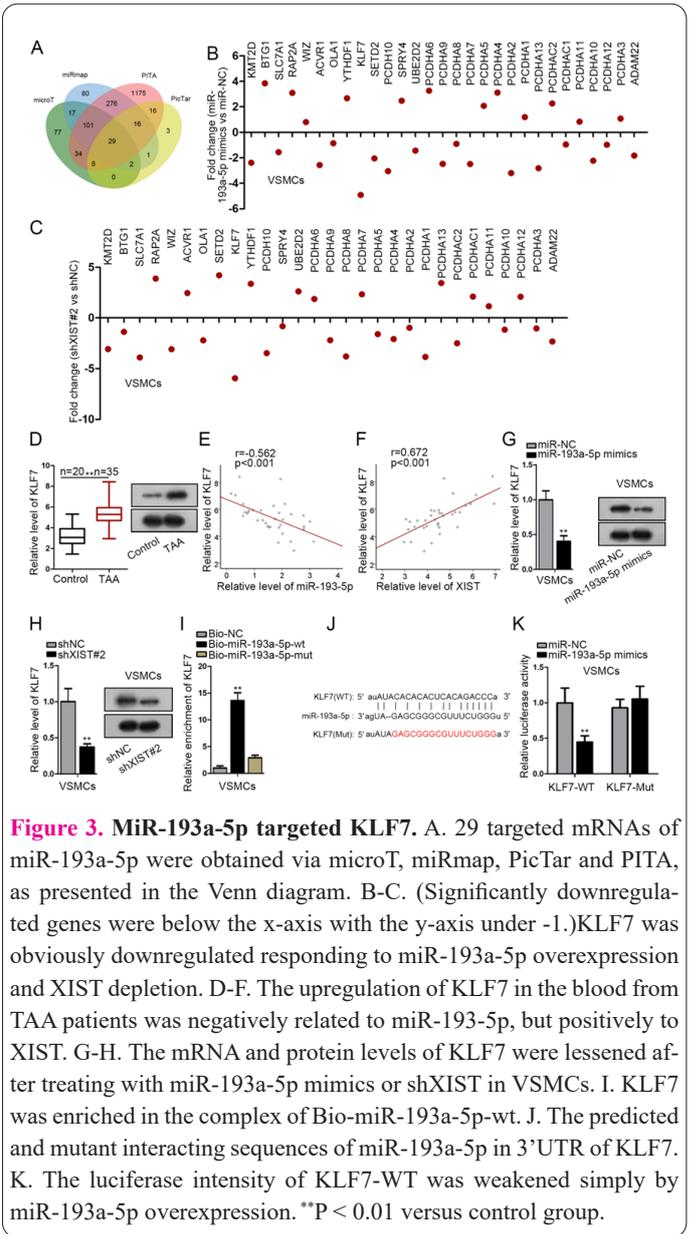


Figure 3. MiR-193a-5p targeted KLF7. A. 29 targeted mRNAs of miR-193a-5p were obtained via microT, miRmap, PicTar and PITA, as presented in the Venn diagram. B-C. (Significantly downregulated genes were below the x-axis with the y-axis under -1.) KLF7 was obviously downregulated responding to miR-193a-5p overexpression and XIST depletion. D-F. The upregulation of KLF7 in the blood from TAA patients was negatively related to miR-193-5p, but positively to XIST. G-H. The mRNA and protein levels of KLF7 were lessened after treating with miR-193a-5p mimics or shXIST in VSMCs. I. KLF7 was enriched in the complex of Bio-miR-193a-5p-wt. J. The predicted and mutant interacting sequences of miR-193a-5p in 3'UTR of KLF7. K. The luciferase intensity of KLF7-WT was weakened simply by miR-193a-5p overexpression. **P < 0.01 versus control group.

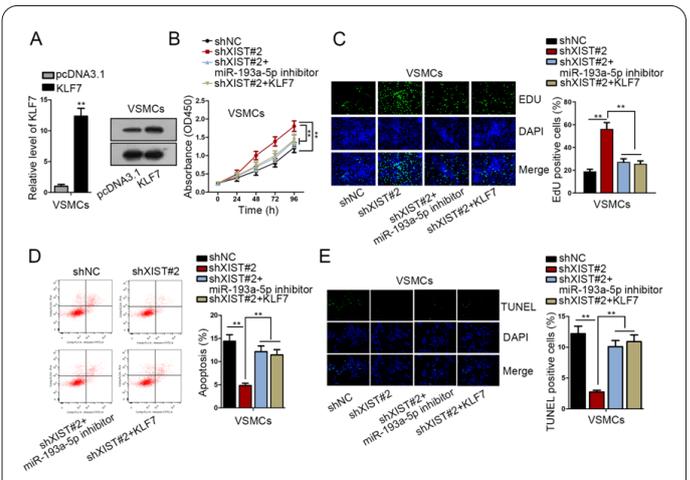


Figure 4. XIST negatively regulated TAA cell proliferation via miR-193a-5p/KLF7. A. KLF7 was upregulated at both mRNA and protein levels in VSMCs. B-C. VSMCs proliferation was promoted by XIST knockdown, but was reduced through inhibiting miR-193a-5p or enhancing KLF7. D-E. MiR-193a-5p depletion or KLF7 overexpression stimulated cell apoptosis which was hindered by silencing XIST before. **P < 0.01 versus control group.

firstly displayed.

Recent papers have indicated the participation of long ncRNAs (lncRNAs) and miRs in multiple diseases, in sequence or competition. For instance, lncRNA H19 restrains endothelial-mesenchymal transition (EMT) in diabetic retinopathy (24); miR-124 prevents focal cerebral ischemia via Usp14-dependent REST degradation (25); lncRNA SNHG16 neutralizes the influences of miR-15a/16 on LPS-induced inflammation (26). Then, we tried to find out the lncRNA relevant to miR-193a-5p in TAA. Among the predicted lncRNAs gained from starBase v3.0, we selected XIST for further exploration since that XIST was detected to be the most up-regulated lncRNA in TAA. Additionally, XIST was once revealed to be highly-expressed in thoracic aortic dissection (21). In this research, we affirmed that XIST was bound with miR-193a-5p and could influence the proliferation capacity in TAA.

Sequentially, we continued to discover the downstream factors underlying XIST and miR-193a-5p. Four bioinformatics prediction databases, including microT, miRmap, PITA and PicTar screened 29 shared target mRNAs of miR-193a-5p. Finally, Kruppel like factor 7 (KLF7) was picked out in that KLF7 exhibited the largest down-regulation in response to either miR-193a-5p addition or XIST inhibition. Several reports have explored the oncogenic role of KLF7 in tumors. For example, STAT3-promoted LINC00668 facilitates cell migration and invasion of non-small cell lung cancer by the miR-193a/KLF7 axis (27). KLF7 enhances polyamine biosynthesis and glioma progression by transcriptional activation of ASL (28). MiR-185 hampers the proliferation and invasion of non-small cell lung cancer by down-regulating KLF7 (29). Subsequent mechanism and rescue assays certified the downstream role of KLF7 underlying the XIST/miR-193a-5p pathway for TAA proliferation.

In this work, we exposed that XIST and KLF7 were relatively high and miR-193a-5p was relatively low in TAA. Also, XIST and miR-193a-5p dysregulation affected the proliferation capacity of TAA cells. In conclusion, these data showed that XIST exhausted miR-193a-5p for the improvement of KLF7, which might make contributions to the deeper comprehension of TAA pathogenesis.

Acknowledgments

We thank all participants.

Interest conflict

The authors declare that they have no conflict of interest.

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