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LncRNA TUG1 promotes proliferation of vascular smooth muscle cell and atherosclerosis through regulating miRNA-21/PTEN axis

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Abstract. – OBJECTIVE: Atherosclerosis is a major risk factor for cardiovascular disease, but its mechanism of progression remained unclear. However, many long non-coding RNAs (lncRNAs) have recently been implicated in different processes for cardiovascular disease. In this study, we mainly focused on the role of lncRNA TUG1 in atherosclerosis.

PATIENTS AND METHODS: qRT-PCR was used to detect the expression of lncRNA TUG1 in atherosclerosis patients and animal model. Moreover, the expression of TUG1 in vascular smooth muscle cell dysfunction model was also measured. Proliferation ability was tested by CCK-8 and cyclin D1 assay, through loss- and gain-of function approaches. Western-blot was used to measure the expression of PTEN, when TUG1 was in different levels.

RESULTS: We found that the lncRNA TUG1 was highly expressed in serum samples from 38 patients with atherosclerosis, compared with 24 healthy volunteers. LncRNA TUG1 was dramatically upregulated in atherosclerotic plaques of ApoE-/- mice. We also found that the expression of TUG1 was upregulated in vascular smooth muscle cell injury model. Through loss- and gain-of function approaches, we showed that TUG1 promotes cell proliferation and induces apoptosis *in vitro*. What's more, TUG1 expression level was reversely correlated with PTEN expression in patients with atherosclerosis. LncRNA TUG1 could compete with PTEN for miR-21 binding.

CONCLUSIONS: We found that lncRNA TUG1 was closely related to the progression of atherosclerosis, which could be a potential target for treating atherosclerosis.

Key Words:
LncRNA TUG1, Vascular smooth muscle cell, Atherosclerosis, miRNA-21/PTEN axis.

Abbreviations

BSA = bovine serum albumin, DMEM=Dulbecco's Modified Eagle Medium, ECL= enhanced chemiluminescence, FBS= fetal bovine serum, GAPDH= glyceraldehyde 3-phosphate dehydrogenase, PMSF= phenylmethylsulfonyl fluoride, PVDF= polyvinylidene difluoride, RIPA = radioimmunoprecipitation assay, RT-PCR= reverse transcriptase-polymerase chain reaction, SDS-PAGE= sodium dodecyl sulphate-polyacrylamide gel electrophoresis, TBST= Tris-buffered saline and Tween 20, TGF-α= Transforming Growth factor-α, PBS= phosphate-buffered saline, PCR= polymerase chain reaction.

Introduction

Atherosclerosis is one of the most common vascular disorders – including stroke and coronary heart disease – and one of the leading causes of mortality worldwide[1-3]. It is considered to be a kind of chronic inflammation resulting from interaction between modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial[4-6]. Moreover, proliferation of vascular smooth muscle cells (VSMCs) and the formation of neo-intima dominate atherosclerosis lesion development[7,8]. Long noncoding RNAs (lncRNAs) are around 200-nucleotide (nt)-long RNA molecules and stably exist in the plasma and urine, with disease and tissue specificity and without protein-coding potential. They constitute a large portion of mammalian transcriptome, since only around 2% of the mammalian genome is composed of genes that encode proteins[9-11]. Recently, lncRNAs have been implied to be frequently expressed aberrantly in lots of cancers and involved in

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a large range of biological processes[12,13]. However, the role of lncRNAs in cardiovascular diseases is less understood. Taurine upregulated gene 1 (TUG1), a 7.1-kb lncRNA, was initially identified as a transcript upregulated by taurine[14]. Previous reports[15-17] showed that TUG1 participated in the development of several cancers, such as non-small cell lung cancer, bladder cancer and osteosarcoma. What's more, the high-expression of TUG1 could remove the reversed effect of tanshinol on oxidized low-density lipoprotein (ox-LDL) by inducing endothelial cells apoptosis [18]. However, the function and mechanism of TUG1 in VSMCs with atherosclerosis are not fully addressed. In this study, we aimed to examine the functional role of lncRNA TUG1 in the pathogenesis of atherosclerosis, as well as to disclose molecular mechanisms. Firstly, we measured the expression of TUG1 in in serum of 38 patients with atherosclerosis. Then, proliferation ability was tested by CCK-8 and cyclin D1 assay, through loss- and gain-of function approaches. After that, the expression of PTEN was measured. Our study, therefore, uncovered a critical function of lncRNA TUG1 in atherosclerosis.

Patients and Methods

Patients

38 serum samples from patients with atherosclerosis and 24 serum samples from healthy volunteers were collected from the Third Affiliated Hospital of Chongqing Medical University Hospital in Chongqing (Chongqing, China) from June 2016 to July 2017. All of the patients were well informed and informed consents were also signed. The experiment protocol was approved by the Institutional Review Board of the Third Affiliated Hospital of Chongqing Medical University (Chongqing, China).

Animals

The whole procedure was performed according to the National Institutes of Health (NIH) Animal Use Guidelines. 20 ApoE-/- mice were obtained from (Beijing HFK Bioscience Co., Ltd., Beijing, China). Mice received high-fat diet, and were kept in the standard SPF environment, with 12 hours light and 12 hours dark cycle at a stable temperature of 20-25°C. These mice were sacrificed after 16 weeks, and the atherosclerotic plaque and adjacent tissue were kept in the liquid nitrogen for further use.

Cell Culture

Human vascular smooth muscle cell line HA-VSMC and mouse mononuclear macrophage cell line RAW264.7 were purchased from ATCC (Manassas, VA, USA) and cultured following the instructions of manufacturers. The cells were culture at 37°C in a humidified incubator with 5% CO_2 and 95% air, and maintained in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Beyotime, Shanghai, China).

RNA Extraction and Real-time Quantitative PCR Assays

Total RNA from clinical samples and cells was extracted by using RNAiso Plus (TaKaRa, Otsu, Shiga, Japan), following the manufacturer's protocol. Reverse transcription polymerase chain reaction (RT-PCR) was performed using PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's protocol (Table I). The levels of mRNA expression were quantified by standard Real-time PCR protocol with SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). GAPDH was used as a reference gene.

Table I. Primers for selected genes.

Gene name	Primers	
	Sense	Antisense
lncRNA TUG1	CTGAAGAAAGGCAACATC	GTAGGCTACTACAGGATTTG
PTEN	CCGAAAGGTTTTGCTACCATTCT	AAAATTATTTCCTTTCTGAGCATTCC
miRNA-21	ACACTCCAGCTGGGTAGCTTATCAG ACTGA	CTCAACTGGTGTCGTGGAGTCGGCAA TTCAGTTGAGTCAACATC
GAPDH	GTCAACGGATTTGGTCTGTATT	AGTCTTCTGGGTGGCAGTGAT

Construction of Plasmid, siRNA and Cell Transfection

The full-length of human TUG1 cDNA was synthesized and sub cloned into a pCDNA3.1 (Invitrogen, Carlsbad, CA, USA) vector, resulting in TUG1-pcDNA for its overexpression. For small interfering RNAs (siRNAs) analysis, three TUG1 siRNAs and negative control siRNA (si-NC) were provided by Invitrogen (Carlsbad, CA, USA), and the siRNA sequences targeting the sequence of TUG1 transcript were as follows: siTUG1-1# 50–GGGAUAUAGCCAGAGAACAAUUCUA-30; siTUG1-2#50-GCUUGGCUUCUAUUCUGAAUCCUUU-30; siTUG1-3#50-CAGCUGUUACCAUUCAACUUCUUAA-30.

Cells were pre-incubated to 40-60% confluence on a six-well plate and then transfected by incubation with plasmids or siRNAs with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. At indicated time point post the transfection, cells were harvested for further analysis.

CCK8 Assays

The target cells were seeded into 96-well plates, with the density of 2000 cells in each well. Three replicate wells were set in each group. CCK8 assay was done as follows: 10 ul of Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) were added into 100 ul of DMEM medium in each well, which were co-cultured at darkness for 2 hours at 37°C. The 96-well plate was placed at the absorbance of 450 nm. The data were collected for 5 days. The whole experiment was repeated three times.

Western Blotting

Target cell was harvested at 80-90% density in cell culture bottle. Phosphate-buffered saline (PBS) was used to wash the target cells for three times to remove the rest cell culture media. Protein in cell was extracted by RIPA lysis buffer containing phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Shanghai, China). The concentration of protein was detected according to the standard bovine serum albumin (BSA) protein quantitation assay. 50 mg of samples were added to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% denaturing gel. The protein was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) after electrophoresis, which were blocked in the 5% non-fat milk for 1 hour at room temperature. TBST (Boster, China) was used to wash these membranes, which were then put into the primary antibody at 4°C overnight. Respective second antibody was used to incubate these membranes. Immunoblots were visualized by ECL chemiluminescent detection system. The protein bands were quantified using densitometry analysis in Quantity One software (Bio-Rad, Hercules, CA, USA), and were analyzed by using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Luciferase Activity

Wt-TUG1/mut-TUG1 or wt-PTEN 3'UTR/mut-PTEN 3'UTR sequences were amplified and cloned into the downstream of the stop codon of the firefly luciferase in PGL3 basic vector (Promega, Madison, WI, USA). HA-VSMC cells were cultured overnight after being seeded into a 24-well plate, co-transfected with the Wt-TUG1/mut-TUG1 or wt-PTEN 3'UTR/mut-PTEN 3'UTR reporter gene plasmid and miR-21 mimics or miR-21 inhibitor. Renilla expression vector was transfected into each group to serve as a normalized control. 48 h after transfection, firefly and Renilla luciferase activities were measured using Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Data were normalized against the activity of the Renilla luciferase gene.

Statistical Analysis

All the data were expressed as the mean±SD; each assay was applied at least three independent experiments or replicates. The significance between groups was analyzed by Student's *t*-test. p-value<0.05 was considered statistically significant. All statistical analyses were performed by LSD post-hoc analysis using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

Results

LncRNA TUG1 was Highly Expressed in Patients with Atherosclerosis

To explore the effect of lncRNA TUG1 in atherosclerosis, we first used qRT-PCR to detect the expression of lncRNA TUG1 in serum samples from 38 patients, compared with those from 24 healthy volunteers. We found that the expression of lncRNA TUG1 was highly expressed in the patients with atherosclerosis (Figure 1A). What's more, we examined the expression of lncRNA TUG1 in aortic atherosclerotic plaques of ApoE-/- mice fed with a high-fat diet, a widely used ani-

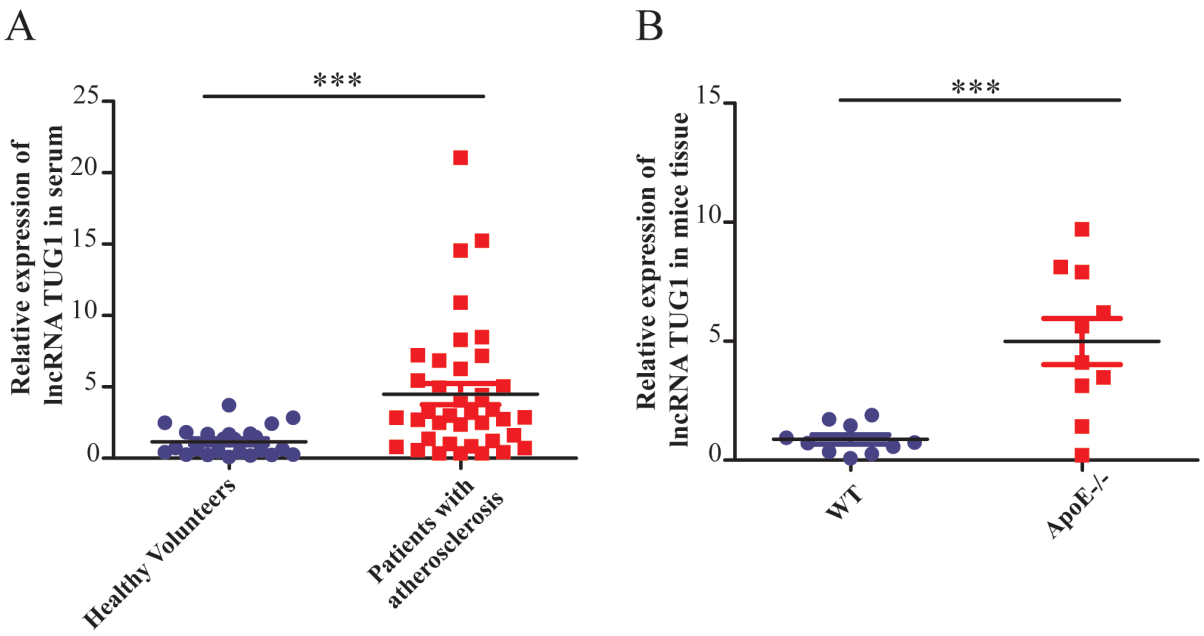

Figure 1. LncRNA TUG1 was highly expressed in the serum of patients with atherosclerosis. ***(A)*** qRT-PCR was used to detect the expression of lncRNA TUG1 in serum samples from 38 patients with atherosclerosis, compared with those from 24 healthy volunteers. *** $p<0.001$. ***(B)*** lncRNA TUG1 transcript expression in atherosclerotic plaques of ApoE knockout mice (ApoE-/-) and wild-type C57 control mice (WT) was measured by qRT-PCR. N=10. ***$p<0.001$.

mal model of atherosclerosis. Indeed, we found that the expression of lncRNA TUG1 was substantially higher in the aortic plaques of ApoE-/- mice when compared with that of wild type control mice (Figure 1B). These results indicated that lncRNA TUG1 was correlated with procession of atherosclerosis, but the effect remained unclear.

LncRNA TUG1 was Upregulated in VSMCs Induced with Hypoxia or TNF-α

The injury of endothelial cells plays an important role on the development of atherosclerosis and CAD. According to the above results, we observed the role of lncRNA TUG1 in injury models. Firstly, human vascular smooth muscle cell line HA-VSMC was induced with hypoxia condition (4% O_2) to simulate the anoxic environment. Being treated for 24 hours, the lncRNA expression profiles were detected using RT-PCR. Results revealed that the expression of lncRNA TUG1 was upregulated in hypoxia induced HA-VSMC (Figure 2A). Then, the HA-VSMC was induced with TNF-αcondition (20 ng/ml), lncRNA TUG1 was also highly expressed (Figure 2B). Moreover, the same results come out with RAW264.7 cells, which were induced with hypoxia or TNF-α condition (Figure 2C-D). These results provide a valuable research orientation for subsequent experiment.

LncRNA TUG1 Improved the Proliferation and Suppressed Apoptosis of HA-VSMC and RAW264.7

To further explore the role of lncRNA TUG1 in atherosclerosis, we investigated the function of lncRNA TUG1 in cell proliferation and apoptosis. We used the human vascular smooth muscle cell line HA-VSMC and the mouse macrophage cell line RAW264.7, both of which have been widely used to investigate atherosclerosis *in vitro*. Firstly, lncRNA TUG1 expression levels in HA-VSMC and RAW264.7 transfected with siRNAs and plasmid were significantly down-regulated or upregulated compared to control group (Figure 3A-B). CCK8 and apoptosis assays were used to detect the proliferation and apoptosis ability of HA-VSMC and RAW264.7. We found that lncRNA TUG1 knockdown could decrease the proliferation ability, while lncRNA TUG1 enforced expression could increase the proliferation ability in both cell lines (Figure 3C-D). What's more, elevated expression of lncRNA TUG1 increased the expression of cyclin D1 (Figure 3E-F) by using the qRT-PCR. In conclusion, results revealed that

lncRNA TUG1 could promote proliferation and inhibit apoptosis of HA-VSMC and RAW264.7, which might accelerate the formation of atherosclerosis.

LncRNA TUG1 Modulates the Expression of PTEN by Targeting miRNA-21

We found that lncRNA TUG1 could promote atherosclerosis via regulating the proliferation and apoptosis, but the molecular mechanism remained unclear. PTEN is a crucial down-regulator of the PI3K/AKT/Mtor pathway, which regulates cell proliferation and migration. To explore the potential relationship between lncRNA TUG1 and PTEN, we detected the expression of PTEN mRNA in the 20 serum samples of atherosclerosis patients and analyzed the correlation. The data showed the expression of PTEN mRNA was negatively correlated with the expression of lncRNA TUG1 (R^2=0.7339, p<0.0001; Figure 4A). Next, we detected the relative protein expression level of PTEN with lncRNA TUG1 by Western blot. Compared with the control groups, transfection with lncRNA TUG1 inhibitor significantly increased the expression of PTEN protein, while transfection with lncRNA TUG1 promoter significantly reduced the expression of PTEN protein (Figure 4B). Previous researches[19, 20] have indicated that miRNA-21, as the most overexpressed miRNA in the vast majority of cancers, targets PTEN to promote tumor growth and proliferation. We also detected the expression of miRNA-21 in the 20 serum samples and analyzed the correlation. The data showed the expression of miRNA-21 was positively correlated with the expression of lncRNA TUG1 (R^2=0.6365, p<0.005; Figure 4C). Then,

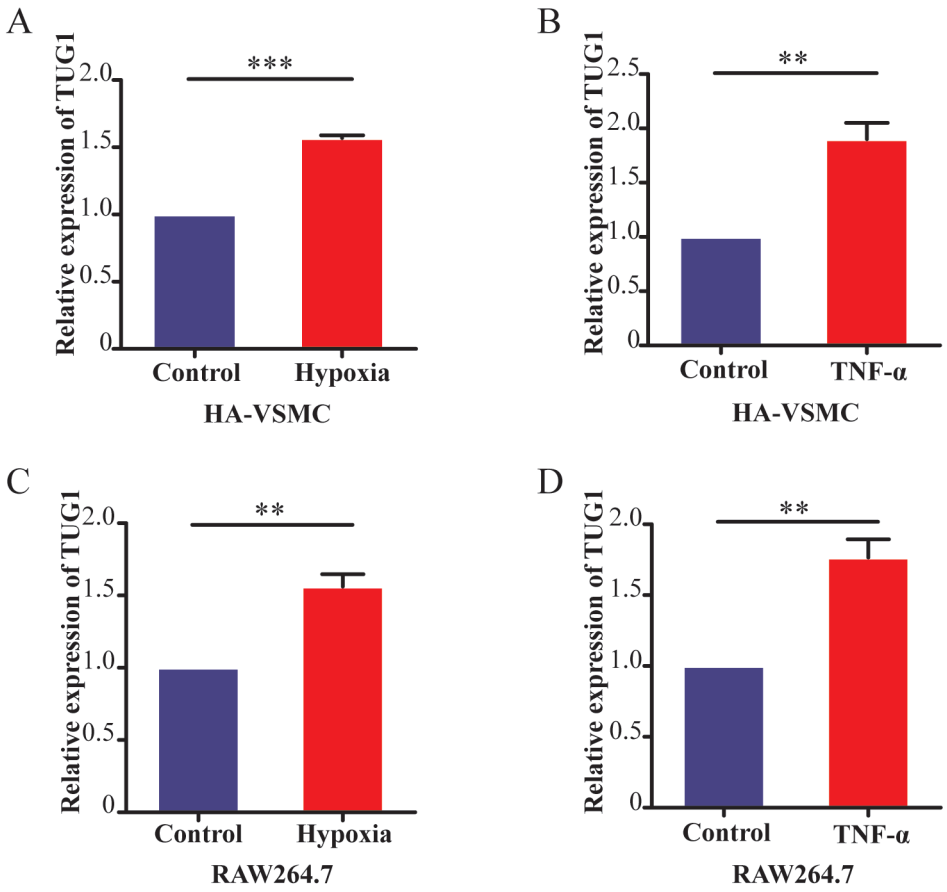

Figure 2. LncRNA TUG1 was up-regulated in HA-VSMC cells induced with hypoxia or TNF-α. ***(A)*** Expression of lncRNA TUG1 was detected by qRT-PCR in hypoxia induced HA-VSMC cells. *** p<0.001. ***(B)*** Expression of lncRNA TUG1 was detected by qRT-PCR in TNF-α (20 ng/ml) induced HA-VSMC cells. ** p<0.01. ***(C)*** Expression of lncRNA TUG1 was detected by qRT-PCR in hypoxia induced RAW264.7 cells. ** p<0.01. ***(D)*** Expression of lncRNA TUG1 was detected by qRT-PCR in TNF-α (20 ng/ml) induced RAW264.7 cells. ** p<0.01.

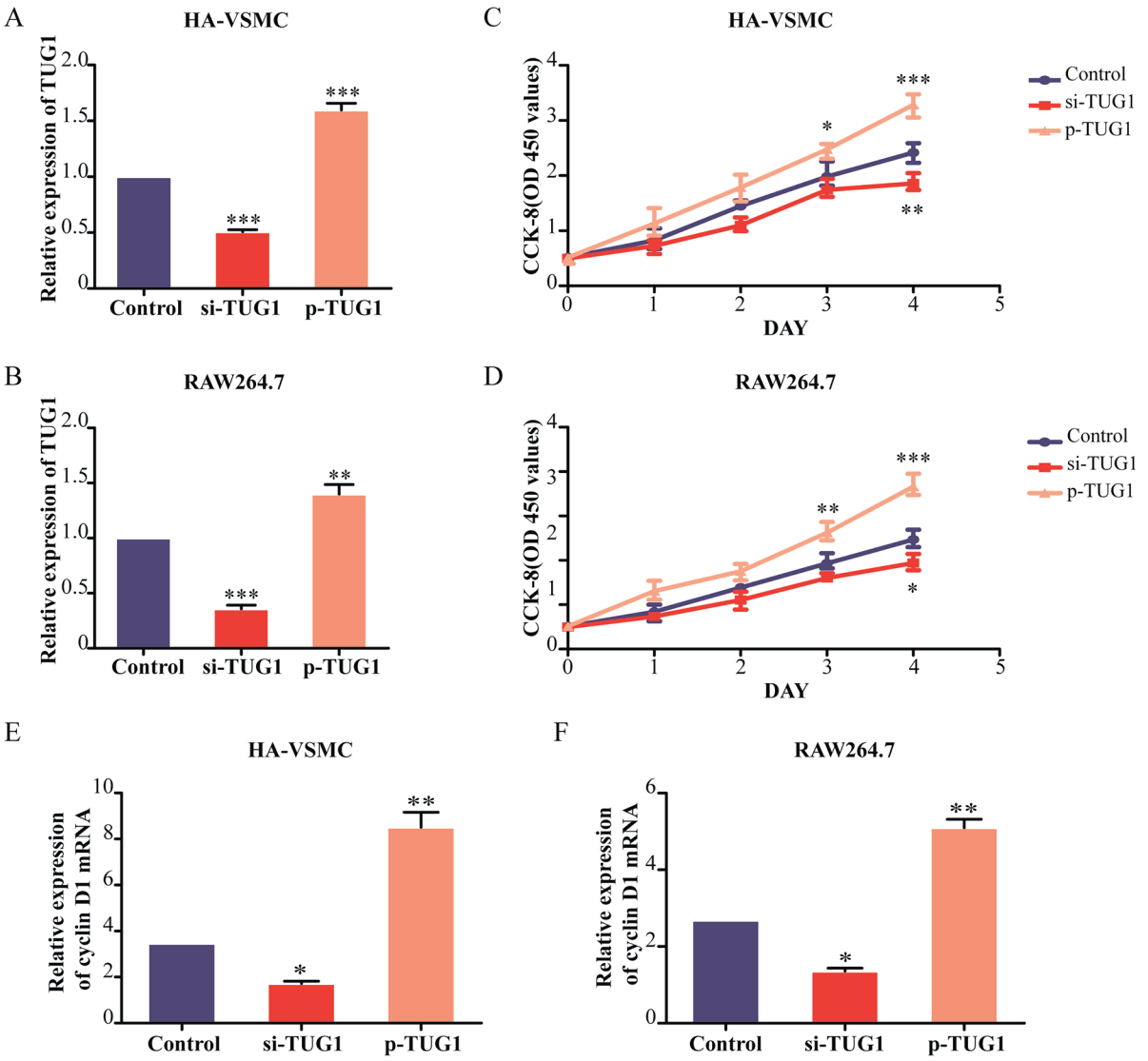

Figure 3. LncRNA TUG1 regulated proliferation and apoptosis of HA-VSMC and RAW264.7 cells. ***(A) (B)*** Expression levels of lncRNA TUG1 in HA-VSMC and RAW264.7 cells transfected with siRNA and plasmids. *** $p<0.001$, ** $p<0.01$. ***(C) (D)*** The proliferation and viability of HA-VSMC and RAW264.7 cells were measured using the Cell Counting Kit-8 (CCK-8) colorimetric assay after lncRNA TUG1 knockdown or overexpressed. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$. ***(E) (F)*** Different levels expression of lncRNA TUG1 regulated the expression of cyclin D1. $^{**}p<0.01$, $^{*}p<0.05$.

the down-regulation of TUG1 noticeably inhibited the expression of miRNA-21 (Figure 4D). To further investigate the association of miR-21 and TUG1, a wt-TUG1 luciferase reporter vector (wt-TUG1), a mut-TUG1 3'UTR luciferase reporter vector (mut-TUG1) with mutations on predicted miR-21 binding site in TUG1, a wt-PTEN 3'UTR luciferase reporter vector (wt-PTEN), and a mut-PTEN 3'UTR luciferase reporter vector (mut-PTEN) with mutations on miR-21 binding site in the 30UTR of PTEN, were constructed (Figure 4E-F). After that, we conducted a luciferase reporter assay. Compared with other groups, co-transfection with miR-21 mimic and wt-TUG1 significantly increased the luciferase activity of HA-VSMC. The data revealed that miR-21 could directly bind to TUG1 binding sites (Figure 4G). Moreover, co-transfection with miR-21 and wt-PTEN 3'UTR significantly reduced the luciferase activity of HA-VSMC (Figure 4H), suggesting that miR-21 directly binds to the 3'UTR seed regions of PTEN and down-regulates the expression of PTEN. All above, these data suggest that lncRNA TUG1 promotes proliferation of vascular

smooth muscle cell and atherosclerosis by regulating PTEN expression and decreasing PTEN activity by targeting miRNA-21.

that the overexpression of circulating exosomes and exosomal lncRNA HIF1A-AS1 could result from the activation of endothelial cells and vascu-

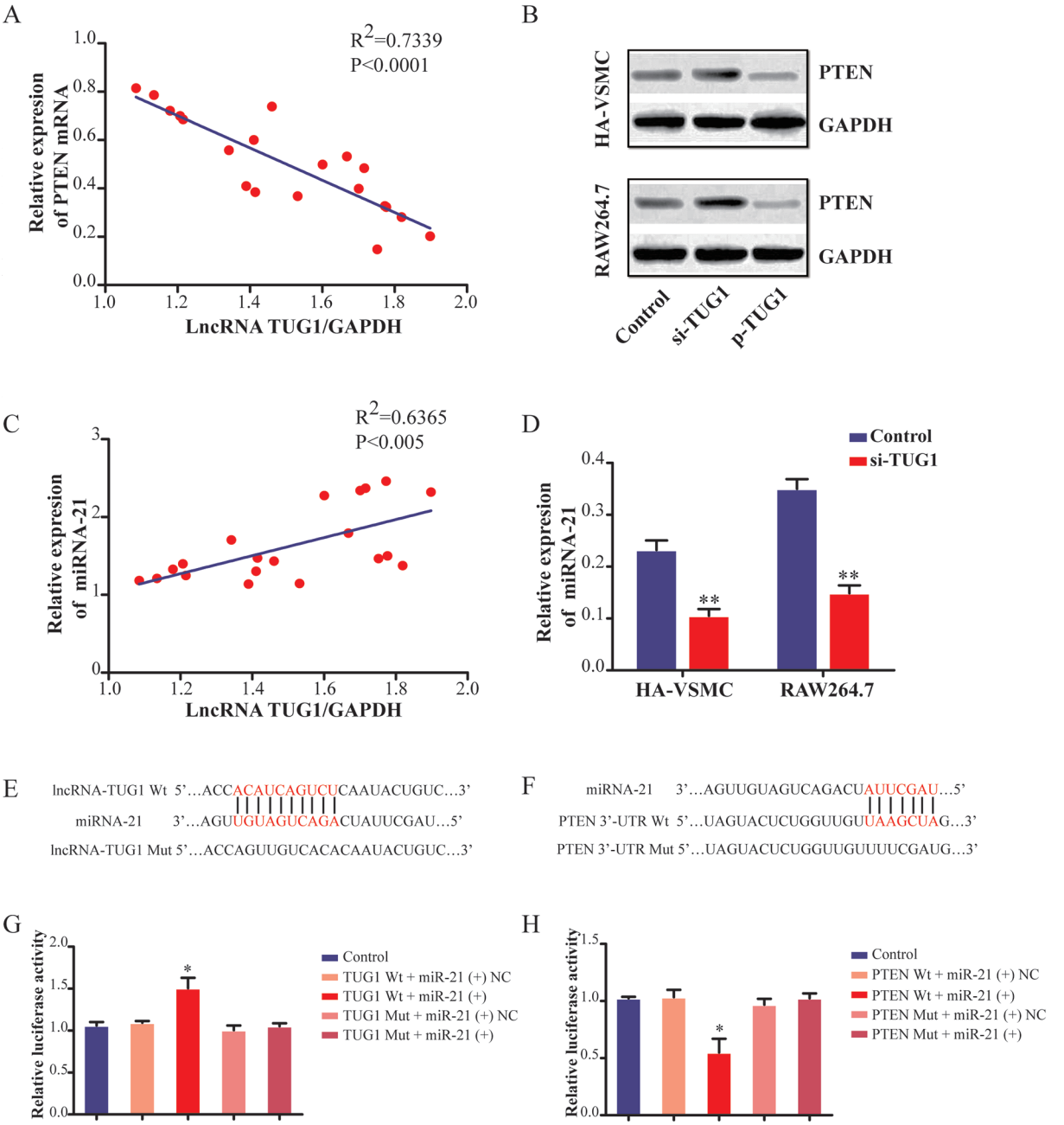

Figure 4. LncRNA TUG1 suppresses the expression of PTEN by targeting miRNA-21. ***(A)*** The LncRNA TUG1 expression level was reversely correlated with PTEN expression in patients with atherosclerosis. R^2=0.7339, p<0.0001. ***(B)*** The expression of PTEN was measured by WB in HA-VSMC and RAW264.7 cells. ***(C)*** The LncRNA TUG1 expression level was positively correlated with miRNA-21 expression in patients with atherosclerosis. R^2=0.6365, p<0.005. (D) Down-regulation of LncRNA TUG1 significantly decreased the expression of the expression of miRNA-21 in HA-VSMC and RAW264.7 cells. ** p<0.01. ***(E)*** Bioinformatics analysis predicted binding sites between TUG1 and miRNA-21. ***(F)*** Predicted seed regions between and PTEN and miRNA-21. ***(G) (H)*** The luciferase reporter assay. Co-transfection with miR-21 and TUG1 Wt significantly increased the luciferase activity of HA-VSMC compared with others. Co-transfection with miR-21 and PTEN Wt significantly reduced the luciferase activity of HA-VSMC compared with others. * p<0.05.

lar smooth muscle cells. Li et al[24] demonstrated that higher expression of BANCR could up-regulate VSMCs proliferation and migration partly through activating the JNK pathway. These studies indicated that lncRNAs were important in regulating atherosclerosis and other cardiovascular diseases. Further investigations are still needed to identify those detailed functions and mechanisms. LncRNA TUG1, a full-length 6.7 kilobase untranslated RNA molecule, was originally identified to contribute to the forming of photoreceptors and played crucial roles in retinal development[25]. Evidence showed that the dysregulation of TUG1 participated in the development of several biological processes. Zhang et al[16] showed that the expression level of TUG1 in osteosarcoma tumor tissue was significantly higher than that of normal tissue. By down-regulating TUG1 in U2OS, the proliferation was inhibited. Liu et al[15] uncovered that low expression of TUG1 inhibited proliferation and induced apoptosis in bladder cancer cells by targeting ZEB2 mediated by miR-142 through the inactivation of Wnt/β-catenin pathway. Chen et al [26] shed light on the role of LncRNA TUG1 as a miRNA sponge for ischemic stroke. Knockdown of TUG1 decreased the ratio of apoptotic cells and promoted cells survival *in vitro*, which may be regulated by the elevated miRNA-9 expression and decreased Bcl2l11 protein. In our study, we found that lncRNA TUG1 was highly expressed in the serum of patients with atherosclerosis, as well as in the aortic plaques of ApoE-/- mice. We also found that the expression of TUG1 was upregulated in VSMCs induced with hypoxia or TNF-α. Through loss- and gain-of function approaches, we showed that TUG1 promotes cell proliferation and induces apoptosis *in vitro*. These findings are remarkable because they indicate that modulation of the activity of non-coding RNAs such as lincRNA TUG1 may be a novel therapeutic approach to treat human cardiovascular disease. PTEN is a well-known tumor suppressor gene at the 10q23 locus. It is a crucial down-regulator of the PI3K/AKT/Mtor pathway, which regulates the cell cycle, proliferation, apoptosis, and migration[27-31]. Extensive studies have indicated that PTEN is associated with a wide spectrum of tumors, while PTEN in atherosclerosis is rarely reported. In this study, we found that the expression of PTEN mRNA was negatively correlated with the expression of lncRNA TUG1. Furthermore, transfection with lncRNA TUG1 inhibitor significantly increased the expression of PTEN protein, while transfection with lncRNA TUG1 promoter significantly reduced the expression of PTEN protein. Additional experiments indicated that TUG1 interacted directly with miR-21, and there was a positive correlation between miR-21 and TUG1. We also found that miR-21 directly targeted PTEN. The results also indicate that there might be a TUG1–miRNA-21–PTEN axis in in HA-VSMC and RAW264.7.

Conclusions

We demonstrated that lncRNA TUG1 was highly expressed in patients with atherosclerosis and atherosclerotic plaque from ApoE-/- mice. Furthermore, our findings firstly uncovered that lncRNA TUG1 could promote proliferation ability of atherosclerosis by suppressing PTEN activity. We indicated that lncRNA TUG1 might be used as a promising prognostic marker and a potential target for treating atherosclerosis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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