LncRNA RASSF8-AS1 knockdown displayed antiproliferative and proapoptotic effects through *miR-188-3p/ATG7* pathway in ox-LDL-treated vascular smooth muscle cells

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Background: Long noncoding RNA (lncRNA)-mediated changes in gene expression contribute to atherosclerosis (AS) development. However, the roles of numerous lncRNAs in AS have not been fully elucidated. Here, we aimed to investigate the potential role of *lncRNA RASSF8-AS1* (*RASSF8-AS1*) in autophagy of human aortic vascular smooth muscle cells (HA-VSMCs).

Methods: *RASSF8-AS1* expression in patients with AS was extracted from the Gene Expression Omnibus (GEO) database. *RASSF8-AS1* and microRNA-188-3p (*miR-188-3p*) expression was analyzed in 20 enrolled patients with AS. HA-VSMCs were treated with oxidized low-density lipoprotein (ox-LDL) (25, 50, 75, and 100 μg/mL) for 24 h. Loss- or gain-of-function of *RASSF8-AS1*, miR-1883p, and autophagy-related 7 (*ATG7*) was studied using the transfected HA-VSMCs. Cell viability was assessed using Cell Counting Kit-8 (CCK-8). Apoptosis was detected with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Relative luciferase reporter assay was used to confirm the targeting relationship of *miR-188-3p* to *RASSF8-AS1* or *ATG7*. Gene expression was detected by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot.

Results: *RASSF8-AS1* was enriched in the serum of patients with AS and ox-LDL-treated HA-VSMCs. Ox-LDL induced proliferation and autophagy while inhibiting the apoptosis of HA-VSMCs, which was abated by *RASSF8-AS1* knockdown. *RASSF8-AS1* downregulated *miR-188-3p* of ox-LDL-treated HA-VSMCs. *RASSF8-AS1* knockdown caused an increase in *miR-188-3p*, which inhibited proliferation and autophagy and induced the apoptosis of ox-LDL-treated HA-VSMCs. *miR-188-3p* inhibited *ATG7* expression in ox-LDL-treated HA-VSMCs. *RASSF8-AS1* elevated *ATG7* and induced autophagy through sponging *miR-188-3p* in ox-LDL-treated HA-VSMCs.

Conclusions: *RASSF8-AS1* regulated autophagy by targeting *miR-188-3p*, a messenger RNA-binding miRNA that increases *ATG7* level, which may be a new target molecule for the prevention and prognosis of AS.

Keywords: *LncRNA RASSF8-AS1*; vascular smooth muscle cells (VSMCs); *miR-188-3p*; autophagy-related 7 (ATG7); atherosclerosis (AS)

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Introduction

Atherosclerosis (AS), which refers to the formation of fibrofatty lesions in the innermost layer of arteries, is the most main underlying pathology of coronary artery disease (1). AS mainly causes cardiovascular diseases, such as stroke, myocardial infarction, and disabling peripheral artery disease, and is a significant cause of death worldwide (2). Low-density lipoprotein (LDL) is a particle that transports cholesterol through the bloodstream and is necessary for the generation of atherosclerotic lesions (2). A growing body of evidence points to the presence of other risk factors, including diabetes mellitus, hypertension, inflammation, clonal hematopoiesis, and cigarette smoking (3). AS is a diffuse and slowly progressive disease that can involve multiple arterial beds (2). Its slow progression leads to the asymptomatic phenotype that can last for decades (2). The clinical presentation of AS involves carotid and cerebral arteries, thoracic aorta, renal arteries, superior and inferior mesenteric arteries, coronary arteries, abdominal aorta, and peripheral arteries (1). A study of the cells and molecules involved in the AS process have advanced the understanding of the link between the risk factors, atheroma development, and clinical manifestations of AS (4).

As the main cell type in the pre-AS intima, smooth muscle cells (SMCs) appear with diffuse intimal thickening and AS plaque generation (5). The phenotype of SMCs changes in response to extracellular lipids and lipoproteins (6). With the development of AS plaque, intimal SMCs exhibit a high proliferative rate, loss of contractility, and an elevated level of proteoglycans (6,7).

Long noncoding RNAs (lncRNAs) with 200 nucleotides can regulate phenotypic effects via their capacity to target DNA, microRNA (miRNA), RNA-binding proteins, and proteins (8-11). Research has discovered that lncRNA expression is altered in the intima of AS lesions (12,13). Macrophage-specific lncRNA MAARS increases 270-fold in the aortic intima with the progression of AS (12), while aortic intimal expression of lncRNA VINAS decreases with AS progression (13). LncRNAs are increasingly considered as significant modulators of the pathophysiological processes of AS (12,13). It has been confirmed that lncRNA MAARS is involved in apoptosis via the regulation of caspase-9, p27, p53, and BCL2 based on the interaction with HuR/ELAVL1 (12). One loss-of-function study proved that lncRNA VINAS mediating the *NF-κB* and *MAPK* signaling pathways regulates vascular inflammation (13). In addition, it has been demonstrated that lncRNA-TCONS_00034812 is significantly up-regulated in arterial tissue samples of AS patients, and lncRNA-TCONS_00034812 promotes human aortic smooth muscle cells proliferation by up-regulating miR-21 (14). Tao *et al.* reported that lncRNA SOX2-OT was outstandingly upregulated in patients with carotid AS, which might be an independent prognostic biomarker for the progression of carotid AS (15). Guo *et al.* demonstrated that lncRNA-FA2H-2 interacted with the promoter of the MLKL gene to lead to the downregulated MLKL, thereby activating inflammation and inhibiting autophagic flow, which in turn aggravates AS (16). Wang *et al.* indicated that lncRNA SNHG16 aggravated atherosclerotic plaque formation and increased ox-LDL-activated vascular smooth muscle cells (VSMCs) growth by upregulating HMGB2 expression via sponging miRNA-22-3p (17). Accumulating evidence have indicated that lncRNAs exert critical roles in AS progression, but their functional roles and regulatory mechanisms remain unclear.

In this study, bioinformatics and quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis confirmed that the upregulation of *lncRNA RASSF8-AS1* (*RASSF8-AS1*) in AS can be used as a diagnostic marker for AS, suggesting that *RASSF8-AS1* may be a potential lncRNA associated with AS development. Furthermore, ox-LDL stimulation was used to construct an

Highlight box

Key findings

- The up-regulation of *RASSF8-AS1* in peripheral blood could be used as a diagnostic marker for AS patients.
- *RASSF8-AS1* directly elevated *ATG7* expression through sponging *miR-188-3p* in ox-LDL-treated HA-VSMCs.
- *RASSF8-AS1* potentiated proliferation and anti-apoptosis of VSMCs-treated by ox-LDL in AS by regulating *miR-188-3p/ATG7*-mediated autophagy pathway.

What is known and what is new?

- LncRNA plays an important role in the development of AS.
- We identified a novel *lncRNA RASSF8-AS1* involved in AS progression and elucidated that *RASSF8-AS1* directly promoted ATG7-mediated autophagy through sponging *miR-188-3p*.

What is the implication, and what should change now?

- Our data confirmed that a novel *RASSF8-AS1/miR-188-3p/ATG7*-mediated autophagy pathway regulated the biological function of VSMCs in AS, suggesting that this pathway could be used as diagnostic tools and novel therapeutic strategies of AS. However, the above signaling pathways need to be further verified by various animal experiments in the future.

Table 1 Clinical characteristics of patients with AS and healthy individuals

Variables	Normal (n=21)	AS (n=20)	P value
Males/females (n)	9/12	9/11	–
Age (years)	58±5	63±11	0.05
Body mass index (kg/m^2)	22.05±5.99	24.11±3.25	0.25
Current smokers	–	6 (30.0)	–
Hypertension	–	15 (75.0)	–
Diabetes mellitus	–	7 (35.0)	–
Total cholesterol (mmol/L)	4.44±0.97	4.80±0.99	0.06
Triglyceride (mmol/L)	0.96±0.31	1.33±0.78	0.3
HDL-C (mmol/L)	1.46±0.0.30	1.27±0.31	0.07
LDL-C (mmol/L)	2.08±0.67	2.93±1.00	3.00E-03

Data are shown as n (%) or mean ± SD. AS, atherosclerosis; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

AS cell model in order to confirm the effect of *RASSF8-AS1* on the biological function of human aortic vascular smooth muscle cells (HA-VSMCs). Moreover, previous studies have revealed the crosstalk between the biogenesis of proteins and lncRNA-mediated processing of miRNAs in AS (18,19). Hence, we also analyzed the pathogenesis of AS from the perspective of lncRNA-miRNA-messenger RNA (mRNA) axis, and we identified a novel *lncRNA RASSF8-AS1* directly promoted ATG7-mediated autophagy and proliferation of HA-VSMCs through sponging *miR-188-3p*. these founding suggested that novel *RASSF8-AS1/miR-188-3p/ATG7*-mediated autophagy pathway could be used as diagnostic tools and novel therapeutic strategies of AS. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6457/rc).

Methods

Data extraction and bioinformatical analysis

Transcriptomic profiles of lncRNAs in patients with AS from the Gene Expression Omnibus (GEO) database under the accession number GSE146882 (http://www.ncbi.nlm.nih.gov/geo/) were obtained. The expression of lncRNAs was normalized, and $\log_2$ transformation was applied for each matrix. lncRNAs with significant differences between AS patients and healthy individuals were screened with |log(fold change)| more than 1.5 and P values less than 0.05. The target genes of *miR-188-3p* were predicted using miRWalk (http://mirwalk.umm.uni-heidelberg.de/).

Sample collection

This study enrolled 20 patients with AS but no history of treatment and 21 healthy individuals. The clinical features of patients with AS and healthy participants are summarized in *Table 1*. Peripheral blood (10 mL) was collected for gene expression analysis. The clinical parameters are shown in *Table 1* and include LDL cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C). The inclusion criteria for healthy individuals were as follows: no AS symptoms, malignant and inflammatory diseases, autoimmune diseases, or infections within the previous 1 month. All participants who participated in this study signed an informed consent form, and this study was approved by the Ethics Committee of the First Affiliated Hospital of Guangdong Pharmaceutical University (No. 202112). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell culture and treatment

HA-VSMCs were provided by the American Type Culture Collection (ATCC; Rockville, MD, USA). HA-VSMCs were maintained in SMC medium (SMCM; ScienCell, Carlsbad, CA, USA) and cultured in an incubator containing 5% CO_2 at 37 ℃. HA-VSMCs were passaged every 3–4 days. The medium was added with 1% penicillin-streptomycin and 2% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). HA-VSMCs were incubated for 24 h. Oxidized LDL (ox-LDL) was prepared at concentrations of 25, 50, 75, and 100 μg/mL.

Cell transfection

The short-hairpin RNA targeting *RASSF8-AS1* (sh-lncRNA#1, sh-lncRNA#2, and sh-lncRNA#3) and corresponding negative control (sh-NC) were designed and generated by GenePharma (Shanghai, China). To construct the vectors overexpressing *RASSF8-AS1* or *ATG7*. The full-length sequence of *RASSF8-AS1* or *ATG7* mRNA was synthesized and constructed into the pcDNA3.1 vector (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). For the NC, the cells were transfected with the empty pcDNA3.1 vector. *miR-188-3p* inhibitor and NC inhibitor,

Table 2 The sequences of *RASSF8-AS1* shRNA, *miR-188-3p* mimic, *miR-188-3p* inhibitor and their negative control

Item	Sequence (5'-3')
RASSF8-AS1 shRNA#1	CCGGCAGTTGTGACTATATTCTACTCGAGTAGAATAT
	AGTCACAACTGTTTTTG
RASSF8-AS1 shRNA#2	CCGGGATTGTCAATGAATCAGAACTCGAGTTCTGAT
	TCATTGACAATCTTTTTG
RASSF8-AS1 shRNA#3	CCGGGGACCATAAGAAAGTGAAACTCGAGTTTCAC
	TTTCTTATGGTCCTTTTTG
NC shRNA	CCGGTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGA
	GGGCGACTTAACCTTAGGTTTTTG
miR-188-3p mimics	CUCCCACAUGCAGGGUUUGCA
NC mimics	UUGUACUACACAAAAGUACUG
miR-188-3p inhibitor	UGCAAACCCUGCAUGUGGGAG
NC inhibitor	CAGUACUUUUGUGUAGUACAA

shRNA, short hairpin RNA; NC, negative control.

and *miR-188-3p* mimic and NC mimic were obtained from GenePharma. The transfection was implemented with Lipofectamine 3000 (Invitrogen) in accordance with manufacturer protocol. The sequences of *RASSF8-AS1* short hairpin RNAs (shRNAs), *miR-188-3p* mimics/inhibitor, NC mimic, and NC inhibitor are provided in *Table 2*.

Cell viability

HA-VSMCs were seeded into 96-well plates at 5×10^3 cells each well and precultured overnight. Cell viability of HA-VSMCs was assayed using Cell Counting Kit-8 (CCK-8). After 24 h of intervention with ox-LDL, the medium was replaced with 100 µL of fresh medium supplemented with CCK-8 reagent (10 µL; Beyotime, Shanghai, China). After 4 h of incubation, the supernatants were discarded. Next, 200 µL of dimethyl sulfoxide was added to each well in the 96-well plate. A microplate reader (Thermo Fisher Scientific) was used to detect the optical value at 450 nm.

Apoptosis assay

HA-VSMCs were collected in pre-cooled phosphate-buffered saline (PBS) and then dissociated by trypsinization. The cells were harvested by centrifugation (3,000 r/min, 5 min) and resuspended in binding buffer (1×). Then, 100 µL of HA-VSMCs were incubated into 96-well plates (1×10^5 cells each well). After supplementation with Annexin V-conjugated fluorescein isothiocyanate (FITC; 5 µL) and propidium iodide (PI; 5 µL; Sigma-Aldrich, St. Louis, MO, USA), the cells were incubated for 15 min in the dark at room temperature. Next, binding buffer (1×; 400 µL) was added into the culture. Flow cytometry (Becton Dickinson, San Jose, CA, USA) was used to detect apoptotic cells.

Dual-luciferase reporter assay

The *RASSF8-AS1* sequence or the *ATG7* 3' untranslated region (3'-UTR) sequence harboring the predicted binding sites of *miR-188-3p* [*RASSF8-AS1*-wild type (WT) or *ATG7*-WT] or designed mutant binding sites [*RASSF8-AS1*-mutant (MUT) or *ATG7*-MUT] were inserted into pmirGLO reporter vectors (Promega, Madison, WI, USA), respectively. For luciferase reporter assays, the vectors and *miR-188-3p* mimics or NC mimics were co-transfected into HA-VSMCs. HA-VSMCs were incubated for 48 h before luciferase assay. Then, the Dual-Luciferase Reporter System (Promega) was used to detect luciferase activity after 48 h of transfection. The relative expression of the luciferase reporter was analyzed by the ratio of firefly and Renilla luciferase signals.

Table 3 Specific primer sequences for qRT-PCR

Item	Sequence (5'-3')
RASSF8-AS1	Forward primer, GGTATGTGGTGCTGGCAGGAAT
	Reverse primer, AGAGGTGGAAAGACGGGTGAGA
miR-188-3p	RT primer, 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCG GCAATTGCACTGGATACGACTGCAAAC-3'
	Forward primer, GCAATATTACTCCCACATGCAGG
	Reverse primer, GTCGTATCCAGTGCGTGTC
ATG7	Forward primer, CGTCACCGAGAGCCTCTTATG
	Reverse primer, AATCACCACTGGCTGAGAACC
U6	RT primer, AAAATATGGAACGCTTCACGAATTTG
	Forward primer, CTCGCTTCGGCAGCACATATACT
	Reverse primer, ACGCTTCACGAATTTGCGTGTC
GAPDH	Forward primer, AAGTATGACAACAGCCTCAAG
	Reverse primer, TCCACGATACCAAAGTTGTC

qRT-PCR, quantitative real-time reverse-transcription polymerase chain reaction; RT, reverse transcription.

qRT-PCR

Total RNA was isolated from HA-VSMCs using MagMAX *mir*Vana Total RNA Isolation Kit (Thermo Fisher Scientific). For *ATG7* and *RASSF8-AS1*, complement DNA (cDNA) was generated using PrimeScript RT Master Mix Kit (Takara, Dalian, China). SYBR Premix Ex Taq Kit (Takara Bio) was used for qRT-PCR. The reaction was performed on a Bio-Rad IQ5 qPCR system (Bio-Rad Laboratories, Hercules, CA, USA). *ATG7* and *RASSF8-AS1* expression was normalized to GAPDH. TaqMan MicroRNA Assay and TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) were used for the amplification of *miR-188-3p*. *miR-188-3p* expression was normalized to U6. qRT-PCR was carried out with the specific primers for *RASSF8-AS1*, *miR-188-3p*, and *ATG7* (*Table 3*). Relative quantification of *RASSF8-AS1*, *miR-188-3p*, and *ATG7* was based on the $2^{-\Delta\Delta CT}$ formula.

Western blot analysis

HA-VSMCs cells were washed in PBS, lysed with the pre-cooled RIPA buffer (including protease inhibitor cocktail), and collected by centrifugation (12,000 r/mim; 10 min; 4 ℃). The pellets were collected in the mixture of 0.125 Tris-hydrochloride, 20% sodium dodecyl sulfate (SDS), 10% glycine, and 2-mercaptoethanol. After being separated by 10% SDS-polyacrylamide gel electrophoresis, the proteins were blotted onto polyvinylidene fluoride (PVDF) membranes (Roche, Switzerland), blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween20 (TBST), maintained at 4 ℃ overnight, washed with TBST buffer, probed with primary antibodies, and co-incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at 25 ℃. The enhanced chemiluminescence (Thermo Fisher Scientific) and ImageJ software (National Institutes of Health, Bethesda, MD, USA) were used for protein visualization and quantification. β-actin was selected for normalization.

Statistical analysis

Values are presented as the mean ± standard error of the mean. GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used for the statistical analysis. The receiver operating characteristic (ROC) curve was used to measure the diagnostic power of *RASSF8-AS1* and *miR-188-3p* transcript levels for categorizing patients. The correlations of *RASSF8-AS1* expression with LDL-C level and *miR-188-3p* expression were evaluated by Spearman rank correlation coefficient. The differences between the two groups were confirmed by Student *t*-test. P values <0.05 indicated statistically significant difference. All experiments were repeated 3 times.

Results

Serum of patients with AS and ox-LDL-treated HA-VSMCs showed increased expression of RASSF8-AS1

The lncRNA dataset GSE146882 consisting of patients with AS and sex- and age-matched normal participants was downloaded from the GEO database for analysis. It was found that serum *RASSF8-AS1* of patients with AS was significantly increased compared to healthy participants (*Figure 1A*). ROC analysis indicated that *RASSF8-AS1* expression predicted the presence of AS (AUC =0.77; P value =0.04; 95% CI =0.55–0.99; *Figure 1B*). Peripheral blood samples from patients with AS (n=20) showed increased level of *RASSF8-AS1* compared to those of healthy participants (*Figure 1C*), which was in line with the results from the analysis of the GSE146882 dataset. Recent studies have demonstrated

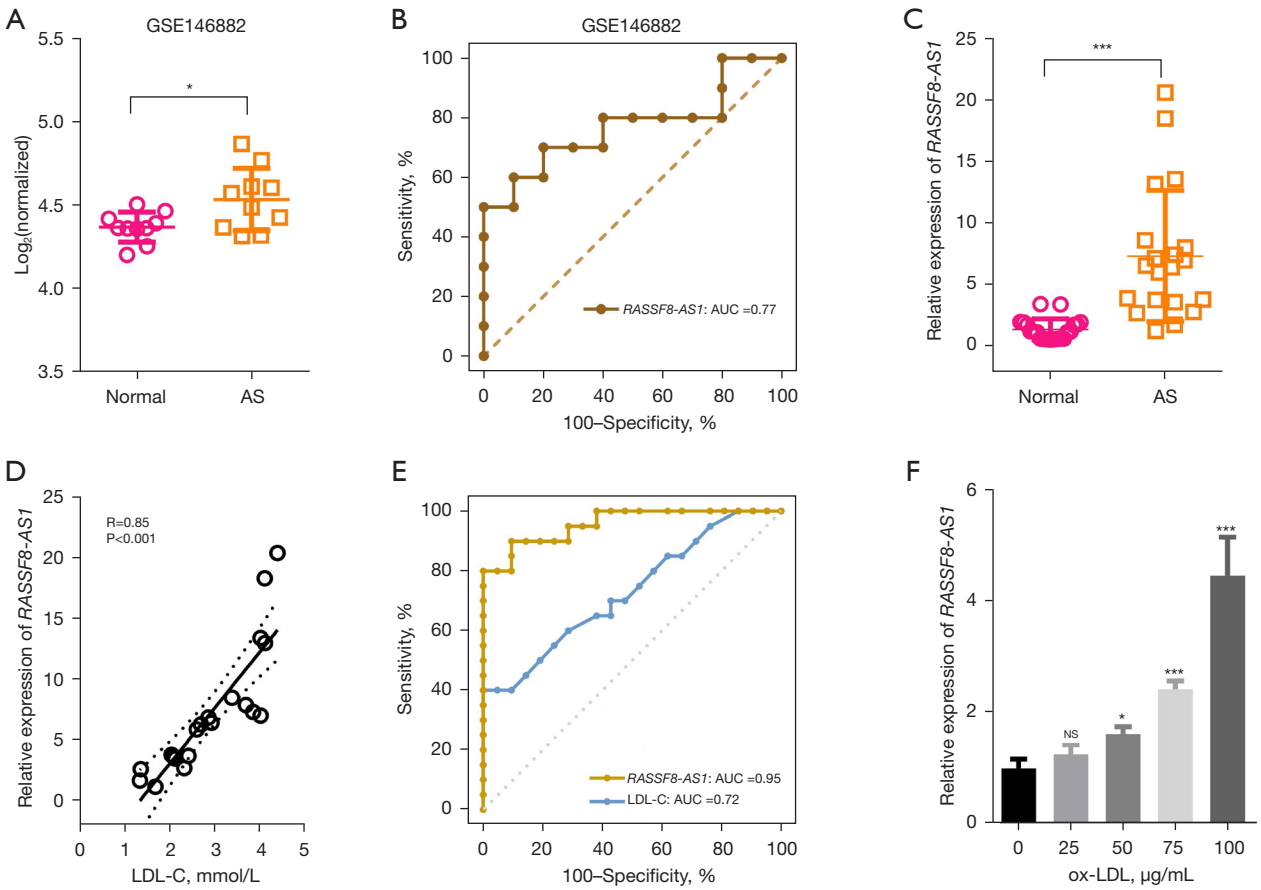


Figure 1 Patients with AS and ox-LDL-treated HA-VSMCs exhibited increased level of *RASSF8-AS1*. (A) *RASSF8-AS1* was detected with high expression in patients with AS compared with healthy participants. Dataset GSE146882 was downloaded from GEO database. The Y-axis represents the signal intensity of gene expression (log_2-transformed). P value =0.01; N=10. *P<0.05. (B) ROC curve analysis of *RASSF8-AS1* value in diagnosis of AS. AUC =0.77. (C) Healthy participants (normal; N=20) and patients with AS (N=20) were enrolled for assaying *RASSF8-AS1* expression in peripheral blood. ***P<0.001 (*t*-test). (D) Relationship between *RASSF8-AS1* and LDL-C. R=0.85; P<0.001. (E) ROC curve analysis of *RASSF8-AS1* value in predicting LDL-C-treated HA-VSMCs. *RASSF8-AS1* AUC =0.95; ox-LDL AUC =0.72. (F) HA-VSMCs (N=3) were assayed for *RASSF8-AS1* expression after administration with ox-LDL (0, 25, 50, 75, and 100 μg/mL). ^{NS}P>0.05; *P<0.05; ***P<0.001 (*t*-test). HA-VSMC, human aortic vascular smooth muscle cell; AS, atherosclerosis; ox-LDL, oxidized low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; GEO, Gene Expression Omnibus; ROC, the receiver operation characteristic; AUC, area under the receiver operating characteristic curve.

that AS cardiovascular disease (ASCVD) is associated with elevated LDL-C (20,21). Therefore, we analyzed the correlation between serum *RASSF8-AS1* expression level and LDL-C content in patients with AS and found that *RASSF8-AS1* expression was significantly positively associated with LDL-C levels in the peripheral blood of patients with AS (*Figure 1D*). ROC curve analysis also demonstrated that both *RASSF8-AS1* and LDL-C could distinguish patients with AS, and the specificity of *RASSF8-AS1* was higher than that of LDL-C (*RASSF8-AS1*: AUC =0.95; P value <0.001, 95% CI =0.90–1.01; LDL-C: AUC =0.72, P value =0.01, 95% CI =0.58–0.88; *Figure 1E*). Finally, *RASSF8-AS1* in HA-VSMCS treated by ox-LDL (0, 25, 50, 75, and 100 μg/mL) was detected by qRT-PCR. *RASSF8-AS1* showed an increased expression in HA-VSMCs treated by ox-LDL in a concentration-dependent manner (*Figure 1F*). As *RASSF8-AS1* was upregulated in the peripheral blood of patients with AS and VSMCs treated by ox-LDL, it may serve as a diagnostic marker for AS.

RASSF8-AS1 knockdown inhibited cell proliferation and promoted apoptotic effects in ox-LDL-treated HA-VSMCs

To confirm the role of *RASSF8-AS1* in ox-LDL-treated HA-VSMCs, shRNA targeting *RASSF8-AS1* was designed to knockdown its expression in HA-VSMCs. sh-*RASSF8-AS1*#1 and sh-*RASSF8-AS1*#2 evidently downregulated *RASSF8-AS1* expression in HA-VSMCs, especially sh-*RASSF8-AS1*#1 (*Figure 2A*). Hence, sh-*RASSF8-AS1*#1 (sh-*RASSF8-AS1*) was used to knock down the expression of *RASSF8-AS1*. Furthermore, ox-LDL increased cell viability in HA-VSMCs, while RASSF8-AS knockdown inhibited the ox-LDL-induced viability of HA-VSMCs (*Figure 2B,2C*). However, ox-LDL promoted the protein expression of proliferating cell nuclear antigen (PCNA) and Kiel 67 antigen (Ki-67) in HA-VSMCs, and *RASSF8-AS1* knockdown reduced the expression of PCNA and Ki-67 (*Figure 2D,2E*). ox-LDL inhibited the apoptosis of HA-VSMCs, which was inversely increased by *RASSF8-AS1* knockdown (*Figure 2F,2G*). Overall, *RASSF8-AS1* deficiency inhibited the proliferative and antiapoptotic activity of HA-VSMCs treated by ox-LDL.

RASSF8-AS1 sponged miR-188-3p to downregulate miR-188-3p in HA-VSMCs treated by ox-LDL

For determining the molecular mechanism of *RASSF8-AS1*, RNA nucleocytoplasmic separation combined with qRT-PCR was carried out to analyze the localization of *RASSF8-AS1* in HA-VSMCs. The results showed that a small amount of *RASSF8-AS1* was detected in the nucleus, and *RASSF8-AS1* was mainly localized in the cytoplasm (*Figure 3A*). DIANA-LncBase Predicted v.2 was further used predicted the miRNAs targeted to *RASSF8-AS1*. It was found that *miR-188-3p* and *RASSF8-AS1* had targeted binding sites. Patients with AS and ox-LDL-treated HA-VSMCs exhibited decreased expression of *miR-188-3p* (Figure S1A and *Figure 3B*). *miR-188-3p* levels showed the potential of predicting the risk for AS (AUC =0.78; Figure S1B). Moreover, our data revealed a negative correlation between serum *miR-188-3p* and *RASSF8-AS1* in patients with AS (R=–0.75; P<0.001; *Figure 3C*). To prove *miR-188-3p* was targeted by *RASSF8-AS1*, a mutant type of *RASSF8-AS1* was produced (*Figure 3D*). Dual-luciferase reporter assay demonstrated that *RASSF8-AS1* resulted in targeted inhibition of *miR-188-3p* transcription (*Figure 3E*), which was further confirmed in HA-VSMCs with high or low expression of *RASSF8-AS1* (*Figure 3F*). The ox-LDL induced the down-regulation of miR-188-3p in HA-VSMCs, the effect was significantly reversed by downregulation of *RASSF8-AS1* (*Figure 3G*). The results demonstrated that *RASSF8-AS1* inhibited *miR-188-3p* expression in HA-VSMCs by sponging *miR-188-3p* in response to ox-LDL.

RASSF8-AS1 knockdown inhibited the cell proliferation and apoptosis in ox-LDL-treated HA-VSMCs by up-regulating miR-188-3p

To determine whether *RASSF8-AS1* affects the biological function of HA-VSMCs by regulating *miR-188-3p* in response to ox-LDL, HA-VSMCs were next incubated with ox-LDL after transfection of sh*RASSF8-AS1* alone or with *miR-188-3p* inhibitor. *RASSF8-AS1* knockdown increased *miR-188-3p* expression, which was significantly inhibited by *miR-188-3p* inhibitor (*Figure 4A*). *RASSF8-AS1* knockdown apparently decreased cell viability of HA-VSMCs after ox-LDL treatment, which could be weakened by silencing *miR-188-3p* (*Figure 4B*). Moreover, *RASSF8-AS1* knockdown reduced PCNA and Ki-67 expression in HA-VSMCs treated by ox-LDL, which could be retarded by silencing *miR-188-3p* (*Figure 4C* and Figure S2). *RASSF8-AS1* knockdown significantly elevated cell apoptosis of HA-VSMCs in response to ox-LDL, but silencing *miR-188-3p* abrogated these effects (*Figure 4D*). Together, the above data suggested that knockdown of *RASSF8-AS1* suppressed proliferation and promoted apoptosis via upregulating *miR-188-3p* in HA-VSMCs treated by ox-LDL.

ATG7 was targeted by miR-188-3p in HA-VSMCs in response to ox-LDL

The targeted genes of *miR-188-3p* were predicted using miRWalk database. The results revealed 2 binding sites to *miR-188-3p* at the 3'-UTR of *ATG7*. This finding indicated that *ATG7* was a downstream targeted gene of *miR-188-3p*. Furthermore, ox-LDL induced the mRNA and protein expression of *ATG7* in HA-VSMCs (*Figure 5A*). High expression of *ATG7* mRNA was confirmed in patients with AS (*Figure 5B*). *miR-188-3p* overexpression decreased the transcript level of *ATG7*, while *miR-188-3p* knockdown intensified the transcription of *ATG7* (Figure S3 and *Figure 5C*). This result indicated that *ATG7* expression was negatively modulated by *miR-188-3p*. Luciferase reporter assay confirmed the targeting relationship between *ATG7* and *miR-188-3p* in both the WT and MUT of *ATG7*

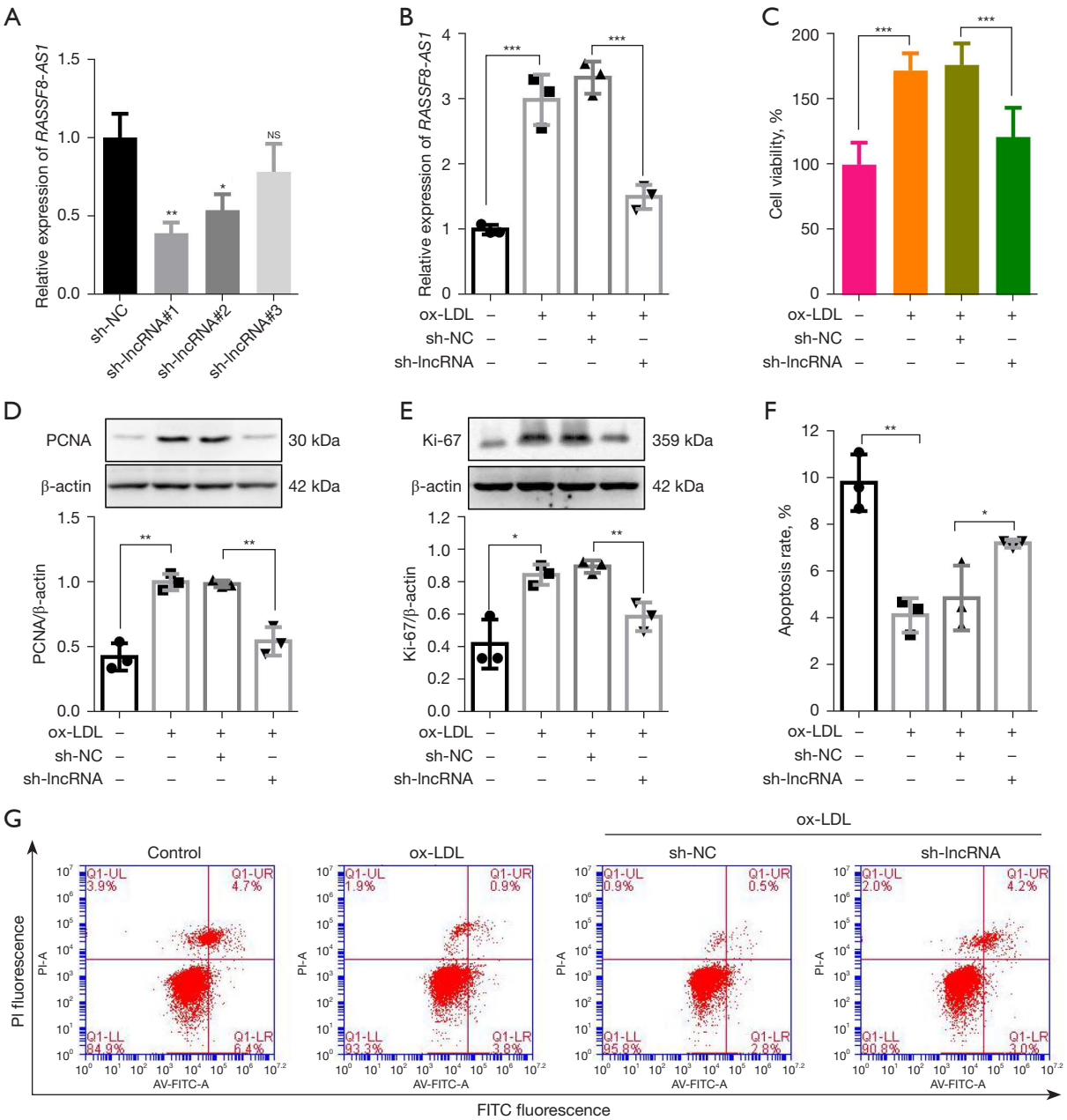


Figure 2 ox-LDL increased cell viability and inhibited apoptosis in HA-VSMCs expressing *RASSF8-AS1*. (A) *RASSF8-AS1* expression in HA-VSMCs. shRNA targeting *RASSF8-AS1* (sh-lncRNA#1, #2, and #3) was introduced into HA-VSMCs. *P<0.05; **P<0.01; ^{NS}P>0.05 (*t*-test). N=3. (B) *RASSF8-AS1* expression in HA-VSMCs transfected with sh-lncRNA#1 and treated by 75 μg/mL of ox-LDL. ***P<0.001 (*t*-test). N=3. (C) Cell viability of sh-lncRNA#1-treated HA-VSMCs. HA-VSMCs were treated by ox-LDL (75 μg/mL). ***P<0.001 (*t*-test). N=3. (D,E) HA-VSMCs were assayed for PCNA and Ki-67 expression after transfection with sh-lncRNA#1 and administration with ox-LDL (75 μg/mL). *P<0.05; **P<0.01 (*t*-test). N=3. (F,G) HA-VSMCs were detected for apoptosis rate after transfection with *RASSF8-AS1* shRNA. *P<0.05; **P<0.01 (*t*-test). N=3. HA-VSMC, human aortic vascular smooth muscle cell; ox-LDL, oxidized low-density lipoprotein; sh-lncRNA, short hairpin *RASSF8-AS1*; sh-NC, short hairpin negative control; PCNA, proliferating cell nuclear antigen; FITC, fluorescein isothiocyanate; PI, prodium iodide.

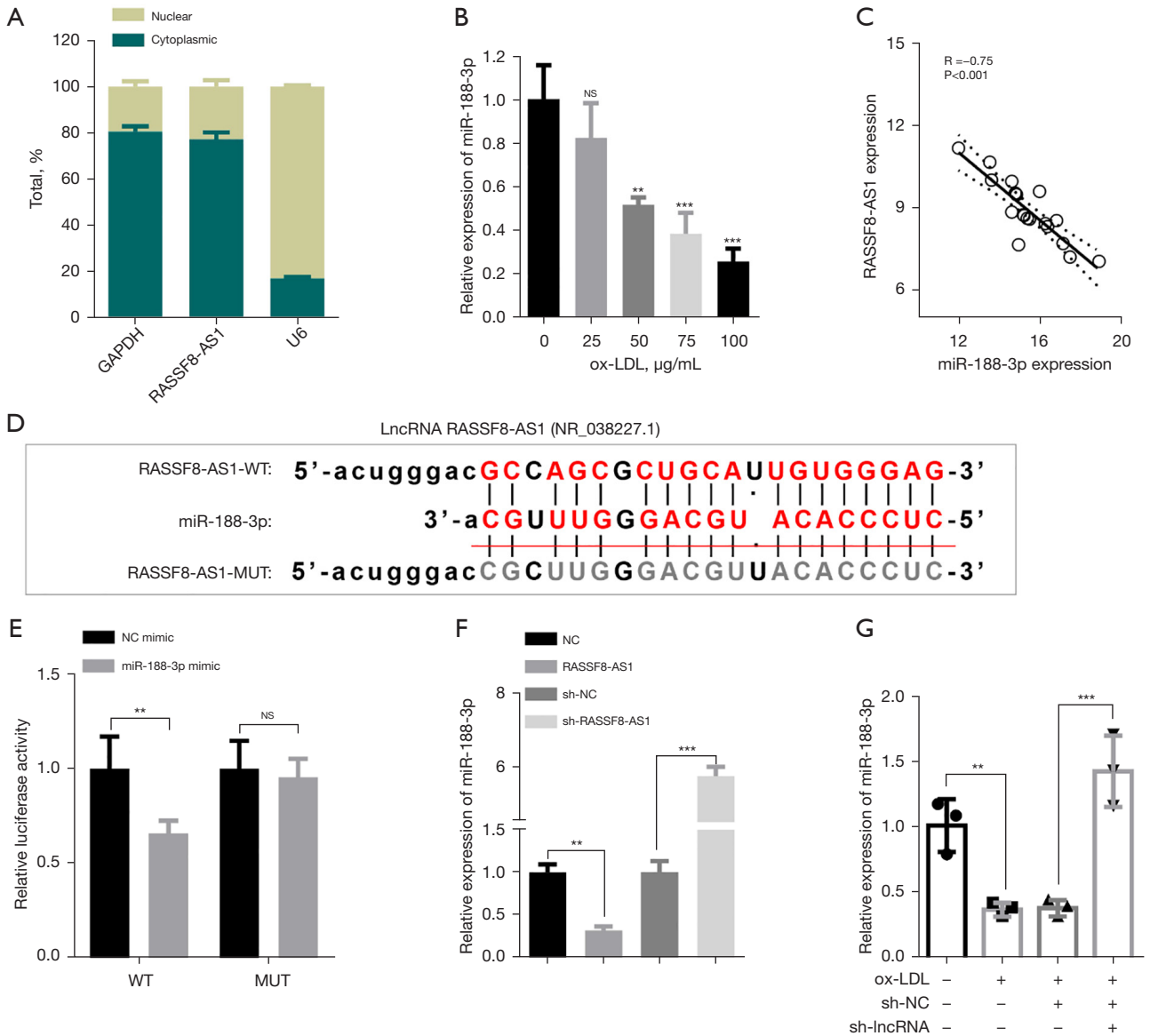


Figure 3 *RASSF8-AS1*-induced targeted inhibition of *miR-188-3p* expression by *RASSF8-AS1* in HA-VSMCs treated by ox-LDL. (A) Nuclear and cytoplasmic RASSF8-AS1 in HA-VSMCs (N=3). (B) HA-VSMCs were assayed for *miR-188-3p* expression after the treatment of ox-LDL (0, 25, 50, 75, and 100 μg/mL). ^{NS}P>0.05; **P<0.01; ***P<0.001. N=3. (C) The linear relationship based on *miR-188-3p* and *RASSF8-AS1* expression in patients with AS. N=20; R =–0.75; P<0.001. (D) *miR-188-3p*-interacted *RASSF8-AS1* (wild type and mutant type). (E) Confirmation of the interaction between *RASSF8-AS1* and *miR-188-3p* by relative luciferase reporter assay. ^{NS}P>0.05; **P<0.01 (*t*-test). N=3. (F) *miR-188-3p* level in HA-VSMCs high or low expressing *RASSF8-AS1*. **P<0.01; ***P<0.001 (*t*-test). N=3. (G) *miR-188-3p* expression in *RASSF8-AS1*-deficient HA-VSMCs after ox-LDL treatment. **P<0.01; ***P<0.001 (*t*-test). N=3. HA-VSMC, human aortic vascular smooth muscle cell; ox-LDL, oxidized low-density lipoprotein; AS, atherosclerosis; sh-lncRNA, short hairpin RASSF8-AS1; sh-NC; short hairpin negative control; NC, negative control; WT, wild type; MUT, mutant type.

(*Figure 5D*). *miR-188-3p* overexpression inhibited the relative luciferase activities of *ATG7*-WT (4,691–4,712 nt) in HA-VSMCs, but conferred no significant effects on the relative luciferase activities of *ATG7*-MUT (4,691–4,712 nt), *ATG7*-WT (4,651–4,670 nt), or *ATG7*-MUT (4,651–4,670 nt; *Figure 5E*), suggesting *miR-188-3p* directly

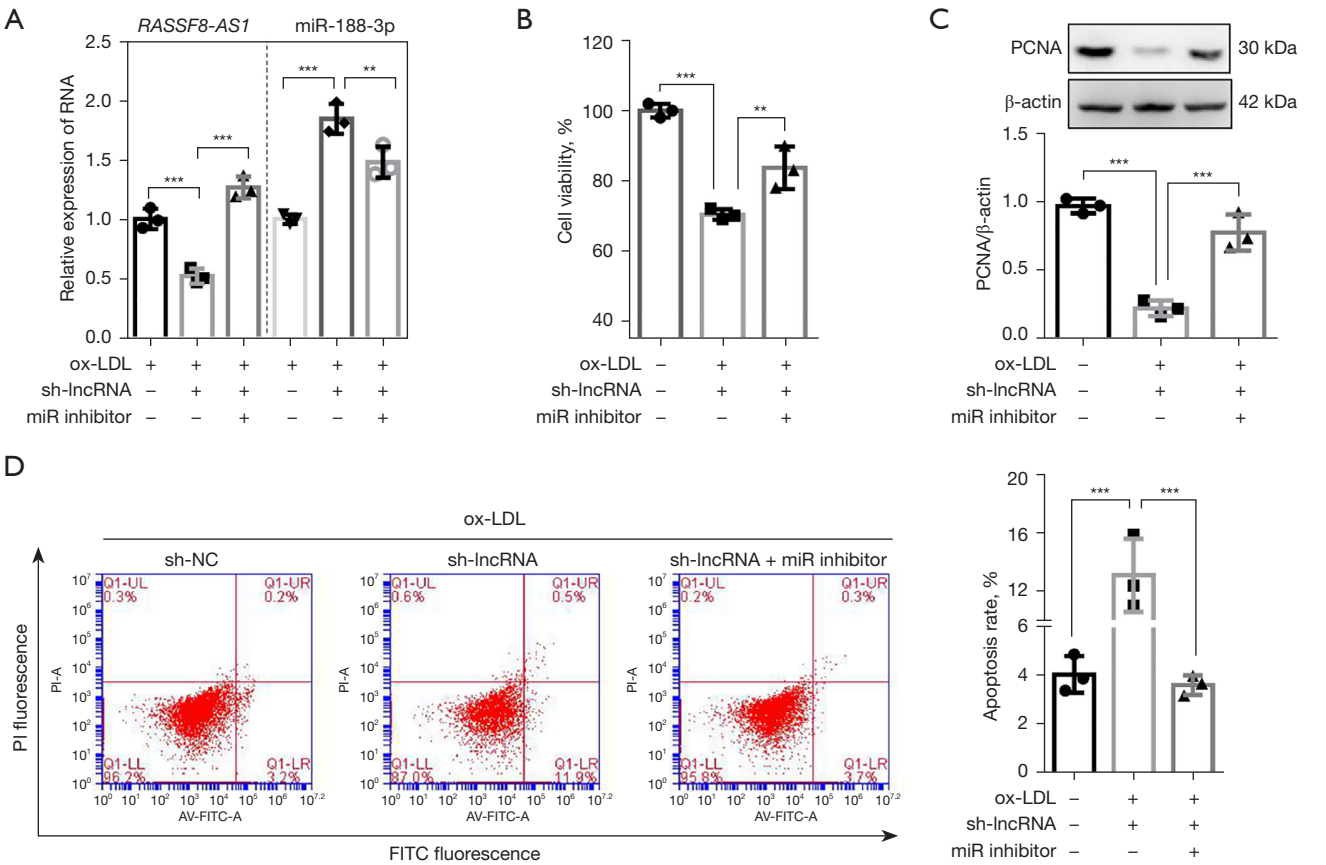


Figure 4 Targeted sponging of *miR-188-3p* by *RASSF8-AS1* regulated proliferative activity and apoptotic process of HA-VSMCs in response to ox-LDL. (A) *RASSF8-AS1* and *miR-188-3p* expression in HA-VSMCs treated by ox-LDL after transfection with sh-lncRNA#1 or miR inhibitor. **P<0.01; ***P<0.001 (*t*-test). N=3. (B) HA-VSMCs were assayed for cell viability after transfection with sh-lncRNA#1 or miR inhibitor or treatment by ox-LDL (75 μg/mL). **P<0.01; ***P<0.001 (*t*-test). N=3. (C) *PCNA* expression in HA-VSMCs after transfection with sh-lncRNA#1 or miR inhibitor and treatment of ox-LDL (75 μg/mL). ***P<0.001 (*t*-test). N=3. (D) Apoptosis rate of HA-VSMCs after transfection with sh-lncRNA#1 or miR inhibitor and treatment with ox-LDL (75 μg/mL). ***P<0.001 (*t*-test). N=3. *PCNA*, proliferating cell nuclear antigen; shRNA, short hairpin RNA; HA-VSMC, human aortic vascular smooth muscle cell; ox-LDL, oxidized low-density lipoprotein; sh-lncRNA, short hairpin RASSF8-AS1; miR inhibitor, miR-188-3p inhibitor; NC, negative control; FITC, fluorescein isothiocyanate; PI, prodium iodide.

binds to *ATG7*-3UTR (4,691–4,712 nt) to regulate *ATG7* transcriptional activity. *miR-188-3p* overexpression inhibited *ATG7* expression in HA-VSMCs in response to ox-LDL (*Figure 5F* and Figure S4). Hence, *ATG7* is a downstream targeted gene of *miR-188-3p*, and its expression was mediated by *miR-188-3p* in HA-VSMCs after ox-LDL treatment.

RASSF8-AS1 elevated ATG7 expression and autophagy through sponging miR-188-3p in HA-VSMCs treated by ox-LDL

ATG7 is an autophagy-related gene, and so we hypothesized that *RASSF8-AS1* regulates *ATG7* and thus autophagy via *miR-188-3p*. To confirm the above hypothesis, Western blot was performed to ascertain the protein expression of *ATG7* and autophagy markers (LC3 I, LC3 II, Beclin-1, and p62). In HA-VSMCs after ox-LDL treatment, *RASSF8-AS1* knockdown markedly decreased *ATG7* protein expression, reduced LC3 II and Beclin-1 expression, decreased the ratio of LC3 II to LC3 I, and increased P62 expression. However, these effects were reversely abrogated by silencing *miR-188-3p* (*Figure 6A-6F*). The above results suggest that knockdown of *RASSF8-AS1* suppresses *ATG7* expression and autophagy by upregulation of *miR-188-3p* in

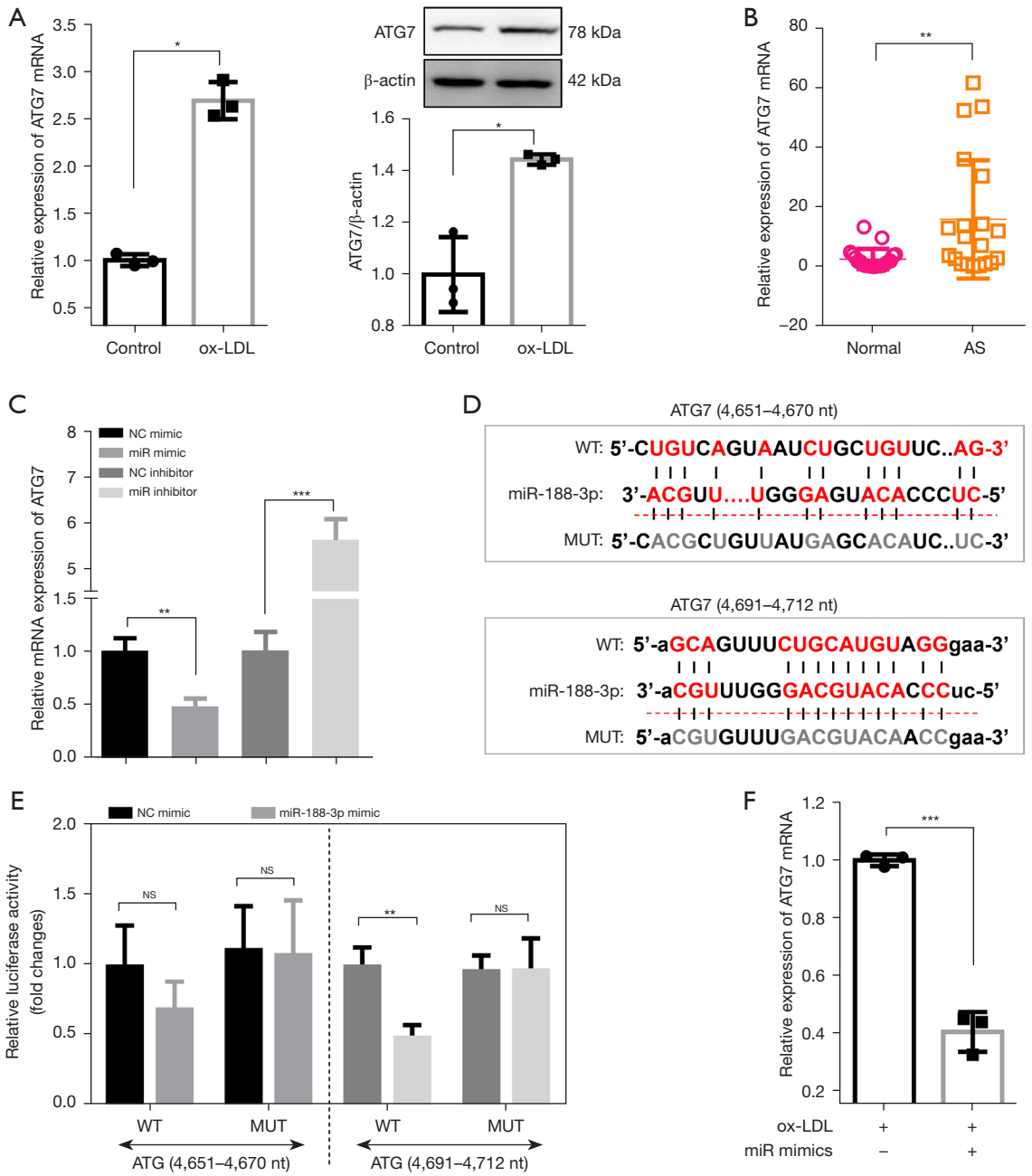


Figure 5 *ATG7* was a downstream target of *miR-188-3p* in HA-VSMCs. (A) HA-VSMCs were assayed for *ATG7* mRNA and protein after ox-LDL treatment (75 μg/mL). *P<0.05 (*t*-test). N=3. (B) Serum *ATG7* mRNA in healthy individuals (normal) and patients with AS. **P<0.01 (*t*-test). N=20. (C) HA-VSMCs were assayed for AGT7 mRNA after transfection with miR mimic or inhibitor. **P<0.01; ***P<0.001 (*t*-test). N=3. (D) Targeted sites between the wild type or mutant type of *miR-188-3p* and *ATG7*. (E) The direct interaction between *ATG7* mRNA and *miR-188-3p* was confirmed by relative luciferase reporter assay. ^{NS}P>0.05; **P<0.01 (*t*-test). N=3. (F) *miR-188-3p* mimic decreased *ATG7* transcription in ox-LDL-treated HA-VSMCs. ***P<0.001 (*t*-test). N=3. ox-LDL, oxidized low-density lipoprotein; *ATG7*, autophagy-related gene 7; HA-VSMC, human aortic vascular smooth muscle cell; AS, atherosclerosis; sh-lncRNA, short hairpin RASSF8-AS1; miR inhibitor, miR-188-3p inhibitor; miR mimic, miR-188-3p mimic; NC, negative control; WT, wild type; MUT, mutant type.

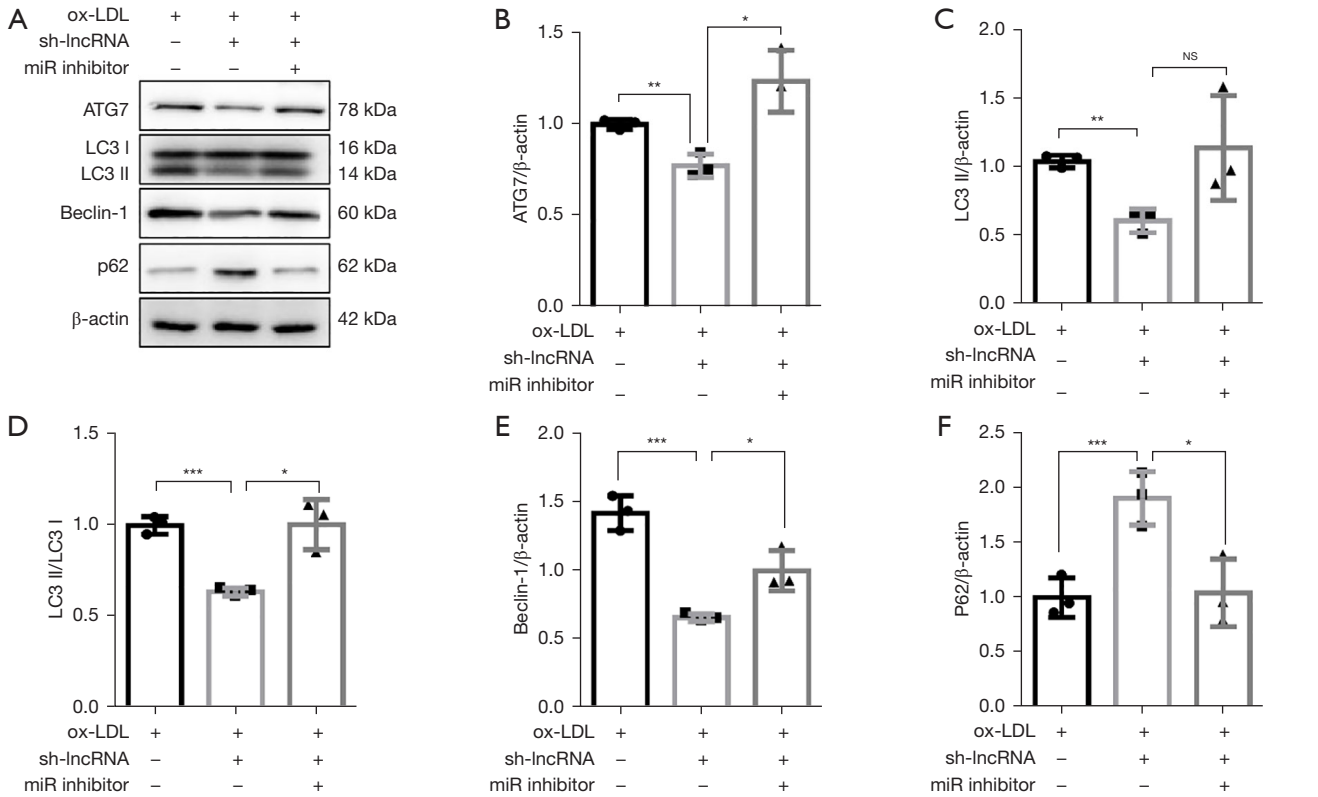
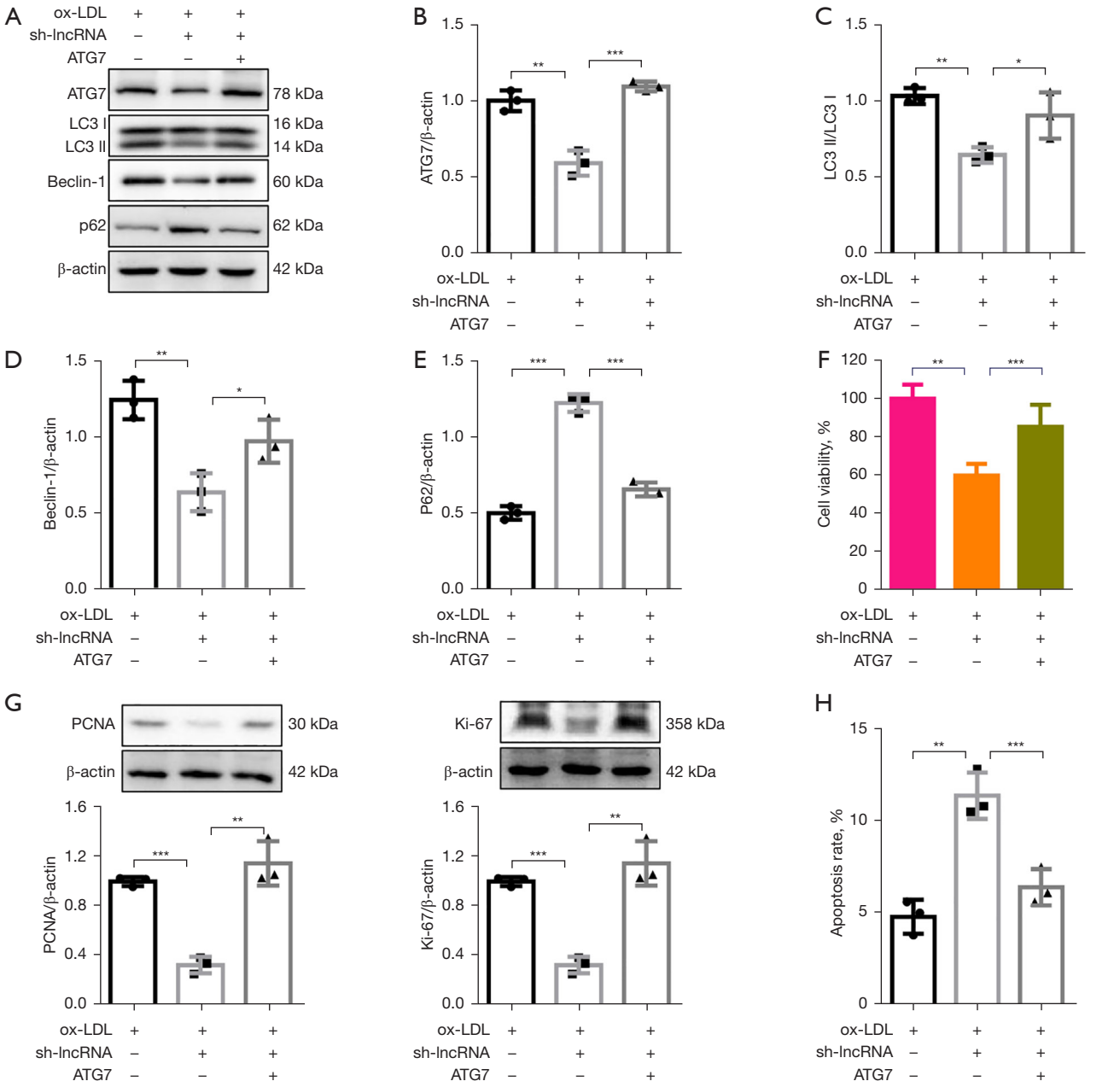


Figure 6 *ATG7* expression and autophagy induced by *RASSF8-AS1*-mediated sponging of *miR-188-3p* in HA-VSMCs after ox-LDL treatment. (A) Western blotting showing autophagy protein expression in HA-VSMCs transfected with the indicated vehicles and treated by ox-LDL. (B-F) Quantification of *ATG7* (B), LC3 II (C), Beclin-1 (E), and P62 (F) protein, as well as the ratio of LC3 II and LC3 I (D). ^{NS}P>0.05; *P<0.05; **P<0.01; ***P<0.001 (*t*-test). N=3. ox-LDL, oxidized low-density lipoprotein; *ATG7*, autophagy-related gene 7; HA-VSMC, human aortic vascular smooth muscle cell; sh-lncRNA, short hairpin RASSF8-AS1; miR inhibitor, miR-188-3p inhibitor.

HA-VSMCs after ox-LDL treatment.

RASSF8-AS1 knockdown restrained the cell proliferative and anti-apoptosis via downregulation of ATG7 in ox-LDL-treated HA-VSMCs

Previous studies have shown that *ATG7* mediates autophagy to affect biological function of VSMCs (22-26). Therefore, we speculated that *RASSF8-AS1* affected the biological function of HA-VSMCs in AS by regulating *ATG7* expression. As shown in Western blot (*Figure 7A*), by, knockdown of *RASSF8-AS1* in ox-LDL-treated HA-VSMCs significantly decreased the protein expression of *ATG7* and Beclin-1, repressed the ratio of LC3 II and LC3 I, as well as increased the expression protein of P62, and these effects were abrogated by overexpression of *ATG7* (*Figure 7A-7E*), suggesting that *RASSF8-AS1* knockdown promoted autophagy of HA-VSMCs induced by ox-LDL by regulating *ATG7*. Subsequently, to determine whether *RASSF8-AS1* affected the biological function of HA-VSMCs by regulating *ATG7* in response to ox-LDL, HA-VSMCs were treated with ox-LDL after transfection of sh*RASSF8-AS1* alone or with *ATG7*. *RASSF8-AS1* knockdown decreased cell viability of HA-VSMCs treated by ox-LDL, which was relieved by ATG overexpression (*Figure 7F*). Meanwhile, *RASSF8-AS1* knockdown decreased PCNA and Ki-67 expression in ox-LDL-treated HA-VSMCs, while this inhibitory effect was reserved by *ATG7* upregulation (*Figure 7G*). Moreover, results from flow cytometry indicated that *RASSF8-AS1* knockdown caused apoptosis of HA-VSMCs treated by ox-LDL, and this change was significantly relieved by *ATG7* overexpression (*Figure 7H,7I*). The above results indicate that knockdown of *RASSF8-AS1* inhibits the proliferation and anti-apoptosis via the regulation of *ATG7*-mediated autophagy in HA-VSMCs treated by ox-LDL.



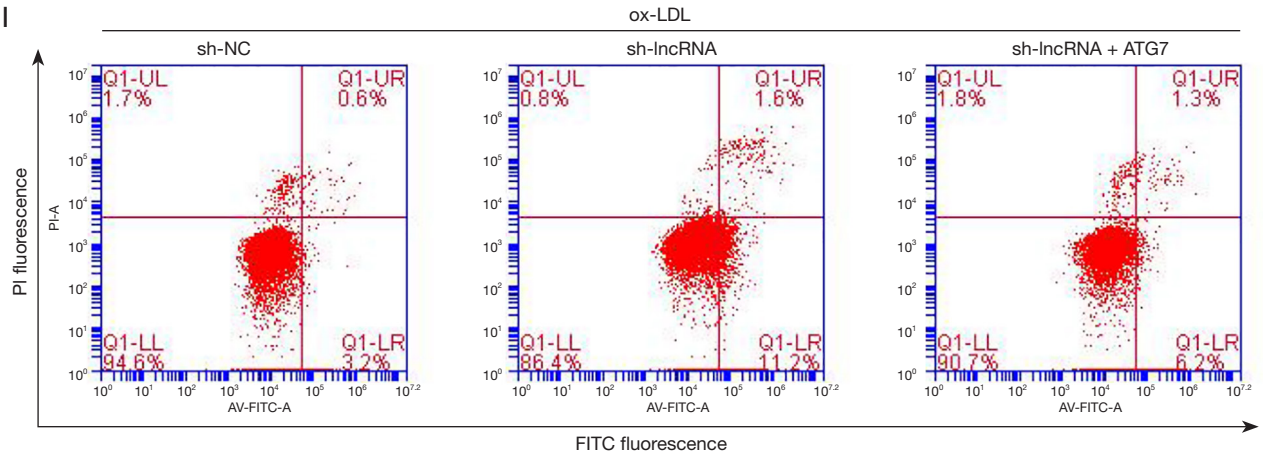


Figure 7 *RASSF8-AS1* modulated cell proliferation and apoptosis by regulating the *ATG7*-mediated autophagy pathway. (A**)** Western blotting showing autophagy protein expression in HA-VSMCs after transfection with the indicated vehicles (sh-lncRNA and *ATG7*) and treated by ox-LDL. (B-E) Quantification of the protein expression of *ATG7* (B), Beclin-1 (D), and P62 (E), and the ratio of LC3 II and LC3 I (C). *P<0.05; **P<0.01; ***P<0.001 (*t*-test). N=3. (F) Cell viability of HA-VSMCs after transfection with sh-lncRNA or *ATG7* and treatment with ox-LDL (75 μg/mL). **P<0.01; ***P<0.001 (*t*-test). N=3. (G) PCNA and Ki-67 protein expression in HA-VSMCs after transfection with sh-lncRNA or *ATG7* and treatment with ox-LDL (75 μg/mL). **P<0.01; ***P<0.001 (*t*-test). N=3. (H,I) Apoptosis of HA-VSMCs transfected with sh-lncRNA or *ATG7* and treated by ox-LDL (75 μg/mL). **P<0.01; ***P<0.001 (*t*-test). N=3. PCNA, proliferating cell nuclear antigen; sh-lncRNA, short hairpin *RASSF8-AS1*; ox-LDL, oxidized low-density lipoprotein; *ATG7*, autophagy-related gene 7; HA-VSMC, human aortic vascular smooth muscle cell; NC, negative control; FITC, fluorescein isothiocyanate; PI, prodium iodide.

Discussion

Autophagy is involved in the cellular stress response and phenotypic switching of VSMCs (27). It has been speculated that autophagy protects VSMCs against atherogenic stressors and massive stimuli and stressors such as lipid species and reactive oxygen, which is an adaptive mechanism found so far (27). Atherogenic oxidized lipids like ox-LDL increase autophagy-related protein expression and autophagosome accumulation in VSMCs, inducing a strong activation of autophagy (28). Whether autophagy is a protective or deleterious mechanism in vascular pathology is still controversial. However, there is a general consensus that VSMC survival is promoted by successful autophagy under conditions associated with extreme lipid peroxidation. Overactivated autophagy can induce the autophagic death of VSMCs, leading to reduced collagen synthesis and thus plaque instability (29). Here, we provide evidence that *RASSF8-AS1* modulates autophagy by targeting *miR-188-3p*, an mRNA-binding miRNA that increases the expression of *ATG7*.

Genomewide transcription profiling of AS and other cardiovascular diseases has provided new insights into the molecular mechanisms involving lncRNAs (30-32). The aberrantly expressed lncRNAs regulate the ubiquitinated proteasome pathways in AS-induced ischemic stroke (31), affect gene expression relevant to inflammation in coronary artery disease (30), and mediate T cell receptor signaling pathway in carotid AS (32). Altered lncRNA transcriptomic profiles suggest that *RASSF8-AS1* is increased in patients with AS. Consistent with this finding, we detected an increase in *RASSF8-AS1* expression in 20 enrolled patients with AS. It has been well-established that high serum concentrations of LDL-C are fundamental in the initiation and development of AS (33). Our study observed that increased expression of *RASSF8-AS1* was associated with a high concentration of LDL-C in patients with AS. *RASSF8-AS1* expression in ox-LDL-treated HA-VSMCs was directly proportional to the ox-LDL concentration. ROC analysis suggested that the level of ox-LDL in peripheral blood of patients with AS was positively correlated with the expression of *RASSF8-AS1*. These findings identify *RASSF8-AS1* as a potent indicator and a risk factor for AS.

ox-LDL exerts mitogenic effects on VSMCs via the activation of Ras/Raf/MEK/MAPK pathway (34). A previous study has provided evidence that mildly oxidized LDL functions synergistically with angiotensin II in

inducing the DNA synthesis of VSMCs (35). This study found that *RASSF8-AS1* silencing inhibited cell viability and induced apoptosis with decreased PCNA and Ki-67 expression in response to ox-LDL stimulation. It has been reported that symptomatic patients with carotid obstructive plaques are commonly detected with PCNA protein, showing a progressive alteration of the cellular homeostasis and unstable plaque (36). Ki-67 has been shown to be useful for the identification of replicative SMCs functioning in the development of intimal thickening during AS or after arterial lesion (37). VSMCs are considered to be the primary source of the extracellular matrix in diffuse intimal thickenings, which likely causes the enhancement of thickness of the intima, accounting for the progression of AS (38). These findings suggest that *RASSF8-AS1* serves as a positive feedback regulator when HA-VSMCs are stimulated by ox-LDL, which may cause the development and progression of AS plaque.

Direct binding of lncRNA and miRNA appears to alter the cellular function of downstream molecules, leading to abnormalities of vascular endothelial cells and SMCs (39-42). *miR-188-3p* was predicted to be targeted by *RASSF8-AS1* based on bioinformatic analysis. However, the interaction of *RASSF8-AS1* and *miR-188-3p* has not been experimentally validated. In this study, we confirmed *miR-188-3p* as a target of *RASSF8-AS1*. Patients with AS showed decreased expression of *miR-188-3p*, which is consistent with the results from apolipoprotein E (ApoE) knockout mice (43,44). There is evidence that *miR-188-3p*, which targets fibroblast growth factor 1 and decreases its expression, inhibits the proliferation and migration of VSMCs (43). Additionally, Zhang *et al.* characterized *miR-188-3p* as a regulator alleviating macrophage inflammatory response and reducing intravascular lipid accumulation in ApoE-deficient mice (44). Here, we identified *RASSF8-AS1* sponging *miR-188-3p* and *RASSF8-AS1* silencing enhanced *miR-188-3p* expression in ox-LDL-treated HA-VSMCs. Hence, we assumed *RASSF8-AS1* exerts its AS effect by targeting *miR-188-3p*. Our experimental results suggested that *miR-188-3p* downregulation induced by *RASSF8-AS1* increased cell viability, decreased apoptosis, and increased PCNA and Ki-67 expression in HA-VSMCs treated by ox-LDL.

Autophagy is tightly governed by highly conserved autophagy-related genes and mediated by lysosome in the turnover of damaged cytosolic materials. VSMCs from AS plaques were observed under transmission electron microscopy to have characteristics of autophagy (45,46). Autophagy is considered a crucial determinant in the cellular simulative response and phenotypic switching of VSMCs following vascular injury (29). *ATG7* is an essential gene for the ATG conjugation systems involved in amino acid supply and autophagosome formation. In vascular disease, prolonged stress conditions such as hypoxia/ischemia or oxidative stress dysregulate autophagy homeostasis and may activate autophagic cell death (47). Here, *ATG7* expression was increased in patients with AS compared with healthy participants. ox-LDL induced autophagy and increased the expression of *ATG7*. Most interestingly, *RASSF8-AS1* enhanced *ATG7* expression and autophagy in ox-LDL-treated HA-VSMCs by sponging *miR-188-3p*. Consequently, we emphasize the possibility that *RASSF8-AS1* regulates cell proliferation and apoptosis by regulating *ATG7*-mediated autophagy pathway.

Conclusions

Our data revealed that the upregulation of *RASSF8-AS1* in AS can be used as a diagnostic marker for AS and that *RASSF8-AS1* knockdown inhibited proliferation, promoted apoptosis, and suppressed autophagy in HA-VSMCs treated by ox-LDL. Notably, mechanistic analysis confirmed that a newly discovered *RASSF8-AS1/miR-188-3p/ATG7*-mediated autophagy pathway regulated the biological function of VSMCs in AS, and intervention of this pathway could be incorporated into diagnostic tools and novel therapeutic strategies in AS.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-6457/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-6457/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6457/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All participants who participated in this study signed an informed consent form, and this study was approved by the Ethics Committee of the First Affiliated Hospital of Guangdong Pharmaceutical University (No. 202112). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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