

CTBP1-AS2 inhibits proliferation and induces autophagy in ox-LDL-stimulated vascular smooth muscle cells by regulating miR-195-5p/ATG14

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Abstract. Atherosclerosis (AS) is a chronic progressive disease caused by injury and functional changes in vascular smooth muscle cells (VSMCs). Long non-coding RNAs (lncRNAs) are pivotal regulators in AS development. The present study aimed to explore the roles and molecular mechanisms of lncRNA *CTBP1-AS2* in AS progression. A dual-luciferase reporter assay confirmed that miR-195-5p is a downstream target miRNA of lncRNA *CTBP1-AS2* and miR-195-5p was increased in AS. The expression levels of miR-195-5p and *CTBP1-AS2* in the serums of patients with AS and human aorta vascular smooth muscle cells was increased or decreased, respectively, following treatment with oxidized low-density lipoprotein (ox-LDL). Functional experiments showed that the overexpression of lncRNA *CTBP1-AS2* inhibited the proliferation of HA-VSMCs and promoted their autophagy following ox-LDL treatment. This effect could be reversed by treatment with ROC-325, the inhibitor of autophagy, or miR-195-5p mimics. Autophagy related 14 (ATG14) was identified to be a target of miR-195-5p, and lncRNA *CTBP1-AS2* promoted ATG14 expression by serving as a competing endogenous RNA of miR-195-5p. The present study revealed that lncRNA *CTBP1-AS2* may serve a role in AS by inhibiting the proliferation and promoting the autophagy of VSMCs through ATG14 modulation via miR-195-5p. These data may provide a novel therapeutic target for AS.

Introduction

Cardiovascular disease (CVD) is one of the leading causes of mortality, with an annual mortality rate of >17.3 million worldwide (1). Atherosclerosis (AS) constitutes a large

subgroup of CVD and is the biggest cause of heart attack and stroke, accompanied by inflammation, and characterized by lipid accretion and fibrous cap and necrotic core formation (2). The formation of AS lesions involves a diverse range of cells, including endothelial cells (ECs), smooth muscle cells (SMCs), and macrophages (3). It is well-known that vascular SMCs (VSMCs) are the major components of the arterial wall. Previous studies have also demonstrated that the abnormal proliferation and migration of VSMCs are important pathophysiological processes in the development of atherosclerosis and that adequate VSMC function is crucial in protecting the vessel wall against atherosclerosis (4,5). The abnormal proliferation, apoptosis, autophagy, and migration of VSMCs are closely associated to AS advancement (6,7). Depending on this, VSMCs were selected in the present study to explore the role of *CTBP1-AS2* on cell function. Autophagy also serves an important role in AS (8). Oxidized low-density lipoprotein (ox-LDL), a primary factor in the promotion of AS progression, causes VSMC proliferation, invasion, and apoptosis (9). Therefore, the regulatory mechanisms involved in AS were explored using ox-LDL-stimulated VSMCs as an AS cell model.

Long non-coding RNAs (lncRNAs) measure 200 nucleotides (nt) long and are a category of non-coding (nc)RNAs that serve as core modulators in manifold complex diseases, including AS (10,11). lncRNAs associated with AS have received extensive attention due to their involvement in the inflammatory response, lipid metabolism, cell proliferation, adhesion, apoptosis and migration (12). For example, lncRNA p21 exhibits an overtly decreased expression in AS plaques of Apolipoprotein E^{-/-} mice and strengthens p53 activity to curb VSMCs and mononuclear macrophage cells of mice to proliferate and boost their apoptosis (13). The downregulation of lncRNA *RNCR3* accelerates AS progression by inhibiting the migration and proliferation of ECs and VSMCs, thereby resulting in their apoptosis *in vitro* (14). lncRNA *CTBP1-AS2* is an lncRNA that acts a novel modulator of cardiomyocyte hypertrophy by regulating TLR4 and an oncogene in papillary thyroid cancer (15). However, its role in AS has not been explored.

MicroRNAs (miRNAs) are small ncRNAs measuring ~22 nt that post-transcriptionally modulate genes (16). Numerous miRNAs are pivotal modulators in AS pathological

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processes, including immune responses, lipid and cholesterol biosynthesis, endotheliocyte vascular and biological functions, cholesterol effluence, and lipoprotein metabolism (17). miR-195-5p is located on chromosome 17p13.1 and belongs to the miR-15a/b/16/195/497 family (18). miR-195-5p is aberrantly expressed and serve a role as tumor suppressor in a number of types of cancer in humans, including prostate (19) and breast cancer (20), and hepatocellular carcinoma (21); however, its functions in AS is rarely known.

In the present study, lncRNA *CTBPI-AS2* was downregulated among patients with AS, and miR-195-5p was identified as a potential target gene. The study aimed to investigate the possible effects and molecular characteristics of *CTBPI-AS2* in VSMC progression, to determine the potential targets of AS treatment.

Materials and methods

Clinical samples. The present study was approved by The First Affiliated Hospital of Anhui Medical University. All samples were collected between February 2015 and October 2019. The subjects, aged between 50-70 years (mean \pm standard deviation, 61.21 ± 1.302 years), including 21 males and 3 females, provided written informed consent. Then, 10 ml blood samples were collected from 24 patients with untreated AS and 24 healthy volunteers (age range, 50-70 years; mean \pm standard deviation, 58.88 ± 1.081 years; 20 males and 4 females) into anticoagulant-free centrifuge tubes in the hospital. The healthy volunteers also provided written informed consent. The enrollment criteria for healthy volunteers were as follows: Individuals without AS, inflammatory diseases, malignant tumors, autoimmune diseases, or recent infection (<1 month). The collected blood samples were preserved at room temperature for 1 h and centrifuged to extract serum at $1,006 \times g$, 4°C for 5 min. TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for RNA isolation from the serum.

Cell culture. Human aorta VSMCs (HA-VSMCs) were preserved in F-12 K medium acquired from American Type Culture Collection, and the medium contained 10% fetal bovine saline (Invitrogen; Thermo Fisher Scientific, Inc.), 0.05 mg/ml ascorbic acid, 0.01 mg/ml transferrin, 0.01 mg/ml insulin, 10 ng/ml sodium selenite, 10 mM TES and 10 mM HEPES (Sigma-Aldrich; Merck KGaA), and 0.03 mg/ml endothelial cell growth supplement purchased from Cell Applications Inc., in a humid incubator under 5% CO_2 and 37°C .

Cell transfection and treatment. Full length *CTBPI-AS2* sequences were amplified via PCR and subcloned into pcDNA3.1 vectors (Invitrogen; Thermo Fisher Scientific, Inc.) to generate pcDNA-*CTBPI-AS2* overexpression (OE) plasmids. The small interfering RNAs (siRNAs) specific to *CTBPI-AS2*, miR-195-5p mimic, and miR-195-5p inhibitor and their corresponding negative controls si negative control (NC), mimic NC, and inhibitor NC were designed and synthesized by Shanghai GenePharma Co. Ltd. Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was utilized for cell transfection following the manufacturer's protocol. HA-VSMCs were treated with ox-LDL (0, 25, 50 and $100 \mu\text{g}/\text{ml}$; Beijing Biosynthesis Biotechnology Co., Ltd.) for

1 day to determine its effects on *CTBPI-AS2* and miR-195-5p expression levels. ROC-325 (MedChemExpress), a novel inhibitor of autophagy, was dissolved in double distilled water (ddH_2O) and applied to inhibit cell autophagy. Following treatment with $5 \mu\text{M}$ ROC-325 for 24 h, the levels of autophagy proteins LC3/II and Beclin1 were examined.

Reverse transcription-quantitative (RT-q)PCR. TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for total RNA isolation following the manufacturer's guidelines. Then, $1 \mu\text{g}$ RNA from each serum or cell sample was reverse transcribed into first-strand cDNA using RT primers (miR-195-5p and U6 small nucleolar RNA) or random primers (*CTBPI-AS2* and β -actin) and M-MLV reverse transcriptase from Invitrogen; Thermo Fisher Scientific, Inc. *CTBPI-AS2*, autophagy related 14 (ATG14), GAPDH, miR-195-5p and U6 expression levels were examined using qPCR primers and SYBR Green Real-Time PCR Master Mix provided by Toyobo Life Sciences. The $2^{-\Delta\Delta\text{C}_q}$ method (22) was used to calculate the relative gene expression normalized by GAPDH and U6. The sequences of the primers were listed below: *CTBPI-AS2* forward, 5'-GAGCCCTGATTTACCGTCCG-3' and reverse, 5'-AAGGGGAGACGTAGGCTTCT-3'; ATG14 forward, 5'-CATAACAACCCCGCTACAC-3' and reverse, 5'-TGC GTTCAGTTTCCTCACTG-3'; miR-195-5p forward, 5'-GGG GTAGCAGCACAGAAAT-3' and reverse, 5'-TCCAGTGCG TGTCGTGGA-3'; U6 forward, 5'-CTCGCTTCGGCAGCA GCACATATA-3' and reverse, 5'-AAATATGGAACGCTT CACGA-3'; GAPDH forward, 5'-GAAGAGAGAGACCCT CACGCTG-3' and reverse, 5'-ACTGTGAGGAGGGGAGAT TCAGT-3'.

Western blot analysis. RIPA lysis buffer (Beyotime Institute of Biotechnology) and a Pierce bicinchoninic acid Protein Assay kit (Thermo Fisher Scientific, Inc.) were used to isolate and quantify whole proteins, respectively. Then, proteins ($50 \mu\text{g}$) were separated using 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). The membranes were blocked with fat-free milk (5%) for 1 h at room temperature and incubated at 4°C overnight with primary antibodies against the following: Proliferating cell nuclear antigen (PCNA; cat. no. ab92552; 1:1,000), GAPDH (cat. no. ab181602; 1:2,500), proliferation marker protein Ki-67 (cat. no. ab92742; 1:2,000), Beclin-1 (cat. no. ab62557; 1:1,000) and microtubule-associated proteins 1A/1B light chain 3B (LC3; cat. no. ab51520; 1:1,000). All primary antibodies were from Abcam. The membranes were then incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibody (cat. nos. ab97040 and ab97080, respectively; 1:3,000) from Abcam. Clarity Max[™] Western ECL Substrate provided by Bio-Rad Laboratories, Inc., was used to amplify certain protein signals. Image J (V1.8.0.112; National Institutes of Health) was used to perform the densitometric analysis.

CCK-8 experiment. Subsequent to seeding onto 96-well plates at a density of $\sim 1 \times 10^4/\text{well}$ overnight, HA-VSMCs were transfected for 0, 24, 48 or 72 h, and then $10 \mu\text{l}$ Cell Counting Kit-8 solution (MedChemExpress) was added to each well and incubated at 37°C for 3 h. Cell proliferation was evaluated by measuring the absorbance (A_{450}).

Colony formation experiment. Cells in the logarithmic growth phase were digested into a single cell layer using 0.25% trypsin and then suspended in F-12 K medium. Each group was inoculated with 200 cells per dish. Following cultivation for 15 days in complete medium, the transfected HA-VSMCs were fixed using 75% methanol for 15 min at room temperature and stained with 0.1% crystal violet solution (Sigma-Aldrich; Merck KGaA) at room temperature for 20 min. Finally, after washing, images of the cells were captured and cells were counted using light microscopy (magnification, x10), and the colony-forming efficiency was calculated using the following formula: Colony forming efficiency=clone number/inoculated cell number x100%.

Intracellular protein segregation. A Cytoplasmic and Nuclear RNA Purification kit (Norgen Biotek Corp.) was used for separation and purification of RNAs in the nucleus and cytoplasm of HA-VSMCs according to the manufacturer's guidelines. Next, *CTBPI-AS2*, *GAPDH* and *U6* expression levels in nuclear and cytoplasmic segments were independently examined via RT-qPCR.

Bioinformatics analysis. DIANA tools-miRGen v3 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=mirgen v3%2Findex) was applied to predict the target gene of *CTBPI-AS2*.

Luciferase reporter assay. PCR amplification was implemented for specific segments of *CTBPI-AS2* and *ATG14* 3' untranslated regions (UTRs) with miR-195-5p binding sites, and these segments were created into psiCHECK-2 vector (Promega Corporation). Therefore, wild-type lncRNA *CTBPI-AS2* and *ATG14* reporter plasmids were generated. A QuikChange Multi Site-Directed Mutagenesis kit (Stratagene; Agilent Technologies, Inc.) was used to generate mutated lncRNA *CTBPI-AS2* and *ATG14* reporters with mutational miR-195-5p binding sites. HA-VSMCs were then transfected with miRNAs or plasmids and the created luciferase reporters, independently. After 48 h, a dual-luciferase reporter assay kit (Promega Corporation) was utilized to detect cell luciferase activity according to the protocols of the manufacturer.

Statistical analysis. SPSS 22.0 (IBM Corp.) was used for statistical analysis of all data. Each experiment was repeated a minimum of three times, and data are presented as mean \pm standard error of the mean. Pearson's analysis was performed to analyze the correlation between miR-195-5p and lncRNA *CTBPI-AS2*. Comparisons between groups were performed using Student's t-test or one-way ANOVA followed by Bonferroni's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CTBPI-AS2 level is decreased and miR-195-5p is increased in AS serum and HA-VSMCs treated with ox-LDL. *CTBPI-AS2* and miR-195-5p expression levels in the serum of patients with AS and HA-VSMCs treated with ox-LDL were examined. RT-qPCR experiments revealed that *CTBPI-AS2* level decreased significantly (Fig. 1A) and miR-195-5p level

increased significantly (Fig. 1B) in the serum of patients with AS compared with that of healthy volunteers. Treatment with ox-LDL dose-dependently decreased *CTBPI-AS2* expression levels (Fig. 1C) and increased miR-195-5p expression levels (Fig. 1D) in HA-VSMCs. *CTBPI-AS2* and miR-195-5p may be pivotal factors in AS advancement.

OE of CTBPI-AS2 inhibits proliferation in HA-VSMCs treated with ox-LDL and triggers their autophagy. The effects of *CTBPI-AS2* on the proliferation and autophagy of HA-VSMCs treated with ox-LDL was detected by the use of OE-*CTBPI-AS2*. The RT-qPCR results indicated that OE-*CTBPI-AS2* markedly increased *CTBPI-AS2* expression compared with the negative control (NC) in HA-VSMCs treated with ox-LDL (Fig. 2A). The CCK-8 assay results suggested that *CTBPI-AS2* markedly decreased the level of proliferation in HA-VSMCs treated with ox-LDL compared with the NC (Fig. 2B). This result was corroborated by the results from the colony formation experiment (Fig. 2C). PCNA and Ki-67 expression levels in HA-VSMCs treated with ox-LDL were examined, and their expression levels exhibited a marked decrease subsequent to *CTBPI-AS2* overexpression (Fig. 2D and E). The overexpression of *CTBPI-AS2* markedly promoted autophagy, and resulted in significant increases in the lipid-modified LC3/GAPDH ratio and in the Beclin-1 protein expression level in HA-VSMCs compared with the NC (Fig. 2F). Rescue experiments were conducted to determine the association between VSMC proliferation and autophagy. As demonstrated in Fig. 2G, treating cells with 5 μ M ROC-325, an inhibitor of autophagy, in combination with *CTBPI-AS2* OE plasmids partly reversed the ox-LDL-mediated increase in cell proliferation. *CTBPI-AS2* inhibited the proliferation of HA-VSMCs treated with ox-LDL by increasing the levels of autophagy.

CTBPI-AS2 prevents miR-195-5p expression by direct interaction. Bioinformatics analysis was used to ascertain the potential targets of *CTBPI-AS2*. Complementary sites between *CTBPI-AS2* and miR-195-5p were identified (Fig. 3A). As demonstrated in Fig. S1, other potential target miRNAs were also detected; however, no significant difference between the patients with AS and the controls was identified (Fig. S1A-F). *CTBPI-AS2* was primarily located in the cytoplasm of HA-VSMCs, as verified by the intracellular segregation experiment, indicating the potential interaction between *CTBPI-AS2* and miR-195-5p due to their shared location (Fig. 3B). The luciferase reporter assay verified that treatment with the miR-195-5p mimic decreased the luciferase activity in wild-type *CTBPI-AS2* reporter plasmid by ~48% compared with that in the scramble control (Fig. 3C). Nevertheless, miR-195-5p did not affect the luciferase activity of the mutated *CTBPI-AS2* reporter plasmid, which had mutated assumed binding sites between *CTBPI-AS2* and miR-195-5p (Fig. 3C). An RT-qPCR assay was implemented to determine the effects of *CTBPI-AS2* on miR-195-5p expression. *CTBPI-AS2* upregulation decreased miR-195-5p expression, whereas si-*CTBPI-AS2* transfection significantly increased miR-195-5p expression (Fig. 3D and E). miR-195-5p expression exhibited an inverse correlation with *CTBPI-AS2* levels in the serum of 24 patients with AS (Fig. 3F). These data

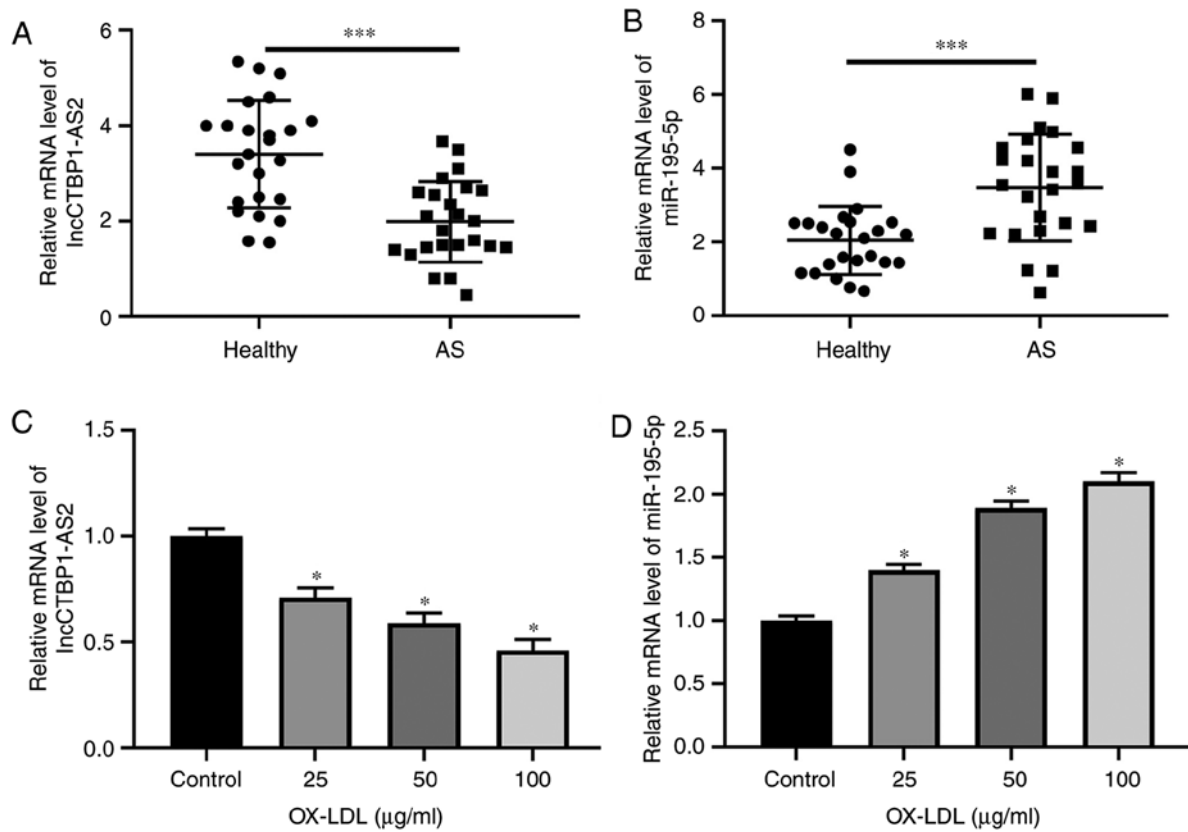


Figure 1. CTBP1-AS2 level is decreased and miR-195-5p is increased in AS serum and HA-VSMCs treated with ox-LDL. The expression of levels of (A) *CTBP1-AS2* and (B) miR-195-5p in the serum of 24 patients with AS and 24 healthy volunteers. HA-VSMCs were treated with ox-LDL (0, 25, 50 and 100 $\mu\text{g/ml}$) for 1 day, and the expression levels of (C) *CTBP1-AS2* and (D) miR-195-5p were measured. * $P<0.05$ and *** $P<0.001$. miR, microRNA; AS, atherosclerosis; HA-VSMCs, human aorta vascular smooth muscle cells; ox-LDL, oxidized low-density lipoprotein; lnc, long non-coding RNA.

confirmed that *CTBP1-AS2* inhibited miR-195-5p expression by direct reciprocal action.

miR-195-5p mimic counteracts the effects of CTBP1-AS2 on the levels of proliferation and autophagy in HA-VSMCs treated with ox-LDL. The RT-qPCR assay results demonstrated that the miR-195-5p mimic induced miR-195-5p expression and promoted miR-195-5p expression via *CTBP1-AS2* in HA-VSMCs treated with ox-LDL (Fig. 4A). Co-treatment with miR-195-5p and ox-LDL promoted HA-VSMCs to proliferate and form colonies (Fig. 4B and C). The CCK-8, colony formation and western blot assays results showed that the effect of *CTBP1-AS2* on cell growth was remarkably abrogated by the miR-195-5p mimic, as demonstrated by increased cell proliferation (Fig. 4B), colony formation (Fig. 4C), and PCNA and Ki-67 expression levels (Fig. 4D). miR-195-5p significantly counteracted *CTBP1-AS2*-mediated autophagy, as shown by the increased level of the autophagy-associated proteins (Fig. 4E). miR-195-5p can modulate the *CTBP1-AS2*-mediated effects by inhibiting proliferation and promoting autophagy in HA-VSMCs treated with ox-LDL.

ATG14 is a target of miR-195-5p. Multiple studies have revealed that lncRNAs modulate target mRNA expressions via their function as competing endogenous RNAs (ceRNAs) of miRNAs (23-25). Bioinformatics methods were used to find the potential targets of miR-195-5p. The results revealed that potential binding sequences of miR-195-5p were present in the

ATG14 3'UTR (Fig. 5A). Concomitantly, the relative expression levels of other potential target genes in AS were also detected, but no significant difference was observed (Fig. S1G-L). Subsequently, the dual luciferase reporter assay showed that miR-195-5p evidently inhibited the luciferase activity of the wild-type ATG14 reporter plasmid, but did not change that of the mutated ATG14 reporter (Fig. 5B). The data from the clinical samples indicated that ATG14 exhibited a high expression level in the serum of healthy volunteers compared with those of patients with AS (Fig. 5C). The RT-qPCR assay data demonstrated that the ox-LDL-mediated decrease in ATG14 was dose-dependent (Fig. 5D). The RT-qPCR results also showed that ATG14 expression was positively correlated with *CTBP1-AS2* expression (Fig. 5E) but inversely correlated with miR-195-5p expression (Fig. 5F) in the serum of 24 patients with AS. In addition, *CTBP1-AS2* induced ATG14 expression, and si-*CTBP1-AS2* decreased ATG14 expression in HA-VSMCs treated with ox-LDL (Fig. 5G and H). miR-195-5p upregulation decreased ATG14, but its inhibitor increased ATG14 (Fig. 5I and J). Western blot analysis demonstrated that decreased *CTBP1-AS2* or miR-195-5p markedly inhibited ATG14 expression compared with their respective control (si-NC or mimic-NC). Ectopically expressed *CTBP1-AS2* abrogated the miR-195-5p-mediated inhibition of ATG14 expression in HA-VSMCs when the cells were treated with ox-LDL (Fig. 5K). Collectively, these results suggested that *CTBP1-AS2* promoted ATG14 expression by serving as a ceRNA for miR-195-5p in HA-VSMCs.

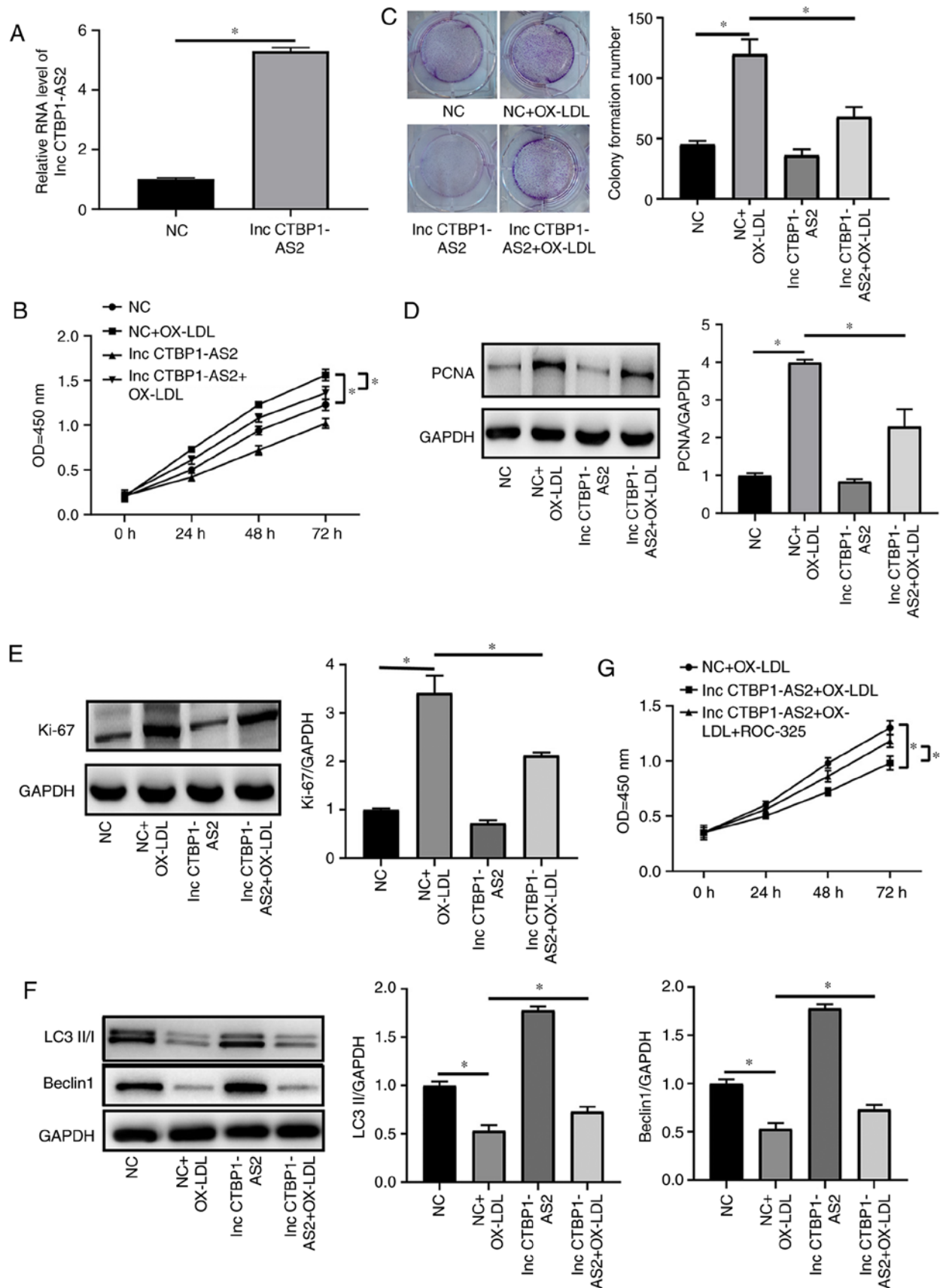


Figure 2. Overexpression of CTBP1-AS2 inhibits the level of proliferation in HA-VSMCs treated with ox-LDL and triggers their autophagy. *CTBP1-AS2*-treated HA-VSMCs were treated with 50 μ g/ml ox-LDL, and (A) *CTBP1-AS2* expression, (B) cell formation, (C) colony formation, (D) PCNA and (E) Ki-67 expression, and (F) autophagy-associated proteins LC3 and Beclin-1 expression were measured. (G) The proliferation of cells co-transfected with *CTBP1-AS2* and ROC-325 was detected. Data are presented as mean \pm standard error of the mean of at least 3 independent experiments. * $P < 0.05$. HA-VSMCs, human aorta vascular smooth muscle cells; ox-LDL, oxidized low-density lipoprotein; PCNA, proliferating cell nuclear antigen; Ki-67, proliferation marker protein Ki-67; LC3, microtubule-associated proteins 1A/1B light chain 3B; LC3I, cytoplasmic LC3; LC3II, lipid-modified LC3; NC, negative control; OD, optical density; Inc, long non-coding RNA.

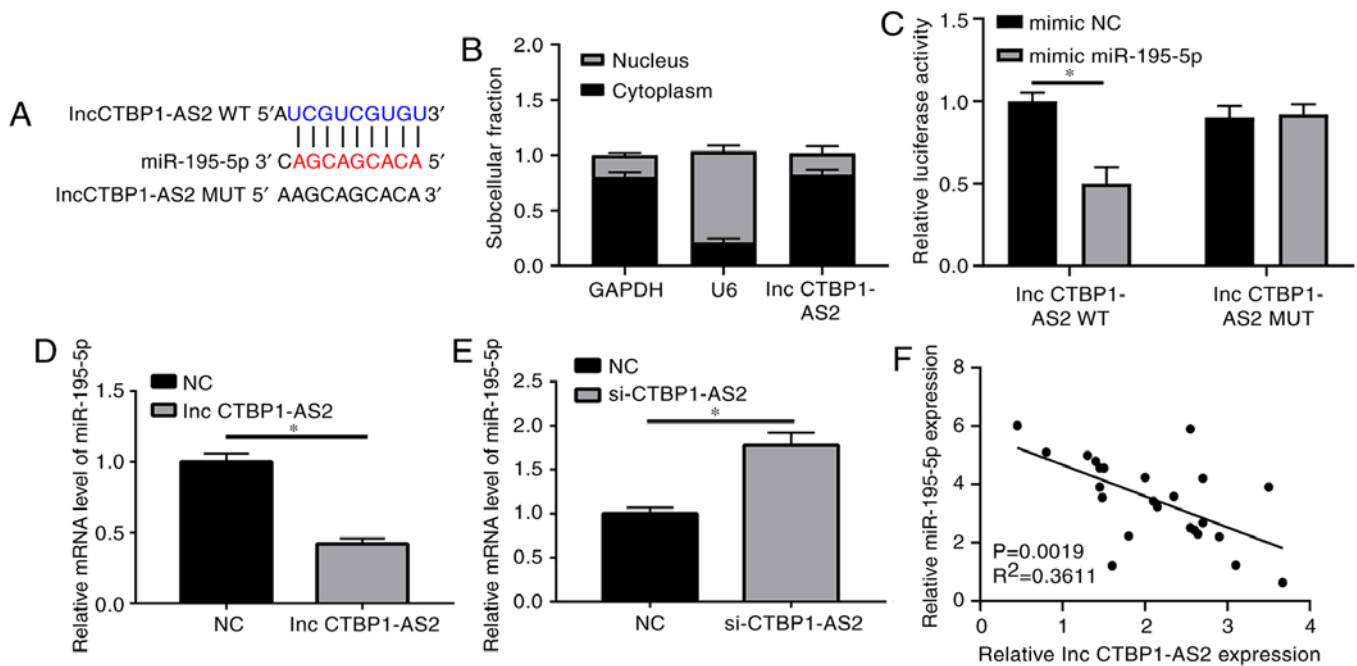


Figure 3. *CTBP1-AS2* prevents miR-195-5p expression by direct interaction. (A) Predicted binding sites between *CTBP1-AS2* and miR-195-5p, and the mutated sites in the MUT *CTBP1-AS2* reporter plasmid. GAPDH, U6 and *CTBP1-AS2* expression levels in the cytoplasm and nucleus of HA-VSMCs. GAPDH was used as a transcript control in the cytoplasm and U6 as a transcript control in the nucleus. (B) The percentage fractions of GAPDH, U6 and *CTBP1-AS2* proteins in the cytoplasm and nucleus. (C) Luciferase activity of HA-VSMCs treated with WT or MUT *CTBP1-AS2* reporter plasmids and miR-195-5p or mimic NC. (D) Decreased miR-195-5p expression following upregulation of *CTBP1-AS2* expression, and (E) increased miR-195-5p expression following downregulation of *CTBP1-AS2* expression. (F) *CTBP1-AS2* was inversely correlated with miR-195-5p expression in patients with atherosclerosis. Data are presented as mean \pm standard error of the mean of at least 3 independent experiments. * $P<0.05$. miR, microRNA; WT, wild-type; MUT, mutant; HA-VSMCs, human aorta vascular smooth muscle cells; NC, negative control; si, small interfering RNA; Inc, long non-coding RNA.

Discussion

Despite substantial advances in research investigating its diagnosis and treatment methods, AS remains a leading threat to human health with high incidence and mortality rates worldwide (26). ncRNAs, including miRNAs and lncRNAs, exert pivotal functions in AS development (27,28). In the present study, bioinformatics analysis was used to identify the possible targets of *CTBP1-AS2* and further investigate its molecular mechanism. The results suggested that miR-195-5p may act as a downstream gene of *CTBP1-AS2*. miR-195-5p, belonging to the miR-195 family, modulates core processes in cells, including cell cycle progression, proliferation, invasion, migration and differentiation (29,30). miR-195 regulates the proliferation and migration of VSMCs, induces cytokine secretion mode, and prevents neointimal generation in the cardiovascular system (31).

Ox-LDL can trigger AS by promoting EC activation and dysfunction, VSMC behavior, foam cell generation and other mechanisms (32). As a core vascular cell type, VSMCs are implicated in arterial wall reconstruction, which are important for maintaining blood flow in vessels (33,34). As such, the present study was designed to determine the role of *CTBP1-AS2* in AS regulation by controlling miR-195-5p in HA-VSMCs treated with ox-LDL. A decreased level of *CTBP1-AS2* and increased level miR-195-5p in the serum of patients with AS were detected. Furthermore, when VSMCs were treated with ox-LDL, the decrease of *CTBP1-AS2* and increase of miR-195-5p were ox-LDL dose-dependent.

Autophagy is a self-degradative process mediated by lysosomes and has a critical effect on maintaining cell

homeostasis (35); it is implicated in multiple states of vascular diseases, such as hypertension, atherosclerosis and restenosis (36). Autophagy restrained the formation of ox-LDL-provoked VSMCs (37). In the present study, it was identified that *CTBP1-AS2* was downregulated in AS and over-expressed *CTBP1-AS2* could promote autophagy in VSMCs. According to a previous study (38), enhanced autophagy can inhibit AS, and inhibition of autophagy may aggravate the occurrence of AS (35). Therefore, the low expression levels of *CTBP1-AS2* may promote the development of AS by inhibiting autophagy. In addition, the abnormal proliferation of VSMCs is an important pathophysiological process in the development of atherosclerosis, and the inhibition of VSMCs proliferation may delay disease progression (39). In the present study, it was also identified that the upregulated *CTBP1-AS2* impaired the proliferation of VSMCs; therefore, the downregulation of *CTBP1-AS2* may accelerate the progression of AS by promoting the proliferation of VSMCs. The results of the present study also demonstrated that excessive autophagy may trigger autophagy-mediated cell death. However, a systematic review reported that active autophagy is able to induce autophagic death of VSMCs, resulting in decreased synthesis of collagen leading to the destabilization of plaque (8). These contradicting results may be due to the fact that the present study explored the progress of AS from a different perspective. The present study focused on the association between proliferation and autophagy of VSMCs, however the aforementioned systematic review may focus on the relationship between autophagy and the synthesis of collagen, which is related to the stability of plaque. However,

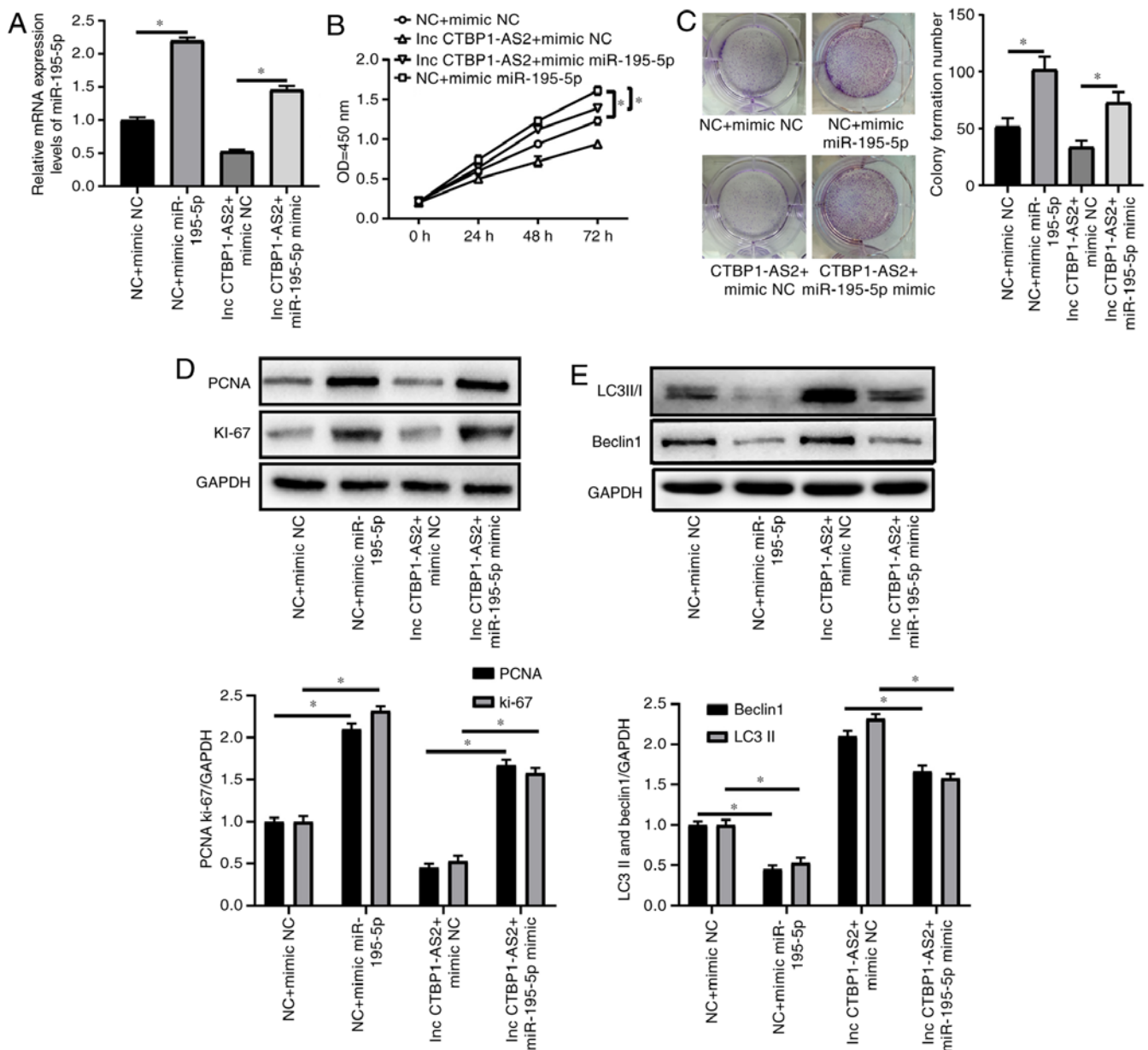


Figure 4. miR-195-5p mimic counteracts the effects of CTBP1-AS2 on the levels of proliferation and autophagy in HA-VSMCs treated with ox-LDL. (A-C) HA-VSMCs were transfected with si NC + mimic NC, si NC + mimic miR-195-5p, *CTBP1-AS2* + mimic NC, or *CTBP1-AS2* and mimic miR-195-5p and then treated with ox-LDL (50 μ g/ml) for 1 day, and (A) miR-195-5p, (B) cell proliferation (C) and colony formation were assessed. (D and E) HA-VSMCs were transfected with si NC + mimic NC, si NC + mimic miR-195-5p, *CTBP1-AS2* + mimic NC, or *CTBP1-AS2* and mimic miR-195-5p and then treated with ox-LDL (50 μ g/ml) for 1 day, and the levels of (D) PCNA and Ki-67, and (E) LC3 and Beclin-1 expression were detected. Data are presented as mean \pm standard error of the mean of at least 3 independent experiments. * P <0.05. miR, microRNA; HA-VSMCs, human aorta vascular smooth muscle cells; NC, negative control; si, small interfering RNA; PCNA, proliferating cell nuclear antigen; Ki-67, proliferating cell nuclear antigen; LC3, microtubule-associated proteins 1A/1B light chain 3B; LC3I, cytoplasmic LC3; LC3II, lipid-modified LC3; OD, optical density; Inc, long non-coding RNA.

the role of autophagy in AS is not clear at present (8,40). In addition, it may exert its roles through different ways. The results of the present study provide new insights concerning the association between autophagy and AS. This topic should also be investigated in more detail to improve understanding on the mechanism of autophagy during AS progression. This may help find a balance point where autophagy inhibits the proliferation of VSMCs but does not cause the destabilization of plaques in the development of AS.

The mechanism of *CTBP1-AS2* in AS was verified by determining whether its effects of the proliferation and autophagy of HA-VSMCs was mediated through miR-195-5p. According

to the results of the present study, the anti-proliferation and pro-autophagy effects modulated by *CTBP1-AS2* were markedly inhibited by the decrease in miR-195-5p expression in HA-VSMCs treated with ox-LDL.

miRNAs modulate the translation or stability of target mRNAs. Therefore, the possible target mRNAs of miR-195-5p were predicted, and the role of ATG14 as the potential target of miR-195-5p was verified by the luciferase reporter assay. ATG14 is an essential autophagy-specific regulator of the class III phosphatidylinositol 3-kinase complex (41). Autophagy occurs in developing atherosclerotic plaques (42). ATG14 mRNA expression was demonstrated to exhibit a positive

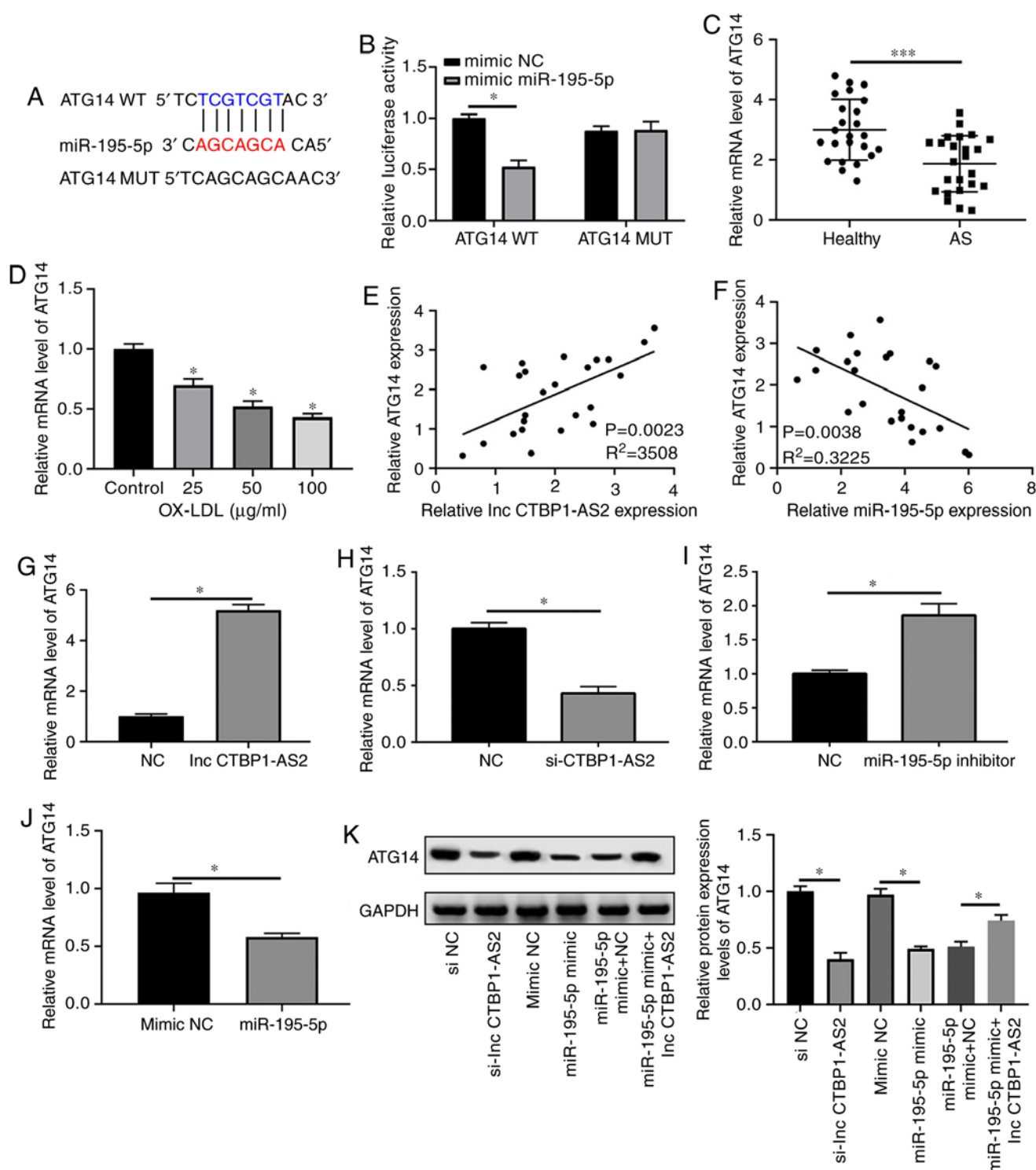


Figure 5. ATG14 is a target of miR-195-5p. (A) Predicted binding sequences between miR-195-5p and ATG14 3' untranslated region and the mutated sites in mutated ATG14 reporter plasmid. (B) Luciferase activity for 48 h after co-transfection with WT or MUT ATG14 reporter and mimic miR-195-5p/NC into HA-VSMCs. (C) ATG14 expression in the serum of 24 healthy volunteers and 24 patients with AS. (D) HA-VSMCs were treated with ox-LDL (0, 25, 50 or 100 μ g/ml) for 1 day, and ATG14 levels were measured. (E and F) Correlation analysis between ATG14 and (E) CTBP1-AS2 or (F) miR-195-5p in the serum of 24 patients with AS. (G) Increased ATG14 expression following the increase in CTBP1-AS2 expression, and (H) decreased ATG14 expression following the downregulation of CTBP1-AS2 expression. (I) Downregulation of miR-195-5p increased ATG14 expression levels. (J) Upregulation of miR-195-5p decreased ATG14 levels. (K) Effects of *CTBP1-AS2* knockdown (si *CTBP1-AS2*), miR-195-5p overexpression (miR-195-5p), and *CTBP1-AS2* overexpression on ATG14 protein expression assessed using western blot analysis at 48 h after transfection in HA-VSMCs treated with ox-LDL. Data are presented as mean \pm standard error of the mean of at least 3 independent experiments. * P <0.05 and *** P <0.001. ATG14, autophagy related 14; miR, microRNA; WT, wild-type; MUT, mutant; NC, negative control; HA-VSMCs, human aorta vascular smooth muscle cells; ox-LDL, oxidized low-density lipoprotein; si, small interfering RNA; Inc, long non-coding RNA.

correlation with that of *CTBP1-AS2* but a negative correlation with that of miR-195-5p in the serum of patients with

AS. miR-195-5p upregulation decreased the ATG14 protein expression level, and *CTBP1-AS2* increased the level slightly.

Therefore, *CTBP1-AS2* may increase ATG14 expression via its role as a ceRNA for miR-195-5p in HA-VSMCs.

However, there were certain limitations in the present study; for example, only the proliferation and autophagy of VSMCs were detected in the cell function experiments without cell cycle and apoptosis. However, it is important to note that, to the best of our knowledge, there have only been few studies investigating the association between proliferation and autophagy of VSMCs; therefore, the present study aimed to reveal the association between proliferation and autophagy of VSMCs. Finally, as there was no access to a working flow cytometer, the levels of apoptosis could not be examined. When this has been resolved, it will be included in our future studies.

The results of the present study demonstrated the involvement of *CTBP1-AS2*/miR-195-5p/ATG14 regulatory axis in the proliferation and autophagy of HA-VSMCs and suggest the potential use of *CTBP1-AS2* in AS prevention. However, the use as a drug target and mechanisms of *CTBP1-AS2* in VSMCs and AS should be studied further in *in vivo* animal models.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YW and WHG performed the experiments and generated data. CXZ analyzed the data. SLG designed the experiments. YW and WHG wrote the manuscript. SLG and CXZ revised the manuscript. All authors gave final approval of the version to be published and agreed to be 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 authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The First Affiliated Hospital of Anhui Medical University (Hefei, China). All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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