

ZNF295-AS1 inhibits autophagy *via* the ZNF295-AS1/miR-508-5p/ATG7 axis in AS

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Abstract. – **OBJECTIVE:** As a result of gene-environment interactions, the incidence of atherosclerosis (AS) is rapidly increasing worldwide. Autophagy in endothelial cells is a key process of AS and is difficult to control when it becomes excessive in the end stage of AS.

MATERIALS AND METHODS: In this study, we found increased expression levels of ZNF295-AS1 in the serum of AS patients, as well as in ox-LDL-treated HUVECs. The autophagy level was also upregulated in both samples. We demonstrated that ZNF295-AS1 may interact directly with miR-508-5p to act as a miR-508-5p sponge. The negative relationship between ZNF295-AS1 and miR-508-5p indicated that ZNF295-AS1 may be an upstream suppressor of miR-508-5p.

RESULTS: ATG7 plays a critical role in autophagy and was predicted to be a target of miR-508-5p. Therefore, we overexpressed miR-508-5p, which reduced the expression level of ATG7, enhanced cell proliferation and prevented autophagy. These data indicated that the ZNF295-AS1/miR-508-5p/ATG7 axis may participate in autophagy regulation in ox-LDL-treated HUVECs. The subsequent rescue experiments revealed the specificity of the ZNF295-AS1/miR-508-5p/ATG7 axis in the contribution of ZNF295-AS1 to autophagy.

CONCLUSIONS: Overall, our findings demonstrate a novel mechanism by which ZNF295-AS1 silencing regulates ATG7 reduction and inhibits autophagy, which may delay the progression of AS. The ZNF295-AS1/miR-508-5p/ATG7 axis may be of therapeutic significance in AS.

Key Words:

Atherosclerosis, Autophagy, ZNF295-AS1.

cells from damage, while the failure or excess of autophagy results in endothelial cell apoptosis, leading to the breakdown of the integrity of the endothelium, which facilitates local lipid deposition leading to atherogenesis, plaque instability, and even acute coronary occlusion and sudden death⁵⁻⁷. Increasing evidence⁸ shows that autophagy is of great importance in AS. Nevertheless, our understanding of the mechanisms that govern autophagy in endothelial cells is still limited.

Long noncoding RNAs (lncRNAs) are found throughout the genome and serve as key regulatory hubs in AS progression¹. Previous transcriptome profiling of lncRNA expression in ox-LDL-treated HUVECs showed a significant upregulation of ZNF295-AS1 and led us to propose a possible role in AS⁹. ZNF295-AS1, also known as C21 or f121, is one of the antisense transcripts of the ZNF295 gene. It is located on chromosome 21q22.3, whose function is only partially understood¹⁰. LncRNAs function predominantly to bind miRNAs, and their interplay is an interesting topic of research due to the different cellular mechanisms involved^{11,12}. Salmena et al¹³ found ZNF295-AS1 to be a ceRNA that plays a major role in promoting directed differentiation of stem cells to neuronal cells mainly by binding to miRNAs.

In this study, we analyzed the role of ZNF295-AS1 in AS and investigated the modulatory role of ZNF295-AS1 and its downstream miRNA axis. Identification of a new mechanism for autophagy may provide strategies to delay or prevent the development of AS.

Introduction

Atherosclerosis (AS) is a diffuse, systemic process and the most common cause of cardiovascular disease, cerebral infarction and peripheral vascular disease¹⁻³. Endothelial cell injury is a major step in the pathological progression of AS⁴. Upon injury, endothelial cell autophagy may occur to protect

Materials and Methods

Cell Culture and Treatment

Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Bank of Chi-

nese Academy of Sciences (Shanghai, China). HUVECs were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and cultured at 37°C in a 5% CO₂ atmosphere. Where indicated, cells were treated with ox-LDL (0, 25, 50, 100 µg/ml) (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours.

RNA Extraction and Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. Then, RNA was treated with DNase I to remove genomic DNA. Reverse transcription was carried out to generate first-strand cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). qPCR was performed using SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China) in the StepOne Plus system (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocols. The expression of ZNF295-AS1, miR-508-5p and ATG7 was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression of miRNAs was normalized against that of GAPDH. The primers used were as follows: for ZNF295-AS1, CCCAGGAGGGAGGTGATA (forward) and TGGGTAGCTTGTGAACCACC (reverse); for ATG7, 5'-ACCCAGAAGAAGCTGAACGA-3' (forward) and 5'-CTCATTTGCTGCTTGTTCCTCA-3' (reverse); and for GAPDH, 5'-ACAGTCCATGCCATCACTG (forward) and 5'-GTGAGGGTCTCTC TCTTCCT-3' (reverse). The expression of RNAs was calculated using the comparative Ct method.

Transient Transfection

Transfection of ZNF295-AS1 siRNA was performed using Lipofectamine iMAX (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. Double-stranded miRNA mimics and their respective negative control mimics (NC) (GenePharma, Shanghai, China) were transfected into cells at a final concentration of 50 nM. After 24 h, cells were exposed to ox-LDL (100 µg/ml) (Sigma-Aldrich, Shanghai, China) for additional 24 h.

Western Blotting (WB)

Protein samples were prepared by lysing cells in modified radio-immunoprecipitation assay

(RIPA) buffer. Identical quantities of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose filter membranes. After incubation with antibodies specific to ATG7 (Abcam, Hong Kong, China), LC3 (Abcam, Hong Kong, China), or GAPDH (Abcam, Hong Kong, China), the blots were incubated with anti-rabbit IgG H&L (HRP) or anti-mouse IgG H&L and were detected using ChemiDoc Imaging Systems (Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control for WB assays.

MTT Assay

Cell proliferation was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 5 × 10⁴ cells/ml were seeded in a 96-well plate. After treatment, 100 µl Dulbecco's Modified Eagle's Medium (DMEM) and 100 µl diluted reagent were added to each well for incubation at 37°C. Absorbance at 570/650 nm was evaluated by a SpectraMax Paradigm Multi-Mode Detection Platform (Molecular Devices, San Jose, CA, USA). Cell proliferation was calculated as the ratio relative to the proliferation of the dimethyl sulfoxide (DMSO) group (set as 1.0).

Transmission Electron Microscopy

HUVECs were fixed in 2.5% glutaric dialdehyde overnight at 4°C and washed with phosphate-buffered saline (PBS) three times, then post-fixed in 1% osmium tetroxide for 1-2 hours, dehydrated in a graded series of ethanol concentrations, and embedded in Sparr resin. Sections of 50-70 nm thickness were placed on copper grids that were double stained with uranyl acetate and lead citrate. Samples were examined with a H-7650 transmission electron microscope (Hitachi, Japan).

Luciferase Reporter Assays

A Luciferase reporter containing ZNF295-AS1 and miR-508-5p mimics (experimental group) or NC mimics (control group) were cotransfected into 293T cells. Cells were collected, and then a Dual-Luciferase reporter assay was performed to detect the Luciferase activity of the two groups of cells. Consequently, the relationship between ZNF295-AS1 and miR-508-5p was identified. The same assay was performed for miR-508-5p and ATG7.

Statistical Analysis

All statistical analyses were performed using the Statistical Product and Service Solution (SPSS) 18.0 software package (Chicago, IL, USA). For comparisons, Student's *t*-test, Wilcoxon signed-rank test, and Pearson's correlation analysis were performed as indicated. *p*-value < 0.05 was defined as statistically significant.

Results

ZNF295-AS1 is Upregulated During AS

To determine whether ZNF295-AS1 is involved in the regulation of human AS, we first detected ZNF295-AS1 expression in the serum of AS patients and in a cell model. In our study, a well-established experimental model of AS employing cultured HUVECs was adopted. Different concentrations of ox-LDL (100 μ M) were added to the culture medium of HUVECs for 24 h to evaluate its effect on cell proliferation and autophagy levels. At the end of incubation with ox-LDL, cell proliferation was evaluated by measuring cellular MTT, which showed a dose-dependent effect on the growth of HUVECs (Supplementary Figure 1A). In addition, the number of autophagosomes was increased by ox-LDL treatment (Supplementary Figure 1B). RT-PCR revealed that the mRNA expression level of ZNF295-AS1 was significantly increased in AS (Figure 1A). Additionally, the same results were found in ZNF295-AS1 in response to ox-LDL in HUVECs (Figure 1B). Given the critical role of autophagy in the maintenance of endothelial cell morphology and

function, we validated a concentration-dependent increase in autophagosomes and upregulation of autophagic markers, including ATG7, p62 and LC3 I/II. The results showed that ox-LDL significantly activated autophagy (Figure 1C). These results provide further support for the potential link between ZNF295-AS1 and AS.

Silencing of ZNF295-AS1 Inhibited the Autophagy Induced by ox-LDL

To further validate the important role of ZNF295-AS1 in ox-LDL-induced autophagy, we characterized the effectiveness of si-ZNF295-AS1#1 and si-ZNF295-AS1#2 in knocking down the lncRNA. Our data revealed that both si-lncRNAs resulted in ZNF295-AS1 silencing (Figure 2A). The MTT assay showed that transfection of si-ZNF295-AS1#1 and #2 effectively enhanced the proliferation of ox-LDL-treated HUVECs at selected time points (Figure 2B). The number of autophagosomes was reduced after ZNF295-AS1 silencing (Figure 2C). Decreased expression of the autophagic markers ATG7 and LC3 II/I also indicated a decreased level of autophagy (Figure 2D). These results suggest that silencing ZNF295-AS1 may inhibit ox-LDL-induced autophagy and increase the proliferation of ox-LDL-treated HUVECs.

ZNF295-AS1 Targets MiR-508-5p and Suppresses MiR-508-5p

Using a bioinformatic database (RegRNA 2.0), we predicted the potential miRNA binding sites in ZNF295-AS1 (Figure 3A). To con-

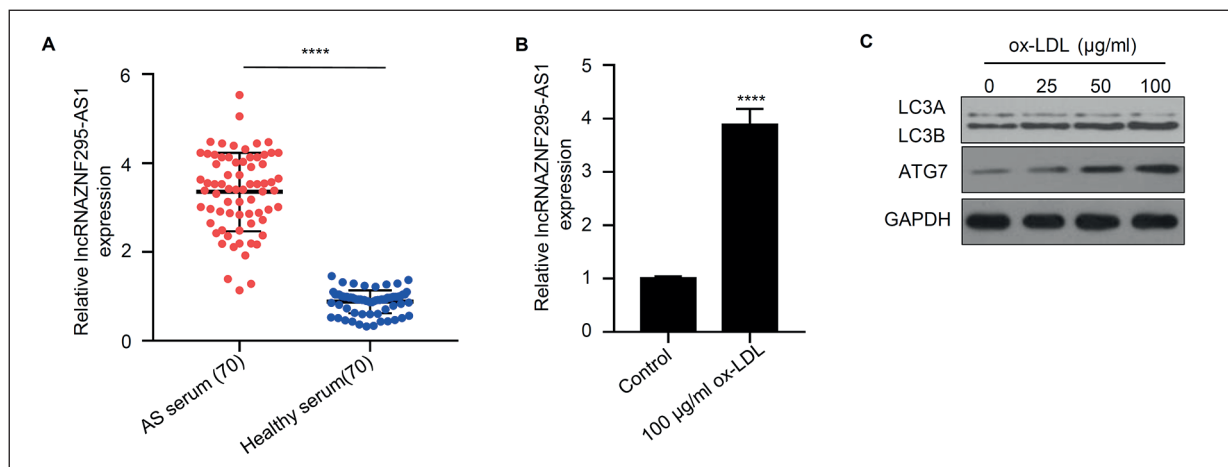


Figure 1. Expression of ZNF295-AS1 is increased in atherosclerosis. A, The mRNA expression of ZNF295-AS1 in normal and atherosclerotic patients. B, The mRNA expression of ZNF295-AS1 in the control and ox-LDL treatment groups. C, The protein expression of LC3 and ATG7. (magnification: $\times 300$). Data are means $\pm$ SD ($n = 3$). **** $p < 0.0001$.

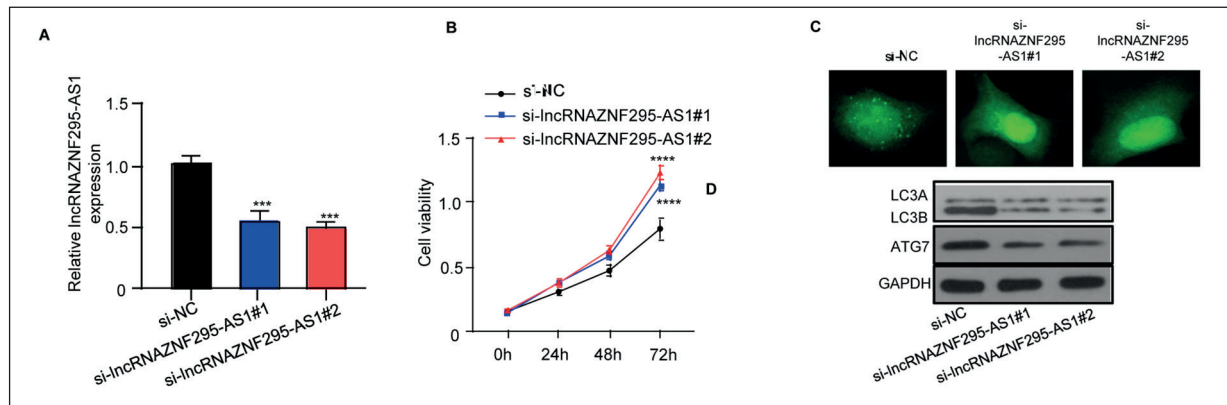


Figure 2. Silencing of ZNF295-AS1 attenuates autophagy in ox-LDL-treated HUVECs. **A**, The mRNA expression of ZNF295-AS1 in ox-LDL-treated HUVECs transfected with 2 siRNAs specific to ZNF295-AS1 after 24 h. **B**, Cell proliferation in ox-LDL-treated HUVECs transfected with 2 siRNAs after 24 h, 48 h, and 72 h. **C**, Number of autophagosomes in HUVECs transfected with 2 siRNAs. (magnification: $\times 300$). **D**, Protein expression of LC3 and ATG7. Data are means $\pm$ SD ($n = 3$). *** $p < 0.001$; **** $p < 0.0001$.

firm the interaction between ZNF295-AS1 and miR-508-5p, luciferase reporter assays were performed and showed that overexpression of miR-508-5p, not the NC, significantly inhibit-

ed the luciferase activities of transfected 293T (Figure 3B), which implied that ZNF295-AS1 is a target of miR-508-5p. The level of miR-508-5p increased after transfection with miR-508-5p

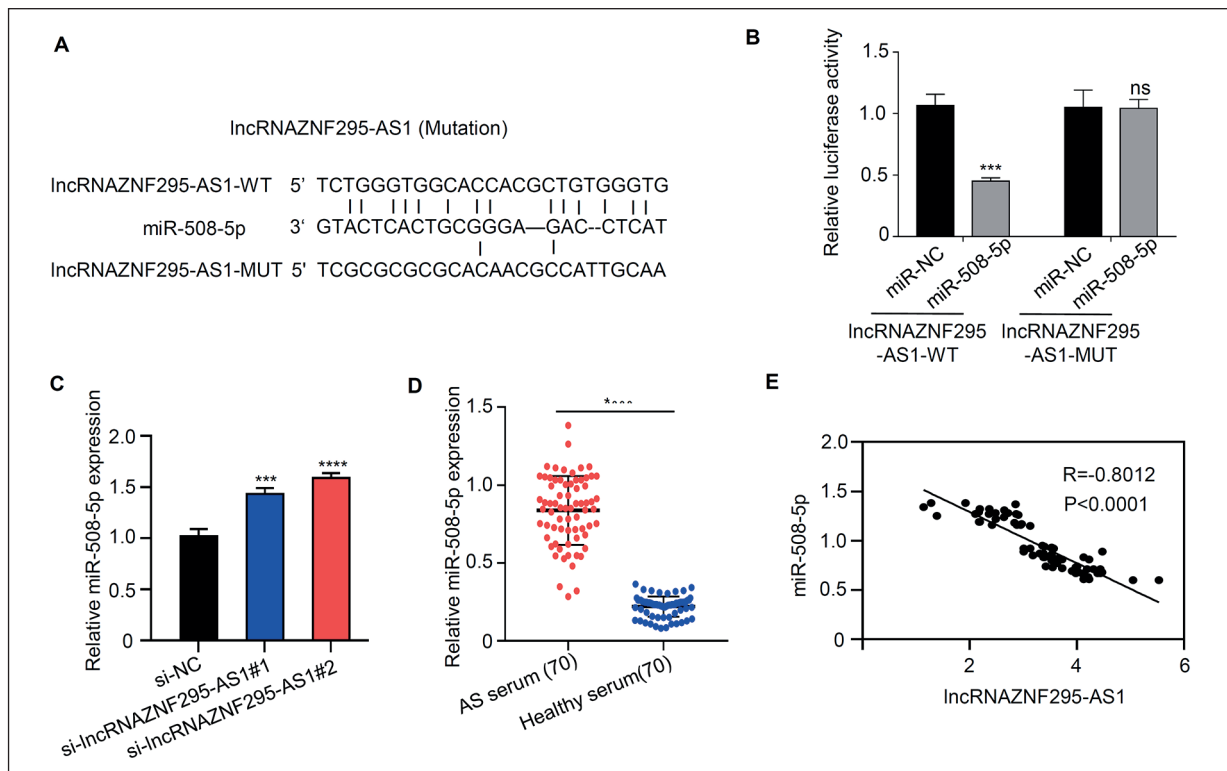


Figure 3. ZNF295-AS1 targets miR-508-5p and suppresses miR-508-5p. **A**, Schematic diagram of the predicted miR-508-5p binding sites in ZNF295-AS1. **B**, Luciferase reporter assay with co-transfection of 293T cells with wild-type or mutant ZNF295-AS1 and miR-508-5p mimic or control. **C**, mRNA expression levels of miR-508-5p. **D**, mRNA expression levels of miR-508-5p in the serum of atherosclerosis patients. **E**, Relationship between ZNF295-AS1 and miR-508-5p levels in the serum. The t -test was used for statistical analysis. Data are means $\pm$ SD ($n = 3$). *** $p < 0.001$; **** $p < 0.0001$.

mimic (Supplementary Figure 2). The results from si-lncRNAZNF295-AS1-treated HUVECs showed Figure a prominent upregulation of miR-508-5p (Figure 3C). RT-PCR revealed that the expression level of miR-508-5p was significantly decreased in AS (Figure 3D). Pearson correlation analysis between the expression levels of miR-508-5p and ZNF295-AS1 showed an inverse correlation (Figure 3E).

Overexpressed MiR-508-5p Inhibits Autophagy by Targeting ATG7

Overexpression of miR-508-5p enhanced the proliferation of ox-LDL-treated HUVECs (Figure 4A). The MTT assay showed that transfection of the miR-508-5p mimic increased the proliferation of ox-LDL-induced HUVECs at selected time points (Figure 2B). The number of autophagosomes was reduced after overexpression of miR-508-5p (Figure 4B). The autophagic markers ATG7 and LC3 II/I also indicated a decreasing level of autophagy (Figure 4C). To determine the precise target of miR-508-5p in autophagy regulation, we searched RegRNA 2.0 and found ATG7 (Figure 4D). A luciferase assay was performed

between miR-508-5p and ATG7 to confirm direct binding of miR-508-5p and ATG7 (Figure 4E). Then, we tested whether miR-508-5p could regulate the expression level of ATG7. The qRT-PCR results showed that overexpression of miR-508-5p led to a reduction in ATG7 in the cell culture model (Figure 4F).

ZNF295-AS1 Indirectly Regulates ATG7 by Sponging MiR-508-5p

Together, these data confirmed the presence of the ZNF295-AS1/miR-508-5p/ATG7 axis. However, the specific mechanism of the ZNF295-AS1/miR-508-5p/ATG7 axis in the ZNF295-AS1 contribution to autophagy remained to be determined. The overexpression of ATG7 was detected by RT-PCR (Figure 5A). The level of ATG7 increased after transfection (Supplementary Figure 3). In ox-LDL-treated HUVECs, the miR-508-5p inhibitor restored the mRNA (Figure 5B) and protein (Figure 5C) expression levels of ATG7, which were reduced upon ZNF295-AS1 silencing, indicating the importance of ZNF295-AS1/miR-508-5p in the regulation of ATG7. ZNF295-

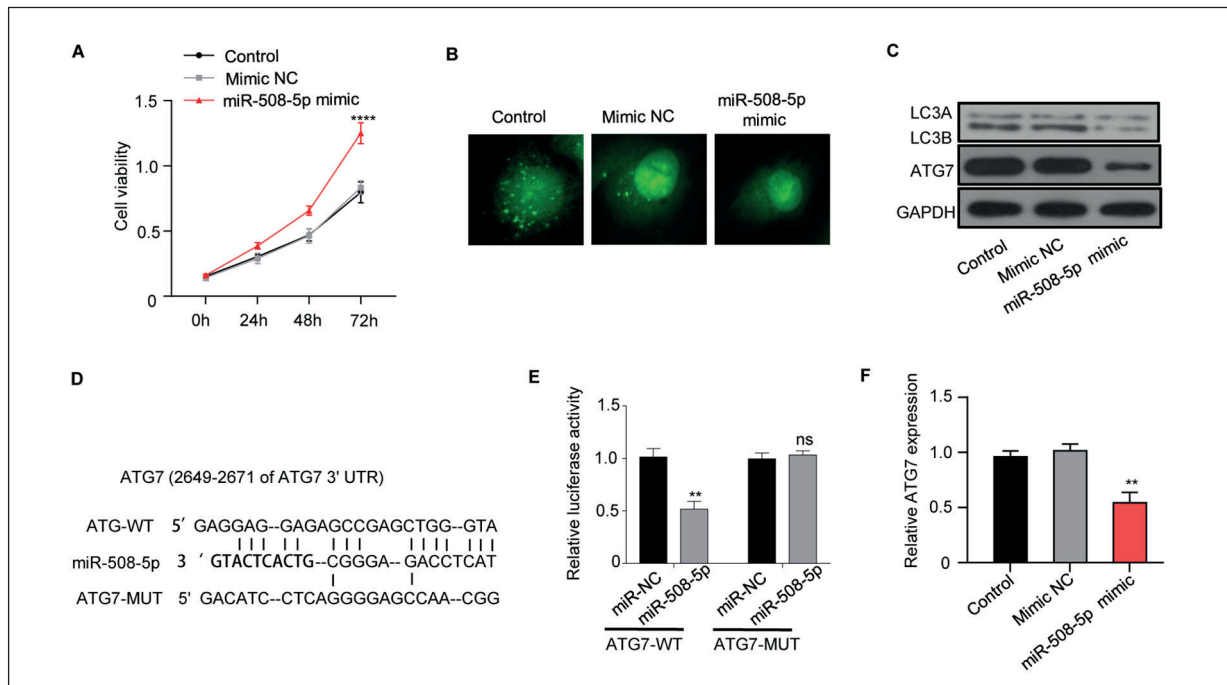


Figure 4. Overexpression of miR-508-5p attenuates autophagy in ox-LDL-treated HUVECs by decreasing ATG7. **A**, Cell proliferation in ox-LDL-treated HUVECs overexpressing miR-508-5p after 24 h, 48 h, and 72 h. **B**, Number of autophagosomes in HUVECs overexpressing miR-508-5p. **C**, Protein expression levels of ATG7 and LC3. (magnification: $\times 300$). **D**, Schematic diagram of the predicted miR-508-5p binding sites in ATG7. **E**, Luciferase reporter assay with co-transfection of 293T cells with wild-type or mutant ATG7 and miR-508-5p mimic or control. **F**, mRNA expression levels of ATG7. Data are means $\pm$ SD (n = 3). ** $p < 0.01$; **** $p < 0.0001$.

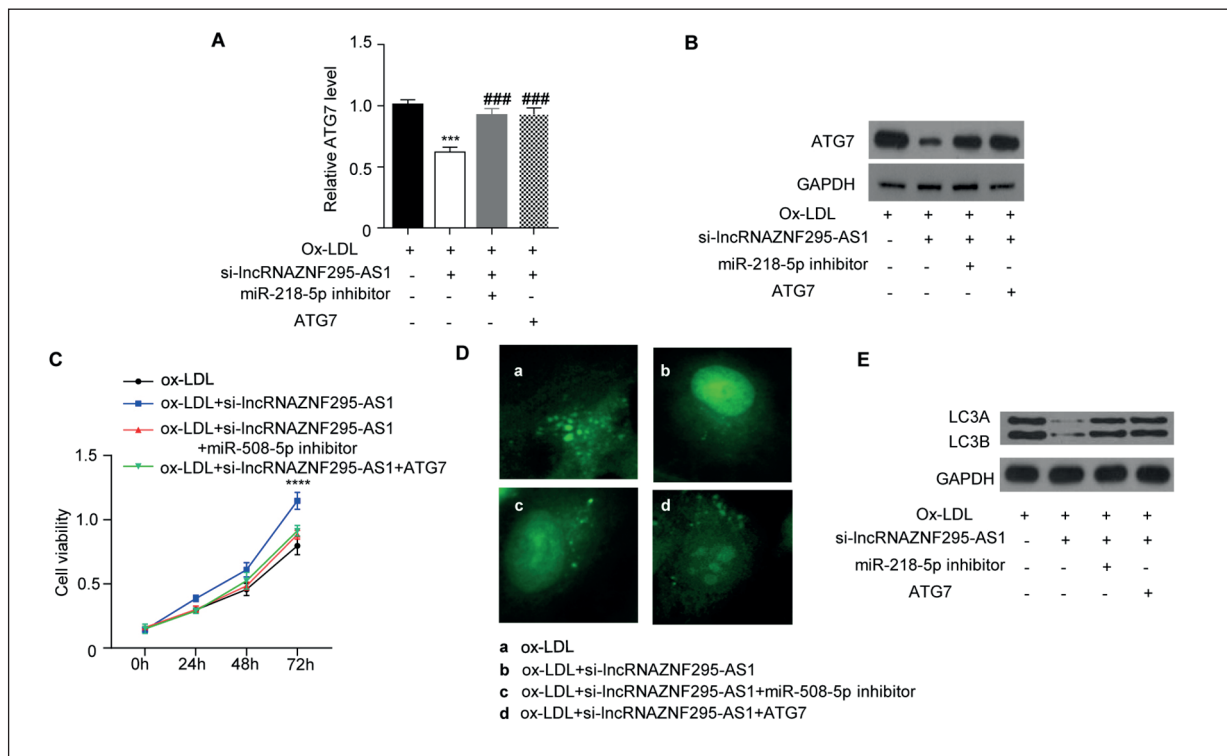


Figure 5. ZNF295-AS1 indirectly regulates ATG7 expression by sponging miR-508-5p. **A**, **B**, mRNA (**A**) and protein (**B**) expression levels of ATG7 in each group. **C**, Cell proliferation of each group after treatment for 24 h, 48 h, and 72 h. **D**, Number of autophagosomes in each group. **E**, The protein expression of LC3. (magnification: $\times 300$). Data are the means $\pm$ SD ($n = 3$). Data are the means $\pm$ SD ($n = 3$). ## Compared to the control group; ** $p < 0.01$; *** $p < 0.001$.

AS1 silencing in ox-LDL-treated HUVECs significantly inhibited cell proliferation and reduced autophagy levels, which was partially reversed by the miR-508-5p inhibitor (Figure 5D). We also estimated the autophagy level by counting the number of autophagosomes and testing the expression of autophagic markers. Similarly, the inhibitory effect of ZNF295-AS1 silencing on autophagy was notably reversed by co-transfection of ox-LDL-treated HUVECs with ZNF295-AS1 siRNA (Figure 5E).

These data indicate that the ZNF295-AS1/miR-508-5p/ATG7 axis can modulate autophagy.

Discussion

AS of vessels is a primary cause of coronary heart disease, stroke and peripheral vascular disease. Vascular endothelial injury is the first step in the pathogenesis of AS. Aberrantly expressed lncRNAs have recently been shown to be involved in the development of AS and endothelial cell survival/death^{1,9}.

In our study, a significant upregulation of ZNF295-AS1 was observed in the serum of patients with AS compared to controls. The same phenomenon was observed in the *in vitro* model, which was in agreement with previous transcriptome profiling of lncRNA expression in ox-LDL-treated HUVECs⁹. ZNF295-AS1 has also been observed to promote autophagy and inhibit the proliferation of HUVECs *in vitro*. A previous study found that ZNF295-AS1 may act as a ceRNA and plays an important role in promoting the directed differentiation of stem cells to neuronal cells mainly by binding to miRNAs¹³.

In combination with bioinformatics prediction and *in vitro* validation, miR-508-5p and ZNF295-AS1 were shown to bind directly. We found that the level of this miRNA was reduced in both AS patient serum and an *in vitro* model, and we found a negative relationship between the lncRNA and miR-508-5p. The inhibition of ZNF295-AS1 by a specific siRNA was able to significantly upregulate the expression of miR-508-5p, indicating that ZNF295-AS1 may be an upstream repressor of miR-508-5p.

In previous reports, miR-508-5p inhibited cell proliferation, tumor growth and chemoresistance¹⁴⁻¹⁶. In the cardiovascular setting, miR-508-5p can cause EPC dysfunction in patients with coronary artery disease, resulting in chronic heart failure¹⁴.

In our study, miR-508-5p did not affect cell proliferation but was also important for autophagy. MiR-508-5p knockdown significantly modulated ox-LDL-treated HUVEC proliferation and autophagy activation. In addition, Shang et al¹⁷ have reported the regulation of miR-216a and ATG5 by endothelial cells after ox-LDL treatment. This prompted us to study the possible interplay of lncRNAs and miRNAs in regulating autophagy-associated proteins during the development of AS. We found that ATG7 is a target of miR-508-5p.

The expression of many important proteins in autophagy is regulated by miRNAs. ATG7, a molecule that participates in the formation of autophagosomes and plays a very important role in autophagy, is one of the regulated proteins¹⁸. Excessive activation of autophagy occurs in the end stage of AS. It causes autophagic cell death, which inhibits collagen production, resulting in lesion rupture and/or subsequent thrombosis¹⁹. Baehrecke et al²⁰ have shown that autophagy can be inactivated *in vivo* by knocking down ATG7. In various diseases, such as cancer, diabetes, and obesity, different miRNAs control the degree of autophagy by regulating ATG7^{21,22}. In our study, the significance of miR-508-5p downregulation was emphasized by the discovery of the negative impact of miR-508-5p on ATG7, leading to the alteration of autophagy in AS.

To determine whether the association of lncRNAs, miRNAs and protein expression is causal in AS, we conducted a number of experiments. Loss-of-function and rescue experiments revealed that miR-508-5p inhibitor could reverse the inhibition of ZNF295-AS1 silencing on autophagy *in vitro*. Therefore, it is possible that ZNF295-AS1 exerts physiological functions through sponging miR-508-5p.

With the development of technology and genomics, emerging studies have demonstrated that lncRNAs are important for the regulation of gene expression by affecting miRNAs. Our study indicated that ZNF295-AS1 and miR-508-5p are closely related to autophagy through ATG7.

Autophagy plays a role in the control of cell death and survival. Autophagy can be modulated in advanced AS plaques by cytokines, reactive lipids, lipopolysaccharides, advanced glycation end products, and microRNAs, which exert both

protective and detrimental functions in vascular disorders^{19,23}. However, previous studies have disputed the exact role of autophagy in the heart under pathological conditions^{24,25}. On the one hand, autophagy is an integral part of the vascular endothelial injury process, which can reduce the increase in ROS levels upon the fragmentation of damaged mitochondria and delay apoptosis to a certain extent. On the other hand, excessive autophagic removal can also lead to an increase in ROS levels, triggering mitochondrial penetration, inducing apoptosis mechanisms in vascular endothelium, reducing vascular endothelial integrity, causing inflammation, and promoting AS. Thus, modulating autophagy represents an attractive future therapeutic target for treating cardiovascular disease. The activation of autophagy is generally considered to be cardioprotective, whereas excessive autophagy can lead to cell death and cardiac atrophy. It is important to understand how autophagy is regulated to identify ideal therapeutic targets for treating diseases. Therefore, the finding of the ZNF295-AS1/miR-508-5p/ATG7 axis may contribute to a new idea for AS therapy.

Conclusions

The observations and evaluations made during this study suggest that ZNF295-AS1 indirectly regulates ATG7 expression by binding to miR-508-5p. Additionally, downregulation of l ZNF295-AS1 or upregulation of miR-508-5p reduces autophagy levels in AS, indicating a novel idea for therapy. ZNF295-AS1 binds to miR-508-5p and acts as a miRNA sponge to modulate the pathophysiological progression of AS, demonstrating the underlying regulation of AS by the ZNF295-AS1/miR-508-5p/ATG7 axis. However, due to the limited time and experimental sample size, the internal mechanism of the ZNF295-AS1/miR-508-5p/ATG5 gene network remains to be further investigated, with the hope to provide a novel target for the treatment of AS.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Authors' Contribution

QWQ, JY and YZL conceived and designed the experiments, LL, JTG and YS analyzed and interpreted the results of the experiments, and ZDL and TZL performed the experiments.

Ethics Approval and Consent to Participate

The animal use protocol listed below has been reviewed and approved by the Animal Ethical and Welfare Committee.

Informed Consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

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