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Circular RNA circHIPK3 as a novel circRNA regulator of autophagy and endothelial cell dysfunction in atherosclerosis

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Abstract. – OBJECTIVE: The aim of the study was to explore the role and mechanism of circHIPK3 in atherosclerosis.

MATERIALS AND METHODS: AS model was constructed *in vivo* and *in vitro* for high fat-fed and ox-LDL treatment. RT-PCR was used to assess the level of circHIPK3. The autophagy level of HUVECs was detected by Western blot, transmission electron microscopy, and LC3II fluorescence intensity. HUVECs lipid accumulation was assessed by oil red staining. Luciferase assay was performed to verify the relationship of circRNA and miRNA, miRNA, and target gene.

RESULTS: The expression of circHIPK3 was downregulated in HFD mice, and ox-LDL treated HUVECs. The level of autophagy was decreased in AS, which was reversed by overexpression of circHIPK3. Meanwhile, forced expression of circHIPK3 would reduce the accumulation of lipid in HUVECs.

CONCLUSIONS: CircHIPK3 could inhibit lipid content in ox-LD-treated HUVECs via activating autophagy. This progression mechanism may target the miR-190b/ATG7 signal pathway, which indicates a suitable role in the pathogenesis of atherosclerosis.

Key Words:
CircHIPK3, Autophagy, Atherosclerosis.

Introduction

Atherosclerotic (AS) is a chronic inflammatory disease, which is the leading cause of coronary heart disease and ischemic stroke[1]. Its mechanism is complex and related to various factors, including environment, smoking, obesity, and hyperlipidemia[2,3]. Besides, genetic factors have also been confirmed to be related to AS. The early stage of As lesion is endothelial dysfunction of the arterial vascular system caused by lipid deposition under the endodermis of the arterial wall and is characterized by inflammatory infiltration of macrophages, dendritic cells, and activated T cells[4,5]. However, unstable atherosclerotic plaques and secondary thrombosis are often seen in late AS. Unstable atherosclerotic plaques are characterized by the thin fibrous cap, vascular microcalcification, large necrotic nucleus, and fragile new microvessels in the plaque. These pathological changes lead to lumen occlusion or wall rupture and bleeding and lead to severe consequences, such as myocardial infarction, stroke, and so on[6,7]. Therefore, there is an urgent need to seek more diagnostic indicators and treatment.

CircRNA is a covalently closed loop structure of endogenous non-coding RNA that is not easily degraded by RNA exonuclease or ribonuclease R[8,9]. It is endogenous, evolutionarily conservative, stable, and highly expressed in the eukaryotic transcriptome, showing tissue or growth stage-specific expression. CircRNAs play an essential role in cell function, life process, occurrence, and development of disease[10,11]. Li et al[12] performed circRNAs microarray analysis in ox-LDL-induced HUVECs and found 943 differentially expressed circRNAs. They found that silencing of circRNA_003575 could improve the angiogenic ability of HUVECs, and speculated that there was a ceRNA mechanism of circ_0003575-miR-199-3p/miR-9-5p/miR-377-3p/miR-141-3p-mRNA. Shen et al[13] found that circRNA_0044073 was upregulated in atherosclerosis and promoted the proliferation and invasion of HUVECs by inhibiting miR-107 and activating the JAK/STAT signal transduction pathway, which provided a new strategy and target for the treatment of AS. It was found that the lipoprotein receptor (Lrp) 6 was a gene highly expressed in blood vessels and involved in vascular pathology. CircLrp6 regulated the migration, proliferation, and differentiation in vascular smooth muscle cells (VSMCs) by acting as a sponge of miR-145

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molecules. The silencing of circLrp6 inhibited the proliferation of VSMCs, promoted differentiation, and reduced the formation of neointima. This finding was consistent with the increase in the biological activity of miR-145 molecules with anti- AS effect[14].

Autophagy is a survival mechanism of cells in the face of a stressful environment, and its protective effect in the development of AS has been confirmed[15,16]. Autophagy can avoid cytochrome C's release and excessive activation of caspase by clearing damaged proteins and mitochondria in time. Autophagy is also involved in the defense process of antioxidant stress, promoting the recovery of plaque cells in the inflammatory environment, preventing their apoptosis, thus improving the stability of plaques[17]. In AS, autophagy and apoptosis are highly environment-dependent, and they can occur in the same cell. In macrophages, apigenin-mediated apoptosis of macrophages can inhibit the over-activation of autophagy and help to improve atherosclerosis[18]. These results suggest that moderate autophagy can protect cells from apoptosis, while excessive autophagy can be counterproductive.

Recently, it has been found that circHIPK3 was highly expressed in tumor tissues, which could promote cell growth[19-21]. Meanwhile, circHIPK3 could promote myocardial repair, heart protection, and regeneration[22]. However, the role and mechanism of circHIPK3 have not been reported in research. Therefore, this study intended to explore the effect of circHIPK3 on AS and further study its regulatory molecules mechanism.

Materials and Methods

Animal and Model

8-week-old ApoE-/- mice, weighing (20±3) g, were purchased from Nanjing Junke Bioengineering Co., Ltd. The animals are raised in a standard laboratory animal room with a temperature of (23±1)°C and a humidity of 55% to 60%. They are free to eat and drink. Twelve ApoE$^{-/-}$ mice were randomly divided into ApoE$^{-/-}$ normal diet group (n = 6) and high fat diet (high fat diet, HFD) group (n = 6)[23]. The mice in the high-fat group were fed with a high-fat diet for 16 weeks. High-fat diet contains 78.85% basic feed, 0.15% cholesterol and 21% fat. After 16 weeks, 6 mice in each group were anesthetized with isopentane inhalation, blood vessels and hearts were taken, and fixed at -80°C or 4% paraformaldehyde. Before collecting samples, the mice fasted for 12 hours. Under the aseptic condition, blood samples were taken from eyeballs to separate plasma and platelets, quickly open the chest and abdominal cavity, and peel off the full-length aorta along with the aortic valve to the branches of the iliac artery. The contents of TC and TAG were determined by enzyme coupling colorimetry, and the contents of HDL-C and LDL-C were determined by selective precipitation. The research was carried out based on the Guidelines' proposals for the Care and Use of Laboratory Animals of the National Institutes of Health. It has been approved by Animal Experiment Ethics of the Cangzhou Central Hospital. All animal experiments took place at the Cangzhou Central Hospital.

RT-PCR

Total RNA of cells and tissues were extracted by the TRIzol method, 1 μg total RNA was reverse transcribed into cDNA, products for circHIPK3, GAPDH amplification, and the experiment was run by PCR instrument. The reaction cycle is 40. The sequence of primers was shown as follows.

CircHIPK3: Forward: 5'-TATGTTGGTGGATCCTGTTCGGCA-3', Toward: 5'- TGGTGGGTAGACCAAGACTTGTGA-3'; GAPDH: Forward: 5'-GTGGAGGGGGACTGGAACACA-3',
Toward: 5'-GTCAAGGGAGGGGGGAAMAA-3'.

Western Blot

After RAPI lysed the cells, the supernatant was taken after centrifugation, and the protein concentration was determined by BCA protein quantitative method. After the protein was quantified, the sample buffer was added, the protein was denatured by heating at 95°C, 6%-15% SDS polyacrylamide gel was used for protein electrophoresis, the film was transferred to the NC membrane with a constant current of 300 mA, incubated with 5% skimmed milk powder at room temperature for 2 hours, diluted with the corresponding primary antibody, and incubated overnight at 4°C, and the membrane was incubated with the fluorescent second antibody after PBST washing. Using Odyssey scanning, the result is expressed as grayscale value/internal reference grayscale value.

H&E Staining

The aorta was fixed with 10% formaldehyde buffer, alcohol gradient dehydration, xylene transparency, paraffin embedding, section, the thickness of 4 μm, and performed H&E staining.

Immunofluorescence

In the culture plate, the slides that had already climbed the cells were washed with PBS 3 times, each time 3 min. Fixed with 4% paraformaldehyde, 15 min, washed 3 times with PBS. 0.2% Triton X-100 room temperature permeable 20 min. PBS washed slides 3 times, absorbed PBS, added goat serum, sealed at room temperature for 30 min, absorbed the sealing solution, incubated with the primary antibody, put it in a wet box, incubated overnight at 4°C. According to the experimental steps, the second antibody was added, DAPI was added to avoid light and incubated for 5 min, to dye the nucleus of the specimen, absorb the liquid from the climbing film, seal the film with the sealing solution containing anti-fluorescence quenching agent, and observe and take pictures with a fluorescence microscope.

Statistical Analysis

The data were analyzed by GraphPad 8.0. (La Jolla, CA, USA) the experimental data were expressed in ±SEM, and the difference was statistically significant by *t*-test or one-way ANOVA. The difference was statistically significant ($p < 0.05$).

Results

CircHIPK3 Was Downregulated in AS

To explore the function of circHIPK3 in AS, ApoE$^{-/-}$ mice were separately fed with HFD and normal diet. The weight of mice was recorded every two weeks (Figure 1A). After 16 weeks, the mice were sacrificed; the blood was isolated. Then, the increased level of serum TG and TC were found in AS mice. The decreased HDL-C level and the increased level of LDL-C were observed in HFD mice (Figure 1B). In the control mice group, the thickness of the aortic wall was uniform, and no abnormality was found in intima, media, and adventitia. In the HFD mice

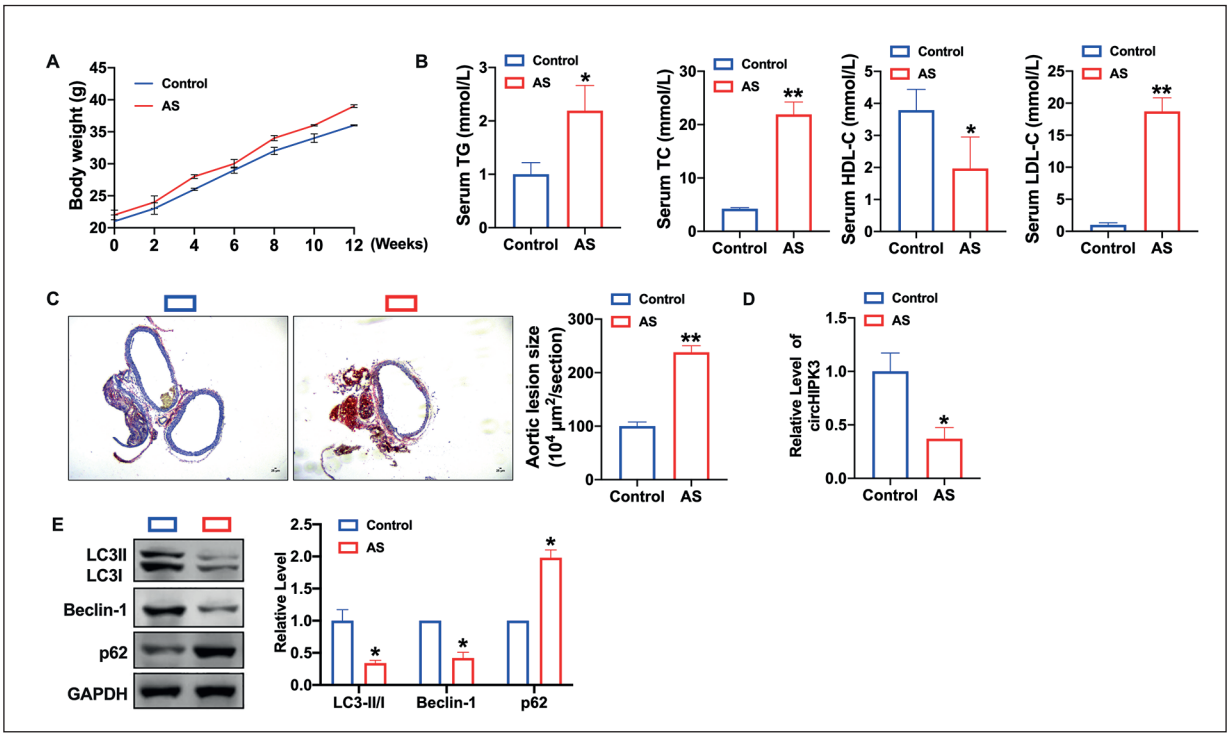

Figure 1. CircHIPK3 was downregulated in AS model. **A,** ApoE$^{-/-}$ mice with a high-fat diet (HFD) is constructed as an atherosclerosis model. The weight was recorded. n=6. **B,** serum TG, TC, and LDL-C, serum HDL-C levels were detected. n=6. *p<0.05. **C,** H&E histological staining of the aortic area presented a marked augment in aortic lesion size. optical microscope showed several marked lipid plaques in the aortic area. n=3. **p<0.01. **D,** The expression of circHIPK3 in HFD mice and control mice. n=6. *p<0.05. **E,** The protein level of LC3, Beclin-1, and p62 were detected by Western blot. n=4. *p<0.05.

group, the intima of the aortic wall was thickened, the thickness of the aortic wall was not uniform, and the focus of AS was evident (Figure 1C). The data indicated that the AS model was established successfully. Then, we found that circHIPK3 was downregulated in AS mice (Figure 1D). Then, the expression of autophagy-associated proteins was detected *via* Western blot. The decreased expression of LC3II/I and Beclin-1 and the increased expression of p62, were found in AS mice (Figure 1E).

Forced Expression of CircHIPK3 Induces Autophagy and Improves AS In Vitro

To explore the function of circHIPK3 in AS, we treated the HUVECs with 100 μg/mL ox-LDL for 12 h, the expression of circHIPK3 was assessed by RT-PCR. The decreased expression of circHIPK3 was observed in ox-LDL treated HUVECs (Figure 2A). The plasmid for overexpression circHIPK3 was constructed, and the transfection efficiency of circHIPK3 plasmid was confirmed (Figure 2B). Then, we detected

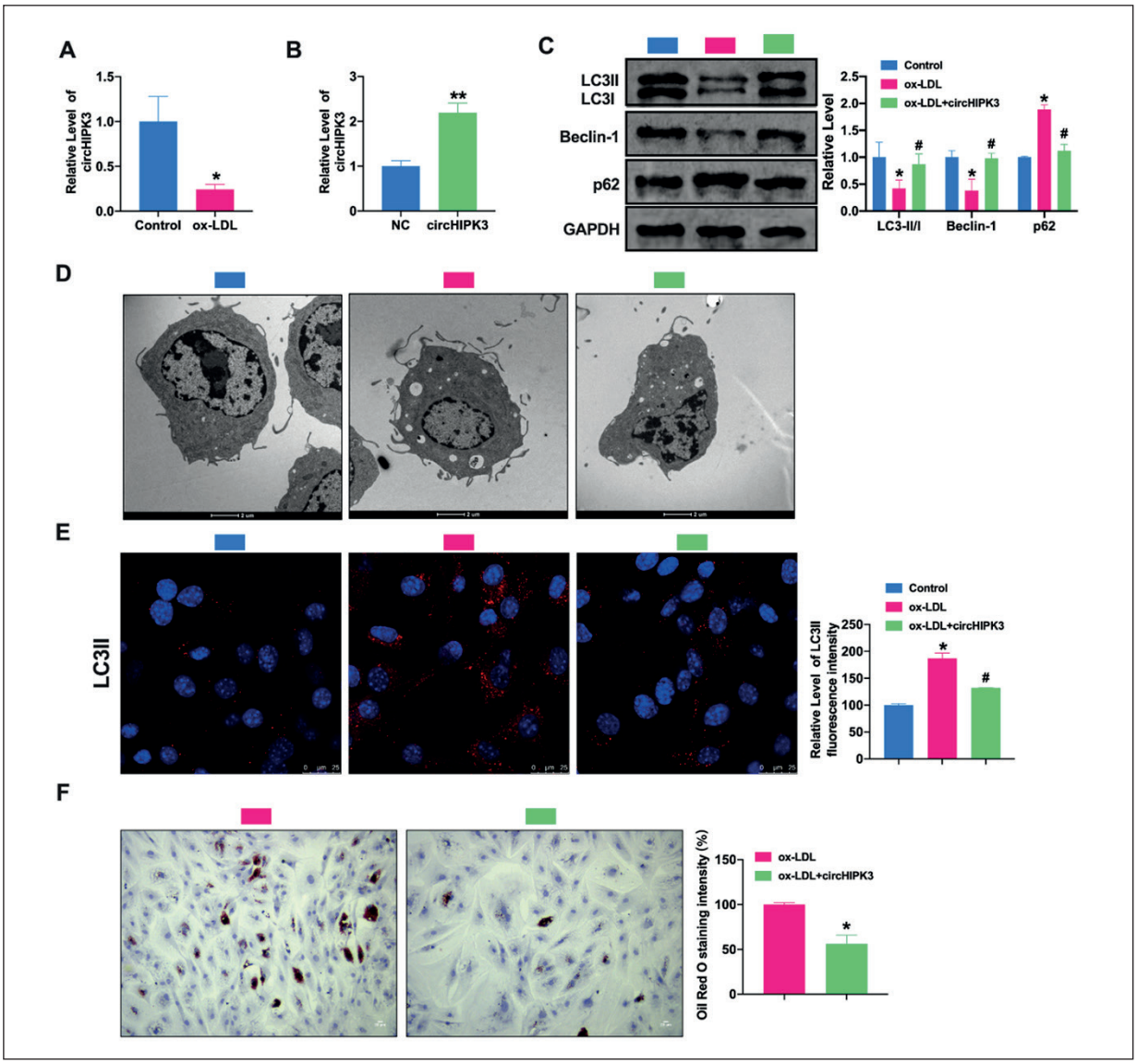

Figure 2. Forced expression of circHIPK3 induced autophagy and lipid accumulation in ox-LDL treated HUVECs. **A,** The expression of circHIPK3 was detected in HUVECs after ox-LDL treated. n=5. *$p<0.05$. **B,** RT-PCR showed the transfection efficiency of circHIPK3 plasmid. n=6.**$p<0.01$. **C,** The protein level of LC3, Beclin-1, and p62 were detected by Western blot. n=4. *$p<0.05$, $^{\#}p<0.05$. **D,** Intracellular autophagosomes visualized by transmission electron microscopy (TEM). **E,** LC3 fluorescence showing LC3B-II punctiform in ox-LDL-treated HUVECs. n=4, (magnification, 200×). *$p<0.05$, $^{\#}p<0.05$. **F,** HUVECs were analyzed for Oil Red O staining. n=4, (magnification, 20×). *$p<0.05$, $^{\#}p<0.05$.

the autophagy-associated proteins level in ox-LDL treated HUVECs after circHIPK3 transfection. Ox-LDL inhibited the protein level of LC3II/I and Beclin-1 and promoted the protein level of p62, which was reversed by overexpression circHIPK3 (Figure 2C). A transmission electron microscope (TEM) was used to observe the autophagosome. The ox-LDL-stimulated HUVECs led to a significant decrease in cytoplasmic autophagosomes, which was prevented by circHIPK3 plasmid (Figure 2D). Similarly, the ox-LDL-stimulated HUVECs led to a considerable reduction in LC3II fluorescence intensity, which was blocked by forcing the expression of circHIPK3 (Figure 2E). It also performed a reduction of ox-LDL uptake of Oil Red O staining in HUVECs after circHIPK3 transfection (Figure 2F). In summary, circHIPK3 induced autophagy and protected the AS in ox-LDL treated HUVECs.

CircHIPK3 Binds With MiR190b

Bioinformatics website predicts that miR-190b can combine with circHIPK3 and the prediction binding sites, as shown in Figure 3A. Then, the cells were co-transfected miR-190b/miR-NC with WT-circHIPK3/mutant-circHIPK3, miR-190b co-transfected with WT-circHIPK3 performed the decreased Luciferase activity, while miR-190b co-transfected with mutant-circHIPK3 did not change the Luciferase activity. The results showed that circHIPK3 could interact with miR-190b (Figure 3B). CircHIPK3-specific probe was used to pull down its interactive miRNAs. CircHIPK3 precipitation complex performed the enrichment of miR-190b (Figure 3C). Then, the expression of miR-190b was detected by RT-PCR. Overexpression of circHIPK3 inhibited the level of miR-190b (Figure 3D), and the silencing of circHIPK3 induced the expression of miR-190b (Figure 3E). Meanwhile, we found a negative

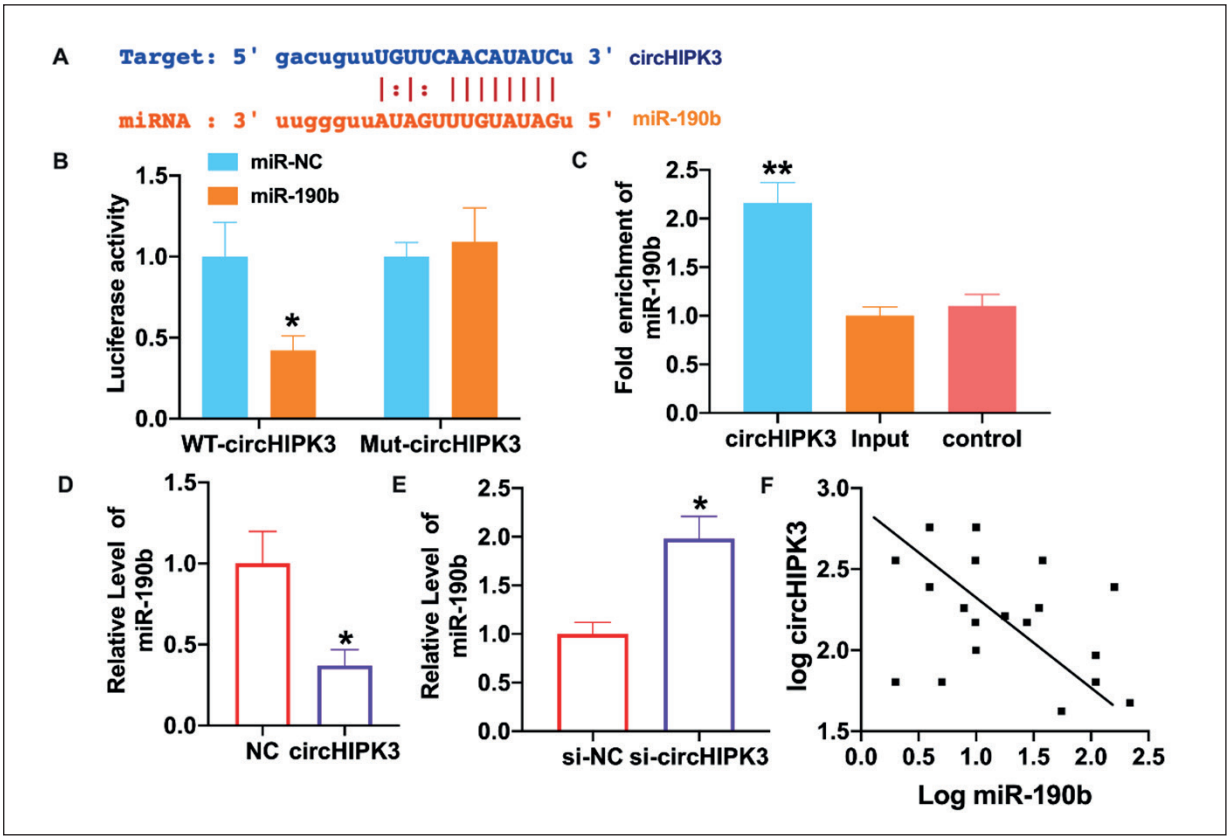

Figure 3. MiR-190b was a target of circHIPK3. **A,** The prediction bind sites between circHIPK3 and miR-190b. **B,** The Luciferase assay was performed to verify the relationship between circHIPK3 and miR-190b. n=3. *p<0.05. **C.** The enrichment of miR-190b by the circHIPK3 probe was performed. n=4. **p<0.01. **D-E,** The level of miR-190b was detected by RT-PCR. n=6. *p<0.05. **F,** The relationship between circHIPK3 and miR-190b in ox-LDL treated HUVECs. n=19. *p<0.05.

correlation between the expression of miR-190b and circHIPK3 in ox-LDL-treated HUVECs cells (Figure 3F).

MiR-190b Prevents the Function of CircHIPK3 on AS In Vitro

To research the downstream function of circHIPK3 in AS, we co-transfected the si-miR-190b with circHIPK3 in ox-LDL treated HUVECs. As Figure 4A shown, circHIPK3 induced the expression of LC3II/I and Beclin-1, which prevented the expression of p62, which was blocked by miR-190b (Figure 4A). CircHIPK3 prevented the decreased cytoplasmic autophagosomes and LC3II fluorescence intensity induced by ox-LDL-stimulated HUVECs, which was controlled by overexpression of miR-190b (Figure 4B and C). It also performed a decrease in ox-LDL uptake of Oil Red O staining in HUVECs after circHIPK3 transfection, which was prevented by miR-190b (Figure 4D). Taken together, the forced expression of miR-190b stopped the function of circHIPK3 on ox-LDL treated HUVECs.

CircHIPK3 Regulates AS Progression by Targeting MiR-190b/ATG7

ATG7, as an activating enzyme, participates in forming two essential autophagy complex-

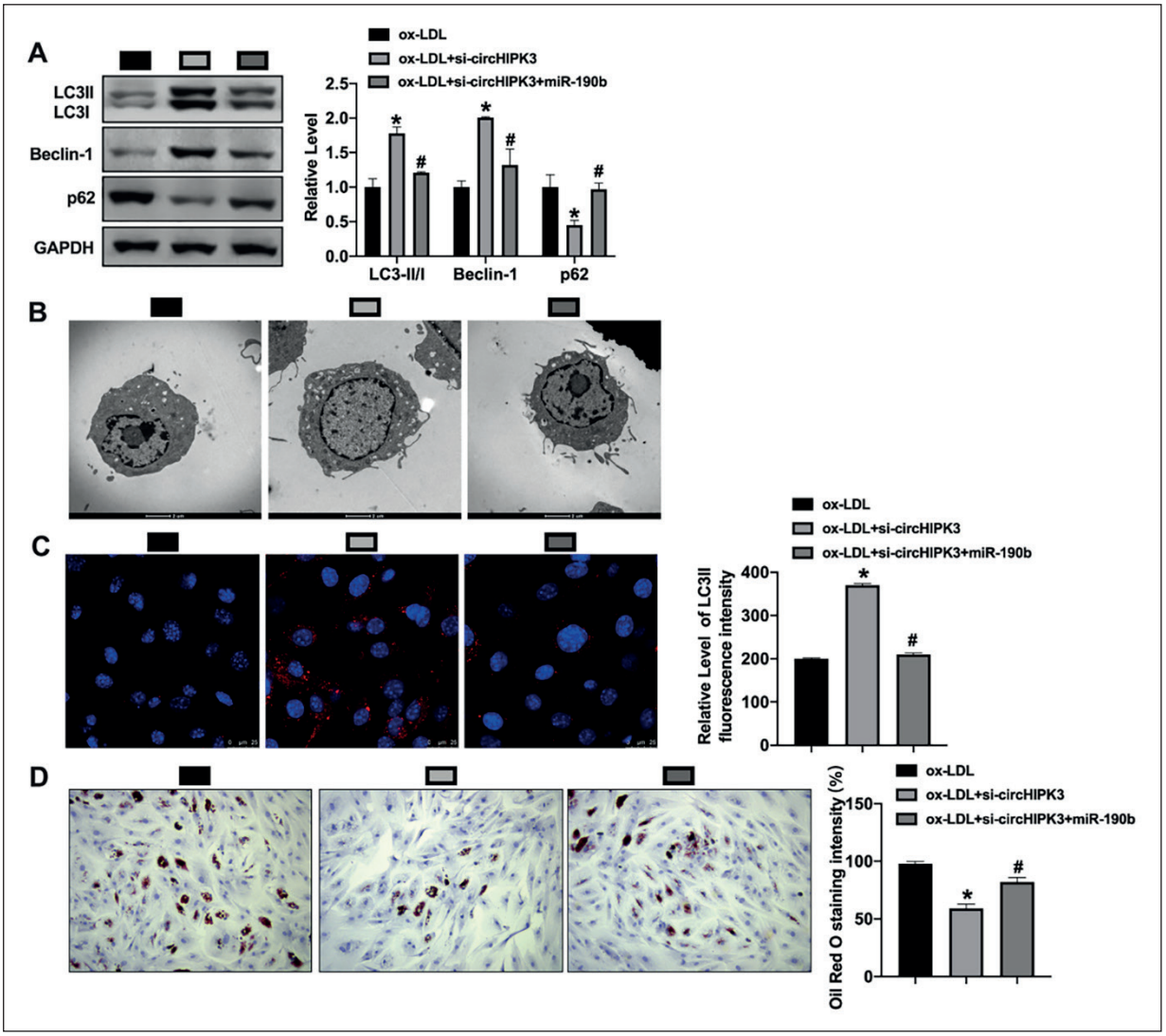

Figure 4. MiR-190b prevents the function of circHIPK3 in ox-LDL treated HUVECs. **A,** The protein level of LC3, Beclin-1, and p62 were detected by Western blot. n=4. *$p<0.05$, $^{\#}p<0.05$. **B,** Intracellular autophagosomes visualized by TEM. **C,** LC3 fluorescence showing LC3B-II punctiform in ox-LDL-treated HUVECs. n=4, (magnification, 200×). *$p<0.05$, $^{\#}p<0.05$. **D,** HUVECs were analyzed for Oil Red O staining. n=4, (magnification, 20×). *$p<0.05$, $^{\#}p<0.05$.

es, ATG12-ATG5-ATG16LL and ATG8-PE, in the process of autophagy formation and is a crucial enzyme. Its decrease will lead to a reduction in the level of autophagy. We predicted that ATG7 could interact with miR-190b (Figure 5A), and Luciferase verified the relationship between miR-190b and ATG7 (Figure 5B). We also found a negative correlation between the expression of miR-190b and ATG7 in ox-LDL-treated HUVECs cells (Figure 5C). The silencing

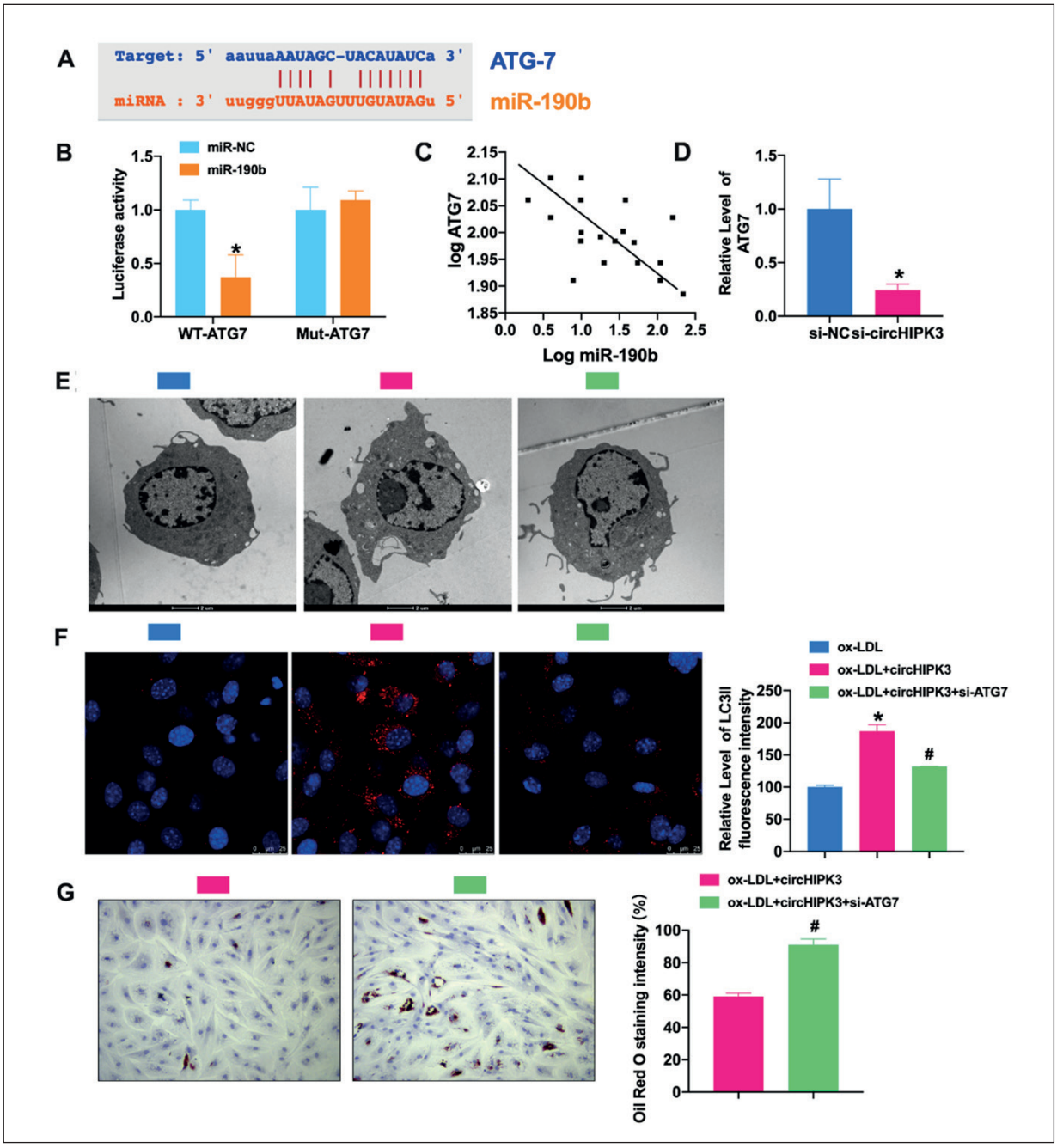

Figure 5. CircHIPK3 regulates AS progression by targeting miR-190b/ATG7. **A,** The prediction bind sites between ATG7 and miR-190b. **B,** The Luciferase assay was performed to verify the relationship between ATG7 and miR-190b. n=3. *p<0.05. **C,** The relationship between ATG7 and miR-190b in ox-LDL treated HUVECs. n=19. *p<0.05. **D,** The expression of ATG7 was detected in HUVECs after si-circHIPK3/si-NC transfection. n=6. *p<0.05. **E,** Intracellular autophagosomes visualized by TEM. **F,** LC3 fluorescence showing LC3B-II punctiform in ox-LDL-treated HUVECs. n=4, (magnification, 200×). *p<0.05, $^{\#}p$<0.05. **G,** Oil Red O staining. n=4, (magnification, 20×). $^{\#}p$<0.05.

of circHIPK3 prevented the expression of ATG7 (Figure 5D). CircHIPK3 prevented the decreased cytoplasmic autophagosomes and LC3II fluorescence intensity induced by ox-LDL-stimulated HUVECs, which was controlled by silencing of ATG7 (Figure 5E and F). It also performed a decreased in ox-LDL uptake of Oil Red O staining in HUVECs after circHIPK3 transfection, which was prevented by si-ATG7 (Figure 5G). Taken together, the knockdown of ATG7 and the target of miR-190b prevented the function of circHIPK3 on ox-LDL treated HUVECs.

Discussion

Atherosclerotic is a chronic inflammatory disease characterized by the formation of lipid plaques in large and medium-sized arteries, which is easy to occur in areas where ECs barrier function is impaired[24]. With the disease's progress, the atherosclerotic plaque rupture can partially or entirely block the lumen and eventually lead to acute cardiovascular events[25]. Although diet, exercise, and other lifestyle changes, the use of lipid-lowering drugs and advanced detection methods, and interventional therapy have significantly prolonged the life span of patients, still could not reverse the trend of the increasing prevalence of AS year by year. Therefore, it is urgent to better understand the root cause of AS and formulate new treatment strategies.

In the past ten years, the related research of circRNA has been gradually deepened, and there have been some achievements in cardiovascular and cancer. CircRNA may play an essential role in the process of life, and as a new clinical diagnosis or predictive marker of the disease, it provides new insights for the treatment of the disease, so it needs in-depth research[26]. At present, there are few studies on circRNA and its regulation related to atherosclerosis. It has been shown that the level of serum circR-284 in patients with carotid plaque rupture is significantly increased, while the level of miR-221 is downregulated. MiR-221 stimulates intimal thickening by downregulating p27Kipi. P27Kipi is a cyclin-dependent kinase inhibitor that can inhibit the proliferation of VSMC. In the early stages of carotid atherosclerotic plaque rupture, the proportion of circR-284: miR-221 significantly increases, which means its potential as a biomarker of rupture susceptibility[27]. Holdt et al[28] found that circANRIL regulated atherosclerosi's shape mainly by controlling the maturation of ribosomal RNA (rRNA). CircANRIL can competitively bind estrogen receptors to regulate antibody homolog 1 (PES1), occupy the binding site between PES1 and pre-rRNA, prevent rRNA maturation mediated by exonuclease, induce nucleolar pressure, lead to p53 activation, and play an anti-atherosclerotic role.

CircHIPK3 is encoded by the second exon of the HIPK3 gene and is mainly located in the cytoplasm. It has been found that in hepatocellular carcinoma cells, circHIPK3 promotes the proliferation and migration of hepatocellular carcinoma cells by competitively binding a variety of miRNAs[29]. The role of circHIPK3 in diabetic retinal vascular function was systematically analyzed. Chen et al[30] found that circHIPK3 promoted the expression of its downstream gene VEGF/WNT2/FZD4 and endothelial cell proliferation by competitively binding to miR-30a-3p. It is suggested that circHIPK3 can promote cell proliferation, migration, and regulate vascular function. However, the regulatory role of circHIPK3 in atherosclerosis has not been reported. This study designed siRNA molecules targeting the circHIPK3 sequence by simulating the atherosclerotic environment *in vivo* and *vitro*. It was found that circHIPK3 can induce autophagy to improve atherosclerosis.

Masuyama et al[31] have found that autophagy is common in atherosclerotic plaques and plays a vital role in the stability of atherosclerotic plaques and the overall development of the disease. The role of autophagy in the occurrence and development of AS has not been fully elucidated. However, it is found that autophagy has both protective and damaging effects. Some research has shown that autophagy protects cells when regulating arterial injury in endothelial cells, which provides a new target for anti-AS therapy. Autophagy related genes (LC3II and Beclin-1) are controlled by ClassIII PI3K and PTEN/Class I PI3K/Akt signal pathways[32]. In most atherosclerosis, autophagy can inhibit apoptosis. Ito et al[33] found that GADD34 is due to the loss of lipopolysaccharide and amino acids and can induce autophagy and inhibit apoptosis in macrophages by regulating the mTOR signal pathway. Similarly, 7-KC, an essential component of oxidized lipoprotein, can protect cells by promoting autophagy in aortic smooth muscle cells while reducing the ability of 7-KC to induce cell death[34]. It was found that ARG2 impairs endothelial autophagy independently of the L-arginine ureahydrolase activity through activation of RPS6KB1 and inhi-

bition of PRKAA, which is implicated in atherogenesis[35]. Here, we found that circHIPK3 could induce autophagy and protect HUVECs caused by ox-LDL.

Conclusions

For the first time, we identified that the downregulation of circHIPK3 was confirmed in AS *in vivo* and *vitro*. Forced expression of circHIPK3 could induce autophagy by directly targeting miR-190b/ATG7 in HUVECs and may play a significant role in atherosclerosis's pathogenesis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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