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Original Paper

Endothelial Cell Autophagy in Atherosclerosis is Regulated by miR-30-Mediated Translational Control of ATG6

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Key Words
Atherosclerosis • Endothelial cell autophagy • ApoE (-/-) • High fat diet (HFD) • ox-LDL • ATG6 • miR-30

Abstract:
Background/Aims: Endothelial cell injury and subsequent death play an essential role in the pathogenesis of atherosclerosis. Autophagy of endothelial cells has a protective role against development of atherosclerosis, whereas the molecular regulation of endothelial cell autophagy is unclear. MicroRNA-30 (miR-30) is a known autophagy suppressor in some biological processes, while it is unknown whether this regulatory axis may be similarly involved in the development of atherosclerosis. Here, we aimed to answer these questions in the current study. ***Methods:*** We examined the levels of endothelial cell autophagy in ApoE (-/-) mice suppled with high-fat diet (HFD), a mouse model for atherosclerosis (simplified as HFD mice). We analyzed the levels of autophagy-associated protein 6 (ATG6, or Beclin-1) and the levels of miR-30 in the purified CD31+ endothelial cells from mouse aorta. Prediction of the binding between miR-30 and 3'-UTR of ATG6 mRNA was performed by bioinformatics analyses and confirmed by a dual luciferase reporter assay. The effects of miR-30 were further analyzed in an *in vitro* model using oxidized low-density lipoprotein (ox-LDL)-treated human aortic endothelial cells (HAECs). ***Results:*** HFD mice developed atherosclerosis in 12 weeks, while the control ApoE (-/-) mice that had received normal diet (simplified as NOR mice) did not. Compared to NOR mice, HFD mice had significantly lower levels of endothelial cell autophagy, resulting from decreases in ATG6 protein, but not mRNA. The decreases in ATG6 in endothelial cells were due to HFD-induced increases in miR-30, which suppressed the translation of ATG6 mRNA via 3'-UTR binding. These *in vivo* findings were reproduced *in vitro* on ox-LDL-treated HAECs. ***Conclusion:*** Upregulation of miR-30 by HFD may impair the protective effects of endothelial cell autophagy against development of atherosclerosis through suppressing protein translation of ATG6.

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Introduction

Atherosclerosis is a chronic inflammatory disease in which lipids and fibrous elements are deposited in the arterial wall large and medium-sized arteries to activate both the innate and adaptive immune systems. Atherosclerosis is the primary cause of heart disease and stroke, which accounts for more than half of all deaths in aged people [1, 2]. The progression of atherosclerosis includes early accumulation of cholesterol-engorged macrophages in the sub-endothelial matrix, accumulation of lipid-rich necrotic debris, elevated numbers of smooth muscle cells (SMCs), and subsequent development of fibrosis, which lead to formation of complex plaques with calcification, ulceration and hemorrhage eventually [1, 2].

Apolipoprotein E (ApoE) is a 34-kDa secreted protein, as a well-known strong suppressor for atherosclerosis [3, 4]. ApoE not only regulates lipoprotein cholesterol transport and controls cellular lipid regulation, but also potently inhibits inflammation [3, 4]. For example, ApoE-deficient (ApoE –/–) mice display enhanced chronic inflammation in response to spontaneous and diet-induced hypercholesterolemia, and display enhanced acute immune response when challenged with bacterial lipopolysaccharide (LPS) [3-9]. High fat diet (HFD) induces development of atherosclerosis in ApoE –/– mice in 12 weeks [10-12].

Endothelial cell injury is a major step for the pathological progression of atherosclerosis [13-17]. Upon injury, endothelial cell autophagy may occur to protect the cells from being damaged, while the failure or inhibition of autophagy results in endothelial cell apoptosis, leading to the break-down of the integrity of endothelium to facilitate the local lipid deposition into atherogenesis, plaque instability, and even acute coronary occlusion and sudden death [13, 18-23]. Nevertheless, our understanding of the mechanisms that control the autophagy of endothelial cells is still limited.

Autophagy is a catabolic pathway that degrades and recycles cellular compartments for cell survival at various stresses, whereas its failure often leads to cell death [24]. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a soluble cellular protein. During autophagy, autophagosomes engulf cytoplasmic components, resulting in conjugation of a cytosolic form of LC3 (LC3-I) to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II). Thus, the ratio of LC3-II to LC3-I represents the autophagic activity [24-26]. Autophagy-associated protein 6 (ATG6, or Beclin-1), ATG7 and p62 are several key autophagy-associated proteins that strongly induce autophagy in an independent or coordinated manner [27]. Autophagy has been shown to play a critical role in endothelial cells to prevent development of atherosclerosis [13, 18-23]. Nevertheless, the underlying mechanisms remain elusive.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate protein translation through their base-pairing with the 3′-untranslated region (3′-UTR) of the target mRNAs [28-34]. MiRNAs play essential roles in regulating atherosclerosis [5, 35, 36]. Among all miRNAs, miR-30 has been shown to regulate carcinogenesis of various cancers [37-40]. Nevertheless, a role of miR-30 in the atherosclerosis has not been studied.

Here, we found that high-fat-diet (HFD) -treated ApoE (-/-) mice developed atherosclerosis in 12 weeks, while the control ApoE (-/-) mice that had received normal diet (simplified as NOR mice) did not. Compared to NOR mice, HFD mice had significantly lower levels of endothelial cell autophagy, resulting from decreases in ATG6 protein, but not mRNA. The decreases in ATG6 in endothelial cells were due to HFD-induced increases in miR-30, which suppressed the translation of ATG6 mRNA via 3′-UTR binding. These *in vivo* findings were reproduced *in vitro* on oxidized low-density lipoprotein (ox-LDL)-treated human aortic endothelial cells (HAECs).

Materials and Methods

Ethics statement

The study was approved by the Animal Care and Use Committee of Chinese PLA General Hospital. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory

Animals, published by the US National Institutes of Health. All experiments were conducted under the supervision of the facility's Institutional Animal Care and Use Committee according to an Institutional Animal Care and Use Committee–approved protocol.

Animal models and quantification of atherosclerotic lesions

Eight-week-old male ApoE−/− mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) or bred in-house and maintained under sterile conditions and standard animal room conditions (temperature, 21 ± 1°C; humidity, 55–60%). The animals were randomly divided into two groups: the normal-diet group (NOR) and the high-fat diet (HFD) group. The animals of the HFD group were maintained for 12 weeks to induce atherosclerosis, after which the aortas were excised from the mice. The aortic roots along with the basal portion of the heart were fixed with 4% paraformaldehyde for 4 hours, cryo-protected in 30% sucrose for 12 hours, and then embedded in OCT compound. The tissue was cross-sectioned into sections of 6μm thickness. Atherosclerotic lesions of the aortic root were examined by H&E staining. Oil red O staining was performed according to the manufacturer's instructions to show the lipid deposition with an Oil red O staining kit (Abcam, Cambridge, MA, USA). Quantification of the images was measured using NIH ImageJ software (Bethesda, MD, USA). The data were calculated from 5 mice for each group. For each mouse, 3 slides that were 20μm apart from each other were used for quantification.

Cell culture, treatment and transfection

Normal human aortic endothelial cells (HAECs) were purchased from American Type Culture Collection (ATCC PCS-100-011, Rockville, MD, USA) and cultured in Endothelial Cell Media supplemented with endothelial cell growth factors, 5% fetal bovine serum (FBS, Invitrogen, CA, Carlsbad, USA) and 1% penicillin/streptomycin (Invitrogen) at 37°C with 5% CO_2. HAECs were transiently transfected with miR-30 mimics, antisense for miR-30 (as-miR-30) or null controls (RiboBio Co., Ltd., Guangzhou, Guangdong, China), using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. The sequences for miR-30: 5'-ACAUUUGUACAUGACAAAUUAUA-3'; as-miR-30: 5'-UAUAAUUUGUCAUGUACAAAUGU-3'. The transfection efficiency was nearly 100%. One day after cell transfection, the cells were treated with or without 100μg/ml oxidized low-density lipoprotein (ox-LDL, Beijing Xiesheng Bio-Technology Limited, Beijing, China). After drug treatment, the cells were used for flow cytometry or protein/RNA extraction.

Cell viability by cell counting kit-8 (CCK-8) assay

The CCK-8 detection kit (Sigma-Aldrich, St. Louis, MO, USA) was used to measure cell viability according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well microplate at a density of $5X10^4$/ml. After 24h, cells were treated with resveratrol. Subsequently, CCK-8 solution (20ml/well) was added and the plate was incubated at 37°C for 2 hours. The viable cells were counted by absorbance measurements with a monochromator microplate reader at a wavelength of 450 nm. The optical density value was reported as the percentage of cell viability in relation to the control group (set as 100%).

Flow cytometry

For analyses and isolation of CD31+ cells, the aorta was dissociated by 10μg/ml trypsin (Sigma-Aldrich) and 10μg/ml DNase (Roche, Nutley, NJ, USA) for 35 minutes. After filtration at 30μm, the single cell digests from mouse aorta were incubated with PE-cy7-CD31 (Becton-Dickinson Biosciences, San Jose, CA, USA) and then sorted on a FACSAria (Becton-Dickinson Biosciences).

Real-time RT-PCR

Aorta intimal RNA was isolated from mouse aortas. After cleaning with ice-cold PBS, mouse aortas were flushed with TRIzol reagent (Invitrogen) using an insulin syringe, and the eluate was collected in a 1.5ml tube and prepared for RNA extraction. Total RNA and miRNAs were extracted from tissue or cultured cells with miRNeasy mini kit or RNeasy kit (Qiagen, Hilden, Germany), respectively. Complementary DNA (cDNA) was randomly primed from 2μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RT-qPCR was subsequently performed in triplicate with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed using 2-ΔΔCt method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to the experimental controls.

Western blotting

The cells were lysed with RIPA buffer containing protease and phosphatase inhibitors (cOmplete ULTRA Tablets, Roche, Nutley, NJ, USA). After centrifugation, the supernatant was collected and quantified. The proteins were then separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk, the membranes were probed with rat anti-CD31 (Becton-Dickinson Biosciences), rabbit anti-p62, rabbit anti-LC3, rabbit-anti-ATG6, rabbit-anti-ATG7 and rabbit-anti-α-tubulin (Cell Signaling Technology, Danvers, MA, USA). Secondary antibodies are HRP-conjugated against rat or rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). The protein levels were first normalized to α-tubulin, and then normalized to the experimental controls. Densitometry of Western blots was quantified with NIH ImageJ software.

MiRNA target prediction and 3'-UTR luciferase-reporter assay

MiRNAs targets were predicted as has been described before, using the algorithms TargetSan (https://www.targetscan.org) [41]. The ATG6 3'-UTR reporter plasmid (pRL-ATG6) was purchased from Creative Biogene (Shirley, NY, USA). Mir-30-modified HAECs were transfected with 0.5μg pRL-ATG6 by Lipofectamine 2000 (5×10^4 cells per well). Cells were collected 48 hours after transfection for assay using the dual-luciferase reporter assay system gene assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Statistical analyses

The data in this study are shown as the mean ± S.D. Differences among groups were analyzed using one-away ANOVA with a Bonferoni correction, followed by Fisher' Exact Test for comparison of two groups (GraphPad Prism, GraphPad Software, Inc. La Jolla, CA, USA). $p < 0.05$ was considered significant.

Result

HFD induces atherosclerosis in ApoE (-/-) mice

Here, we gave a high-fat diet (HFD) to treat ApoE (-/-) mice (simplified as HFD mice) to induce experimental atherosclerosis in 12 weeks, which was confirmed by increases in aortic lesion size (Fig. 1A), and by increases in lipid content in aortic sinus (Fig. 1B), compared to littermate ApoE (-/-) mice that had received normal diet (NOR) as a control. Thus, HFD induces atherosclerosis in ApoE (-/-) mice. In order to analyze endothelial cells, we dissociated mouse aorta and purified endothelial cells based on CD31 labeling by flow cytometry (Fig. 1C).

HFD impairs endothelial cell autophagy in ApoE (-/-) mice

Then we analyzed the autophagy levels in endothelial cells from HFD and NOR mice. We found that the LC3-II/I ratio significantly decreased in endothelial cells from HFD mice, than those from NOR mice, shown by representative immunoblots (Fig. 2A), and by quantification (Fig. 2B). Then we analyzed the levels of major autophagy-activators ATG7, p62 and ATG6 in endothelial cells from these mice, and found no difference in the levels of ATG7 and p62 between endothelial cells from HFD and NOR mice, shown by representative immunoblots (Fig. 2A), and by quantification (Fig. 2C-D). However, we detected a significant decrease in the levels of ATG6 in endothelial cells from HFD mice, compared to those from NOR mice, shown by representative immunoblots (Fig. 2A), and by quantification (Fig. 2E). These data suggest that HFD may inhibit ATG6 to impair endothelial cell autophagy. Interestingly, the mRNA level of ATG6 was not altered in endothelial cells from HFD mice, compared to those from NOR mice (Fig. 2F). These data suggest that the effects of HFD on endothelial cell ATG6 may be through post-transcriptional control.

HFD impairs endothelial cell autophagy in ApoE (-/-) mice through suppressing the translation of ATG6 mRNA by miR-30

Next, we examined the underlying mechanisms. Since post-transcriptional control of gene expression is usually regulated miRNAs, we used bioinformatics analyses to screen all

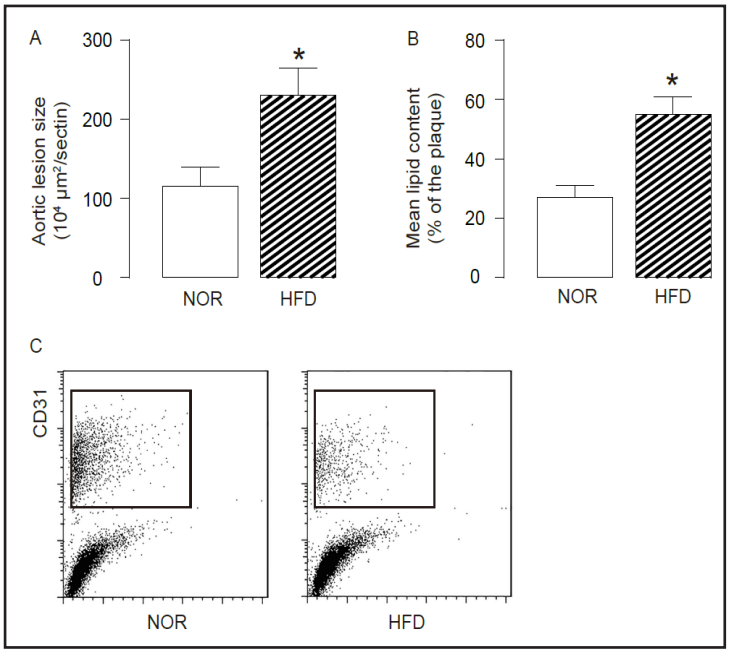

Fig. 1. HFD induces atherosclerosis in ApoE (-/-) mice. We used ApoE (-/-) mice treated with high-fat diet (HFD; simplified as HFD mice) for analyzing endothelial cell apoptosis. ApoE (-/-) mice that had received normal diet (NOR) were used as a control. (A-B) After a 12-week HFD treatment, analysis of H&E-stained histological sections of the aortic sinus showed a significant increase in aortic lesion size (A), and analysis of Oil-red-O-stained histological sections of the aortic sinus showed a significant increase in lipid content (B). (C) The aortas were dissociated and purified for endothelial cells based on CD31 labeling by flow cytometry. *p<0.05. N=5.

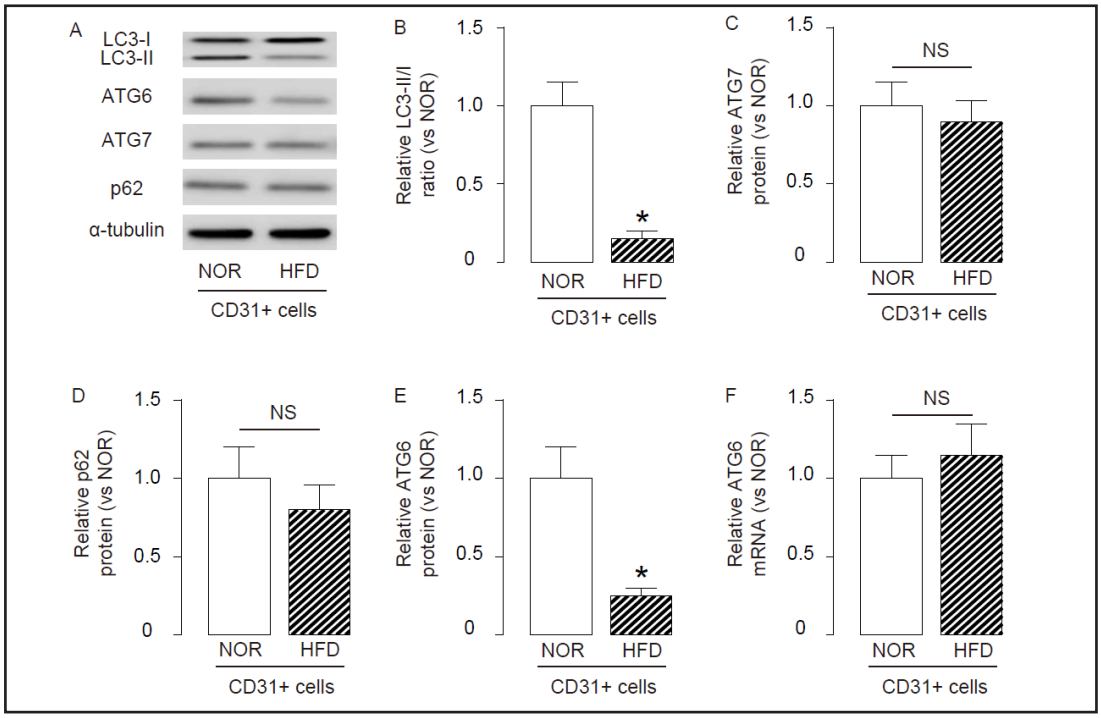

Fig. 2. HFD impairs endothelial cell autophagy in ApoE (-/-) mice. (A-E) Immunoblots for apoptosis-associated genes LC3-II/I, ATG7, p62 and ATG6 in endothelial cells from HFD and NOR mice, shown by representative blots (A), and by quantification for LC3-II/I (B), ATG7 (C), p62 (D) and ATG6 (E). (F) RT-qPCR for mRNA of ATG6. *p<0.05. NS: non-significant. N=5.

miRNAs that target ATG6 and altered their levels in endothelial cells in HFD mice. Specifically, we found that miR-30 targets 3'-UTR of ATG6 mRNA at one binding site of base pair 97th to 106th (Fig. 3A). Moreover, miR-30 levels in endothelial cells significantly increased after HFD treatment (Fig. 3B). Hence, we modified miR-30 levels in human aortic endothelial

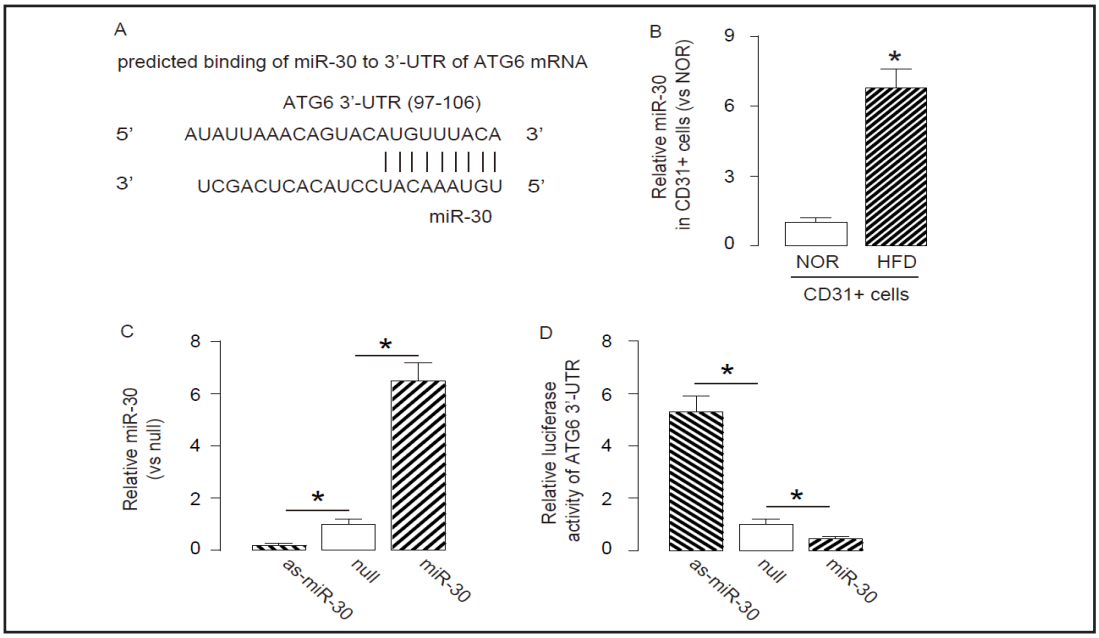

Fig. 3. HFD impairs endothelial cell autophagy in ApoE (-/-) mice through suppressing the translation of ATG6 mRNA by miR-30. (A) Bioinformatics analyses showing that miR-30 targets 3'-UTR of ATG6 mRNA at one binding site of base pair 2820th to 2826th. (B) MiR-30 levels in endothelial cells from HFD or NOR mice. (C) RT-qPCR for miR30 in miR-30-modified human aortic endothelial cells (HAECs), prepared by transfection of the cells with miR-30 mimics, antisense for miR-30 (as-miR-30) or null controls (null). (D) The intact 3'UTR of ATG6 mRNA was cloned into a luciferase reporter plasmid, and used for transfection of miR-30-modified HAECs. The luciferase activities were quantified. *p<0.05. N=5.

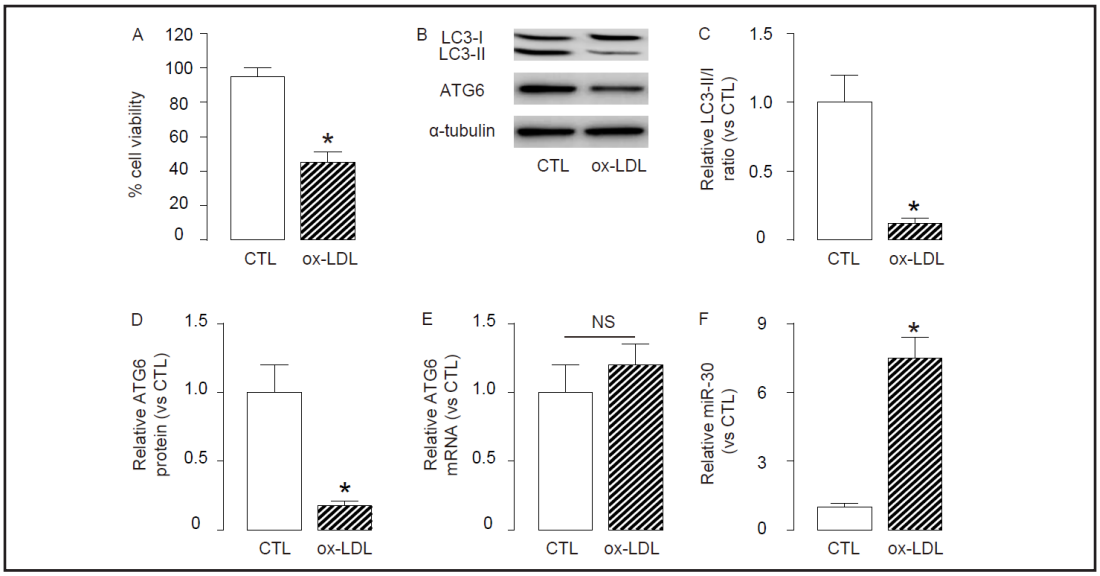

Fig. 4. Ox-LDL-treatment similarly impairs cell autophagy in HAECs. To confirm the findings in HFD mice, we used an *in vitro* model of atherosclerosis, in which HAECs were treated with or without 100μg/ml oxidized low-density lipoprotein (ox-LDL). (A) Quantification of viable cell number in an CCK-8 assay. (B-E) Immunoblots for apoptosis-associated genes LC3-II/I and ATG6 in endothelial cells from HFD and NOR mice, shown by representative blots (B), and by quantification for LC3-II/I (C) and ATG6 (D). (E-F) RT-qPCR for mRNA of ATG6 (E) and miR-30 (F). *p<0.05. NS: non-significant. N=5.

cells (HAECs), by transfection of the cells with miR-30 mimics, antisense for miR-30 (as-miR-30) or null controls (null). First, modification of miR-30 levels in HAECs was confirmed

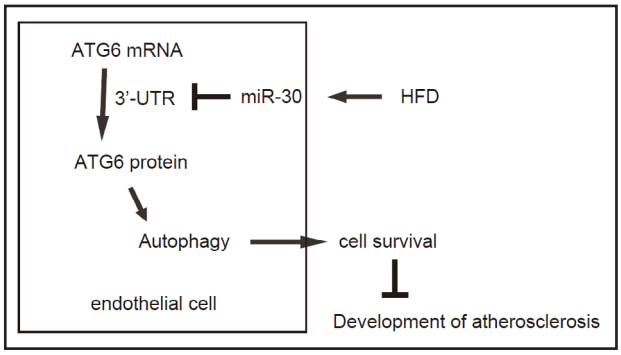

Fig. 5. Schematic of the model. The failure of atherosclerosis-associated endothelial cell autophagy may result from HFD-induced downregulation of ATG6, through increased miR-30 that binds and suppresses translation of ATG6 mRNA.

by RT-qPCR (Fig. 3C). Next, the intact 3'-UTR of ATG6 mRNA was cloned into a luciferase reporter plasmid, and used for transfection of miR-30-modified HAECs. We found that miR-30 significantly reduced the luciferase activity of 3'-UTR reporter for ATG6, while as-miR-30 significantly increased the luciferase activity of 3'-UTR reporter for ATG6, compared to null control (Fig. 3D). Together, these data suggest that HFD impairs endothelial cell autophagy in ApoE (-/-) mice through suppressing the translation of ATG6 mRNA by miR-30.

Ox-LDL-treatment similarly impairs cell autophagy in HAECs

To confirm the findings in HFD mice, we used an *in vitro* model of atherosclerosis, in which HAECs were treated with or without 100μg/ml oxidized low-density lipoprotein (ox-LDL) to induce cell injury similar to *in vivo* model. First, we confirmed that ox-LDL treatment resulted in a significant reduction in viable cell number in an CCK-8 assay (Fig. 4A). Moreover, ox-LDL significantly decreased the ratio of LC3II/I and ATG6 levels (Fig. 4B-D), without altering ATG6 mRNA (Fig. 4E). Finally, the levels of miR-30 were significantly increased by ox-LDL (Fig. 4F). Together, these data suggest that ox-LDL-treated HAECs reproduce our findings in HFD on mice. Thus, our study suggest that the failure of atherosclerosis-associated endothelial cell autophagy may result from HFD-induced downregulation of ATG6, through increased miR-30 that binds and suppresses translation of ATG6 mRNA (Fig. 5).

Discussion

Atherosclerosis is a complex immune-inflammatory disease involving arteries of medium-to-large size and its pathogenesis is coordinated among endothelial cells, macrophages and smooth muscle cells. First of all, injury of the vascular endothelium is a proven initial step in the pathogenesis of atherosclerosis. High glucose is a potent pro-atherosclerotic factor that has been widely used to induce experimental atherosclerosis *in vivo*, while *in vivo* application of ox-LDL is able to cause endothelial cell injury that is observed during development of atherosclerosis.

Aberrantly expressed miRNAs have recently been found to be involved in the development of endothelial cell survival/death, supported by many recent reports. Specially, Pan et al. reported that cardiomyocyte autophagy was regulated by ATG6 and miR-30 in cardiac disease model. They used a dual luciferase reporter assay to confirm that ATG6 was a target gene of miR-30a. These previous studies encouraged us to examine a possible role of miRNAs in the development of atherosclerosis through modulation of autophagy-related proteins.

Here, we found that HFD significantly impaired endothelia cell autophagy, and this failure of autophagy significantly increased endothelial cell death, through augment in cell apoptosis. Importantly, we found that ATG6 was the effector protein as a target of HFD, and most interestingly, its protein but not mRNA was changed by HFD. Of note, here the seemingly slight increases in mRNA (not reaching significance) may be resulting from a feedback of requirement of increasing the protein levels that continuously failed. Since it is unlikely that

ATG6 may be regulated by phosphorylation or acetylation in our experimental setting, we hypothesized that it might be regulated by miRNAs.

All ATG6-targeting miRNAs were then determined by bioinformatics analyses, and we screened all these miRNAs and found that miR-30 levels significantly increased in CD31+ endothelial cells from HFD mice. In addition, we used an *in vitro* atherosclerosis model to confirm this model in endothelial cell apoptosis.

Previous studies have shown controversy on the exact role of autophagy in the heart under pathological conditions [42]. Moreover, some of these studies have demonstrated seemingly different results from our work [43-48]. However, we think that these seemingly contradictory data are actually reflections of the complexity of autophagy in the regulation of development of atherosclerosis. Since autophagy could be a double-edged sword, in which modest levels of autophagy could improve the survival of the cells under insults, while the augment of autophagy level could lead to autophagy-associated cell death. Moreover, the differences in the exact HFD protocols, mouse strains (here ApoE-knockout mice), doses and frequency of ox-LDL, and analyzed target cells (here purified endothelial cells rather than mixed cell types in some studies) may modify the relative levels of autophagy- and apoptosis- associated proteins in various studies, leading to seemingly paradoxical results. Nevertheless, these studies demonstrate a delicate control of the levels of autophagy may be crucial for the development of atherosclerosis.

Together, our study identified a new role for miR-30 in the regulation of endothelial cell autophagy during atherosclerosis, and shed new insight into development of innovative therapy by targeting miR-30 in atherosclerosis treatment.

Disclosure Statement

The authors have declared that no competing interests exist.

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