



The activation of Wnt signaling facilitates autophagy by modulating GSK-3 β -AMPK axis in atherosclerosis

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Abstract

Background Endothelial dysfunction induced by oxidized low-density lipoprotein (ox-LDL) is implicated in the pathogenesis of atherosclerosis (AS). Activated autophagy was reported to improve endothelial functions and alleviate AS development. AMPK is a key protein in the regulation of autophagy, which can be modulated by Wnt signaling pathway.

Objective Our study was designed to explore whether the canonical Wnt activator Wnt3a promotes autophagy in AS through in vitro and in vivo assays.

Results Human umbilical vein endothelial cells (HUVECs) were first treated with ox-LDL at 50 μ g/mL for 24 h and then transfected with Wnt3a-overexpressed plasmid pcDNA3.1/Wnt3a. At 48 h after transfection, HUVECs were treated with 100 μ M 3-MA. To investigate the effects of GSK-3 β inhibition on the activation of AMPK, HUVECs were treated with 10 μ M TWS119. Cell viability was detected via Trypan blue staining. Wnt3a expression, autophagy markers, GSK-3 β inhibition and AMPK activation were examined by western blotting. To induce animal model of AS, male apolipoprotein E deficient (ApoE^{-/-}) mice were fed with high-fat diet. pcDNA3.1/Wnt3a was injected into ApoE^{-/-} mice after high-fat diet induction. Oil Red O staining was performed to examine lipid and plaque deposition in atherosclerotic lesions. In this study, ox-LDL treatment decreased HUVEC viability and downregulated Wnt3a expression. Wnt3a overexpression promoted autophagy, induced GSK-3 β phosphorylation at Ser9, and AMPK phosphorylation at Thr172 in ox-LDL-stimulated HUVECs. Overexpressing Wnt3a ameliorated lipid accumulation in aortic plaque of ApoE^{-/-} mice. Furthermore, Wnt3a overexpression also activated autophagy, and induced GSK-3 β and AMPK phosphorylation in ApoE^{-/-} mice.

Conclusion Wnt3a activates AMPK through inhibiting GSK-3 β , thereby facilitating autophagy in AS.

Keywords Atherosclerosis · Wnt/GSK-3 β signaling · AMPK · Endothelial dysfunction · Autophagy

Introduction

Atherosclerosis (AS), a chronic multifactorial vascular disorder featured by accumulation of lipid and cholesterol in arterial walls, is the prevailing cause of cardiovascular morbidity and mortality (Bäck et al. 2019; Herrington et al. 2016). Vascular endothelial cells play a critical role in maintaining cardiovascular homeostasis (Endemann and Schiffrin 2004). As the precursor of AS, endothelial dysfunction can be triggered by multiple risk factors, including oxidized low-density lipoprotein (ox-LDL), immune response, mechanical

stress and virus infection (Steinberg 2009; Tabas et al. 2007). Ox-LDL increases the level of oxidative stress, enhances endothelial cell adhesion, and induces the release of inflammatory factors in vascular endothelial cells, thereby contributing to endothelial dysfunction and the occurrence of AS (Li et al. 2002; Trpkovic et al. 2015). Thus, the improvement of ox-LDL-induced damage in endothelial cells is a therapeutic direction for AS.

Autophagy is a fundamental catabolic process in which damaged proteins or organelles are transported to lysosomes for degradation, plays an essential role in maintaining cellular energy homeostasis (Hamasaki et al. 2013; Xie and Klionsky 2007). Growing evidence supports the involvement of autophagy in a variety of physiological processes, including immunity, host defense, cellular metabolism and survival, development, and cancer (Bhattacharya and Eissa 2015; Wu et al. 2013; Xia et al. 2021). The beneficial effects

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of autophagy on endothelial functions and the participation of autophagy in cardiovascular diseases have been demonstrated in recent studies (Jiang 2016; Ren and Zhang 2018). The impairment of endothelial cell autophagy can result in multiple adverse consequences, such as decreased vascular permeability and damaged vascular integrity (Zhang et al. 2018a). The activation of endothelial cell autophagy inhibits monocyte invasion and infiltration into the subendothelium byusion, which pushes the pause button on the atherogenic process (Hua et al. 2022). Many studies have proved that enhancing autophagy is an effective measure to delay AS to a certain extent (Meng et al. 2021; Schrijvers et al. 2011). For example, ANGPTL4 enhances endothelial cell proliferation and repressed endothelial cell injury by increasing autophagy, thereby protecting against the development of AS (Zhan et al. 2022). Resveratrol attenuates endothelial inflammation in the pathogenesis of AS by inducing autophagy (Chen et al. 2013) However, current research at home and abroad has not shown effective methods in enhancing the autophagy capacity of endothelial cells.

Wnt signaling mediates multiple biological processes by a canonical or noncanonical pathway, and is involved in a vast spectrum of disorders (Kahn 2014). In the classical pathway, classical Wnt ligands bind to the Frizzled receptor, resulting in GSK-3 β inhibition, which allows β -catenin stabilization and nuclear translocation (Angers and Moon 2009). Nevertheless, new players have recently emerged in the classical Wnt pathway, indicating that the biological effects of β -catenin-independent pathways rely on the rapid regulation of GSK-3 β in the cytoplasm but don't require β -catenin accumulation (Acebron et al. 2014; Acebron and Niehrs 2016). Previously, Li et al. demonstrated that the suppression of the wnt/ β -catenin/GSK3 β pathway inhibited SIRT1 expression to promote autophagy in endothelial progenitor cells, thereby alleviating the coronary atherosclerosis in mice (Li et al. 2022). In addition, Wnt signaling and cellular metabolism can be linked through AMPK (Takatani et al. 2011). AMPK is a key sensor of cellular energetic conditions, which is extensively expressed in various tissues and organs (Hardie et al. 2016). AMPK's cell signaling pathways participate in a variety of physiological processes, including apoptosis, autophagy, transcriptional control, cytoskeleton construction and metabolism (Kahn et al. 2005). AMPK modulates endothelial cell functions and lipid and carbohydrate metabolism, thereby participating in AS development (Dong et al. 2010; Ewart and Kennedy 2011). Reduced AMPK activity and autophagy dysfunction are associated with AS (Ou et al. 2018). When intracellular ATP production is reduced, AMPK is phosphorylated at Thr172, and the activated AMPK can inhibit mTORC-1 complex, thereby enhancing autophagy and promoting catabolism (Chandramouleeswaran et al. 2020). In 2014, Suzuki and

colleagues identified AMPK to be negatively regulated by GSK-3 β , which promotes AMPK phosphorylation at Thr172 by PP2A and subsequent dephosphorylation, leading to the inactivation of AMPK (Suzuki et al. 2013). Previously, Wnt3a, a canonical Wnt ligand, was reported to enhance autophagy in hippocampal neurons via activating AMPK through GSK-3 β inhibition (Ríos et al. 2018).

Based on the above knowledge, we inferred whether the GSK-3 β -AMPK signaling can be regulated by Wnt3a in mediating autophagy in AS. We examined the autophagy role of Wnt3a-GSK-3 β -AMPK axis in ox-LDL-stimulated human umbilical vein endothelial cells (HUVECs) and in apolipoprotein E deficient (ApoE^{-/-}) mice.

Materials and methods

Cell culture, treatment and transfection

HUVECs obtained from Lonza (Walkersville, MD, USA) were maintained in EGM-2 endothelial growth medium (CC-3162; Lonza, Walkersville, MD, USA) containing 2% fetal bovine serum (FBS; Catalog No: 10099141C, Gibco) at 37 °C with 5% CO₂. To induce cell damage, HUVECs were first exposed to ox-LDL (0–100 μ g/mL; Catalog No: AY-1501, Shanghai AngYu Biotechnology Co., Ltd., Shanghai, China) for 24 h to examine the cytotoxic effects to HUVECs. Then, empty pcDNA3.1 or pcDNA3.1/Wnt3a were transfected into HUVECs using Lipofectamine 2000 (Catalog No: 11668019, Invitrogen, Carlsbad, CA, USA) for 48 h following 50 μ g/mL ox-LDL stimulation. The pcDNA3.1/Wnt3a-transfected HUVECs were then treated with 100 μ M 3-MA (the autophagy inhibitor; Catalog No: M9281, Sigma-Aldrich, St. Louis, MO, USA). The control or ox-LDL-stimulated HUVECs were treated with 10 μ M TWS119 (the inhibitor of GSK-3 β ; Catalog No: S1590, Selleck, Houston, TX, USA).

Trypan blue staining for cell viability

Cells were seeded in six-well plates and incubated until about 90% confluence. Cells after different treatment and/or transfection were digested with 0.25% trypsin solution (Catalog No: T4049, Sigma-Aldrich). Then, cells were suspended in PBS (pH 7.4; Catalog No: 806552, Sigma-Aldrich), and 0.4% trypan blue dye (1:1 volume; Catalog No: ST798, Beyotime, Shanghai, China) was added to cell suspension. The unstained living cells were counted by the Countstar automated cell counter (Inno-Alliance Biotech, USA).

Green fluorescent protein (GFP)-LC3 immunofluorescence

HUVECs following indicated treatment or transfection were seeded (8×10^4 cells/mL) into a Petri dish (35 mm diameter) covered with a glass slide and incubated for 24 h. Cells were then transfected with GFP-LC3 plasmid using Lipofectamine 2000. Forty-eight h after transfection, the slides were rinsed three times with PBS, fixed with 4% paraformaldehyde, washed three times with PBS, and mounted with anti-fluorescence quenching mounting medium. Fluorescence images were captured using laser confocal microscopy (Zeiss LSM700, Carl Zeiss, Canada)).

Animals

Animal experiments were reviewed and approved by the Ethics Committee of Wuhan Myhalic Biotechnology Co., Ltd (Approval number: 202109136; Hubei, China). Forty male ApoE^{-/-} mice and thirty wild-type C57BL/6 J controls (6-week-old, 28–32 g) were purchased from Peking University Animal Center (Beijing, China). All mice were maintained in a specific pathogen-free environment with the temperature of 24 °C and the humidity of 55% under a 12 h light/dark cycle.

Induction of AS in mice

The in vivo experiments were comprised of 3 parts. The first part was designed to investigate the effects of Wnt3a overexpression on the lipid accumulation in aortic plaque of AS mice, and included 4 groups: sham + vehicle group ($n = 10$), sham + Wnt3a group ($n = 10$), Model + vehicle group ($n = 10$), and Model + Wnt3a group ($n = 10$). The second part was designed to assess the influence on the autophagy in AS mice, and included 5 groups: sham + vehicle group ($n = 10$), sham + Wnt3a group ($n = 10$), Model + vehicle group ($n = 10$), Model + Wnt3a group ($n = 10$), and Model + Wnt3a + 3-MA group ($n = 10$). The third part was designed to evaluate the effects of Wnt3a overexpression on the GSK-3 β /AMPK axis, and included 6 groups: sham + vehicle group ($n = 10$), sham + Wnt3a group ($n = 10$), sham + TWS119 group ($n = 10$), Model + vehicle group ($n = 10$), Model + Wnt3a group ($n = 10$), and Model + TWS119 group ($n = 10$). After one week of feeding acclimation, ApoE^{-/-} mice were fed on high-fat diet (0.15% cholesterol, 21% fat, and 78.85% of basic mice maintain feed) for 12 weeks to establish a visible AS model. C57BL/6J mice fed a high-fat diet served as sham group. After 12 weeks, ApoE^{-/-} mice or C57BL/6J mice were injected via tail vein with Wnt3a-overexpressed plasmid pcDNA3.1/Wnt3a or the empty pcDNA3.1 vector, or intraperitoneally injected with 30 mg/kg TWS119 (the inhibitor

of GSK-3 β ; Catalog No: S1590, Selleck, Houston, TX, USA) or 15 mg/kg 3-MA (the autophagy inhibitor; Catalog No: M9281, Sigma-Aldrich, St. Louis, MO, USA) After 24 h, the blood samples were collected via cardiac puncture and centrifuged to obtain the serum. Then, mice were euthanized via cervical dislocation, and the hearts with aortic arch were resected, fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 5 μ m-thick sections for Oil Red O staining.

Oil red O staining

The slices (5 μ m) were dried with excess temperature for 20 min and incubated with 100% isopropanol (Catalog No: 563935, Sigma-Aldrich) for 5 min. Then the slices were incubated with 0.5% oil red O staining solution (Catalog No: B1094, Applygen Technologies Inc., Beijing, China) in a 60 °C oven, washed in 85% isopropanol, dyed with hematoxylin (Catalog No: B21220, Shanghai yuanye Bio-Technology Co., Ltd., Shanghai, China), cleaned and sealed. The nucleus was stained light blue and the lipid was stained orange or red. The area of atherosclerotic plaque in each aortic section was evaluated using Image-Pro Plus software (NIH, USA).

Serum analysis of lipids

Serum levels of triglyceride (TG; Catalog No: ml094956), total cholesterol (TC; Catalog No: ml094952), low-density lipoprotein cholesterol (LDL-C; Catalog No: ml076620) and high-density lipoprotein cholesterol (HDL-C; Catalog No: ml092722) were detected using commercially available enzyme kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) with a Hitachi 7600 Automatic Biochemistry Analyzer (Tokyo, Japan).

Western blotting

The total protein was extracted from HUVECs and mouse aortic tissues using RIPA lysis buffer (#P0013B, Beyotime) containing protease and phosphatase inhibitors. Equal amount of protein was separated by 10% SDS-PAGE gels and electro-transferred onto a PVDF membrane (Millipore). The membrane was sealed in 5% non-fat dried milk, and added with primary antibodies against Wnt3a (ab219412, 1: 1000; Abcam, Cambridge, MA, USA), LC3B (ab192890, 1: 2000; Abcam), BECN1 (ab207612, 1: 2000; Abcam), P62 (ab240635, 1: 1000; Abcam), AMPK (#2532, 1:1000; Cell Signaling, Danvers, Massachusetts, USA), pThr172-AMPK (#2535, 1: 1000; Cell Signaling), GSK-3 β (#9315, 1: 1000; Cell Signaling), pSer9-GSK-3 β (#9336, 1: 1000; Cell Signaling), and β -actin (ab8227, 1: 1000; Abcam) at 4 °C overnight. Next day, the membrane was incubated with corresponding secondary antibodies (1: 1000, Abcam)

for 2 h. Protein bands were visualized using an enhanced chemiluminescence kit (#P0018S, Beyotime) and then photographed with a gel imaging system. ImageJ software was used to analyze the gray values of protein bands. Relative quantification of protein was carried out with β -actin as an internal reference.

Statistical analysis

All data from at least three independent experiments were analyzed by SPSS 16.0 software (SPSS, Chicago, IL), and are indicated as mean \pm standard deviation. Disparities between two or among multiple groups were formulated by Student's *t* test or one-way analysis of variance. Statistical significance was established by $p < 0.05$.

Results

Ox-LDL treatment decreases HUVEC viability and downregulates Wnt3a expression

First, HUVECs were stimulated by ox-LDL (0–100 $\mu\text{g}/\text{mL}$) for 24 h and cell viability was assessed by trypan blue staining. The results showed that ≤ 10 $\mu\text{g}/\text{mL}$ ox-LDL stimulation did not influence HUVEC viability (Fig. 1A). When the ox-LDL concentration exceeded 25 $\mu\text{g}/\text{mL}$, cell viability started to decrease, and when the cells were stimulated by ox-LDL at 50 $\mu\text{g}/\text{mL}$, cell viability decreased by nearly 50%, so cells stimulated by 50 $\mu\text{g}/\text{mL}$ ox-LDL were used in the subsequent experiments. Then, pcDNA3.1/Wnt3a was transfected into the ox-LDL-stimulated HUVECs. Western blotting analysis illustrated that ox-LDL considerably reduced Wnt3a expression in HUVECs, which was restored after pcDNA3.1/Wnt3a transfection (Fig. 1B–C).

The ox-LDL-induced reduction in HUVEC viability was recovered by overexpressing Wnt3a (Fig. 1D).

Wnt3a overexpression enhances autophagy in ox-LDL-exposed HUVECs

Next, we evaluated the autophagy in ox-LDL-stimulated HUVECs affected by Wnt3a overexpression. Immunofluorescence staining indicated that Wnt3a overexpression efficiently enhanced the percentage of GFP-LC3 cells, which was reversed by the treatment of 3-MA (Fig. 2A). Furthermore, the expression of autophagy-related proteins (LC3, Beclin1, and P62) in HUVECs after different treatment or transfection was detected by western blotting. The results showed that overexpressing Wnt3a in ox-LDL-stimulated HUVECs resulted in an increase in the LC3-II/LC3-I ratio and BECN1 protein level and a decrease in P62 protein level, which were also recovered after 3-MA treatment (Fig. 2B–E). Neither ox-LDL treatment alone nor pcDNA3.1/Wnt3a transfection alone influenced the percentage of GFP-LC3 cells, the LC3-II/LC3-I ratio, and BECN1 and p62 protein levels. Overall, overexpressing Wnt3a can promote autophagy in ox-LDL-exposed HUVECs.

Wnt3a overexpression inhibits GSK-3 β activity to promote AMPK activation in ox-LDL-stimulated HUVECs

We investigated whether Wnt3a overexpression modulates AMPK activity in HUVECs after ox-LDL stimulation. AMPK and GSK-3 β activities were evaluated by assessing Thr172 and Ser9 phosphorylation, respectively. We found that Wnt3a overexpression or TWS119 treatment further enhanced the ox-LDL-induced AMPK phosphorylation at Thr172 and GSK-3 β phosphorylation at Ser9 (Fig. 3A–C).

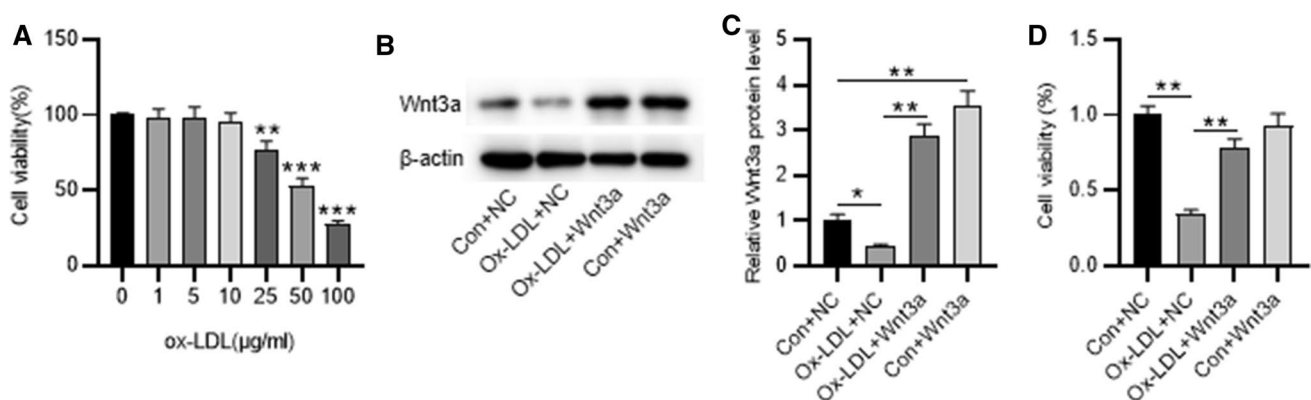


Fig. 1 Influence of ox-LDL stimulation on HUVEC viability and Wnt3a expression. **A** The viability of HUVECs stimulated by ox-LDL (0–100 $\mu\text{g}/\text{mL}$) for 24 h was detected by trypan blue staining. **B** Western blotting analysis of Wnt3a expression in HUVECs follow-

ing ox-LDL treatment or pcDNA3.1/Wnt3a transfection. **C** HUVEC viability influenced by ox-LDL treatment or pcDNA3.1/Wnt3a transfection was measured by trypan blue staining. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

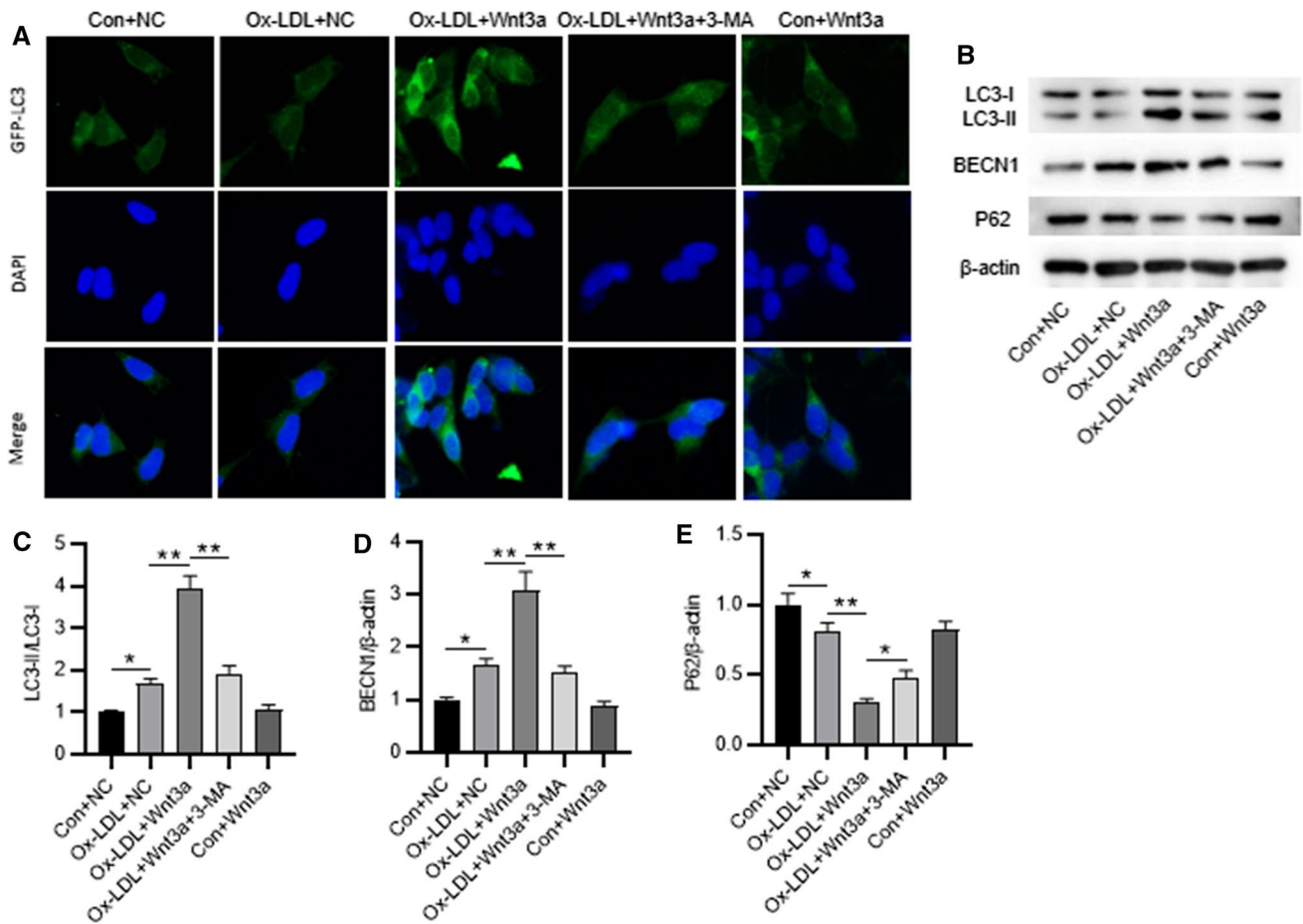


Fig. 2 Autophagy activation in ox-LDL-stimulated HUVECs caused by Wnt3a overexpression. HUVECs were first stimulated by 50 µg/mL ox-LDL for 24 h, and then transfected with pcDNA3.1/Wnt3a for 48 h and treated with 100 µM 3-MA for 24 h. **A** HUVECs were stained with LC3-antibody (green) and Hoechst (blue) to visualize

LC3 puncta and nuclei by confocal laser scanning microscopy. **B–E** HUVECs were harvested for western blotting to examine autophagy markers LC3, BECN1 and P62. Relative quantification of protein was carried out with β-actin as an internal reference. **P* < 0.05, ***P* < 0.01

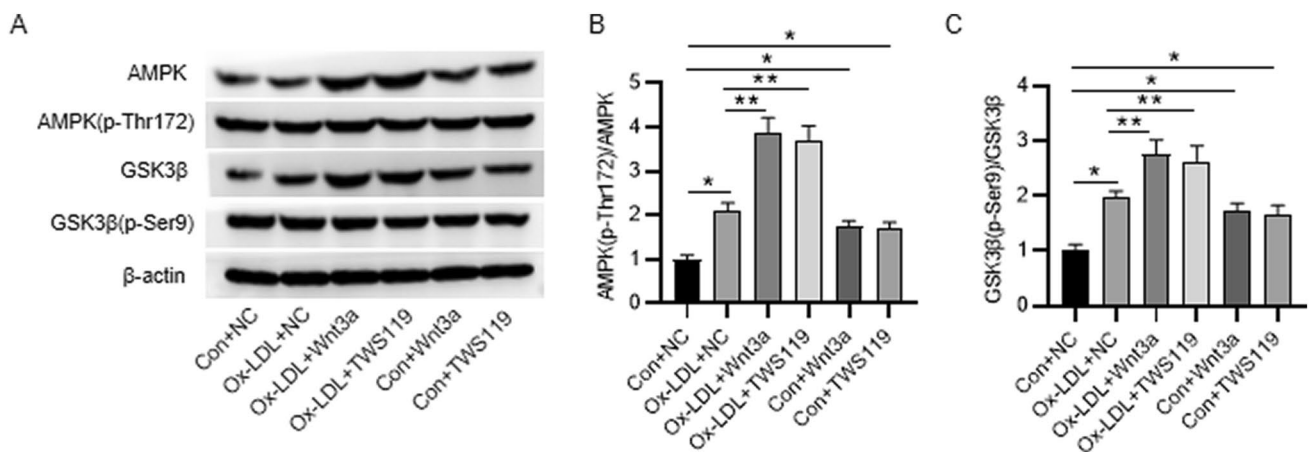


Fig. 3 GSK-3β inhibition and AMPK activation in ox-LDL-stimulated HUVECs caused by Wnt3a overexpression. HUVECs were first stimulated by 50 µg/mL ox-LDL for 24 h, and then transfected with pcDNA3.1/Wnt3a for 48 h or treated with 10 µM TWS119 for 24 h.

A–C In HUVECs, AMPK phosphorylation at Thr172 and GSK-3β phosphorylation at Ser9 were evaluated using western blotting analysis. **P* < 0.05, ***P* < 0.01

Since AMPK can be activated through the phosphorylation at Thr172, and GSK-3 β can be inactivated through the phosphorylation at Ser9, we concluded that overexpressing Wnt3a further stimulated AMPK activation and GSK-3 β inhibition in ox-LDL-stimulated HUVECs. These data imply that Wnt3a causes inhibition of GSK-3 β , thereby promoting AMPK activation.

Overexpressing Wnt3a attenuates lipid accumulation in aortic plaque of ApoE^{-/-} mice

The effects of pcDNA3.1-Wnt3a injection on the lipid content in aortic tissues of mice were assessed utilizing Oil red O staining. Blue nucleus and red fat can be observed in the images. There were hardly any visible lipid deposition and atherosclerotic plaque in arterial vessels of C57BL/6J mice injected with empty pcDNA3.1 or pcDNA3.1-Wnt3a. In contrast, accumulated lipids and obvious areas of atherosclerotic plaque can be observed in experimental ApoE-deficient mice injected with empty pcDNA3.1 compared with C57BL/6J mice pre-injected with empty pcDNA3.1. However, injection with pcDNA3.1-Wnt3 substantially attenuated lipid deposition and the atherosclerotic plaque area

in mouse aorta (Fig. 4A–B). In addition, at the end of the experiment, serum lipid levels were detected. In the serum of ApoE^{-/-} mice, TG, TC and LDL-C levels were evidently elevated, while HDL-C level was considerably reduced, compared with the control C57BL/6J mice. Nevertheless, injection of pcDNA3.1-Wnt3a into ApoE^{-/-} mice obviously lowered TG, TC and LDL-C levels in serum, but exerted no evident impact on HDL-C level (Fig. 4C–F). Taken together, Wnt3a attenuates lipid deposition in aortic plaque of ApoE-deficient mice.

Wnt3a promotes autophagy in ApoE^{-/-} mice by activating the GSK-3 β -AMPK signaling

Finally, whether Wnt3a mediates autophagy and the GSK-3 β -AMPK signaling in ApoE-deficient mice was assessed. The expression of key autophagy-related proteins and the activities of AMPK and GSK-3 β were analyzed using western blotting. The findings revealed that injection of pcDNA3.1-Wnt3a upregulated the LC3-II/LC3-I ratio and BECN1 expression, while downregulated P62 expression in the aorta of ApoE^{-/-} mice, which was antagonized by 3-MA injection (Fig. 5A–D). Furthermore, injection of

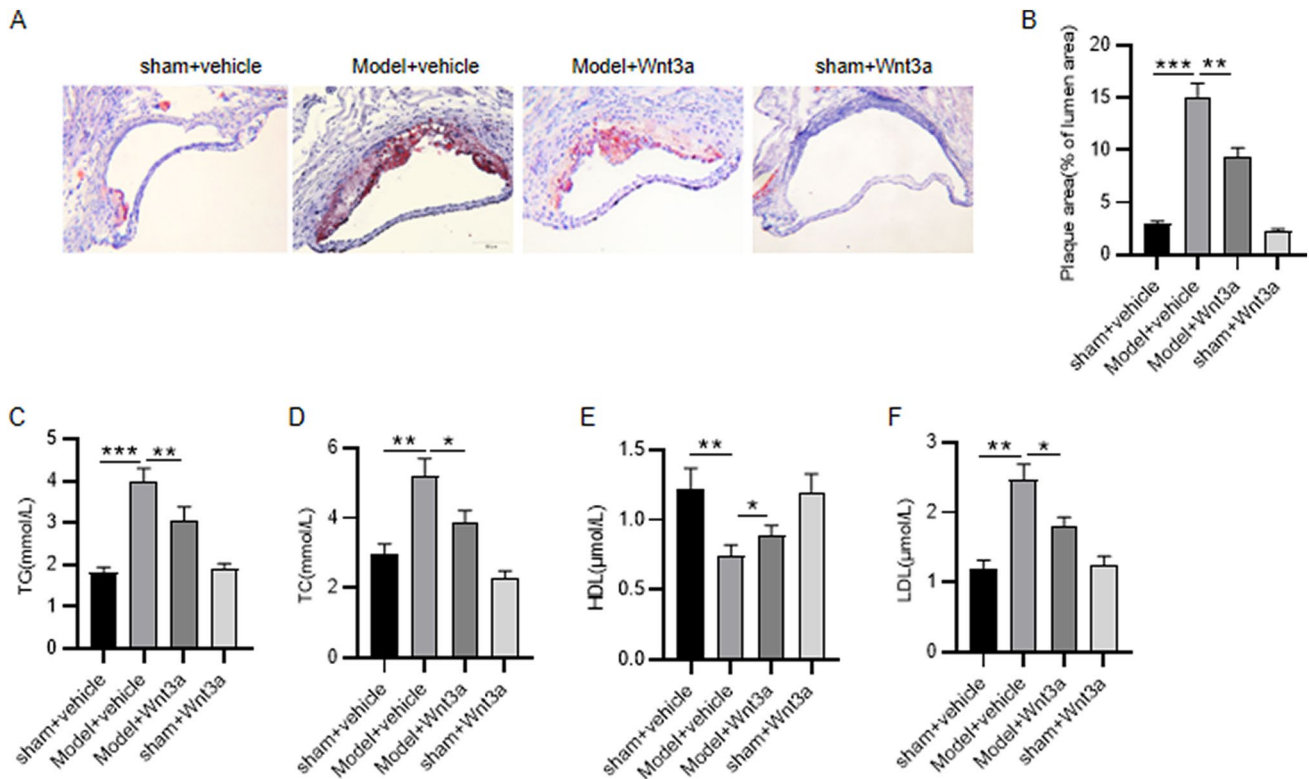


Fig. 4 Improvement of lipid deposition in aortic tissues of ApoE^{-/-} mice by Wnt3a overexpression. After high-fat diet induction for 12 weeks, ApoE^{-/-} mice and C57BL/6J mice were injected with pcDNA3.1/Wnt3a or empty pcDNA3.1. **A** Lipid deposition and atherosclerotic plaque formation were evaluated in each group of mice

adopting Oil Red O staining. **B** Plaque areas in each group were quantified using ImageJ software. **C–F** TG, TC, HDL-C and LDL-C levels in serum of mice in each group were assessed by commercial kits. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

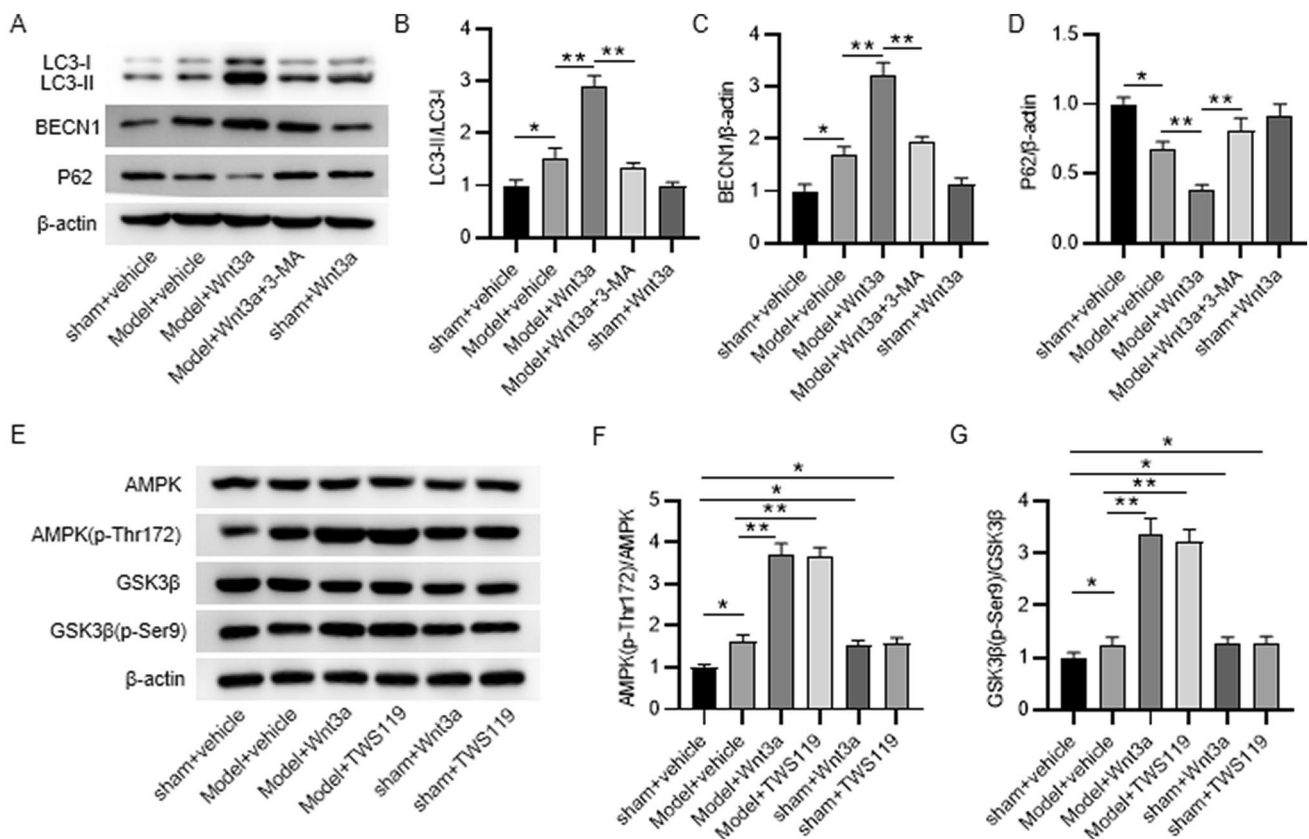


Fig. 5 On activation of autophagy and GSK-3 β -AMPK signaling in ApoE^{-/-} mice by Wnt3a overexpression. After high-fat diet induction for 12 weeks, ApoE^{-/-} mice and C57BL/6J mice were injected with pcDNA3.1/Wnt3a and 15 mg/kg 3-MA to evaluate autophagy, or injected with pcDNA3.1/Wnt3a or 30 mg/kg TWS119 to assess the GSK-3 β -AMPK axis. **A–D** The aortic tissues of ApoE^{-/-} mice

were collected for western blotting to assess autophagy markers LC3, BECN1 and p62. Relative quantification of protein was carried out with β -actin as an internal reference. **E–G** In each group, AMPK phosphorylation at Thr172 and GSK-3 β phosphorylation at Ser9 were investigated using western blotting. * $P < 0.05$, ** $P < 0.01$

pcDNA3.1-Wnt3a or TWS119 further enhanced AMPK phosphorylation at Thr172 and GSK-3 β phosphorylation at Ser9 in ApoE-deficient mice (Fig. 5E–G), indicating that Wnt3a further stimulated AMPK activation and GSK-3 β inhibition in ApoE^{-/-} mice. In conclusion, Wnt3a inhibits GSK-3 β activity to induce the activation of AMPK, thereby promoting autophagy in ApoE-deficient mice.

Discussion

AS is recognized as a worldwide public health problem, in which ox-LDL-induced endothelial damage is a key factor contributing to its initiation and development (Rajagopalan et al. 2004). Ox-LDL can directly damage the endothelial cells on the surface of blood vessels, which result in the increase of the gap between endothelial cells and the enhancement of permeability, facilitating the passage of lipid components through the endothelial layer into the sub-endothelial layer and leading to lipid deposition (Guo et al.

2021). The present study demonstrated Wnt3a, the canonical Wnt activator, attenuated ox-LDL-induced endothelial injury by reinforcing autophagy via inducing GSK-3 β inhibition and the subsequent AMPK activation, enriching our understanding of Wnt signaling in AS progression.

In the past decades, both canonical and non-canonical Wnt pathways have been identified to be implicated in the development of AS (Badimon and Borrell-Pages 2017). In particular, the canonical Wnt/ β -catenin pathway widely participates in regulating endothelial dysfunction and abnormal lipid metabolism in AS. SIRT1 alleviates AS in ApoE^{-/-} mice by inhibiting autophagy in endothelial progenitor cells through activating Wnt/ β -catenin/GSK3 β signaling (Li et al. 2022). LncRNA H19 sponges miR-148b to upregulate WNT1 level, thereby facilitating proliferation and repressing apoptosis in ox-LDL-exposed human aorta vascular smooth muscle cells via triggering Wnt/ β -catenin pathway (Zhang et al. 2018b). Knockdown of Dickkopf-2 stimulates Wnt/ β -catenin pathway, thereby protecting against lipid loading and macrophage polarization and

in ApoE knockout mice (Zhang et al. 2021). However, in recent years, the β -catenin-independent Wnt signaling that depends on the modulation of GSK-3 β in the cytoplasm has attracted people's attention (Cisternas et al. 2016). Wnt3a is a canonical Wnt activator, and Wnt3a ligand treatment was demonstrated to prevent neurotoxic damage through GSK-3 β -mediated inhibition, which emphasized the prominent function of GSK-3 β as a potential master regulator of β -catenin-independent Wnt pathway (Alvarez et al. 2004).

Autophagy, an evolutionarily conserved lysosome-mediated biodegradation process, is the recycling and reuse of cells' own waste and is essential in maintaining cellular homeostasis (Klionsky and Emr 2000). Substantial evidence suggests that autophagy defection is associated with the pathogenesis of various metabolic disorders, including AS, while increased autophagy mitigates the development of AS (Osonoi et al. 2018). Aloe-emodin derivative promotes autophagy in human aortic endothelial cells via enhancing the level of AMBRA1, a key protein associated with autophagosome formation, thereby exerting potent anti-atherosclerosis effects (Tang et al. 2022). Metformin attenuates atherosclerosis and enhances plaque stability in ApoE^{-/-} mice by activating Krueppel-like factor 2-mediated autophagy (Wu et al. 2021). Morin hydrate activates autophagy in HUVECs by inhibiting the NF- κ B pathway and ameliorates vascular inflammation in AS mouse models (Meng et al. 2021). AMPK is a key protein in the regulation of autophagy, and mainly acts as an "energy regulator" in eukaryotic cells (Yan et al. 2022). Previously, inhibition of GSK-3 β induced by Wnt3a ligand was shown to activate AMPK, thereby facilitating autophagy in hippocampal neurons (Ríos et al. 2018). Therefore, our study was designed to confirm the hypothesis that Wnt3a activates autophagy in AS via the GSK-3 β -AMPK axis. We discovered that Wnt3a promoted autophagy, inhibited GSK-3 β activity and activated AMPK activity in both ox-LDL-stimulated HUVECs and in ApoE-deficient mice. Since the use of TWS119 further confirmed that the activity of AMPK can be activated after the treatment of TWS119, the inhibitor of GSK-3 β , we concluded that Wnt3a inhibited GSK-3 β to induce AMPK activation, thereby promoting autophagy in ox-LDL-stimulated HUVECs and in ApoE-deficient mice.

Our paper demonstrated for the first time that Wnt3a enhanced autophagy in AS via modulating GSK-3 β -AMPK axis, shedding light on the mechanism underlying the protection of Wnt signaling activation on endothelial dysfunction induced by ox-LDL and abnormal lipid metabolism. Therefore, the activation of Wnt-GSK-3 β -AMPK-autophagy pathway be a promising therapy for AS.

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Author contributions SQ and BN were the main designers of this study. KZ, NJ, and HL performed the experiments. BN analyzed the

data. SQ drafted the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest Shifang Qu declares that he/she has no conflict of interest. Kuanxin Zhang declares that he/she has no conflict of interest. Nan Jin declares that he/she has no conflict of interest. Han Li declares that he/she has no conflict of interest. Bin Nie declares that he/she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. Animal experiments were granted by the Ethics Committee of Wuhan Myhalic Biotechnology Co., Ltd (Approval number: 202109136; Hubei, China).

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