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Unveiling the protective role of ESM1 in endothelial cell proliferation and lipid reprogramming

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Palmitic acid (PA), being the most prevalent free fatty acid in the human, holds significant implications as a risk factor for atherosclerosis (AS) due to its ability to induce physiological dysfunction in endothelial cells (ECs). Endothelial cell-specific molecule 1 (ESM1), has been identified as a marker for activated ECs. Nevertheless, the mechanisms underlying ESM1-induced endothelial cell proliferation remain elusive. The expression of ESM1, ANGPTL4 and autophagy related protein were confirmed by western blot. Proliferation ability was tested by MTT and EdU. Lipids level was confirmed by Oil red staining. Autophagic flux was confirmed by Monodansylcadaverine (MDC) staining and pCMV-mCherry-GFP-LC3B fluorescence staining assay. The mouse model of AS was used to observe the effect of PA on the ESM1-ANGPTL4-autophagy signaling axis. This study elucidates ESM1-ANGPTL4 axis in maintaining proliferation of ECs and lipid reprogramming. Furthermore, it has been observed that PA has the ability to stimulate EC to autonomously increase the expression of ESM1, which in turn can counteract the detrimental effects of PA on ECs. Conversely, when ESM1 is suppressed, the damaging effects of PA on ECs are exacerbated. Mechanistically, our findings indicate that ESM1 facilitates EC proliferation and lipids homeostasis by up-regulating autophagy through ANGPTL4. This effect of ESM1 on ECs can be attenuated by ATG7 inhibiting. Additionally, the serum levels of ESM1 were found to be elevated in AS mice. ESM1 was found to enhance ECs proliferation and mitigate endothelial cell injury induced by PA through the upregulation of autophagy. This mechanism potentially serves as a protective factor against atherosclerosis progression.

Keywords ESM1, ANGPTL4, Lipid metabolism reprogramming, Endothelial cell injury, Autophagy, Palmitic acid

PA is not only the major component of AS but is also a significant factor in multiple cancer proliferation and metastasis[1,2]. Damage to ECs occurs before PA induces AS[3]. However, the molecular mechanism of endothelial cell interaction with PA remains unknown.

ESM1 is a secreted protein by ECs, which is identified as a marker of activated ECs[4]. ESM1 can promote angiogenesis[5] and EC responses by multiple stress factors and disease[6]. ESM1 can regulate connexin 40 and eNOS in during EC differentiation[7]. Serum ESM1 levels in hypertensive patients are significantly elevated and related to the formation and degree of coronary artery disease[8]. In our previous study, the ESM1 promote the development and progression of ovarian cancer, especially in angiogenesis, which can activate Akt/mTOR

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pathway and interact with Angiopoietin-like 4 (ANGPTL4) to promote ECs proliferation, migration, fatty acid metabolism, and tube formation in ovarian cancer tumor microenvironment[5], which indicated that ANGPTL4 was a significant downstream gene of ESM1. Zhan et al. found that ANGPTL4 could induce autophagy to reduce PA-induced ECs injury[9].

ANGPTL4 was a key role in multiple progression, including metabolic reprogramming, angiogenesis, redox reactions, and cancer development[10]. The most important molecular biological effect of ANGPTL4 is to inhibit the activity of LPL, thus inhibiting the degradation of fatty acids, and thus leading to the accumulation of triglycerides[11]. It has also been pointed out that ANGPTL4 can inhibit the fatty acid uptake of macrophages, and overexpression of ANGPTL4 in ApoE*3-Leiden mice can slow down the development of RA, which does not interfere with the cholesterol and triacylglycerols cycles[12]. In hematopoietic cells, ANGPTL4 can inhibit lipid overload in macrophages and attenuate the AS process[13]. In EC, we found ANGPTL4 can enhance fatty acid and induce angiogenesis by regulating lipid metabolism reprogramming in ovarian cancer tumor microenvironment[14]. Therefore, the function and mechanism of ANGPTL4 in AS remains unknown.

Autophagy, a cellular process in eukaryotes, involves the utilization of lysosomes to degrade cytoplasmic proteins and damaged organelles, regulated by autophagy related genes. This mechanism serves to safeguard cells from harm and react to cytotoxic stimuli[15]. Autophagy serves as a cellular self-defense mechanism, conferring advantages to cellular proliferation and maturation, safeguarding against metabolic stress and oxidative harm, and assuming a pivotal function in upholding cellular homeostasis, as well as in the synthesis, degradation, and recycling of cellular constituents[16]. Nevertheless, an excessive manifestation of autophagy can engender metabolic dysregulation and potentially even cellular demise[17,18]. During the initial phase of AS, the activation of autophagy in ECs is prompted by oxidative factors, thereby preserving the cells through the mitigation of oxidative stress, augmentation of nitric oxide bioavailability, and reduction of vasculitis. The progression of AS is observed to deteriorate gradually in the absence of functional autophagy. Furthermore, impaired autophagy renders cells more susceptible to apoptotic stimuli and inflammatory factors, consequently contributing to plaque instability[19,20]. In the present study, it was observed that ECs exhibit self-repair capabilities through the upregulation of ESM1 secretion upon exposure to PA. The potential molecular mechanism underlying this phenomenon appears to involve ANGPTL4-mediated autophagy and metabolic reprogramming.

Methods

Cell culture and transfection

The HUVEC cell line, obtained from ATCC, was cultured in DMEM with high-glucose, 10% FBS, and 1% penicillin/streptomycin at 37 °C and 5% CO_2. ESM1 shRNA, ANGPTL4 shRNA, and plasmids containing the ESM1 or ANGPTL4 coding region were obtained from HonorGene (Changsha, China). Cell transfection was performed by transfecting the plasmids into HUVEC cell lines at 37 °C for 24 h using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.). Transfection was performed when the cell confluency reached 70-80%, and the cell passage number was strictly maintained within 10 passages to ensure experimental consistency and reproducibility. To establish an in vitro model of lipotoxicity, palmitic acid (PA; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and administered to endothelial cells at a final concentration of 0.2 mM for 48 h. Vehicle controls received equivalent DMSO concentrations (≤ 0.1% v/v). This treatment regimen was selected based on previous reports demonstrating its efficacy in mimicking high-fat-induced endothelial dysfunction.

Western blot

Please refer to our previous article for detailed methods[5]. Tissue or cell samples were lysed with an appropriate volume of RIPA buffer (P0013B, Beyotime) supplemented with PMSF (ST505, Beyotime). After centrifugation, the supernatant was collected, and protein concentration was determined using a BCA assay kit (E112-01, Vazyme). The protein supernatant was mixed with 5× loading buffer (SL1170-1ML, Solarbio) and heated at 100 °C for 5 min for denaturation. Subsequently, a 10% SDS-PAGE gel (SK6010-250T, Coolaber) was assembled in the electrophoresis tank filled with running buffer. Protein samples were loaded into the wells, and electrophoresis was initially performed at 80 V until the samples migrated through the stacking gel and entered the separating gel. The voltage was then increased to 120 V, and electrophoresis continued for 40–50 min until the protein bands reached approximately 1 cm from the bottom of the separating gel. After electrophoresis, the gel was equilibrated in pre-chilled transfer buffer at 4 °C. The gel and a 0.22 μm PVDF membrane, pre-activated with methanol for 1 min, were assembled into a transfer cassette, ensuring no air bubbles between the gel and membrane. Sponges and filter papers were placed on both sides of the cassette, which was then clamped and immersed in pre-chilled transfer buffer. Ice packs were placed around the transfer tank, and transfer was performed at a constant current of 220 mA for 60–90 min. After transfer, the PVDF membrane was washed with TBST and blocked with 5% skim milk at room temperature for 2 h. Following blocking, the membrane was washed with TBST and incubated with primary antibody diluted in blocking buffer at 4 °C on a shaker for 12 h. After primary antibody incubation, the membrane was washed with TBST and incubated with HRP-conjugated goat anti-rabbit IgG (L3012, Solarbio) or goat anti-mouse IgG (L3032, Solarbio) secondary antibody at room temperature for 1 h. Finally, the membrane was washed with TBST, incubated with regular ECL substrate (K-12045-D10, Advansta), and imaged using a chemiluminescence imaging system (MiniChemi610, Saizhi). The primary antibodies used were as follows: ESM1 (Abcam, ab103590), ANGPTL4 (Abcam, ab206420), ATG9A (Abcam, ab108338), ATG7 (Abcam, ab52472), Beclin-1 (Abcam, ab302669), ATG5 (Abcam, ab108327), p62 (Abcam, ab240635), LC3A/B (Abcam, ab62721), and β-actin (Abcam, ab8226). For the raw data of WB, please refer to Supplementary Material 1.

Cell proliferation assay
In the MTT assay, a total of 5000 cells were seeded into individual wells of 96-well plates and incubated for 24, 48, and 72 h, respectively. Following this incubation period, 20 µl of MTT solution (5 mg/ml, Sigma–Aldrich; Merck KGaA) were introduced into each well and allowed to incubate for 4 h. Subsequently, the solution was removed, and 150 µL of DMSO was added to each well to dissolve any precipitates. Finally, the optical density (OD) values were measured at 490 nm using a spectrophotometer. The EdU assay was conducted in accordance with the instructions provided in the EdU kit from RiboBio, Guangzhou, China.

Monodansylcadaverine (MDC) staining
Autophagy activation in cells was assessed using MDC staining (Beyotime, China) followed by fluorescence microscopy. Briefly, cells were treated with Earle's balanced salt solution at 37 °C for 6 h to induce autophagy, followed by incubation with MDC solution at 37 °C for 60 min. After three washes with assay buffer, nuclei were counterstained with DAPI for 10 min. Subsequently, the cells were washed three times with assay buffer and visualized under a fluorescence microscope.

pCMV-mCherry-GFP-LC3B fluorescence staining assay
The pCMV-mCherry-GFP-LC3B plasmid (Beyotime, China) was transfected into cells of appropriate density. Next, 500 µL of the mixture was added to the successfully transfected cells and incubated. After incubation, the cells were fixed using paraformaldehyde. Finally, DAPI staining was completed and the cells were observed and counted by fluorescence confocal microscope (Olympus).

Immunofluorescent staining
Specific methods can be found in our previous research paper. The primary antibodies used were as follows: p62 (Abcam, ab240635) and LC3B (Abcam, ab63817).

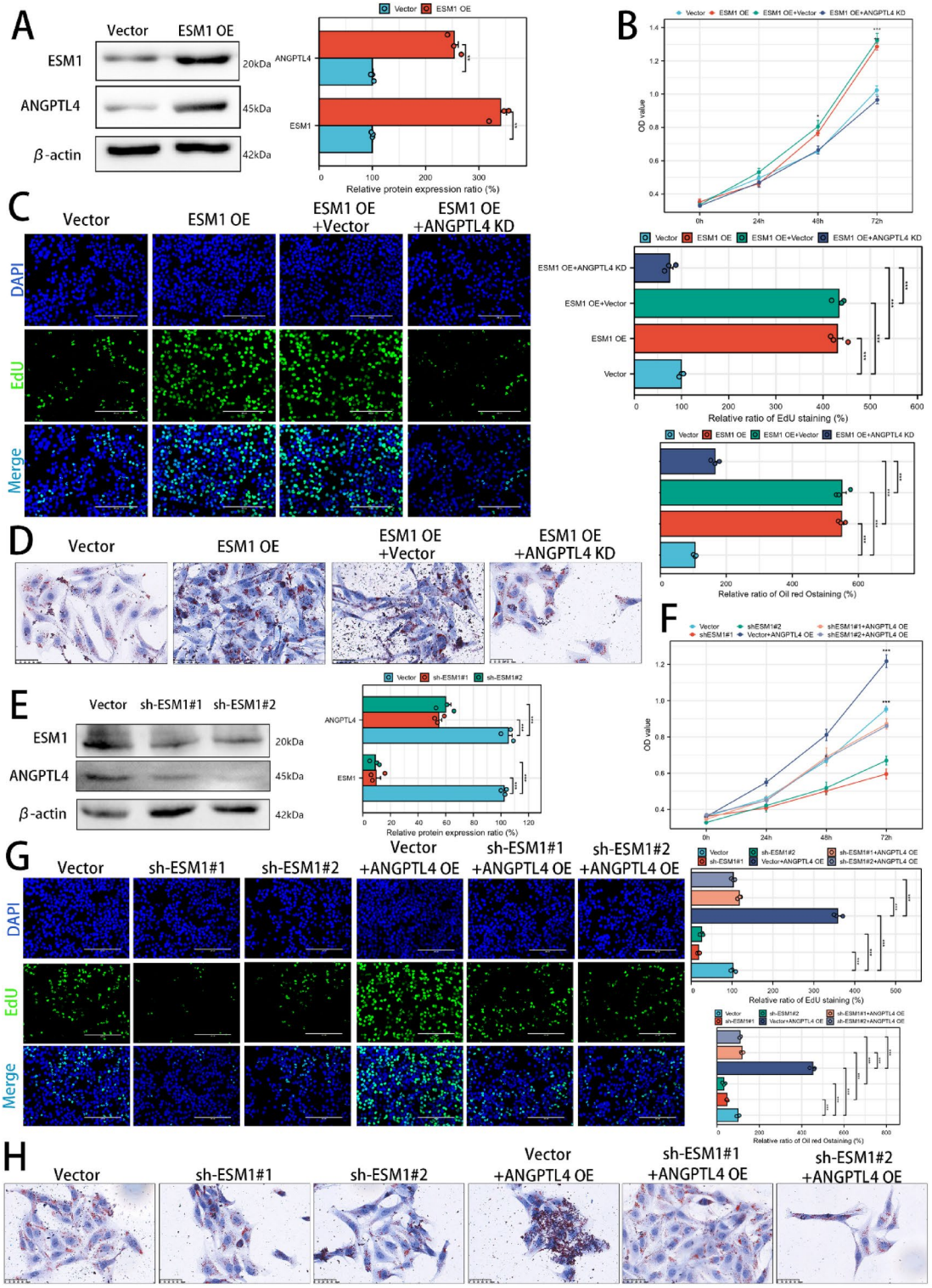
Oil red O staining
The cultured cells were fixed using paraformaldehyde. The cells were then treated using isopropanol. The cells were then stained with the prepared oil red O working solution. Finally, the nuclei were stained with hematoxylin. Subsequent to this, the cells underwent two subsequent washes with distilled water before being employed for photographic documentation and enumeration. Ultimately, ImageJ software was employed to quantify the dimensions of lipid droplets in 100 cells, with measurements expressed in pixels.

AS mice construction
All male mice utilized in this research were of the C57BL/6 J genetic background and carried the ApoE$^{-/-}$ trait. These mice were procured from HonorGene (Changsha, China). They were housed in a specific pathogen-free (SPF) environment and subjected to either a high-fat diet or a control diet for a duration of 8 weeks. The mice were placed in the euthanasia box in turn and carbon dioxide was introduced. After the mice were confirmed to be apnea and heartbeat by pinching and pressing toe reflexes, the corpses were removed. All the mice were euthanized and their aorta was surgically removed. All procedures were approved by the research ethics committee of Xiangya Hospital Zhuzhou Central South University with Institutional Review Board (IRB) approval (ZZCHEC2023059-01).

Statistical analysis
All results were analyzed by t tests and one-way ANOVA with R language.

Results
ESM1 increases the proliferation and lipid production ability of HUVECs
In our previous study, we found ESM1 could upregulate ANGPTL4 to promote angiogenesis in ovarian cancer cell[14]. Therefore, we constructed ESM1 overexpression HUVECs to verify the role of ESM1 in ECs. Western blot showed that ESM1 overexpression could upregulate ANGPTL4 expression in HUVECs (Fig. 1A). Moreover, the proliferation and lipid production ability of HUVECs was significantly increased by ESM1 overexpression, which could be rescued by ANGPTL4 knockdown (Fig. 1B-D). Conversely, ESM1 knockdown could significantly attenuate ANGPTL4 expression in HUVECs (Fig. 1E). The proliferation and lipid production of HUVECs was accordingly decreased by ESM1 knockdown, and ANGPTL4 overexpression could reverse the effects of ESM1 knockdown on HUVECs (Fig. 1F-H). Furthermore, we also found that ANGPTL4 upregulated ESM1 expression (Supplementary Fig. 1A) and promoted HUVECs proliferation (Supplementary Fig. 1B&C) and fatty acid accumulation (Supplementary Fig. 1D).

ECs secrete ESM1 to drive metabolic reprogramming in response to PA-mediated injury
PA can cause endothelial cell dysfunction, and it is widely present in tumor microenvironment and metabolic syndrome microenvironment. MTT analysis showed that PA inhibited the proliferation of HUVECs by a concentration- dependent and time-dependent manner (Fig. 2A). ESM1 and ANGPTL4 was markedly enhanced by PA (Fig. 2B). However, we found that ESM1 overexpression antagonizes the loss of cells due to PA (Fig. 2C&D). Oil red O showed that PA could markedly enhance the lipid level, which ESM1 overexpression could antagonize the lipids level of HUVECs induced by PAs (Fig. 2E). Conversely, ESM1 knockdown enhances PA inhibition of HUVECs (Fig. 2F&G). Oil red O staining showed that ESM1 knockdown further aggravated PA-mediated lipid accumulation in HUVECs (Fig. 2H). These results indicated that ESM1 may act as a self-protection behavior of HUVECs in response to PA injury. By promoting the reprogramming of lipid metabolism in HUVECs, ESM1



increases its lipid tolerance range and enhances lipid metabolism and lipid elimination capabilities, thereby maintaining the homeostasis and function of the vessels and enhancing HUVECs' tolerance to exposure to PA.

ESM1 induces HUVECs autophagy by upregulating ANGPTL4

In previous study, ANGPTL4 was a key role in driving autophagy to antagonize PA-mediated EC injury[9]. Western blot showed that ESM1 overexpression upregulated ANGPTL4 and autophagy relate gene and repressed p62 level (Fig. 3A). Meanwhile, ESM1 knockdown result in the enhancing of p62 and the reducing of ANGPTL4, ATG5, ATG7, ATG9A, LC3B and Beclin1 (Fig. 3B). We also conducted an examination of autophagosome formation through the utilization of mono-dansylcadaverine (MDC) staining. MDC staining enables the labeling of autophagosomes by ion capture, resulting in the manifestation of green fluorescence upon staining. Our findings indicate a substantial augmentation in the quantity of MDC foci within ESM1-overexpressing

◀**Fig. 1**. The effects of ESM1-ANGPTL4 axis on HUVECs proliferation and lipids production. (**A**) Western blot analysis validating ESM1 OE and concomitant ANGPTL4 expression in HUVECs transfected with pcDNA3.1-ESM1 (ESM1 OE). β-actin served as loading control. (**B**,**C**) Proliferation assessment via MTT assay (**B**) and EdU incorporation (**C**) in HUVECs with ESM1 OE alone or combined with ANGPTL4 KD. (**D**) Lipid accumulation quantified by Oil Red O staining in HUVECs transfected with: empty vector, ESM1 OE, ESM1 OE + empty vector, or ESM1 OE + ANGPTL4 KD. Representative images (left) and quantitative analysis (right) shown. (**E**) Western blot confirmation of ESM1 KD efficiency and corresponding ANGPTL4 expression using two distinct shRNAs (shESM1#1 and shESM1#2). (**F**,**G**) Proliferation profiles determined by MTT (**F**) and EdU staining (**G**) following ESM1 KD alone or combined with ANGPTPTL4 OE ($n = 6$). (**H**) Oil Red O staining analysis of lipid deposition in HUVECs treated with: Ctrl, shESM1#1, shESM1#2, Vector + ANGPTL4 OE, shESM1#1 + ANGPTL4 OE, or shESM1#2 + ANGPTL4 OE. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicates statistical significance compared with the control.

cells when compared to control cells, which can be rescued by ANGPTL4 knockdown in HUVECs (Fig. 3C). To further investigate the impact of ESM1 on autophagy, the mCherry-GFP-LC3B flux assay was employed to assess autophagic flux. The mCherry-GFP-LC3 staining technique enabled the differentiation between autophagosomes and autolysosomes, with GFP specifically binding to LC3 in autophagosomes and mCherry detecting both autophagosomes and autolysosomes. Alterations in autophagosomes were indicated by yellow signals, while changes in autolysosomes were represented by red signals. An increase in both yellow and red signals indicated the induction of autophagic flux. Our findings demonstrated that autophagy was enhanced in cells overexpressing ESM1 when compared to control cells, which indicated that ESM1 could enhance autophagy in HUVECs (Fig. 3D).

To investigate the relationship between PA and HUVECs autophagy, cells with either overexpressed or knocked down ESM1 were subjected to PA treatment. Our findings demonstrated that ESM1 overexpression upregulate ANGPTL4, LC3B/3A and Beclin-1, and inhibit p62 expression to promote the initiation of protective autophagy in PA-treated cells (Fig. 3E). However, after knocking down ESM1, the expression of ANGPTL4, LC3B/3A and Beclin-1was suppressed in PA-treated cells, while the expression of p62 was enhanced (Fig. 3F).

ESM1-ANGPTL4 axis induce HUVECs proliferation by driving autophagy

According to the above results, we wanted to know whether ESM1 promotes HUVECs proliferation by regulating autophagy. Autophagy activity was remarkedly inhibited by knockdown of ATG7 (Fig. 4A & Supplementary Fig. 2). We found that ATG7 inhibiting could antagonize the molecular biological role of ESM1 in promoting ECs proliferation (Fig. 4B&C). Moreover, 3-MA (autophagy inhibitor) could repress the proliferation ability of ESM1 overexpression HUVECs (Fig. 4D&E). Hence, our results indicated that ESM1-ANGPTL4 axis accelerate HUVECs proliferation by promoting autophagy.

ESM1-ANGPTL4 axis is correlated with autophagy associated genes (ARGs) in AS mice

Finally, we established AS mice model by feeding $ApoE^{-/-}$ mice with high fat diet. We used ELISA analysis to confirm the serum level of ESM1 in AS model (Fig. 5A). Western blot showed that ESM1, ANGPTL4, and autophagy related proteins were significantly increased in aortic tissues of AS mice (Fig. 5B). These changes in the expression of these proteins further prove that the body will protect its lipid homeostasis and vascular homeostasis in response to the emergency permanence mediated by high lipid environment in vivo.

Discussion

In this study, we confirmed that high level of ESM1 could against PA-mediated EC injury. ESM1-ANGPTL4 axis drove HUVECs proliferation and lipid metabolic reprogramming by regulating autophagy, which might further shed light on the molecular mechanism by which ECs respond to PA injury by ESM1.

In the initial study it was found that ESM1 may have a potential role in the field of EC biology[21], as it was found to be a possible marker of EC activation[22]. It has been reported that ESM1 can affect the viability of endothelial cells by altering the production of nitric oxide by driving eNOS and NOS signals[23]. Most studies generally focus on the reliability of ESM1 as a marker of cardiovascular endothelial system disorders, but the deep molecular mechanism of how ESM1 regulates ECs is still limited[24,25]. However, in the study of malignant tumors, ESM1 has been found to significantly promote the onset and progression of a variety of tumors, especially in the tumor microenvironment of angiogenesis[26–28]. In our previous study, ESM1 can drive PI3K/AKT pathway to promote ovarian cancer progression and angiogenesis[29]. At the same time, the autophagy activity of cancer cells has also been shown to affect the metastasis, invasion[30] and drug resistance[31] of cancer cells. In this study, we found that ESM1 significantly enhances proliferation ability of ECs, which was consistent with the above results of ESM1 on ECs.

In this study, we found ESM1 could induce lipid metabolism reprogramming and attenuate PA induced injury via autophagy in HUVECs. Under physiological conditions, ESM1 promotes fatty acid synthesis of ECs and accelerates EC proliferation. However, in the pathological environment exposed to PA, ESM1 can adapt to the high-lipid microenvironment by inducing EC lipid metabolism reprogramming to maintain its own lipid balance. A variety of exocrine proteins have been confirmed to maintain intracellular metabolic homeostasis in response to microenvironment changes by regulating EC metabolic reprogramming[32,33]. We also found that ESM1 could upregulate ANGPTL4 to promote HUVECs proliferation, migration and lipids production in ovarian cancer microenvironment[14]. Therefore, ESM1 can enhance ANGPTL4 expression by enhancing its stability, and enhance the interaction between ANGPTL4 and LPL to reduce LPL activity and inhibit fatty acid

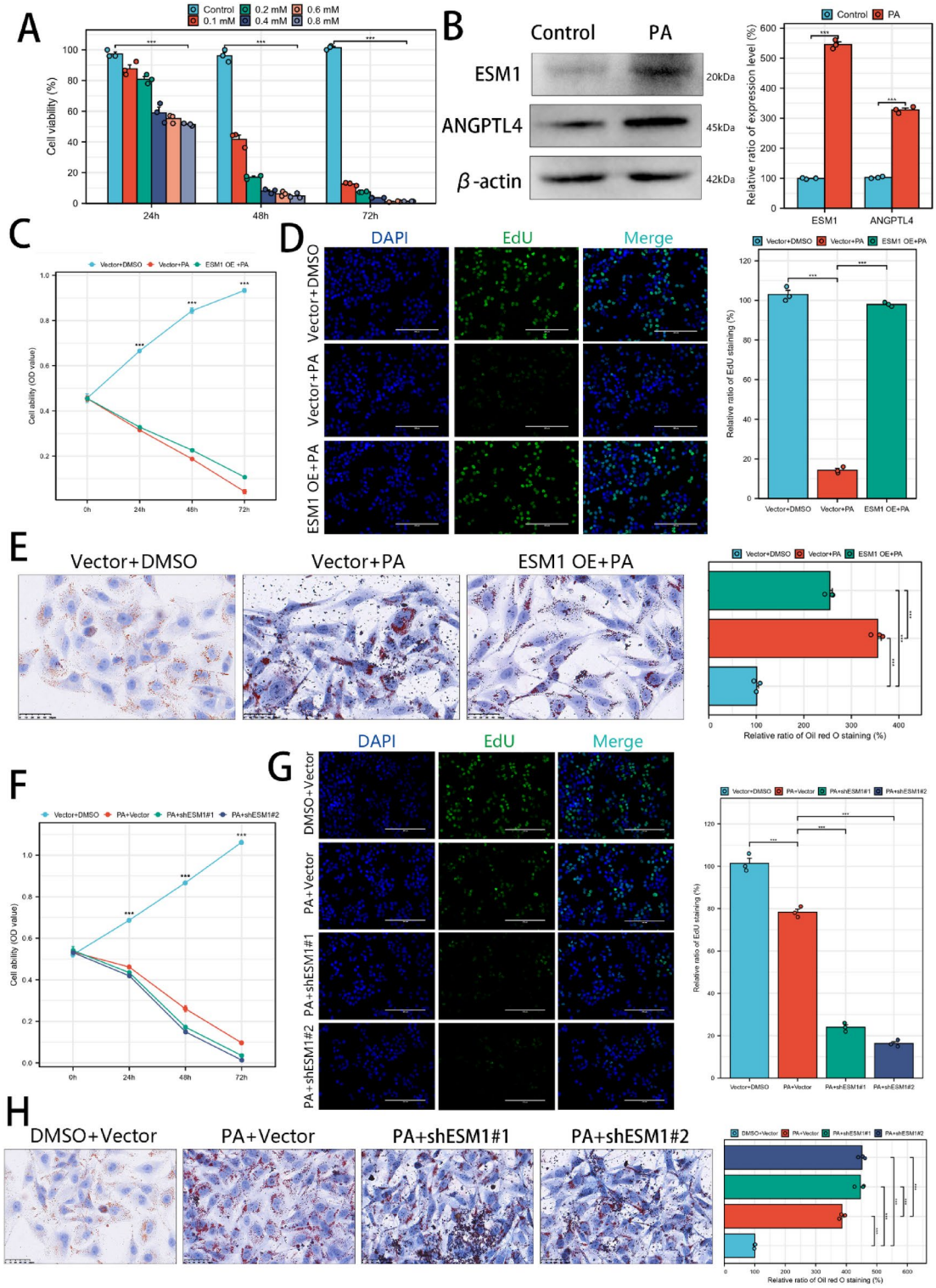


Fig. 2. ESM1 mitigates palmitic acid-induced EC injury. (**A**) Dose-dependent effect of palmitic acid (PA; Sigma-Aldrich) on HUVEC viability assessed by MTT assay after 48 h treatment. (**B**) Western blot analysis of ESM1 and ANGPTL4 expression in HUVECs exposed to 0.2 mM PA for 24 h. β-actin served as loading control. (**C**,**D**) Protective effects of ESM1 OE against PA-induced cytotoxicity: Viability measured by MTT (**C**) and proliferation quantified via EdU assay (**D**) in vector-transfected vs. ESM1 OE cells treated with 0.2 mM PA for 48 h. (**E**) Lipid accumulation analysis by Oil Red O staining in control, PA-treated (0.2 mM), and PA + ESM1 OE groups. (**F**,**G**) Enhanced PA sensitivity following ESM1 knockdown: Viability via MTT (**F**) and proliferation via EdU (**G**) in HUVECs transfected with control vector, shESM1#1, shESM1#2 exposed to 0.2 mM PA or not. (H) Oil Red O staining comparing lipid deposition in PA-treated HUVECs with vector, shESM1#1, or shESM1#2. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicates statistical significance compared with the control.

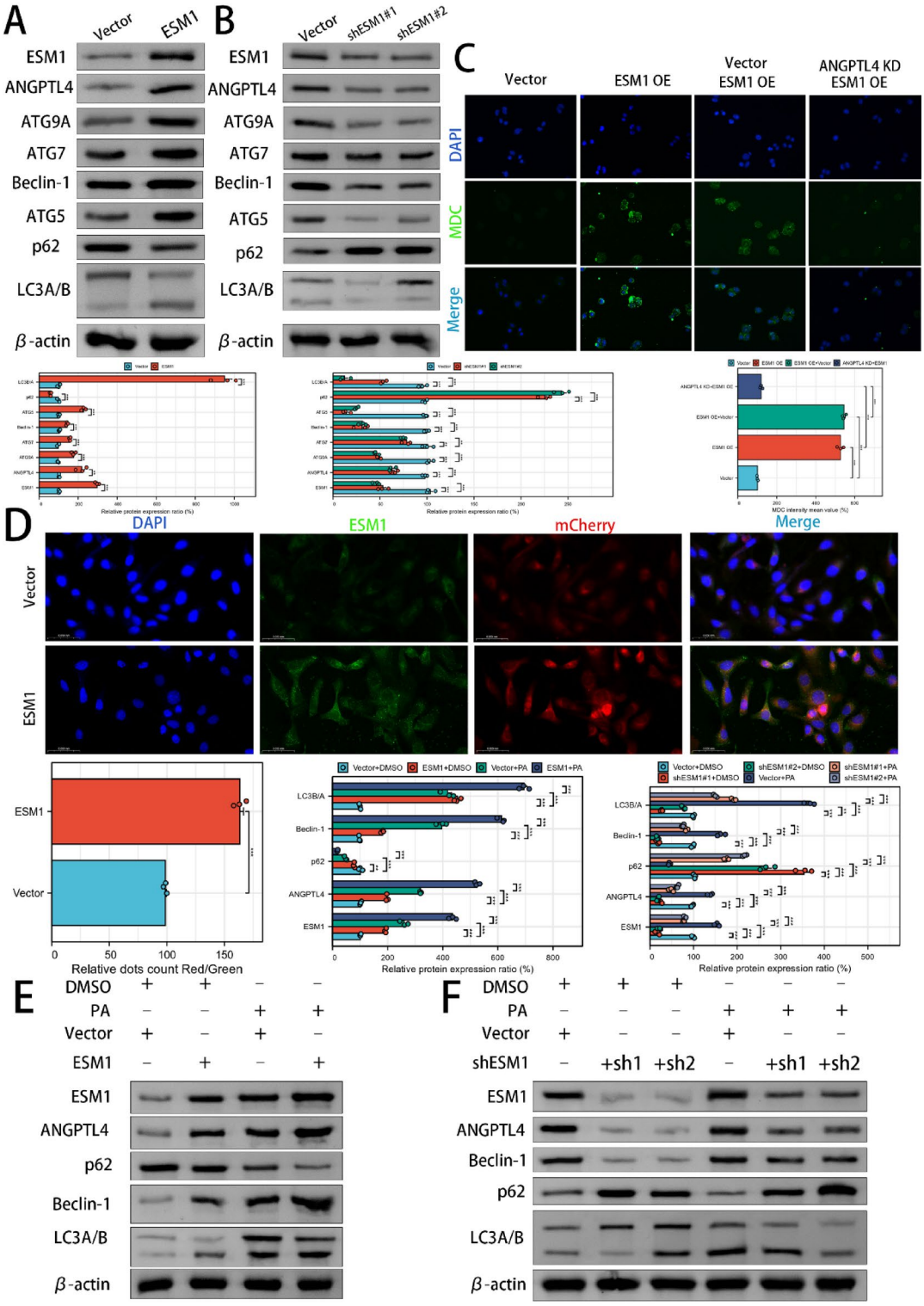


Fig. 3. ESM1 regulates autophagy through ANGPTL4 signaling in HUVECs. (**A**,**B**) Western blot analysis of autophagy-related proteins, including ESM1, ANGPTL4, and autophagy markers (ATG9A, ATG7, Beclin-1, ATG5, p62, and LC3A/B) in **(A)** ESM1 OE and **(B)** ESM1 KD HUVECs. β-actin served as loading control. **(C)** Autophagic flux quantification using MDC staining in: vector control (Ctrl), ESM1 OE, ESM1 OE + vector, and ESM1 OE + ANGPLT4 KD groups. Fluorescence intensity was normalized to Ctrl. **(D)** Dual-fluorescence LC3 reporter assay (GFP-mCherry-LC3) comparing autophagosome (yellow puncta) and autolysosome (red puncta) formation between Ctrl and ESM1 OE groups. Quantification of autophagic vesicles/cell shown. **(E-F)** Western blot analysis of **(E)** ESM1 OE and **(F)** ESM1 KD HUVECs treated with 0.2 mM palmitic acid (PA) for 48 h. Vehicle control (0.1% DMSO) included for comparison. DMSO was used as a solvent control for PA. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicates statistical significance compared with the control.

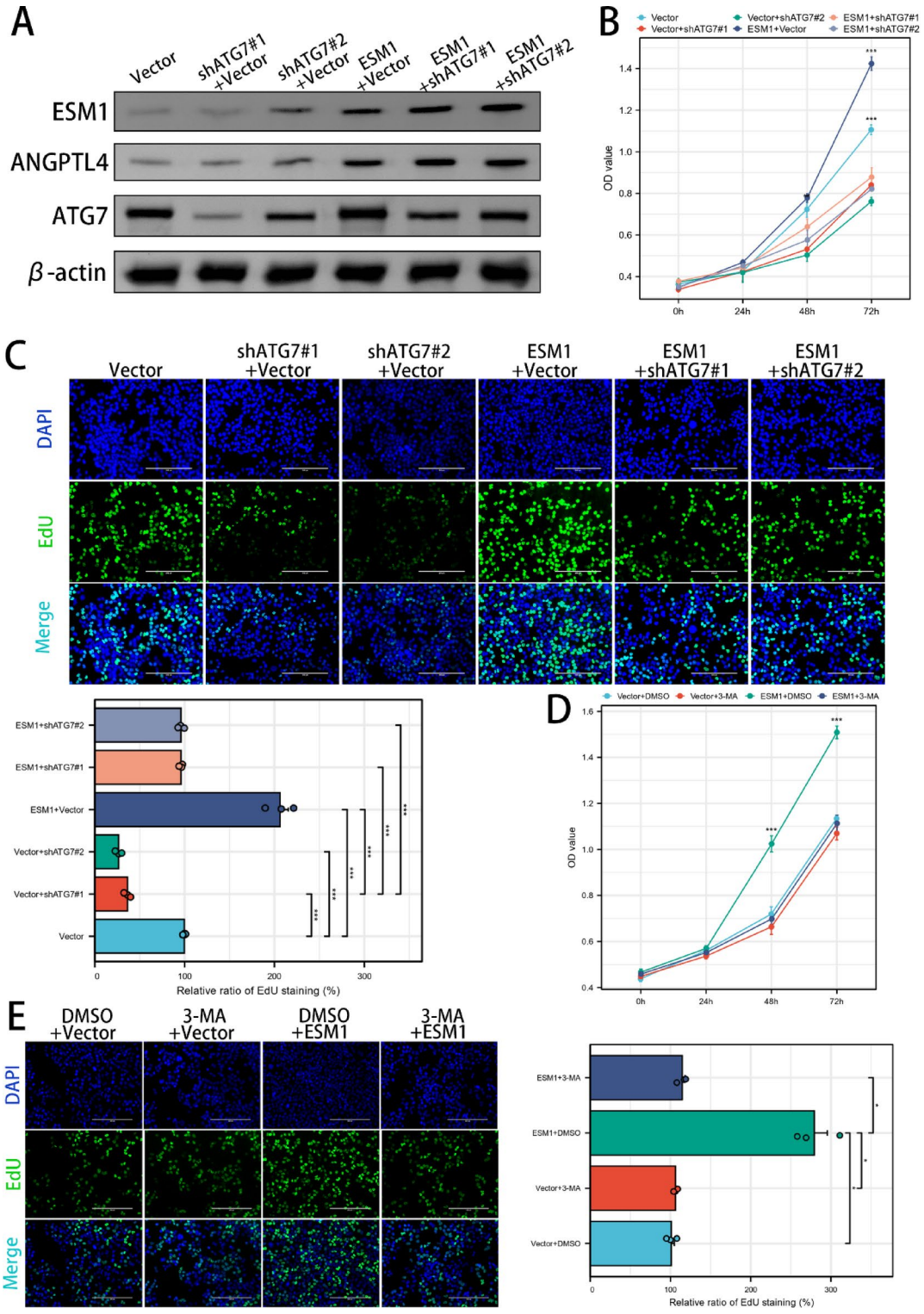


Fig. 4. ESM1/ANGPTL4 Axis Enhances EC Viability Through Autophagy Activation. (**A**) Western blot validation of ATG7 and Autophagy related proteins expression in ESM1 OE HUVECs using two independent shRNAs (shATG7#1 and shATG7#2). β-actin served as loading control. (**B**,**C**) Rescue of ESM1-mediated proliferative advantage by ATG7 KD: Viability assessed by MTT (**B**) and proliferation quantified via EdU incorporation (**C**) in ESM1 OE HUVECs transfected with Vector or shATG7. (**D**,**E**) Pharmacological inhibition of autophagy using 3-methyladenine (3-MA; 5 mM) abrogates ESM1-mediated cytoprotection: MTT (**D**) and EdU (**E**) assays in ESM1 OE HUVECs treated with vehicle (Ctrl) or 3-MA for 48 h. DMSO was used as a solvent control for PA. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicates statistical significance compared with the control.

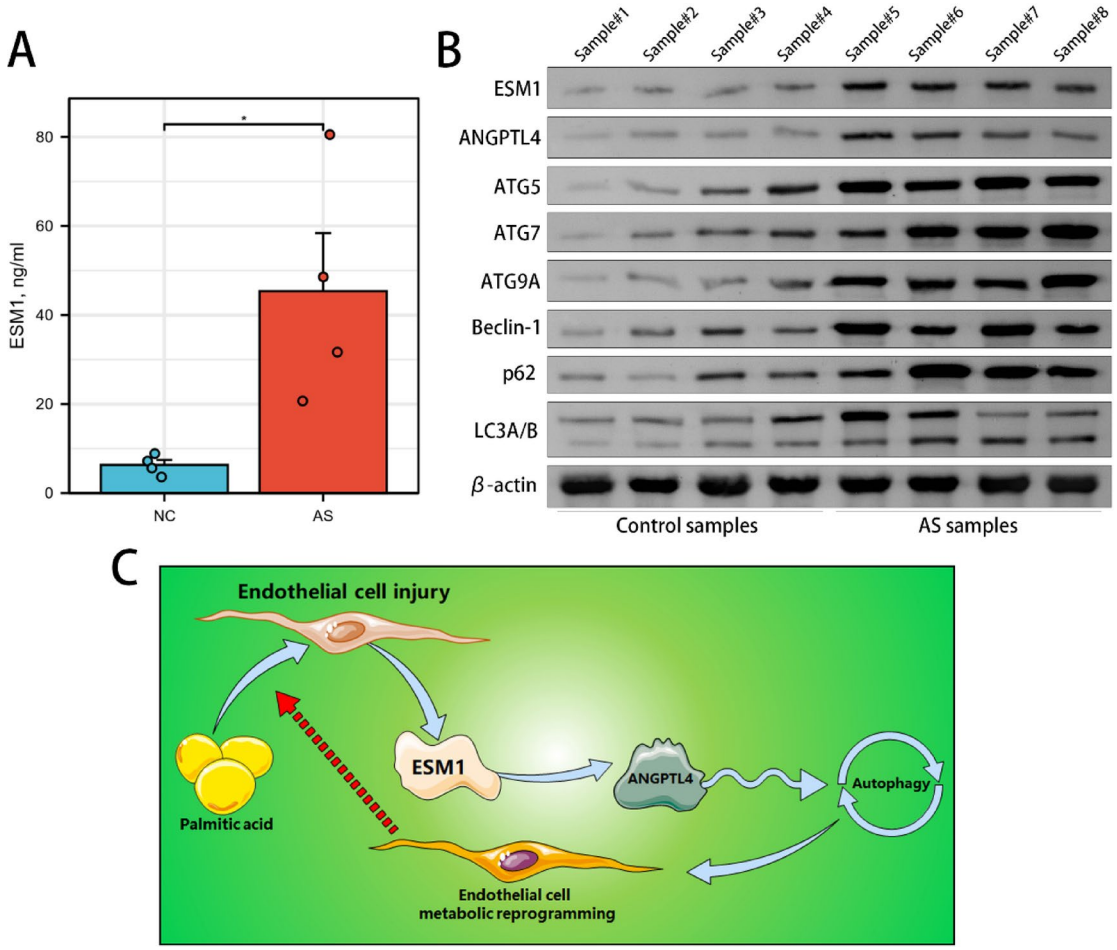


Fig. 5. ESM1/ANGPTL4 axis coordinates autophagy dysregulation in atherosclerotic models. (**A**) Circulating ESM1 levels quantified by ELISA in serum from AS mice versus control mice. (**B**) Representative western blots of aortic lysates showing ESM1, ANGPTL4, and autophagy-related proteins (ATG5, ATG7, ATG9A, Beclin-1, p62, LC3A/B) in AS and WT mice. β-actin normalization shown (three independent experiments). (**C**) Proposed mechanism schematic: ESM1 upregulation activates ANGPTL4-dependent autophagy flux through metabolic reprogramming, attenuating PA-induced endothelial injury (mechanistic model derived from in vitro data). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicates statistical significance compared with the control.

degradation. Moreover, ESM1 could induce AMPK/mTOR pathway to up-regulate FASN and SCD1, promoting fatty acid synthesis through ATP production by PKM2 SUMOylation[34]. These molecular mechanisms may be key to ESM1-induced fatty acid reprogramming in ECs. The effect of ANGPTL4 on EC integrity is still controversial. Some studies suggest that ANGPTL4 can damage ECs integrity, induce EC apoptosis and promote the formation of AS, while other studies suggest that ANGPTL4 can protect ECs integrity and promote angiogenesis and protect proteins in AS[35–38]. Zhan et al. indicated that ANGPTL4 could attenuate PA mediated EC injury by promoting autophagy[9]. Despite being inconclusive, these observations indicated that ESM1 could regulating the development of atherosclerosis through vascular actions that are not dependent on circulating lipid regulation, as the inner endothelium is considered the initial site of atherosclerosis. PA, a saturated fatty acid found in the blood and a major component of atherosclerotic plaque, was found to up-regulate ESM1 expression in this study, leading to EC injury. ESM1 was found to mitigate the functions of PA on HUVECs. ESM1 inhibiting further deteriorate the toxicological effects of PA, which encompassed inhibition of proliferation and heightened accumulation of fatty acids.

Autophagy serves as a cellular self-preservation mechanism in response to external environmental stimuli, enabling the regulation of energy and nutrient synthesis in ECs based on varying external conditions[39]. The metabolic alterations within cells are intricately linked to autophagy, which effectively upholds intracellular lipid homeostasis by modulating the expression of lipid metabolism-associated genes, thereby facilitating cell survival in high-lipid environments[40]. Prior investigations have documented the occurrence of autophagy in endothelial cells upon stimulation by atherogenic factors[41]. In our study, we have observed that the overexpression of ESM1 can stimulate autophagy and sustain EC proliferation by up-regulating ANGPTL4 (Fig. 5C). Moreover, we have found that the promotion of EC proliferation by ESM1 through ANGPTL4 can be counteracted by inhibiting ATG7 or introducing the autophagy inhibitor 3-MA. Consequently, all of the aforementioned findings

collectively substantiate the notion that the ESM1-ANGPTL4 axis augments EC proliferation activity and lipid homeostasis via autophagy.

Autophagy is activated to protect ECs from external stimulus-induced damage, thereby maintaining cell viability and slowing down the development and progression of AS in the early stage[41,42]. However, the inhibition of autophagy has been observed to facilitate the progression of AS. In our investigation, we observed a notable increase in serum ESM1 levels in AS mice, suggesting that endothelial cells may exhibit a self-protective response against damage induced by a high-fat environment. Numerous studies have demonstrated a significant elevation of ESM1 in various vascular disorders, including hypertension, acute ST-segment elevation myocardial infarction, and AS[8,43–46]. Hence, it can be inferred that the upregulation of ESM1 potentially serves as a safeguarding mechanism for ECs against exogenous environmental factors, thereby preserving their functionality and homeostatic balance. Previous research has demonstrated that ANGPTL4 possesses the ability to enhance endothelial barrier function through various mechanisms. Hence, it is postulated that the intervention of ANGPTL4 by ESM1 can potentially decelerate the AS progression by preserving EC integrity. Moreover, ESM1 may contribute to the maintenance of EC lipid homeostasis by modulating EC lipid metabolism. It is hypothesized that this effect is attributed to the activation of protective autophagy mediated by ESM1 through ANGPTL4, thereby impeding the AS process. Our findings demonstrate a significant upregulation of ESM1 and ANGPTL4 in the arterial tissues of AS mice, compared to control group. These phenomena may be due to arterial feedback to AS-related hyperlipidemia stimuli.

Conclusion

In summary, our findings provide confirmation that ESM1 has the ability to enhance the activation of autophagy, thereby facilitating EC proliferation and inducing a reprogramming of lipid metabolism through upregulating ANGPTL4. This mechanism effectively mitigates EC injury induced by PA and hinders the progression of AS.

Data availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
All procedures were approved by the research ethics committee of Xiangya Hospital Zhuzhou Central South University with Institutional Review Board (IRB) approval (ZZCHEC2023059-01). All methods are reported in accordance with ARRIVE guidelines.

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