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MiR-125b-5p ameliorates ox-LDL-induced vascular endothelial cell dysfunction by negatively regulating TNFSF4/TLR4/NF-κB signaling

Wenshuai He[1*], Limin Zhao[1], Pengfei Wang[1], Maojia Ren[2] and Yunfei Han[1]

Abstract

Background Oxidized low-density lipoprotein (ox-LDL)-induced endothelial cell dysfunction plays a crucial role in the progression of atherosclerosis (AS). Although miR-125b-5p is known to be involved in cardiovascular and cerebrovascular disorders, its function in ox-LDL-induced endothelial injury is still not well understood.

Methods An in vitro AS cell model was established by exposing human umbilical vein endothelial cells (HUVECs) to 100 μg/mL ox-LDL for 24 h. A series of functional assays, including CCK-8 assay, flow cytometry, MDA and SOD kits, capillary-like network formation assay and ELISA assay were performed in vitro. TNFSF4/TLR4/NF-κB pathway-related protein expressions were measured by Western blot. Molecular mechanisms were elucidated through quantitative real-time PCR, western blot analysis, and luciferase reporter assays.

Results Our investigation revealed that exposure to ox-LDL led to a downregulation in miR-125b-5p, while upregulating the expression of tumor necrosis factor (ligand) superfamily, member 4 (TNFSF4), TLR4, p-p65 and p-IkBa in HUVECs in a dose-dependent manner. We confirmed TNFSF4 as a direct target of miR-125b-5p. Ox-LDL exposure led to decreased cell viability and angiogenic capacity, along with increased apoptosis, inflammation, and oxidative stress in HUVECs. These effects were reversed by overexpressing miR-125b-5p or knocking down TNFSF4. Overexpression of TNFSF4 significantly reversed the effects brought about by miR-125b-5p in HUVECs exposed to ox-LDL. Moreover, miR-125b-5p inactivated the TLR4/NF-κB signaling pathway by negatively regulating TNFSF4.

Conclusions In summary, our findings demonstrate that miR-125b-5p possessed an anti-inflammatory and anti-apoptosis against ox-LDL-induced HUVEC injury by regulating the TNFSF4/TLR4/NF-κB signaling, indicating that miR-125b-5p may have an important therapeutic function for AS.

Keywords Atherosclerosis, HUVECs, miR-125b-5p, TNFSF4, TLR4/NF-κB signaling

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Background

Atherosclerosis (AS), a chronic and progressively evolving vascular disease, is the predominant cause of morbidity and mortality among cardiovascular and cerebrovascular disorders, such as coronary heart disease, acute myocardial infarction, and stroke [1, 2]. AS is characterized by the thickening of the intima in large and medium-sized arteries, accompanied by the deposition of lipids, calcium, cholesterol, and cellular debris on the inner surface of blood vessels [3]. It is widely recognized that the injury and dysfunction of vascular endothelial cells (VECs) serve as an early indicator of AS [4]. Moreover, overproduction of oxidized low-density lipoprotein (ox-LDL) causes VEC damage, further contributing to the progression of AS [5, 6]. Consequently, exploring the regulatory mechanisms underlying ox-LDL-induced endothelial injury is crucial for the development of preventive and therapeutic strategies for AS.

Recently, research on the association between noncoding RNAs (ncRNAs) and cardiovascular disease has attracted considerable attention [7]. MicroRNAs (miRNAs/miRs), a group of single-stranded RNA molecules with 19 to 22 nucleotides, serve as major post-transcriptional gene regulators that cause degradation or translation inhibition of target genes by recognizing the 3′-untranslated region (3′-UTR) in target mRNAs [8–10]. Among these miRNAs, miR-125b-5p, a major dynamically modulated one, is involved in various pathophysiological processes related to the development of different diseases, including cardiovascular and cerebrovascular disorders [11–13]. For instance, inhibiting miR-125b-5p can reverse the protective effect of SNHG1 silence on cell apoptosis, oxidative stress, and inflammation in Parkinson's disease models [14]. Wu et al. [11] reported that miR-125b-5p enhanced cell viability, yet repressed cell apoptosis and inflammatory factors in oxygen glucose deprivation (OGD)-induced human coronary artery endothelial cells and human cardiac microvascular endothelial cells. Importantly, Lu et al. [15] found that miR-125b-5p was down-regulated in atherosclerotic plaques and could attenuate the secretion of MCP-1 in lipopolysaccharide (LPS)-stimulated THP-1 macrophages. However, the specific functional role and regulatory mechanisms of miR-125b-5p in ox-LDL-induced endothelial injury during AS remain largely unknown.

Tumor necrosis factor (TNF) receptor superfamily, member 4 (TNFRSF4, also known as OX40) is a costimulatory receptor expressed by activated T cells [16]. It is activated by its cognate ligand TNF superfamily, member 4 (TNFSF4, also known as OX40L) [17]. TNFSF4 is found on activated macrophages and mononuclear cells and has the capacity to directly activate intracellular signaling cascades and stimulate cytokine production [18]. It has been documented that TNFSF4 is upregulated in an inflammatory model of condylar chondrocytes induced by IL-1β and TNF-α [19]. Moreover, LPS induces TNFSF4 expression on dendritic cells and B cells. This results in a synergistic activity between TLR4 and TNFRSF4 signals and leads to the production of IL-4 and IL-6, thereby inhibiting Treg development [20]. Based on our previous prediction that TNFSF4 is a potential target of miR-125b-5p and the report by Zhang et al. [21] indicating that the target genes of miR-125b-5p are enriched in inflammation and apoptosis-related signaling pathways, we reason that the miR-125b-5p/TNFSF4 axis may have a significant role in ox-LDL-induced endothelial injury during AS.

In this study, we began by establishing an in vitro model of AS through the stimulation of human umbilical vein endothelial cells (HUVECs) with ox-LDL. Subsequently, we determined the levels of miR-125b-5p/TNFSF4. After analyzing the role of the miR-125b-5p/TNFSF4 axis in the physiological functions of HUVECs, such as inflammatory responses, apoptosis, oxidative stress, and angiogenic capacity, we further explored the underlying function of the TLR4/NF-κB pathway within this axis. The objective of this research is to provide a new target and perspective for the treatment of AS.

Materials and methods

Construction of AS cell model

HUVECs, procured from the Chinese Academy of Medical Sciences (Shanghai, China), were cultivated in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Carlsbad, CA, USA) and 1% antibiotics (Gibco) within a 37 °C humidified atmosphere containing 5% CO_2. For construction of in vitro AS model, HUVECs were exposed to diverse concentrations (25, 50, 100 μg/ml) of ox-LDL (Solarbio, Beijing, China) and were harvested at 24 h for subsequent analyses.

Cell transfection

MiR-125b-5p mimics, small interfering RNA against TNFSF4 (si-TNFSF4), and their corresponding negative controls (miR-NC and si-NC, respectively) were synthesized by RiboBio Co., Ltd. (Guangzhou, China). The TNFSF4 overexpression plasmid (Ov-TNFSF4) was constructed by inserting the TNFSF4 gene into the pcDNA3.1 vector (GenePharma Co., Ltd., Shanghai, China), while an empty vector served as the negative control (Ov-NC). HUVECs were seeded into 6-well plates (1×10^5 cells/well) and transfected with oligonucleotide (60 nM) or vector (0.5 μg) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 48 h, followed by treatment with 100 μg/ml ox-LDL.

Dual-luciferase reporter assay

The sequences of TNFSF4 3′-UTR encompassing the predicted miR-125b-5p target sites and the mutant 3′-UTR of TNFSF4 were synthesized and inserted into the psiCHECK2 vector (Promega) to generate wild-type and mutated type vectors (WT/MUT-TNFSF4). Subsequently, the corresponding reporter vector (50 ng) was co-transfected with 20 nM of miR-125b-5p mimics or miR-NC into HUVECs for 48 h. The luciferase activity was detected using a dual-luciferase reporter assay kit (Promega, USA).

CCK-8 assay

The viability of HUVECs was assessed by means of the Cell counting kit-8 (CCK-8) assay (Dojindo Co., Kumamoto, Japan). Briefly, HUVECs that had undergone different treatments were sub-cultured in 96-well plates at a density of 3×10^4 cells per well. After 24 h, the cells were incubated with 10 µl of CCK-8 reagent for 2 h at 37 °C. Cell viability was determined by measuring the absorbance at 450 nm using a microplate reader (Epoch; BioTek Instruments, Inc.).

ELISA assay

The supernatant was collected from HUVECs that had undergone different treatments and centrifuged at room temperature for 10 min at 2000 r/min. Subsequently, the concentrations of inflammatory cytokine molecules, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), were determined using standard enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc. MN, USA).

Oxidative stress assay

The activities of superoxide dismutase (SOD) and malondialdehyde (MDA) were determined to assess cellular oxidative stress. Briefly, cell lysate from HUVECs subjected to different treatments was mixed with a working solution for half an hour. Subsequently, the samples were treated with the corresponding SOD assay kit and MDA assay kit (both from Beyotime). Then, the absorbance was examined using a microplate reader (Bio-Rad).

Assessment of apoptosis

Cell apoptosis was detected using the Annexin V-FITC Apoptosis Detection kit (Beyotime). Briefly, HUVECs subjected to different treatments were digested by 0.25% trypsin and collected into centrifuge tubes. After 10 min of centrifugation at 2000 rpm, the HUVECs were re-suspended with binding solution and then double-stained with 5 µl Annexin V-FITC and 5 µL propidium iodide (PI) solution. Following 10 to 20 min of incubation in the dark, the percentage of apoptotic cells was determined by flow cytometry (BD Biosciences, San Jose, CA, USA).

Capillary-like tube formation assay

HUVECs that had undergone different treatments were seeded onto 96-well plates pre-coated with growth factor-reduced Matrigel (Corning, Bedford, MA, USA), with a seeding density of 3×10^4 cells per well. After 48 h of incubation, the capillary-like structure was observed using a digital camera attached to a Nikon phase-contrast microscope. The percentage of tumor formation was estimated using ImageJ 64 open software (National Institutes of Health, Bethesda, USA).

Quantitative real-time PCR analysis

RNA samples were obtained with Trizol reagent (Invitrogen). For miRNA, complementary DNA (cDNA) was synthesized using the Mir-X miRNA First-Strand Synthesis Kit by Takara (Tokyo, Japan). For mRNA, a PrimeScript™ RT Reagent Kit by Takara was used. Quantitative real-time PCR was performed for miRNA levels using a Mir-X miRNA qRT-PCR TB Green Kit (Takara) and for mRNA levels using the SYBR™ Green PCR Master Mix (Invitrogen). The primers in Table 1 were synthesized by Shanghai Bioasia Biotech (Shanghai, China). Each RNA amplification was done in triplicate. Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. U6 served as an internal control for miRNA and GAPDH for mRNA.

Western blot analysis

Total protein from HUVECs was extracted using the radioimmunoprecipitation assay (RIPA), and the corresponding concentrations were estimated by BCA Assay Kit (Beyotime). Then, 30 µg of total protein was separated by SDS-PAGE and then transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk in TBST (Tris Buffered Saline with 0.1% Tween) for 2 h, the membranes were washed with TBST thrice using for 5 min each. Subsequently, the membranes were incubated with the diluted primary antibodies for TNFSF4 (1:1000, DF7816, Affinity Biosciences), TLR4 (1:500, A11226, ABclonal), p65 (1:50, #8242, Cell Signaling), p-p65 (1:1000, AP1294, ABclonal), IκBα (1:1000, #4812, Cell Signaling), p-IκBα (1:1000, #2859, Cell Signaling) and GAPDH (1:5000, #5174, Cell Signaling) at 4 °C overnight. After incubation with the

Table 1 Primers for quantitative real-time PCR

Gene	Forward (5′ - 3′)	Reverse (5′ - 3′)
miR-125b-5p	TCCCTGAGACCCTAACTTGTGA	AGTCTCAGGGTCCGAGGTATTC
TNFSF4	CACGTTCCCCTTTTCCATATCT	CCTCCTTTTGGGAAGTGAGGA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
GAPDH	GGTGAAGGTCGGAGTCAACG	GCATCGCCCCACTTGATTTT

horse radish peroxidase (HRP) conjugated secondary antibody (Abcam) for 1 h at room temperature, the membranes were treated with enhanced chemiluminescence (ECL) chromogenic solution (Thermo Fisher Scientific, USA) for 2 min at room temperature. Finally, the target protein bands were visualized using UVP gel imaging system (UVP, MA, USA) and analyzed with Image-J software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data from three independent replicates were presented as mean ± standard deviation (SD) and analyzed using GraphPad Prism 8.0 software. For multiple groups, one-way ANOVA followed by Dunnett's or Tukey's post hoc test was used. For two groups, unpaired Student's t-test was employed. A p-value < 0.05 was regarded as statistically significant.

Results

Expression levels of miR-125b-5p TNFSF4 and TLR4/NF-κB signaling factors in an AS model

First, an AS cell model was constructed in HUVECs by exposing them to different concentrations (25, 50, 100 μg/ml) of ox-LDL, which was validated by CCK-8 assay. As depicted in Fig. 1A, treatment with ox-LDL dramatically dose-dependently decreased the viability of HUVECs. Subsequently, quantitative real-time PCR revealed a significant increase in miR-125b-5p expression (Fig. 1B), while the expression of TNFSF4 significantly decreased compared to the control group (Fig. 1C) in a dose-dependent manner. Western blot analysis confirmed a dose-dependent increase in TNFSF4, TLR4, p-p65 and p-IκBα levels in HUVECs treated with ox-LDL (Fig. 1D), suggesting a possible connection between these abnormally expressed genes and ox-LDL-induced dysfunction in HUVECs. Based on these results, 100 μg/ml

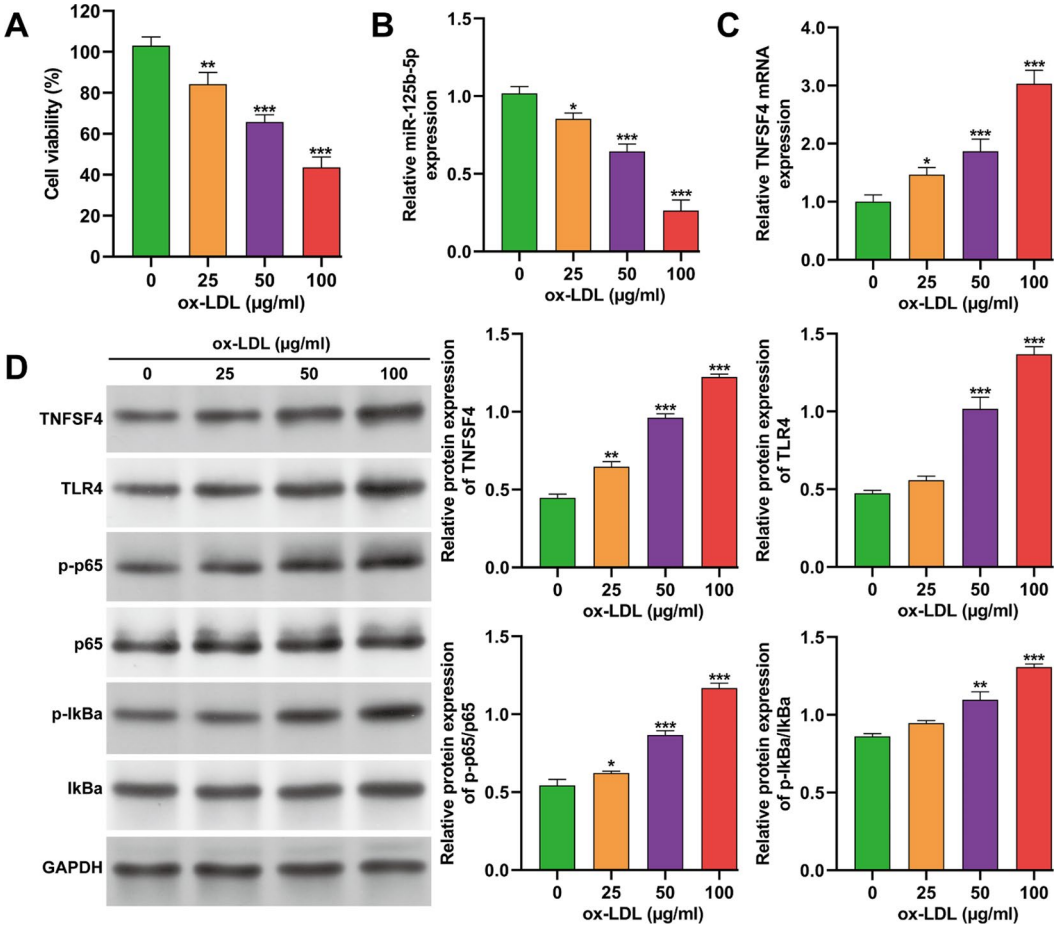

Fig. 1 Expression levels of miR-125b-5p, TNFSF4 and TLR4/NF-κB signaling factors in an in vitro AS model. HUVECs were exposed to diverse concentrations (25, 50, 100 μg/ml) of ox-LDL for 24 h. (**A**) Cell viability of HUVECs was determined using CCK-8 assay. (**B**-**C**) The expression of miR-125b-5p and TNFSF4 mRNA was measured using quantitative real-time PCR analysis in HUVECs. (**D**) The protein levels of TNFSF4, TLR4, p-p65, p65, p-IκBα and IκBα were visualized and quantified via western blot analysis in HUVECs. All experiments were carried out three times with three repetitions. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with 0 μg/ml

ox-LDL produced more obvious effects and was therefore selected as the appropriate treatment dosage.

Targeting relationship between TNFSF4 and miR-125b-5p

The observation that the expression trend of miR-125b-5p was contrary to that of TNFSF4 (Fig. 1B-C), in conjunction with the prediction of TargetScan 7.1, implied the existence of target binding sites between the 3′-UTR of TNFSF4 and miR-125b-5p (Fig. 2A). To confirm this targeting binding, we performed the dual-luciferase reporter assay. As shown in Fig. 2B, ectopic expression of miR-125b-5p significantly reduced the luciferase activity of TNFSF4 3′-UTR-WT, yet it had no impact on TNFSF4 3′-UTR-MUT. Additionally, the effect of miR-125b-5p expression on the TNFSF4 level was investigated. The results showed that the expression of TNFSF4 mRNA (Fig. 2C) and protein (Fig. 2D) was elevated in ox-LDL-treated HUVECs, and this increase was suppressed by overexpression of miR-125b-5p. These findings suggest that miR-125b-5p targets TNFSF4 in HUVECs.

Overexpression of miR-125b-5p alleviated ox-LDL-induced damage in HUVECs

To investigate the functional role of miR-125b-5p in an AS cell model, quantitative real-time PCR was employed to confirm the effective overexpression of miR-125b-5p in ox-LDL-induced HUVECs through transfection with miR-125b-5p mimics (Fig. 3A). The CCK-8 assay demonstrated that overexpression of miR-125b-5p significantly restored the impaired cell viability of HUVECs exposed to ox-LDL (Fig. 3B). The increased concentrations of pro-inflammatory cytokines (TNF-α and IL-1β) resulting from ox-LDL treatment were notably attenuated upon miR-125b-5p overexpression (Fig. 3C). Additionally, it was found that overexpression of miR-125b-5p suppressed the oxidative stress reaction, as indicated by increased superoxide dismutase (SOD) activity and decreased malondialdehyde (MDA) activity in ox-LDL-treated HUVECs (Fig. 3D). Moreover, the ox-LDL-induced apoptosis was significantly inhibited after transfection with miR-125b-5p mimics in HUVECs (Fig. 3E). Furthermore, the impaired tube formation ability in ox-LDL-treated HUVECs was largely restored following miR-125b-5p overexpression (Fig. 3F).

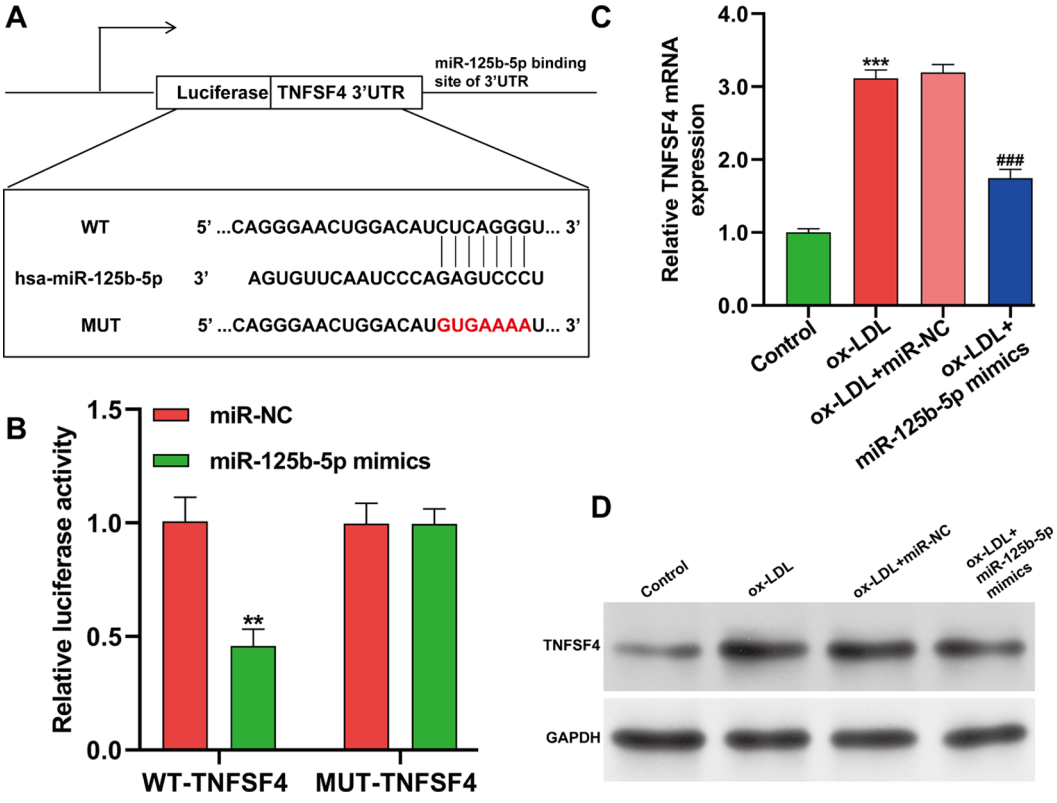

Fig. 2 Targeting relationship between TNFSF4 and miR-125b-5p. (**A**) The target bindings sites between miR-125b-5p and the 3′-UTR of TNFSF4 gene. (**B**) MiR-125b-5p overexpression inhibited the luciferase activity of HUVECs transfected with wild-type sequence of TNFSF4, while did not affect those with mutant sequence of TNFSF4. $^{**}p < 0.01$, compared with miR-NC; (**C**-**D**) The regulatory role of miR-125b-5p mimics on the expression of TNFSF4 mRNA and protein in ox-LDL-induced HUVECs. All experiments were carried out three times with three repetitions. $^{***}p < 0.001$, compared with control; $^{###}p < 0.001$, compared with ox-LDL + miR-NC

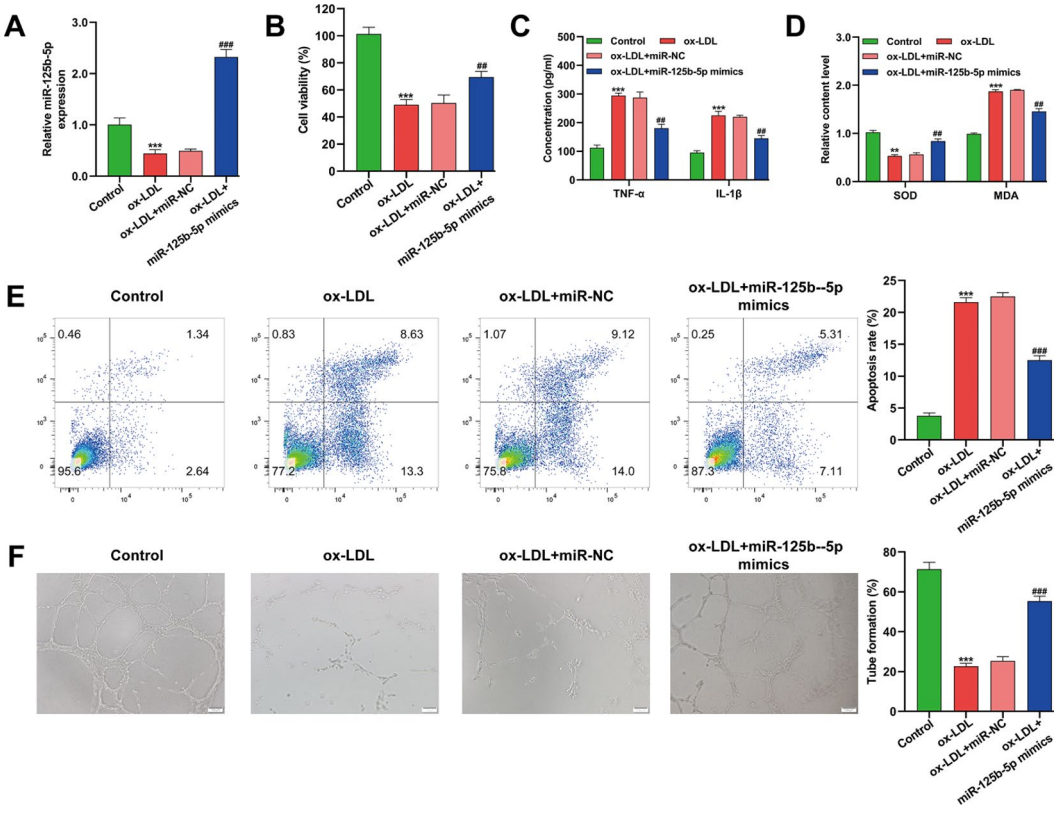

Fig. 3 Overexpression of miR-125b-5p alleviated ox-LDL-induced damage in HUVECs. HUVECs were transfected with miR-125b-5p mimics or miR-NC for 48 h, followed by treated with 100 μg/ml of ox-LDL for 24 h. (**A**) The expression of miR-125b-5p was determined using quantitative real-time PCR analysis. (**B**) Cell viability of HUVECs was assessed using CCK-8 assay. (**C**) The concentrations of inflammation-associated cytokines (TNF-α and IL-1β) were detected by ELISA. (**D**) The production of SOD and MDA was assessed using their matching kits. (**E**) Cell apoptosis was determined using flow cytometry assay. (**F**) The ability of tube formation was analyzed through capillary-like network formation assay in vitro. All experiments were carried out three times with three repetitions. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with ox-LDL + miR-NC

Collectively, our data suggest that overexpression of miR-125b-5p significantly alleviates the dysfunction in HUVECs caused by ox-LDL.

Knockdown of TNFSF4 diminished ox-LDL-induced injury in HUVECs

Given that TNFSF4 is the target of miR-125b-5p and is negatively regulated by miR-125-5p in HUVECs, we subsequently conducted loss-of-function assays to analyze the function of TNFSF4 in ox-LDL-induced injury. As depicted in Fig. 4A, the addition of ox-LDL led to an increase in TNFSF4 mRNA expression. However, this trend was reversed upon transfection of si-TNFSF4 into the cells. Western blot analysis further validated the knockdown of TNFSF4 in ox-LDL-treated HUVECs (Fig. 4B). The CCK-8 assay (Fig. 4C) and ELISA assay (Fig. 4D) indicated that ox-LDL inhibited cell viability and promoted inflammation, but these effects were altered by TNFSF4 downregulation. Moreover, ox-LDL treatment significantly diminished SOD activity and enhanced MDA activity in HUVECs, and both of these changes were reversed by the knockdown of TNFSF4 (Fig. 4E). Additionally, the knockdown of TNFSF4 significantly reversed the increased apoptosis (Fig. 4F) and decreased tube formation (Fig. 4G) induced by ox-LDL in HUVECs.

Overexpression of TNFSF4 weakened the protective effects of miR-125b-5p against ox-LDL-mediated HUVEC injury

Next, we conducted rescue experiments to determine whether TNFSF4 served as the downstream regulator involved in the protective effects mediated by miR-125b-5p against ox-LDL-induced damage. Firstly, we demonstrated the overexpression of TNFSF4 at both the mRNA and protein levels in ox-LDL-treated HUVECs following transfection with Ov-TNFSF4 (Fig. 5A-B). A series of functional experiments revealed that overexpression of TNFSF4 counteracted the effects mediated by miR-125b-5p mimics on cell viability (Fig. 5C), inflammatory cytokines (Fig. 5D), oxidative stress (Fig. 5E), apoptosis (Fig. 5F), and tube formation (Fig. 5G) in ox-LDL-induced HUVECs. Taken together, upregulation of miR-125b-5p safeguards HUVECs from ox-LDL-induced

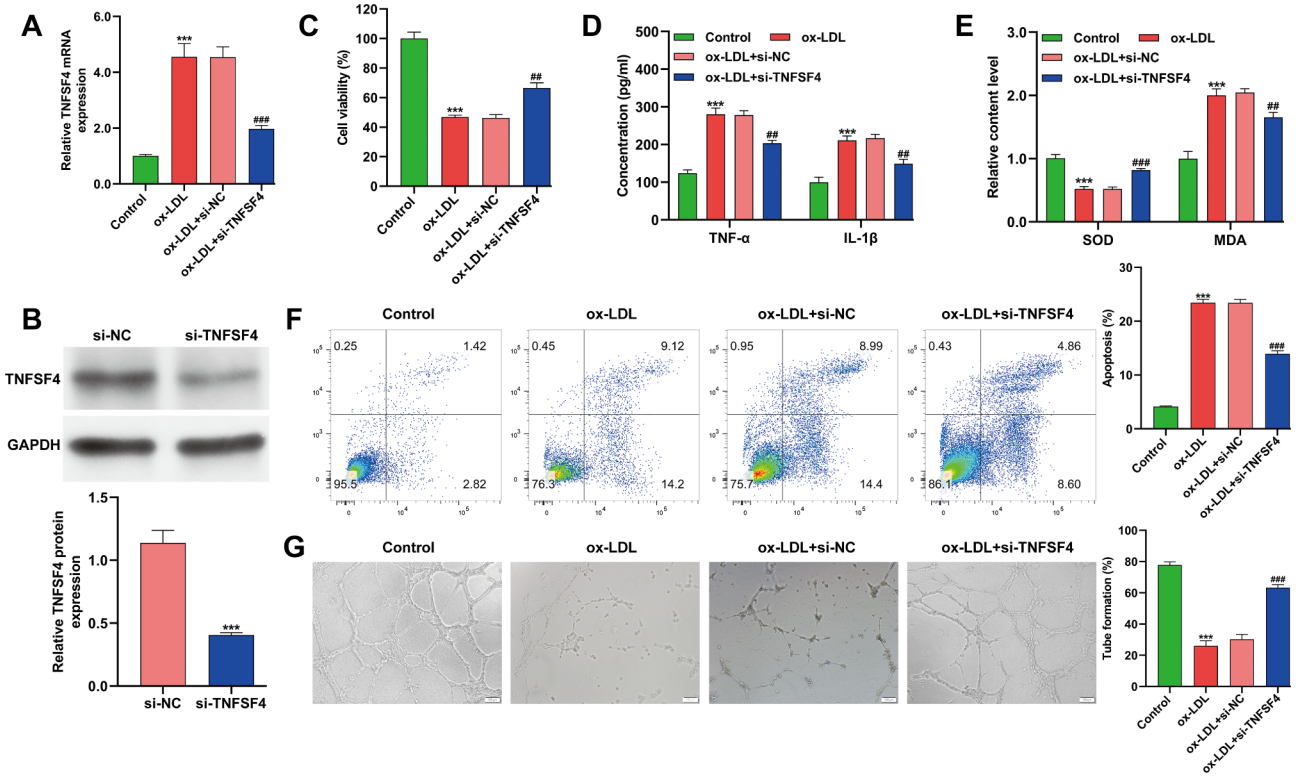

Fig. 4 Knockdown of TNFSF4 diminished ox-LDL-induced injury in HUVECs. HUVECs were transfected with si-TNFSF4 or si-NC for 48 h, followed by treated with 100 μg/ml of ox-LDL for 24 h. (**A**-**B**) The expression of TNFSF4 mRNA and protein was determined using quantitative real-time PCR and western blot analysis, respectively. (**C**) Cell viability of HUVECs was assessed using CCK-8 assay. (**D**) The concentrations of inflammation-associated cytokines (TNF-α and IL-1β) were detected by ELISA. (**E**) The production of SOD and MDA was assessed using their matching kits. (**F**) Cell apoptosis was determined using flow cytometry assay. (**G**) The ability of tube formation was analyzed through capillary-like network formation assay in vitro. All experiments were carried out three times with three repetitions. $^{***}p < 0.001$, compared with control or si-NC; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with ox-LDL + si-NC

dysfunction, which is partly due to the downregulation of TNFSF4.

MiR-125b-5p overexpression suppressed the TLR4/NF-κB pathway by negatively regulating TNFSF4 in ox-LDL-treated HUVECs

To further investigate the molecular mechanisms underlying the miR-125b-5p/TNFSF4 axis in ox-LDL-induced HUVEC injury, we focused on the TLR4/NF-κB pathway due to its crucial role in AS. As shown in Fig. 6, either overexpression of miR-125b-5p or knockdown of TNFSF4 significantly reduced the protein levels of TLR4, the p-p65/p65 ratio, and the p-IκBα/IκBα ratio. Significantly, forced expression of TNFSF4 remarkably reversed the effects of miR-125b-5p overexpression on these protein levels. These data suggested that ectopic expression of miR-125b-5p inhibits the activation of the TLR4/NF-κB pathway by negatively regulating TNFSF4 in ox-LDL-treated HUVECs.

Discussion

In this study, an in vitro model of AS was successfully established by treating HUVECs with 100 μg/ml ox-LDL for 24 h. This treatment led to significant inflammatory, oxidative, apoptotic, and angiogenic damages, which is in line with previous studies [22, 23]. Notably, ox-LDL treatment markedly activated the TLR4/NF-κB pathway, as evidenced by the dose-dependent upregulation of TLR4, the p-p65/p65 ratio, and the p-IκBα/IκBα ratio. As far as we know, vascular endothelial cells play a crucial role in regulating vascular homeostasis [24]. Ox-LDL is a well-known atherogenic factor, and several in vitro studies have reported that it induces endothelial cell injury [25, 26]. The apoptosis of vascular endothelial cells is the main form of endothelial injury involved in the occurrence and development of AS [27]. Additionally, oxidative stress [28], angiogenic damages [29], and especially inflammation [30] have been demonstrated to be crucial throughout the progression of AS, from endothelial dysfunction to plaque rupture and thrombosis. Since ox-LDL treatment activates the TLR4/NF-κB pathway and is known to cause endothelial cell injury with various manifestations, it can be inferred that the TLR4/NF-κB

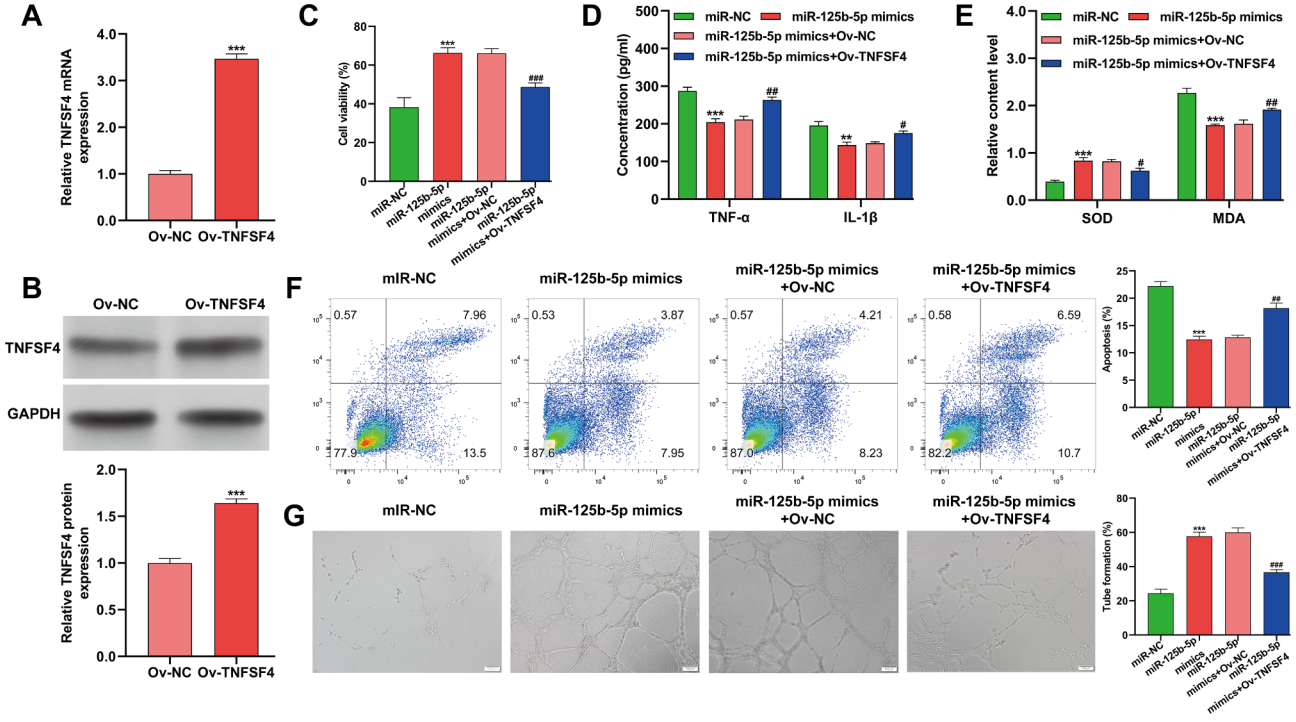

Fig. 5 Overexpression of TNFSF4 weakened the protective effects of miR-125b-5p against ox-LDL-mediated HUVEC injury. (**A**-**B**) The overexpression of TNFSF4 at mRNA and protein mRNA and protein levels was confirmed in ox-LDL-treated HUVECs after transfection with Ov-TNFSF4. $^{***}p < 0.001$, compared with Ov-NC; HUVECs were transfected with miR-125b-5p mimics alone or in combination with Ov-TNFSF4 for 48 h, followed by treated with 100 μg/ml of ox-LDL for 24 h, which were applied for CCK-8 assay (**C**), ELISA assay (**D**), oxidative stress assay (**E**), flow cytometry assay (**F**) and capillary-like network formation assay (**G**), respectively. All experiments were carried out three times with three repetitions. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with miR-NC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with miR-125b-5p mimics + Ov-NC

pathway is likely involved in the detrimental effects of ox-LDL on endothelial cells. This pathway may contribute to the inflammatory, oxidative, apoptotic, and angiogenic damages observed in the in vitro model of AS, suggesting a potential relationship between ox-LDL treatment and the activation of the TLR4/NF-κB pathway in the pathogenesis of AS.

Furthermore, our study revealed a significant decrease in miR-125b-5p levels in ox-LDL-induced HUVECs. The reduction in miR-125b-5p levels upon ox-LDL treatment implies its potential as a biomarker for AS. Overexpression of miR-125b-5p demonstrated several beneficial effects. It enhanced cellular viability and tube formation while suppressing cellular apoptosis, oxidative stress, and inflammation response. These protective effects in the HUVEC injury model suggest that miR-125b-5p may serve as a therapeutic target for AS. Comparable findings have been reported by Reithmair et al. [31] who showed that miR-125b-5p was inhibited in non-survivors with sepsis, and Guo et al. [32] identified that miR-125b was down-regulated in peripheral blood and splenocytes from LPS-induced ALI mice [33]. Another study illustrated that miR-125b-5p attenuates apoptosis and oxidative stress in β-amyloid (Aβ)-treated MCN and N2a cells, providing novel targets for the treatment of Alzheimer's disease (AD) [34]. Additionally, it has been established that miR-125b-5p plays an indispensable role in maintaining blood-brain barrier (BBB) integrity by targeting TLR4 and inhibiting NF-κB signaling in astrocytes [35]. The similarity of miR-125b-5p's actions in different disease models suggests a conserved mechanism of action. In the context of AS, miR-125b-5p's ability to suppress ox-LDL-induced upregulation of Endothelin-1 (ET-1), a potent vasoconstrictive peptide expressed in vascular endothelial cells [36], further highlights its importance in regulating vascular function. Overall, miR-125b-5p holds promise as a key player in the pathogenesis and treatment of AS.

As miRNAs regulate cellular processes by binding to the 3′-UTR of target genes, the TargetScan tool was employed to predict the associated targets of miR-125b-5p. Among them, TNFSF4 was identified as a candidate target, and the targeted relationship between miR-125b-5p and TNFSF4 was validated. AS is a chronic (auto-)inflammatory disease involving T cell activation. It has been demonstrated that blocking the interaction between TNFRSF4 (OX40) and TNFSF4 (OX40 ligand) reduces atherogenesis by inhibiting IL-4 mediated Th2-induced isotype switching [37]. TNFSF4 has been reported to be implicated in various cardiovascular and

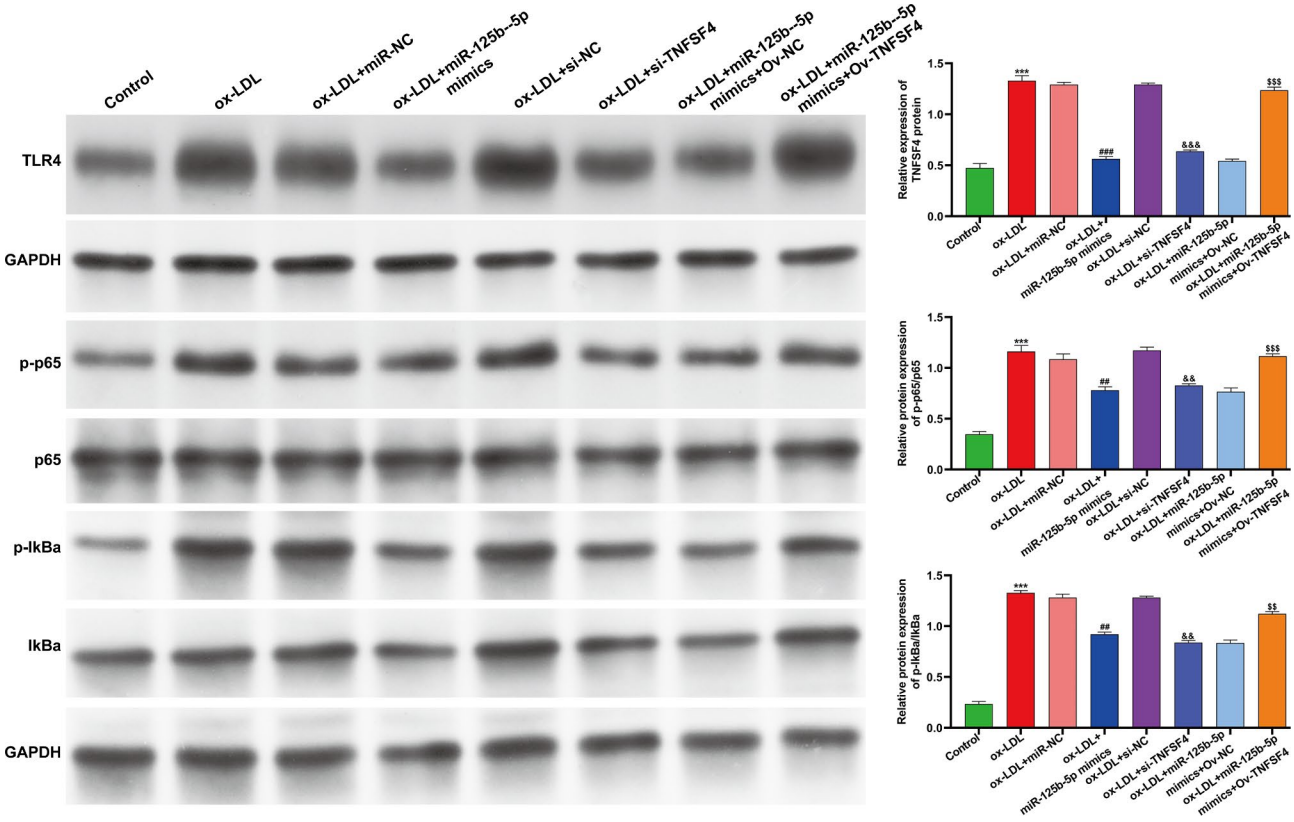

Fig. 6 MiR-125b-5p overexpression suppressed the TLR4/NF-κB pathway by targeting TNFSF4 in ox-LDL-treated HUVECs. HUVECs were transfected with si-TNFSF4 alone, miR-125b-5p mimics alone or in combination with miR-125b-5p mimics and Ov-TNFSF4 for 48 h, followed by treated with 100 μg/ml of ox-LDL for 24 h. The protein levels of TLR4, p-p65, p65, p-IκBα and IκBα were visualized and quantified via western blot analysis. All experiments were carried out three times with three repetitions. $^{***}p < 0.001$, compared with control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with ox-LDL + miR-NC; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, compared with ox-LDL + si-NC; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, compared with ox-LDL + miR-125b-5p mimics + Ov-NC

cerebrovascular diseases such as coronary heart disease [38], stroke [39], and cerebral infarction [40]. A previous study showed that the expression of TNFSF4 proteins and mRNAs is positively correlated with the stability of coronary atherosclerotic plaques and sudden coronary death [41]. Moreover, the upregulation of TNFSF4 is involved in regulating physical association with TLR2 and NF-κB activation [42]. Similarly, in our study, we found that TNFSF4 protein levels were increased in ox-LDL-stimulated HUVECs. Knockdown of TNFSF4 mimicked the protective effects of miR-125b-5p, while overexpression of TNFSF4 could rescue the protective effects of miR-125b-5p against ox-LDL-mediated HUVEC injury. This indicates that the protective effects of miR-125b-5p on AS progression can be partially suppressed by overexpressing TNFSF4. Overall, the miR-125b-5p/TNFSF4 axis may provide a new perspective for understanding and treating AS. Nevertheless, this research is merely an initial exploration of the role of the miR-125b-5p/TNFSF4 axis in AS. Further investigations are required to gain a better understanding of this axis in AS. For instance, the role of the miR-125b-5p/TNFSF4 axis in ox-LDL-induced endothelial cell injury has not been analyzed in AS animal models. Additionally, it remains to be determined whether the overexpression of TLR4/NF-κB signaling is associated with the effect of the miR-125b-5p/TNFSF4 axis on ox-LDL-induced HUVECs injury. Moreover, whether the miR-125b-5p/TNFSF4 axis plays a role in predicting the prognosis of patients with AS needs further analysis. These issues will be addressed in our future research endeavors.

Conclusion

In conclusion, the current findings offer evidence that miR-125b-5p safeguards against ox-LDL-induced endothelial injury by suppressing oxidative stress and inflammatory response. This protection is linked to the inhibition of TNFSF4-mediated TLR4/NF-κB signaling. The data from this study may provide a novel theoretical foundation and strategy for the diagnosis and treatment of AS.

Supplementary Information

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Supplementary Material 1: Figure S1 All the original and unprocessed protein bands

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Author contributions
HWS designed and performed the experiments and wrote the manuscript. ZLM and WPF analyzed the data. ZLM, RMJ and HYF designed the experiments and analyzed the data. ZLM performed the experiments. All authors read and approved the final version of the manuscript.

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Data availability
All data included in this study are available upon request by contact with the corresponding author.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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