MicroRNA-125a-5p alleviates the deleterious effects of ox-LDL on multiple functions of human brain microvessel endothelial cells

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Abstract

MicroRNA-125a-5p (miR-125a-5p) could participate in the pathogenesis of vascular diseases. In this study, we investigated the role of miR-125a-5p in oxidized low density lipoprotein (ox-LDL) induced functional changes in human brain microvessel endothelial cells (HBMEC). The reactive oxygen species (ROS) production, nitric oxide (NO) generation, senescence, apoptosis and functions of HBMEC were analyzed. For mechanism study, the epidermal growth factor receptor (EGFR)/ extracellular regulated protein kinases (ERK)/ p38 mitogen-activated protein kinase (p38 MAPK), and phosphatidylinositol-3-kinase (PI3K), serine/threonine kinase (Akt), endothelial nitric oxide synthase (eNOS), and p–Akt were analyzed. Results showed that: 1) MiR-125a-5p expression was reduced in ox-LDL treated HBMEC. 2) Overexpression of miR-125a-5p prevented HBMEC from ox-LDL induced apoptosis, senescence, ROS production, and NO reduction. 3) Overexpression of miR-125a-5p increased HBMEC proliferation, migration and tube formation, while decreased HBMEC adhesion to leukocytes, as well as counteracted the effects of ox-LDL on those functions. 4) The levels of EGFR/ERK/p38 MAPK pathway, PI3K/Akt/eNOS pathway, cleaved caspase 3 and adherent molecular ICAM-1 and VCAM-1 were associated with the effects of ox-LDL on
these HBMEC functions. In conclusion, miR-125a-5p could counteract the effects of ox-LDL on various HBMEC functions via regulating the EGFR/ERK/p38 MAPK and PI3K/Akt/eNOS pathways and cleaved caspase 3, ICAM-1 and VCAM-1 expression.

Keywords
MiR-125a-5p; oxidized low density lipoprotein; human brain microvessel endothelial cells; cell function; signal pathway,

Introduction
Endothelial cells (EC) play primary roles in the modulation of vascular structure and function (66). EC dysfunction has been suggested as the common pathophysiology and a key therapeutic target for various vascular diseases (55). The uniqueness of the brain endothelium is important in maintaining blood-brain barrier (BBB) function and the homeostasis of the brain vasculature (2, 9). Dysfunction of brain EC is known as an essential step in the pathogenesis of cerebrovascular diseases such as ischemic stroke and subarachnoid hemorrhage (6, 48). The death or apoptosis of brain EC can result in catastrophic failure of BBB’s integrity (19). Previous studies have suggested that BBB disruption is implicated in the onset and progression of ischemic stroke (59, 78). Thus, protecting and enhancing brain microvessel endothelial cells functions should be important in maintaining BBB intergrity and alleviating brain
damage after ischemic stroke.

Metabolic imbalances and inflammatory cytokines contribute to EC dysfunction (1). Accumulating evidence demonstrate that microRNAs (miRNA), such as miR-126, miR-146 and miR-222, are key factors in the regulation of EC functions (5, 52, 64). Oxidized low density lipoprotein (Ox-LDL) is well known as one of the risk factors for various vascular diseases, by inducing pro-inflammatory and pro-atherogenic responses (23). Evidence indicate that ox-LDL could compromise EC functions by increasing oxidative stress, characterized by reactive oxygen species (ROS) overproduction and nitric oxide (NO) reduction (17, 18). Ox-LDL can also mediate endothelial cell inflammation by increasing the level of inflammatory factors, including ICAM-1, tumor necrosis factor-alpha (TNF-α), IL-6 and IL-8 (23, 37) and abnormally regulate cell viability and apoptosis (14, 29, 30, 41).

MiRNA are small noncoding RNAs of 19-25 nucleotides that have been reported to play crucial roles in many biological processes, including development, differentiation, proliferation and apoptosis in various cells (4). MiR-125a-5p is the main functional form of miR-125a (51). It has been reported to be abnormally expressed and regulated the proliferation, migration or apoptosis of cancer cells (20, 33). MiR-125a-5p was found
to mediate lipid uptake and to decrease the secretion of some inflammatory cytokines (e.g. interleukin-2, interleukin-6, TNF-α, transforming growth factor-beta) in ox-LDL stimulated monocyte-derived macrophages (13). Recent studies showed that miR-125a-5p was highly expressed in EC, could suppress the expression of endothelin-1 and contribute to the pathogenesis of atherosclerosis (24, 35). Moreover, miR-125a-5p is expressed at low levels in the aorta of stroke-prone rats and in the blood of mice with ischemic stroke (35, 40). These suggest that the abnormal expression of miR-125a-5p in arteries, most probably in EC, might be related to vascular diseases.

In this study, we investigated the ability of miR-125a-5p to modulate the function of human brain microvessel endothelial cells (HBMEC) under physiological or ox-LDL treatment and the underlying mechanisms with a particular focus on the EGFR/ERK/p38 MAPK and PI3K/Akt/eNOS pathways.

Materials and Methods

Cell lines and cell culture
HBMEC (ScienCell Research Laboratories, Inc.) were cultured on 100-mm cell culture dishes in DMEM (Hyclone), supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin and 100 U/ml streptomycin in a 37 °C incubator with humidified atmosphere of 5% CO₂ / 95% air.

**Lentivirus preparation and in vitro infection**

Lentiviruses expressing green fluorescent protein (GFP) marker and miR-125a-5p (lenti-miR-125a-5p) or with scrambled control (lenti-SC) were purchased from GenePharma Biotech Co. Ltd. HBMEC cultured in 6-well plates with 75% confluence were incubated with 1×10⁷ infectious units of lenti-miR-125a-5p (HBMEC_{miR-125a-5p}) or lenti-SC (HBMEC_{SC}) in culture medium for 24 h. The viruses-containing medium was then removed and replaced with fresh medium. Cells were observed under a fluorescence microscope and the efficiency of miR-125a-5p over expression was confirmed by real-time quantitative polymerase chain reaction.

**Cell proliferation assay**

Proliferative capability of HBMEC was tested by MTT 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) (Sigma, 5 mg/ml) assay (47). HBMEC_{miR-125a-5p} and HBMEC_{SC} were respectively
seeded at $2 \times 10^3$/96-well plate and cultured in 100 μL DMEM (supplemented with 10% FBS) with ox-LDL (10 μg/mL) or PBS (vehicle). After 3 days incubation, the cells were treated with MTT solution (20 μL) for 4 h at 37 °C, before the addition of 150 μL DMSO to each well and further incubation for 20 min at 37 °C. The optical density (OD) of the cells was read at 490 nm in a microplate reader (BioTek, USA) in triplicate. Results were calculated from the values obtained in 3 independent experiments.

HBMEC senescence assay

Cellular aging was determined with a Senescence β-Galactosidase Staining Kit (Beyotime, China). Briefly, after washing with PBS, HBMEC^{miR-125a-5p} and HBMEC^{SC} treated with ox-LDL (10 μg/mL) or PBS (vehicle) were fixed for 6 min in 2% formaldehyde and 0.2% glutaraldehyde in PBS and then incubated for 12 h at 37 °C without CO2 with fresh X-gal staining solution (1 mg/mL X-gal, 5 mmol/L potassium ferrocyanide, 5 mmol/l potassium ferricyanide, and 2 mmol/L MgCl2; pH 6). After staining, blue-stained cells and total cells were counted and the percentage of β-galactosidase positive cells was calculated.

Migration Scratch Assay

Migration capacity of HBMEC was measured using a scratch assay.
HBMEC\textsuperscript{miR-125a-5p} and HBMEC\textsuperscript{SC} were grown to confluence on 6-well cell culture plates. A scratch was made through the cell monolayer using a 200 μL pipette tip. After washing with PBS, cells were cultured in 0.5 % FBS maintenance medium with ox-LDL (10 μg/mL) or PBS (vehicle). Photographs of the wounded area were taken immediately (0 h time point) and 16 h after making the scratch, to monitor the invasion of cells into the scratched area (magnification, 40×).

**Apoptosis assay**

Cell apoptosis was analyzed by Annexin V-PE/7-AAD staining as previously described (50). In brief, a serum-deprived (SD) medium was used for inducing apoptosis. HBMEC\textsuperscript{miR-125a-5p} and HBMEC\textsuperscript{SC} seeded on sterile cover glasses were placed in 6-well plates with ox-LDL (10 μg/mL, SD+ox-LDL) or not (SD) for 48 h. The apoptosis assay of HBMEC was then performed using an Annexin V-PE/7-AAD apoptosis detection kit (BD Biosciences). Briefly, cells were washed with PBS, re-suspended with 100 μL 1X annexin-binding buffer, incubated with 5μL PE-conjugated annexin V and 5 μL 7-AAD for 15 min in the dark before being analyzed by flow cytometry. Cells stained with both annexin V-PE and 7-AAD were considered to be late apoptotic HBMEC, while those stained only with Annexin V-PE were considered to be early apoptotic cells. The experiment was repeated three times.
**Adhesion assay**

After 48 h lentivirus infection, HBMEC were seeded on fibronectin-coated 24-well plates. After cells reached confluence, ox-LDL (10 μg/mL) or PBS (vehicle) was added for 24 h. HL60 cells (1×10^4/well) labeled with acridine orange (Sigma) were then added into each well. One hour later, non-adherent HL60 cells were washed away twice with PBS. The number of HL60 cells adhering to HBMEC was counted from 5 images of random fields captured by a microscope. Cell adhesion ability was defined as the average number of HL60 cells/field.

**Tube formation assay**

The tube formation ability was evaluated using a tube formation assay kit (Chemicon) according to the manufacturer’s instructions. Briefly, the ECMatrix solution was thawed on ice overnight, mixed with 10× ECMatrix diluents and placed in a 96-well tissue culture plate at 37 °C for 1 h to allow the matrix solution to solidify. HBMEC incubated with endothelial cell growth medium-2 (EGM-2) (Longza) containing ox-LDL (10 μg/mL) or PBS (vehicle) were placed on top of the solidified matrix (1×10^4 cells / well) and incubated for 24 h at 37 °C. Tube formation was evaluated with an inverted light microscope and the average number of tubes per field was determined. Five independent fields were assessed for
Measurement of ROS

Intracellular ROS production was determined by dihydroethidium (DHE) (Beyotime, China) staining followed by flow cytometric analysis (68). HBMEC treated with ox-LDL (10 μg/mL) or PBS (vehicle) were grown to confluence on 6-well cell culture plates, incubated with 5 μM DHE solution at 37 °C for 2 h and washed with PBS twice. The fluorescence intensity of cells was analyzed by flow cytometry. For pathway blocking experiments, cells were pre-incubated with PI3K inhibitor LY294002 (20 μM) for 2 h.

Measurement of NO

As described previously (68), total NO production in cells treated with ox-LDL (10 μg/mL) or PBS (vehicle) was determined by measuring the concentration of nitrate and nitrite, a stable metabolite of NO, in the culture medium, using a Nitric Oxide Assay kit (Beyotime). For pathway blocking experiments, cells were pre-incubated with LY294002 (20 μM) for 2 h.

RNA analysis

Total RNA from cell lines was extracted using TRIzol reagent (Invitrogen,
Carlsbad, CA, USA). The miR-125a-5p cDNA were synthesized from 3 μg total RNA using hairpin-it™ miRNA RT-PCR Quantitation kit (Genepharma, Shanghai, China) based on the manufacturer’s instructions (25 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min). Real-time PCR analysis of miR-125a-5p was as follows: 95 °C for 3 min, 40 cycles were performed at 95°C for 12 s, 60 °C for 40 s. Human miR-125a-5p-RT (5′-GTC GTA TCC AGT GCA GGG TCC GAG GTG CAC TGG ATA CGA CCT GCA G-3′) and U6-RT (5′-GTC GTA TCC AGT GCA GGA CCA GGA TTA TAG CCA CTT GAT A C-3′) were used for cDNA synthesis. The obtained cDNA was used for amplification of both mature miR-125a-5p and the endogenous control (U6 snRNA) by PCR. The corresponding PCR primers were as follows: 5′-ACA CTC CAG CTG GGT CCC TGA GAC CCT TAA-3′ and CTC AAC TGG TGT GGA GT-3′) for miR-125a-5p; 5′-CTC GCT TCG GCA GCA CA-3′ and 5′-AAC GCT TCA CGA AYY YGC GT-3′ for U6. U6 was chosen as housekeeping gene for normalizing the data of miR-125a-5p expression.

The cDNA of ICAM-1 and VCAM-1 were synthesized from 1 μg total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) at 42 °C for 60 min, 70 °C for 5 min. Real-time PCR was carried out on a LightCycler480-II System (Roche Diagnostics, Penzberg, Germany) using SYBR Premix Ex TaqTM (TAKARA, Japan) at 95 °C.
for 30 s, 40 cycles (95 °C for 5 s, 60 °C for 20 s). PCR primers: 5’-CAA AGG AGT ACG CAA AC-3’ and 5’-ACA GGA TTT TCG GAG CAG G-3’ for VCAM-1; 5’-CAA TGT GCT ATT CAA ACT GCC C-3’ and 5’-CAG CGT AGG GTA AGG TTC TTG-3’ for ICAM-1; 5’-GAA GGG CTC ATG ACC ACA GTC CAT-3’ and 5’-TCA TTG TCG TAC CAG GAA ATG AGC TT-3’ for GAPDH. Each experiment was repeated 3 times. The relative quantification of the gene expression was determined using the comparative CT method ($2^{\Delta\Delta Ct}$).

**Western blotting**

For western blot analysis, total cell protein (30 µg) extracted from each group, were separated by 12% SDS-PAGE on tri-glycine gels (invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore Corp, Bedford, MA). After blocking at room temperature (RT) in TBS (50 mM Tris, 150 mM NaCl, pH 7.6, 5% fat-free dry milk) for 1 h, the membranes were washed in TBST (0.5% Tween20 in TBS) at RT. Primary antibody was added over night at 4 °C. Following extensive washing, membranes were incubated with secondary antibody (1:50,000, EarthOx, San Francisco, CA, USA) for 1 h at RT. After washing 3 times for 30 min with TBST, immunoreaction was visualized by ECL solution (Amersham, Sweden). Beta actin (1:1000, EarthOx, San Francisco, CA, USA) was used to normalize protein loading. The following primary antibodies were
used: EGF receptor and phosho-EGF receptor (Tyr1068) (1:1000, Invitrogen, Carlsbad, CA, USA), Erk1/2 and phosphor-Erk1/2 (Thr202/Tyr204) (1:1000, CST, USA), p38 MAPK and pho-p38 MAPK (1:1000, CST, USA), Caspase-3 (1:1000, CST, USA), PI3 kinase p110a (1:1000, CST, USA), Akt and phosphor-Akt (1:1000, CST, USA), eNOS (1:1000, abcam, USA).

**Statistical analysis**

Data were expressed as mean ± standard error of mean (SEM). Multiple comparisons were performed by 1- or 2-way ANOVA. Comparisons for 2 groups were performed by Student’s t-test (GraphPad Prism 5 software). For all tests, P<0.05 was considered statistically significant.

**Results**

**MiR-125a-5p is down-regulated by ox-LDL in HBMEC**

The level of miR-125a-5p in ox-LDL treated HBMEC was analyzed by using quantitative RT-PCR for miRNA. Our results show that ox-LDL significantly down-regulated miR-125a-5p expression in HBMEC by 32.7 ±3.1% (vs. vehicle; P<0.05; Fig. 1A).

**Lentivirus infection successfully over-expressed miR-125a-5p in**
Lenti-miR-125a-5p was successfully expressed into HBMEC cells as indicated by the presence of GFP (Fig. 1B). The efficiency of miR-125a-5p overexpression was evaluated by RT-PCR analysis (Fig. 1A). The value of miR-125a-5p in the HBMEC infected with lenti-SC (HBMEC\textsuperscript{SC}) was set as the control. The level of miR-125a-5p in HBMEC infected with lenti-miR-125a-5p (HBMEC\textsuperscript{miR-125a-5p}) was significantly increased to about 6.5 ± 0.7-fold of HBMEC\textsuperscript{SC} (\textit{vs.} HBMEC\textsuperscript{SC}; \(P<0.05\); Fig. 1A).

Over-expression of miR-125a-5p increases NO production and decreases ROS production via PI3k/Akt/eNOS signaling pathway

Ox-LDL (10 \(\mu\)g/mL) induced ROS production while decreasing NO generation in both HBMEC\textsuperscript{miR-125a-5p} and HBMEC\textsuperscript{SC} (\textit{vs.} vehicle; \(P<0.05\); Fig. 2). Over-expression of miR-125a-5p reduced ROS production and increased NO production in both vehicle (PBS) and ox-LDL treated cells (\textit{vs.} HBMEC\textsuperscript{SC}; \(P<0.05\); Fig. 2). In addition, the PI3k inhibitor LY294002 (20 \(\mu\)M) almost completely reversed the effects of miR-125a-5p on NO generation in both vehicle and ox-LDL treated while having more modest effects on ROS production (\textit{vs.} HBMEC\textsuperscript{miR-125a-5p}; \(P<0.05\); Fig. 2). The results suggest that PI3K signaling is involved in the effects of
miR-125a-5p and ox-LDL on NO production and to a smaller extent ROS generation in HBMEC.

To determine the signal pathways associated with miR-125a-5p in regulating NO and ROS production, the expression of PI3K, Akt, p-Akt and eNOS was analyzed by Western blotting. Results show that ox-LDL decreased the levels of PI3K, p-Akt, Akt, and eNOS in both HBMEC<sup>SC</sup> and HBMEC<sup>miR-125a-5p</sup> (vs. vehicle; *P*<0.05; Fig. 2C). Overexpression of miR-125a-5p increased the levels of PI3K, p-Akt/Akt and eNOS in both ox-LDL and vehicle treated HBMEC cells (vs. HBMEC<sup>SC</sup>; *P*<0.05; Fig. 2C). Furthermore, the effects of miR-125a-5p were inhibited by PI3K inhibition (vs. HBMEC<sup>miR-125a-5p</sup>; *P*<0.05; Fig. 2C). Taken together, these data suggest that miR-125a-5p increases NO production and decreases ROS generation in HBMEC cells via activating PI3K/Akt signaling pathway.

**Over-expression of miR-125a-5p reduces apoptosis and senescence of HBMEC and down-regulates caspase-3**

Annexin V-PE/7-AAD analysis revealed that ox-LDL (10 μg/mL) increased the SD-induced apoptotic rate in both HBMEC<sup>SC</sup> and HBMEC<sup>miR-125a-5p</sup> (vs. SD; *P*<0.05; Fig. 3A). In addition, we assessed the level of cleaved caspase-3, which is associated with cell apoptosis. Western blot results showed that cleaved caspase-3 expression was
significantly increased after ox-LDL treatment (vs. SD; $P<0.05$; Fig. 3B). As expected, overexpression of miR-125a-5p decreased both cell apoptosis and cleaved caspase-3 expression (vs. HBMEC$^{SC}$; $P<0.05$; Fig. 3) in SD and SD+ox-LDL-treated HBMEC. These results suggest that miR-125a-5p might protect HBMEC from ox-LDL- or SD-induced apoptosis by inhibiting caspase-3 activation.

We then used β-galactosidase as a biomarker of senescence (15). We observed that ox-LDL induced senescence in both HBMEC$^{SC}$ and HBMEC$^{miR-125a-5p}$ (vs. vehicle; $P<0.05$; Fig. 4). However, miR-125a-5p overexpression significantly decreased by 50% the percentage of senescence-associated β-galactosidase-positive HBMEC in both vehicle and ox-LDL treated groups (vs. HBMEC$^{SC}$; $P<0.05$; Fig. 4). Taken together, these data suggest that miR-125a-5p protects HBMEC cells from senescence under physiological and pathophysiological conditions.

**Over-expression of miR-125a-5p increases the proliferation and tube formation abilities of HBMEC**

The MTT cell proliferation assay showed that ox-LDL (10 μg/mL) markedly increased the proliferation of HBMEC$^{SC}$ (vs. vehicle; $P<0.05$; Fig. 5A). Overexpression of miR-125a-5p increased the proliferation of HBMEC, independently of ox-LDL treatment (vs. HBMEC$^{SC}$; $P<0.05$;
An *in vitro* angiogenesis assay was performed with HBMEC to investigate the effect of miR-125a-5p on HBMEC neovascularization. As shown in Figure 5B, overexpression of miR-125a-5p increased the tube formation ability of HBMEC (vs. HBMEC<sup>SC</sup>; *P*<0.05), while Ox-LDL (10 μg/mL) decreased the tube formation ability of HBMEC (vs. vehicle; *P*<0.05; Fig. 5B). However, miR-125a-5p could improve the tube formation ability of HBMEC impaired by ox-LDL (vs. HBMEC<sup>SC</sup>; *P*<0.05; Fig 5B). Taken together, these data suggest that miR-125a-5p is effective on promoting proliferation and neovascularization of HBMEC under normal and ox-LDL treated conditions.

**Overexpression of miR-125a-5p increases the migration of HBMEC and the level of EGFR/p-EGFR, ERK1/2/p-ERK1/2, p38/p-p38 MAPK**

The effect of miR-125a-5p on HBMEC migration was evaluated using a scratch assay analysis. After 16 h of culturing, results show that migration of HBMEC<sup>miR-125a-5p</sup> was increased compared with HBMEC<sup>SC</sup> in both group (vs. HBMEC<sup>SC</sup>; *P*<0.05; Fig. 6A). Ox-LDL (10 μg/mL) promoted the migration ability of HBMEC<sup>SC</sup> and HBMEC<sup>miR-125a-5p</sup> (vs. vehicle; *P*<0.05; Fig. 6A).

Phosphorylation of EGFR and its downstream MAP kinases ERK1/2 and
p38 MAPK are critical in regulating cell migration (72). We investigated the signal pathways associated with the ability of miR-125a-5p in regulating HBMEC migration by using Western blotting analysis. We observed that ox-LDL (10 μg/mL) increased the levels of EGFR, ERK1/2, and the phosphorylation of EGFR (p-EGFR), ERK1/2 (p-ERK1/2) and p38 MAPK (p-p38 MAPK) in both HBMECsc and HBMECmiR-125a-5p (vs. vehicle; P<0.05; Fig. 6B). Overexpression of miR-125a-5p increased the levels of those proteins in HBMEC cells in both vehicle and ox-LDL treated cells (vs. HBMECsc; P<0.05; Fig. 6B). Meanwhile, the level of p-EGFR/EGFR, p-ERK1/2/ERK1/2 and p-p38 MAPK/p38 MAPK were up-regulated following overexpression of miR-125a-5p (vs. HBMECsc; P<0.05; Fig. 6B). These data suggest that miR-125a-5p improves the migration ability of HBMEC by activating EGFR/ERK/p38 MAPK signaling pathway.

**MiR-125a-5p over-expression decreases HBMEC adhesion to HL60 cells and ICAM-1 and VCAM-1 mRNA expression**

An HL60 cell adhesion assay was used to measure the influence of miR-125a-5p on the leukocytes recruitment of EC. As expected, ox-LDL (10 μg/mL) increased the adhesion ability of HBMEC with HL60 cells in both HBMECsc and HBMECmiR-125a-5p (vs. vehicle; P<0.05; Fig. 7A). However, miR-125a-5p significantly decreased the number of HL60 cells attached to HBMEC independently of ox-LDL treatment (vs. HBMECsc;
Since ICAM-1 and VCAM-1 are important adherent molecules released by EC which promotes leukocytes adhesion (45), we further measured ICAM-1 and VCAM-1 mRNA expression by RT-PCR. Our results show that ox-LDL (10 μg/mL) increased ICAM-1 and VCAM-1 mRNA expression in both HBMEC<sup>SC</sup> and HBMEC<sup>mIR-125a-5p</sup> (vs. vehicle; \( P<0.05 \); Fig. 7B). Meanwhile, ICAM-1 and VCAM-1 mRNA was decreased significantly after over expressing miR-125a-5p in both HBMEC and HBMEC treated with ox-LDL (vs. HBMEC<sup>SC</sup>; \( P<0.05 \); Fig.7B). These observations suggest that miR-125a-5p may decrease the leukocytes recruitment of HBMEC via downregulating ICAM-1 and VCAM-1 expression in HBMEC cells.

**Discussion**

In the present study, we investigated the effects of miR-125a-5p on various activities of HBMEC under normal and ox-LDL induced oxidative stress situations. The major findings of this study are that the level of miR-125a-5p in ox-LDL treated HBMEC was down-regulated. Moreover, in normal and ox-LDL treated situations, miR-125a-5p could promote NO generation and to a smaller extent reduce ROS production via the PI3K/Akt signaling pathway. Its overexpression decreased cell apoptosis and senescence, increased cell proliferation and enhanced the
abilities in migration and tube formation, associated with down-regulating of cleaved caspase-3 expression and up-regulation of p-EGFR/EGFR, p-p38/p38, p-ERK1/2/ERK1/2. Furthermore, miR-125a-5p could inhibit the adhesion of HBMEC with HL60 cells.

Oxidative stress is an important mechanism of vascular disease (34). ROS, an important signaling molecule for oxidative stress, is known to impair cell function and contribute to diseases (5). Excessive ROS can oxidize various molecules, induce intracellular calcium overload and DNA fragmentation, and inactivate NO, resulting in endothelial dysfunction (21). NO is a pivotal inhibitor factor in oxidative stress of EC, playing a primary role in vascular protection (61). Endothelium derived NO has been reported to confer significant vasoprotective effects, including inhibition of both platelet aggregation and inflammatory cell adhesion to EC, disruption of proinflammatory cytokine-induced signaling pathways in EC, and inhibition of EC apoptosis (27, 65). It was reported that NO can reduce the production of ROS (25). Thus, ROS overproduction and NO reduction could contribute to the mechanism of endothelial dysfunction. In this study, we found that miR-125a-5p overexpression could promote NO generation and reduce ROS production, indicating the protective effect of miR-125a-5p. Moreover, ox-LDL overstimulation can induce ROS generation in Human Umbilical Vein Endothelial Cells (7).
Our data also highlight that miR-125a-5p could resist ox-LDL-induced ROS production and NO reduction. Previous studies indicate that NO can regulate cerebral blood flow (CBF) and vascular tone and protects against ischemic stroke by increasing collateral flow to the ischemic area (39), whereas excessive ROS was reported to participate in the pathogenesis of ischemic stroke (3). Therefore, miR-125a-5p deficiency might contribute to the occurrence and development of ischemic stroke via increasing ROS production and reducing NO generation. To be noted, we detected ROS production by use of DHE followed with flow cytometric analysis. DHE as an intracellular probe is commonly used for the detection of superoxide (O$_2^-$) formed in cells and tissues (8, 10, 56). DHE enters the cells and is oxidized by superoxide to form ethidium (E$^+$), which binds to DNA to produce the fluorescent E$^+$-DNA that displays red fluorescence (44). It has been widely used to index intracellular ROS level (12, 16, 46). However, the reaction of O$_2^-$ and DHE could form another fluorescent product, 2-OH-E$^+$, whose optimal excitation and emission wavelengths differ from those of ethidium (E$^+$). The commonly employed filter detecting primarily E$^+$ fluorescence is not capable of estimating the relative concentration of 2-OH-E$^+$ (77). Thus, other methods, such as oxidationsensitive fluorescent probe, 2′7′-dichlorofluorescin diacetate (DCFH-DA) assay (43, 60) can be used to confirm the ROS level. In addition, the PI3K/Akt pathway has been shown to be closely related to
ROS/NO production by us and others (1, 22, 42). Herein, we demonstrated that miR-125a-5p modulates NO generation and ROS production via the PI3K/Akt/eNOS signaling cascade in HBMEC.

It has been reported that endothelial cell senescence induces endothelial dysfunction, which leads to impairment of endothelium-dependent vasodilation, angiogenesis and the antithrombogenic properties of endothelium, suggesting a critical role of endothelial cellular senescence in vascular pathology (25). Our results demonstrate that miR-125a-5p overexpression could inhibit HBMEC cell senescence under normal and ox-LDL induced conditions. This raises the possibility that miR-125a-5p deficiency contribute to cell senescence during vascular dysfunction, which might add to the pathogenesis of ischemic stroke. Studies have shown that, in senescent vascular EC the production of NO is reduced, whereas ROS production is increased (25), which is consistent with our findings that miR-125a-5p overexpression could inhibit HBMEC cell senescence while promoting NO generation and reducing ROS production.

The effects of miR-125a-5p on HBMEC function were also investigated. MiR-125a-5p was reported to prompt the activation and proliferation of hepatic stellate cells (34), and enhance proliferation and migration of
nasopharyngeal carcinoma cell lines, prostate cancer cells and multiple myeloma cells (20, 21, 33). However, the proliferation and/or migration inhibitory effects of miR-125a-5p were also demonstrated in cervical carcinoma cells, glioblastoma cells, gastric cancer cells and colon cancer cells (53, 63, 71, 74). This highlights the diverse effects of miR-125a-5p in various cells/tissues. In our study, we found that miR-125a-5p overexpression could increase proliferation, migration and tube formation of HBMEC under normal and ox-LDL treatment conditions, with up-regulation of EGFR, p38 MAPK, and ERK1/2 activities. Recent studies have reported that miR-125a-5p could modulate cancer cell migration by targeting ABL proto-oncogene 2, MAPK pathway, or PI3K/AKT/mTOR signaling pathway (52, 58, 62). Phosphorylation of EGFR and its downstream MAP kinases ERK1/2 and p38 MAPK are critical in regulating cell migration (42). This is the first demonstration that miR-125a-5p might regulate HBMEC function via up-regulating the EGFR, ERK1/2 and p38MAPK pathways. Noteworthy, ox-LDL was found to increase proliferation and migration while decreasing tube formation in HBMEC. In fact, previous studies have reported the effects of ox-LDL on promoting proliferation and/or migration of human coronary artery smooth muscle cells and EC (29, 41). However, we noted that ox-LDL also induced HBMEC apoptosis, which might lead to the decrease in tube formation. Moreover, our data suggest an anti-apoptotic
effect of miR-125a-5p overexpression on HBMEC, which is further supported by the observation that miR-125a-5p significantly decreases the protein expression of cleaved caspase-3, an apoptosis-promoting factor. Since the proliferation, migration, tube formation abilities and apoptosis of EC are associated with angiogenesis (4, 73), our findings indicate that miR-125a-5p could play an important role in vascular repair following ischemic stroke. It should be noted that newly formed blood vessels might lead to plaque formation and plaque rupture (34, 67). Therefore, miR-125a-5p might participate in the occurrence and development of ischemic stroke.

Leukocyte trafficking from the blood into the CNS is a key event in the pathogenesis of neurological diseases involving acute and chronic inflammation (54). The first step of the event is often described as capture (tethering) of leukocyte, mediated by leukocyte-endothelial interaction (55). It has been reported that activated EC express adherent molecules which promote leukocytes adhesion (51). In this study, we found that miR-125a-5p overexpression decreased the number of HL60 cells adhered to HBMEC, which was associated with the downregulation of important adherent molecules such as ICAM-1 and VCAM-1. It has been reported that excess ox-LDL could induce inflammation in endothelial cells (76). Ox-LDL could activate EC by up-regulating the expression of
several cell surface adhesion molecules which mediate the adhesion of
blood leukocytes (50). Our data also highlight that miR-125a-5p could
inhibit ox-LDL-induced HL60 cells adherence to HBMEC. This indicates
that miR-125a-5p deficiency could increase EC inflammation, which
contributes significantly to ischemic stroke. MicroRNA-125a-5p has also
been found to mediate lipid uptake and decreases the secretion of some
inflammatory cytokines (interleukin-2, interleukin-6, tumor necrosis
factor-alpha, etc.) in oxLDL-stimulated monocyte-derived macrophages,
reflecting the anti-inflammation role of miR-125a-5p in these cells (13).

Our data showed that level of miR-125a-5p in ox-LDL treated HBMEC
was down-regulated. However, the underlying mechanisms remains
unknown. As we know, the expression of miR is regulated by Dicer and
Drosha (57). During miR biogenesis, Drosha leads to the generation of
shorter stem-loop precursors (pre-miR) (32). The pre-miR is recognized
by Exportin-5, and exported from the nucleus to the cytoplasm, where
Dicer, in concert with other partner binding proteins, generates typically
20-22 nt long mature miR. It has been demonstrated that the miR key
regulators Drosha, Dicer, Drosha co-factor DGRC8, and Exportin-5 are
significantly downregulated during the inflammatory process in the skin
(26), indicating that inflammation induce changes in the level of miR
maturation machinery. Moreover, miR has been shown to be associated
with inflammation, such as downregulation (miR-29b,106b, 125b, and 198) in apical periodontitis (75) and upregulation (miR-155 and miR-146a) in patients with rheumatoid arthritis (49). Thus, ox-LDL, as a major proinflammatory factor, may regulate miR-125a-5p expression via the inflammatory response, probably partly by influencing the expression of Drosha and Dicer in EC.

Moreover, the primary target of miR-125a-5p in the present study needs more investigation. MiR-125a has been found to regulate various targeted mRNA expression. It could mediate lipid uptake and decrease the secretion of inflammatory cytokines in ox-LDL stimulated THP-1 cells by targeting oxysterol binding protein like 9 (ORP9) mRNA (13), and promote the activation and proliferation of hepatic stellate cells (HSCs) by targeting the 3'-untranslated region of factor inhibiting hypoxia-inducible factor 1 (FIH1) mRNA (36). In addition, miR-125a-5p has been predicted to target Furin in human dermal microvascular EC (31), and proven to regulate the expression of bone morphogenetic protein receptor 2 (BMPR2) and the tumor suppressor cyclin-dependent kinase inhibitors 1A (CDKN1A) in human pulmonary artery EC, resulting in a proproliferative phenotype of these cells (28). MiR-125a-5p could suppress endothelin-1 expression of murine cardiac microvascular cells by directly targeting preproET-1 mRNA (35), and promote human
umbilical vein EC angiogenesis by repressing the expression of the angiogenic inhibitor delta-like 4 (DLL4) via targeting its 3’ untranslated region (38). One study reported that miR-125a-5p impaired angiogenesis of thoracic aortas EC of aging mice by targeting related transcriptional enhancer factor-1 (RTEF-1) (11). In our present study, we investigated whether miR-125a-5p modulates the EGFR/ERK/p38 MAPK and PI3K/Akt/eNOS signaling pathways, which have been proven to play important roles in regulating HBMEC proliferation, migration, tube formation abilities, and ROS/NO production (42, 70). Our findings in HBMEC are not in agreement with the concept that miR-125a-5p to impair angiogenesis of thoracic aortas EC of aging mice by targeting RTEF-1 (11). The reason for this might be related to different species and different tissue. Additional studies are warranted to investigate the directly targeting of miR-125a-5p by luciferase reporter assay to clarify the underlying mechanisms.

As described above, limited studies have shown functions of miR-125a in vascular EC (11, 28, 38). This study is the first to demonstrate that miR-125a-5p could regulate multiple functions of HBMEC under physiological and ox-LDL-induced pathological conditions. Our findings are supported by recent studies showing that miR-125a-5p could promote human umbilical vein EC angiogenesis (38), and accelerate self-renewal
and long-term multi-lineage repopulation of murine/human multipotent progenitors in transplanted recipient mice (69). Taken together, miR-125a-5p has vascular protective effects and might be a promising target for tissue repair therapy. This will provide a new therapeutic target for brain vascular damage before/after ischemic stroke. Of note, the beneficial effects of miR-125a-5p should be further validated in various animal models.

In conclusion, miR-125a-5p exerts important protective effects on brain EC against ox-LDL-induced endothelial dysfunction via its protein targets including cleaved caspase 3 and ICAM-1/VCAM-1 and through EGFR/ERK/p38 MAPK and PI3K/Akt/eNOS pathways.

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DISCLOSURES
The authors declare that there is no conflict of interests regarding the
publication of this paper.

AUTHOR CONTRIBUTIONS

Qunwen Pan, Xiaorong Liao, Hua Liu, Yan Wang, Xiaotang Ma performed experiments; Qunwen Pan, Xiaotang Ma, Yi Yang wrote the manuscript; Qunwen Pan, Xiaorong Liao, Hua Liu, Yanfang Chen, Bin Zhao, Eric Lazartigues, Yi Yang, Xiaotang Ma contributed to manuscript preparation; All authors read and approved the final version of this manuscript for submission.

Figure legends

**Fig.1** Analysis of miR-125a-5p expression in HBMEC. (A) Real-time PCR results show that the level of miR-125a-5p in ox-LDL treated HBMEC was decreased and miR-125a-5p in HBMEC infected with lenti-miR-125a-5p (HBMECmiR-125a-5p) was significantly increased compared with HBMEC infected with lenti-SC (HBMECSC). *P*<0.05, vs. HBMEC; #*P*<0.05, vs. HBMECSC; n=3/group. (B) Microscopy images of GFP marker expression in HBMEC after lentivirus infection. Scale bars=50 μm.

**Fig.2** Effects of miR-125a-5p overexpression on ROS and NO production, PI3K and p-Akt/Akt expression of HBMEC with ox-LDL(10μg /mL) treatment or not (vehicle). (A) ROS production of HBMEC under
different treatment. (B) NO generation of HBMEC. (C) Western blot showing expression of PI3K, p-Akt/Akt and eNOS of HBMEC pretreated with PI3K inhibitor LY294002 or not. $^\wedge P<0.05$, vs. HBMEC$^{SC}$, vehicle; $^\wedge P<0.05$, vs. HBMEC$^{miR-125a-5p}$, vehicle; $^* P<0.05$, vs. HBMEC$^{SC}$, vehicle; $^\# P<0.05$, vs. HBMEC$^{SC}$, ox-LDL; $^\wedge P<0.05$, vs. HBMEC$^{miR-125a-5p}$, vehicle; $^\wedge P<0.05$, vs. HBMEC$^{miR-125a-5p}$, ox-LDL; n=3/group.

**Fig.3** MiR-125a-5p decreases apoptosis and cleaved capase-3 expression of HBMEC. (A) HBMEC apoptosis determined by Annexin V-PE/7-AADstaining followed by flow cytometric analysis. (B) Cleaved caspase-3 expression as detected by Western blotting. $^* P<0.05$, vs. HBMEC$^{SC}$, SD; $^\wedge P<0.05$, vs. HBMEC$^{SC}$, SD; $^\wedge P<0.05$, vs. HBMEC$^{miR-125a-5p}$, SD; $^\wedge P<0.05$, vs. HBMEC$^{miR-125a-5p}$, ox-LDL; n=3/group.

**Fig.4** Effects of miR-125a-5p on HBMEC senescence under ox-LDL (10μg/mL) treatment or not (vehicle). HBMEC were analyzed by senescence associated acidic β-galactosidase activity assay. $^* P<0.05$, vs. HBMEC$^{SC}$, vehicle; $^\wedge P<0.05$, vs. HBMEC$^{SC}$, vehicle; $^\wedge P<0.05$, vs. HBMEC$^{miR-125a-5p}$, vehicle; $^\wedge P<0.05$, vs. HBMEC$^{miR-125a-5p}$, ox-LDL; n=3/group. Scale bars=400 μm.

**Fig.5** MiR-125a-5p overexpression enhances proliferation and tube formation ability of HBMEC. (A) MiR-125a-5p increases proliferation of
HBMEC. (B) Representative images and quantification results of HBMEC tube formation. \(*P<0.05, \text{vs. } \text{HBMEC}^\text{SC}, \text{vehicle} \); \(^\sim P<0.05, \text{vs. } \text{HBMEC}^\text{SC}, \text{vehicle} \); \(^\wedge P<0.05, \text{vs. } \text{HBMEC}^\text{miR-125a-5p}, \text{vehicle} \); \(^#P<0.05, \text{vs. } \text{HBMEC}^\text{SC}, \text{ox-LDL} \); n=3/group. Scale bars=600 μm.

**Fig. 6** Effects of miR-125a-5p on HBMEC migration and expression of EGFR/p-EGFR, ERK1/2/p-ERK1/2 and p38/p-p38 MAPK. (A). MiR-125a-5p increased migration of HBMEC (vehicle) or ox-LDL (10μg/mL) treated HBMEC. (B). MiR-125a-5p increased the level of p-EGFR/EGFR, p-p38/p38, p-ERK1/2/ERK1/2 in normal or ox-LDL-treated HBMEC. \(*P<0.05, \text{vs. } \text{HBMEC}^\text{SC}, \text{vehicle} \); \(^\sim P<0.05, \text{vs. } \text{HBMEC}^\text{SC}, \text{vehicle} \); \(^\wedge P<0.05, \text{vs. } \text{HBMEC}^\text{miR-125a-5p}, \text{vehicle} \); \(^#P<0.05, \text{vs. } \text{HBMEC}^\text{SC}, \text{ox-LDL} \); n=3/group.

**Fig.7** Effects of miR-125a-5p overexpression on the adhesion of HBMEC with HL60 cells and on the expression of ICAM-1 and VCAM-1 mRNA. (A) Representative images and quantification data of HL60 cells adhered to HBMEC. (B) Quantitative Real time PCR analysis of mRNA for ICAM-1 and VCAM-1. \(*P<0.05, \text{vs. } \text{HBMEC}^\text{SC}, \text{vehicle} \); \(^\sim P<0.05, \text{vs. } \text{HBMEC}^\text{SC}, \text{vehicle} \); \(^\wedge P<0.05, \text{vs. } \text{HBMEC}^\text{miR-125a-5p}, \text{vehicle} \); \(^#P<0.05, \text{vs. } \text{HBMEC}^\text{SC}, \text{ox-LDL} \); n=3/group. Scale bars=50 μm.

**Reference**


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Fig. 1

A

miR-125a-5p expression (fold of change)

HBMEC
ox-LDL treated
HBMEC
HBMEC
HBMEC
HBMEC

B

HBMEC
HBMEC

* 

#
Fig. 2

A

B

C

ROS production (fold of control)

NO production (fold of control)

PI3K/actin

p-Akt/Akt

eNOS/actin

HBMEC Sc

HBMEC

mR-122a-5p

vehicle

+

ox-LDL

LY294002

110KDa

43KDa

60KDa

60KDa

140KDa

+
Fig. 3

A

HBMEC<sup>sc</sup> vs HBMEC<sup>miR-125a-5p</sup>

- SD
  - Q1: 2.53%
  - Q2: 6.88%
  - Q3: 6.01%
  - Q4: 79.9%

- SD+ox-LDL
  - Q1: 6.04%
  - Q2: 21.3%
  - Q3: 7.94%
  - Q4: 64.7%

PE 7-AAD

7-AAD percentage of apoptotic cells:

- SD
  - Q1: 6.04%
  - Q2: 21.3%
  - Q3: 7.94%
  - Q4: 64.7%

- SD+ox-LDL
  - Q1: 2.53%
  - Q2: 10.4%
  - Q3: 10.3%
  - Q4: 76.4%

B

Cleaved caspase-3/Actin

- SD
  - Pro-caspase-3: 35KDa, 43KDa
  - Cleaved caspase-3: 19KDa, 17KDa
  - Actin: 43KDa

- SD+ox-LDL
  - Pro-caspase-3: 35KDa, 43KDa
  - Cleaved caspase-3: 19KDa, 17KDa
  - Actin: 43KDa
HBMEC Senescence (% of β-galactosidase-positive cells)

vehicle

HBMEC<sup>SC</sup> HBMEC<sup>miR-125a-5p</sup>

ox-LDL

HBMEC<sup>SC</sup> HBMEC<sup>miR-125a-5p</sup>

---

Fig. 4
Fig. 5

A

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B

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Tubes/field

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**Fig. 7**

**A**

HBMEC\textsuperscript{Sc}  
HBMEC\textsuperscript{miR-125a-5p}  

ox-LDL  
vehicle  

**B**

Relative mRNA expression  

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<tr>
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<th>VCAM-1</th>
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<tr>
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<td>1.0</td>
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<td>+</td>
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