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MiR-34a-5p inhibition attenuates LPS-induced endothelial cell injury by targeting *FOXM1*

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Abstract. – OBJECTIVE: This study aims to investigate the role of miR-34a-5p in the regulation of lipopolysaccharide (LPS)-induced injury of vascular endothelial cells (ECs).

MATERIALS AND METHODS: Human umbilical vein ECs (HUVECs) were exposed to LPS to stimulate endothelial injury *in vitro*. miRNA microarray analysis was carried out to identify miR-34a-5p expression in HUVECs. MTT and flow cytometry analyses were used to determine cell viability and apoptosis rate, respectively. Furthermore, enzyme-linked immunosorbent assay (ELISA), qRT-PCR, and Western blot were used to examine the factors involved in inflammation and their relative gene expression. Additionally, Matrigel-based tube formation assay was carried out to assess the vasculogenic activity of HUVECs. Luciferase reporter assay was used to analyze the possible relationship between miR-34a-5p and *FOXM1*.

RESULTS: MiR-34a-5p expression was significantly enhanced in HUVECs after 24 h of LPS treatment. LPS treatment led to a dramatic inhibition of cell viability, enhanced apoptosis, increased production of pro-inflammatory cytokines, and inhibited the vasculogenic activity of HUVECs. MiR-34a-5p inhibitor attenuated LPS-induced damage. MiR-34a-5p directly inhibited the expression of *FOXM1*, and its overexpression alleviated the protective effect of *FOXM1* on cell viability, apoptosis, inflammation factor production, and vasculogenic activity. The activity of the NRF2/HO-1 pathway was inhibited by miR-34a-5p, possibly *via FOXM1*.

CONCLUSIONS: MiR-34a-5p inhibition attenuates LPS-induced EC injury by targeting *FOXM1 via* activation of the NRF2/HO-1 pathway.

Key Words:
Sepsis, Endothelial cells, MiR-34a-5p, *FOXM1*.

Introduction

Sepsis, one of the leading causes of mortality worldwide, is induced by exogenous bacteria and results in infection and inflammation and subsequent damage to the multiple organ system[1,2]. Endothelial dysfunction has been considered as an important contributor to several patho-physiological inflammatory conditions by aberrantly producing pro-inflammatory molecules, such as cytokines, chemokines, and adhesion molecules, thus resulting in leukocyte accumulation and the development of sepsis-related organ failure[3]. However, the underlying mechanisms involved in the inflammatory response induced by sepsis remain unclear.

MicroRNAs (miRNAs), 18- to 28- nucleotide non-coding RNAs, regulate physiological and pathological processes, including cell differentiation, the cell cycle, and apoptosis, by post-transcriptional regulation of mRNAs[4,5]. MiRNAs have been demonstrated to play critical roles in vascular integrity and the inflammatory response[6]. It was found that the levels of many miRNAs in the blood and organs of patients with sepsis were increased[7]. MiRNAs can therefore act as diagnostic and prognostic biomarkers for sepsis. MiR-1246 plays a crucial role in lipopolysaccharide (LPS)-exposed pulmonary microvascular endothelial cell apoptosis by targeting angiotensin-converting enzyme 2 (ACE2). Depletion of miR-1246 restored the pulmonary expression of ACE2 and attenuated LPS-induced lung inflammation, neutrophil infiltration, and vascular permeability[8]. MiR-147b mediates LPS-induced endothelial barrier dysfunction during inflammation and sepsis by targeting A-disintegrin and metalloproteinase 15 (ADAM15)[9]. The aberrant expression of miR-155 has been found to be involved in LPS-induced septic cardiac dysfunction and apoptosis in mice[10]. Exos mediates protection against LPS-induced acute lung injury by down-regulating Sprouty-related EVH1 domain-containing protein 1 (SPRED1) and upregulating the

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RAF/ERK signaling pathway[11]. MiR-29b mediates LPS-induced endothelial cell inflammation and apoptosis *via* regulation of the NF-kB and JNK signaling pathways[12].

MiR-34a-5p acts as a tumor suppressor in a variety of tumors and plays a significant role in cell proliferation, apoptosis, epithelial mesenchymal transition, and metasis[13,14]. MiR-34a-5p knockdown attenuates intestinal ischemia/reperfusion-induced (I/R-I) accumulation of reactive oxygen species (ROS) and apoptosis *via* targeting the sirtuin (SIRT1)-mediated antioxidant pathway[15]. Plasma miR-34a-5p can distinguish Alzheimer's disease (AD) from Parkinson's disease and can therefore serve as a potential biomarker in the early identification of AD[16]. However, the role of miR-34a-5p in endothelial injury during sepsis remains to be investigated.

Forkhead box M1 (*FOXM1*), a winged helix/forkhead class of transcription factor, belongs to a large FOX family that includes more than 55 members, and is primarily associated with G1 to S phase transition and controls G2 to M phase transition in embryonic and fetal development[17]. It has been shown that endothelial cell-restricted *FOXM1* mice (*FOXM1* CKO mice) are viable with no aberrant phenotypes, whereas under LPS-induced vascular injury, *FOXM1* CKO mice exhibit a significant protracted increase in lung vascular permeability and a dramatic increase in mortality[18]. Moreover, *FOXM1* was demonstrated to play a crucial role in the regulation of endothelial regeneration and barrier repair following lung injury induced by polymicrobial sepsis in mice[19]. However, the underlying mechanistic link between the different factors present upstream and downstream of *FOXM1* for regulating EC recovery remain unknown.

In the present investigation, we used miR-34a-5p to study its effect on the expression of inflammatory cytokines in LPS-induced HUVECs injury and apoptosis. MiR-34a-5p expression was significantly enhanced in LPS-induced HUVECs and inhibition of miR-34a-5p alleviated LPS-induced HUVEC injury. Furthermore, we illustrated that miR-34a-5p targets *FOXM1* and inhibits its expression. Meanwhile, overexpression of *FOXM1* inhibits LPS-induced HUVEC injury. We found that the downstream target of *FOXM1* was the NRF2/HO-1 pathway. MiR-34a-5p mediated the LPS-induced inflammatory response and apoptosis in HUVECs by targeting the *FOXM1* and NRF2/HO-1 pathways. Thus, miR-34a-5p might act as a potential biomarker for patients with sepsis.

Materials and methods

Cell Cultivation and LPS Treatment

This study was approved by the Ethics Committee of Zhejiang Provincial Hospital. Written informed consent was obtained from all the concerned authorities prior to conducting the study. The cell line of HUVECs was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and were cultured in DMEM low-glucose medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Rockville, MD, USA) using standard cell culture conditions (37°C, 5% CO_2). A series of gradient dilutions of LPS (0, 2.5, 5, and 10 µg/ml) were used to induce HUVEC inflammation injury by treating them at different time points: 0, 3, 6, 12, and 24 h.

Expression Vector Construction and Cell Transfection

MiR-34a-5p mimic, inhibitor, and scramble miRNAs (NC-miRNAs) were obtained from GenScript (Nanjing, China) and used to study the expression of miR-34a-5p in HUVECs. For overexpression of *FOXO1*, the corresponding cDNA for *FOXM1* was amplified and ligated into a pcDNA vector (Invitrogen, Carlsbad, CA, USA) to obtain pcDNA/FOXO1 (pcDNA-FOXO1) and the empty vector (pcDNA) was used as a control. Lipofectamine 3000 reagent (Life Technologies, Carlsbad, CA, USA) was utilized in line with the manufacturer's protocol. All the primers or relevant sequences are listed in **Supplementary Table I**.

Cell Viability Assay

HUVECs were inoculated in 96-well plates at a density of 2×10^3 cells/well for 24 h, and the medium was replaced with different concentrations of LPS (0, 2.5, 5, or 10 µg/ml) for culture (0, 3, 6, 12, 24 h). The cell number in each plate was adjusted to 5000 cells/well, then 20 µl/well of 5 mg/ml MTT was added following a 4-h incubation. Then, the supernatant was replaced with dimethyl sulfoxide (200 µl/well) following shaking for 5 min. The MTT formazan product was detected using a microplate

reader (Synergy 2; BioTek Instruments, Ltd., Winooski, VT, USA), and the absorbance was measured at 490 nm.

Cell Apoptosis Assay

Cell apoptotic quantification was performed using an annexin V–FITC/propidium iodide (PI) apoptosis detection kit (Sigma Aldrich, Shanghai, China). Cells were immediately analyzed with a flow cytometer (Beckman Coulter, Brea, CA, USA). Cells were adjusted to the concentration of 10^5 cells/well and cultured for 24 h. Then, PBS-washed cells were stained successively with PI and fluorescein isothiocyanate (FITC)-conjugated annexin V (FITC–annexin V) following the manufacturer's protocol. Finally, the apoptotic cells were sorted using a FACScan flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

ELISA

After the indicated treatments, the supernatant of HUVECs was collected from plates and the relative production of inflammation cytokines, including TNF-α, IL-1β, and IL-6 were measured by specific ELISA kits (R&D Systems, Abingdon, UK).

RNA Extraction and RT-qPCR Analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After treatment with RQ1 RNase-free DNase I (Promega, Madison, WI, USA), first-strand cDNA synthesis was carried out using SuperScript II Reverse Transcriptase (Toyobo Ltd., Osaka, Japan). To determine the miR-34a-5p level, a TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II (Thermo Fisher Scientific, Waltham, MA, USA) were applied according to manufacturer's instructions. The relative expression was examined using the $2^{-\Delta\Delta Ct}$ cycle threshold method. U6 was used as an internal control for miR-34a-5p expression, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control for *FOXM1*. The products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining under UV light. The primer sequences used for qRT-PCR are listed in **Supplementary Table I**. All experiments were repeated in triplicate and three technical repetitions were performed.

Dual-Luciferase Reporter Assay

The FOXO1-WT and FOXO1-MT were obtained by cloning a fragment of FOXO1 3′UTR with the putative miR-135a-5p binding sites (FOXO1-WT) or the mutated sites (FOXO1-MT) into the pGL3 vector (Promega, Madison, WI, USA). Then, HUVECs were transfected with the aforementioned vectors together with miR-34a-5p mimics or NC-miRNA. Forty-eight hours after the transfection, the Luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western Blot

Cells were lysed with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) in the presence of protease inhibitor (Roche, Guangzhou, China). Protein concentration was measured by BCA™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and separated by 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were blocked in non-fat milk and incubated at 4°C overnight with primary anti-rabbit FOXM1 (1:1000 dilution, ab180710, Abcam, Cambridge, CA, USA), NRF2 (1:1000 dilution, ab62352, Abcam, Cambridge, CA, USA), HO-1 (1:2000 dilution, ab68477, Abcam, Cambridge, CA, USA), or GAPDH (1:5000 dilution, Ab8245, Abcam, Cambridge, CA, USA) antibodies, which were all purchased from Abcam Company (Cambridge, MA, USA). After rinsing with Tris-buffered saline containing Tween 20, the membranes were incubated with secondary anti-rabbit antibody (1:5000 dilution, A0208, Beyotime, Shanghai, China) at room temperature for 1 h. Finally, the blots were observed using enhanced chemiluminescence solution (Thermo Fisher Scientific, Waltham, MA, USA) and the signals were analyzed by Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

Tubule Formation Assay

In vitro neovascularization assays were performed to analyze tube formation in human fibrin matrices as described previously[20]. In brief, after 24 h of LPS treatment, HUVECs were seeded onto Matrigel-coated plates (BD Bioscience, Franklin Lakes, NJ, USA) in EBM medium and incubated at 37°C for 24 h. The tubular structures of the HUVECs in the Matrigel were examined by phase-contrast microscopy. To quantify the length of newly formed tubes, three random phase-contrast photomicrographs per well were

taken, and the length of each tube was measured using Adobe Photoshop software. Tube length obtained from control cells was set to 100.

Statistical Analysis

Data were displayed as mean ± SD. The statistical analyses were carried out using GraphPad 6.0 software. Comparison between the two groups were made by Student's *t*-test. *p*-values < 0.05 were considered statistically significance. Three independent biological repeats were used for each analysis.

Results

LPS-Induced Upregulation of MiR-34a-5p in HUVECs

To determine the optimal concentration of LPS responsible for EC injury, HUVECs were incubated with various concentrations of LPS for 24 h. Cell viability was examined using MTT assay. HUVECs treated with LPS (10 μg/ml) showed a significant reduction in cell viability compared with controls (Figure 1A). We also explored the optimal treatment time (3, 6, 12, 24 h) of 10 μg/ml LPS. LPS treatment for 24 h significantly reduced cell viability as compared to 0 h (Figure 1B). For further analysis we selected 10 μg/ml LPS treatment for 24 h.

To detect the miRNAs that are aberrantly expressed in HUVECs following 10 μg/ml LPS treatment for 24 h, miRNA microarray analysis was carried out. Remarkably, when HUVECs were subjected to 10 μg/ml LPS treatment for 24 h, miR-34a-5p expression was significantly increased (Figure 1C). Additionally, the qRT-PCR analysis showed similar results (Figure 1D), suggesting that miR-34a-5p may play a pivotal role in LPS-induced EC injury.

MiR-34a-5p Inhibition Reduces LPS-Induced HUVEC Injury

To determine the role of miR-34a-5p in LPS-induced HUVEC injury, miR-34a-5p inhibitor and NC-miRNA (negative control) were obtained and transfected into HUVECs. After confirming the inhibitory effect of miR-34a-5p inhibitor on the expression of miR-34a-5p in HUVECs (Figure 2A), we determined the effect of miR-34a-5p on cell viability (Figure 2B). LPS treatment mark-

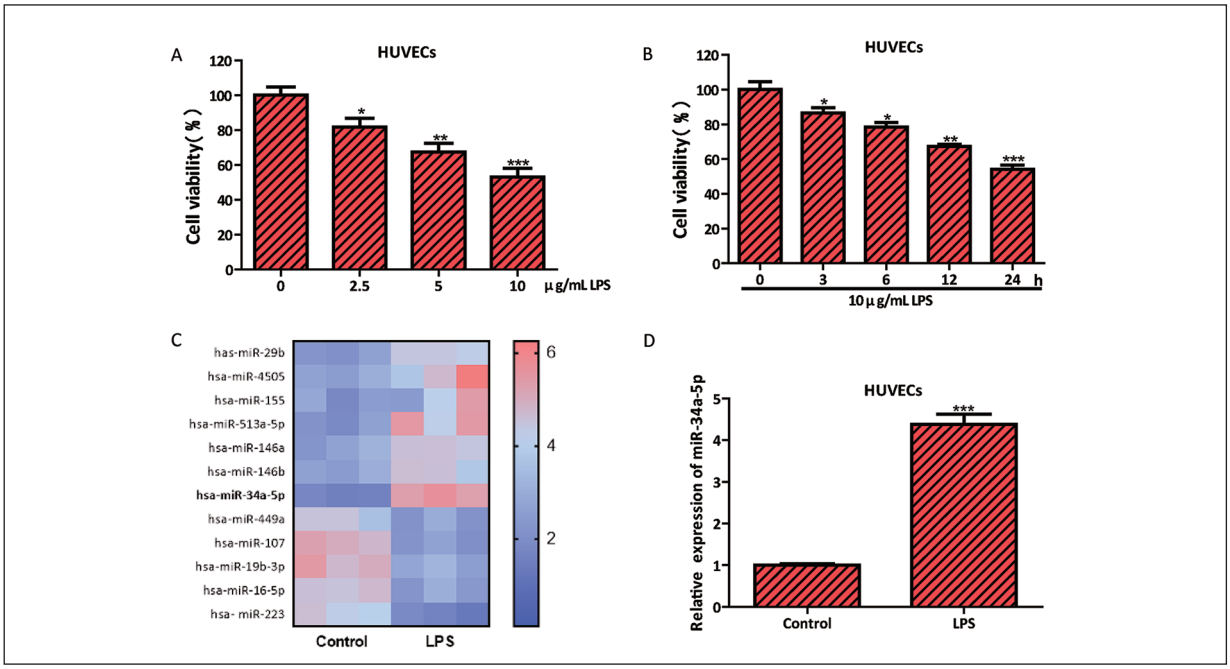

Figure 1. LPS treatment increased miR-34a-5p expression in HUVECs. **A,** Cell viability measurement of HUVECs treated with 0, 2.5, 5, or 10 μg/ml LPS for 24 h using MTT assay. **B,** Cell viability measurement of HUVECs treated for 3, 6, 12, and 24 h with 10 μg/ml LPS using MTT assay. **C,** MiRNA expression profile analysis in HUVECs treated with or without 10 μg/ml LPS for 24 h using microarray. **D,** MiR-34a-5p expression detection in HUVECs treated with or without 10 μg/ml LPS for 24 h using qRT-PCR. Transcript levels were normalized against U6 expression. All experiments were performed in triplicate. Data are represented as means ± SD. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

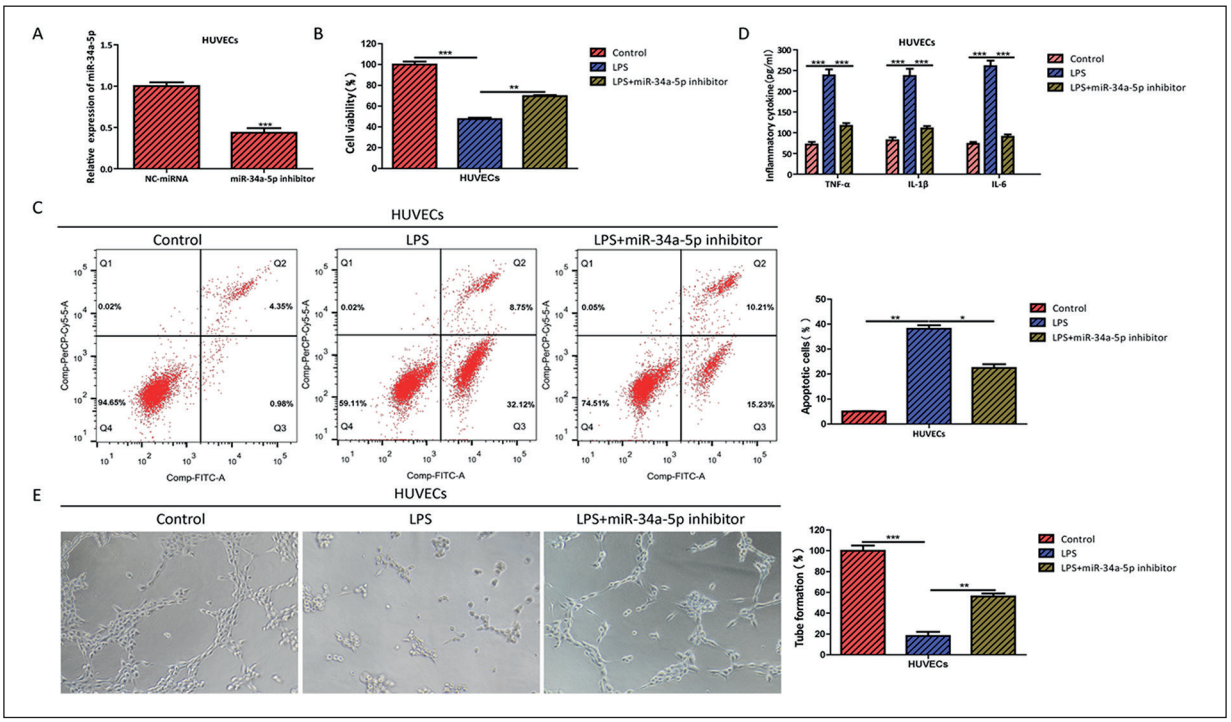

Figure 2. Effect of miR-34a-5p on LPS-induced HUVEC injury. **A,** qRT-PCR analysis of miR-34a-5p expression in HUVECs transfected with miR-34a-5p inhibitor or NC-miRNA. Transcript levels were normalized against U6 expression. **B-E,** Cell viability (**B**), apoptosis rate (**C**), inflammatory factors (TNF-α, IL-1β, and IL-6) expression (**D**), as well as the vasculogenic activity (**E**) detection following exposure to 10 μg/ml LPS for 24 h with or without pre-transfection of miR-34a-5p inhibitor for 48 h using MTT, FITC staining flow cytometry assay, ELISA, and Matrigel-based tube formation assays (20×), respectively. Three biological repeats. Data are represented as means ± SD. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

edly inhibited HUVEC viability, but pre-transfection of HUVECs by miR-34a-5p inhibitor potentially reversed the inhibiting effect of cell viability by LPS.

We also examined the functional role of miR-34a-5p in LPS-induced apoptosis and inflammatory responses in HUVECs. LPS treatment significantly enhanced the percentage of apoptotic cells in HUVECs as compared with the control, indicating that cell viability was severely affected; however, pre-transfection with miR-34a-5p inhibitor led to ~45% inhibition of LPS-induced apoptosis (Figure 2C). In addition, miR-34a-5p inhibitor could significantly reduce the production of inflammatory cytokines (TNF-α, IL-1β, and IL-6) induced by LPS (Figure 2D). To further explore the regulatory effects of miR-34a-5p on HUVECs, a Matrigel-based tube formation assay was carried out to analyze the effect of miR-34a-5p on the vasculogenic activity of HUVECs (Figure 2E). The tube formation capability of HUVECs was significantly decreased after exposure to LPS, however, downregulation of miR-34a-5p expression by transfecting miR-34a-5p inhibitor profoundly reversed the inhibition effect of LPS on EC tube formation. Altogether, our results confirm that miR-34a-5p is involved in LPS-triggered HUVEC injury.

FOXM1 Is a Direct Target of MiR-34a-5p in LPS-Induced HUVECs

To gain further insight into the underlying mechanistic link between different factors of miR-34a-5p and LPS-induced HUVEC injury, we used bioinformatics to search for the potential target of miR-34a-5p using the algorithm provided on microrna.org (http://www.microrna.org). The algorithm predicted that the transcriptional factor FOXM1, which plays a pivotal role in EC injury recovery[21], was a candidate target of miR-34a-5p (Figure 3A). We performed a Dual-Luciferase reporter assay to identify whether the 3′-UTR of FOXM1 was targeted by miR-34a-5p. The 3′-UTR that included the predicted miR-34a-5p recognition site (FOXM1-WT) or the mutated sequences (FOXM1-MT) was inserted into the

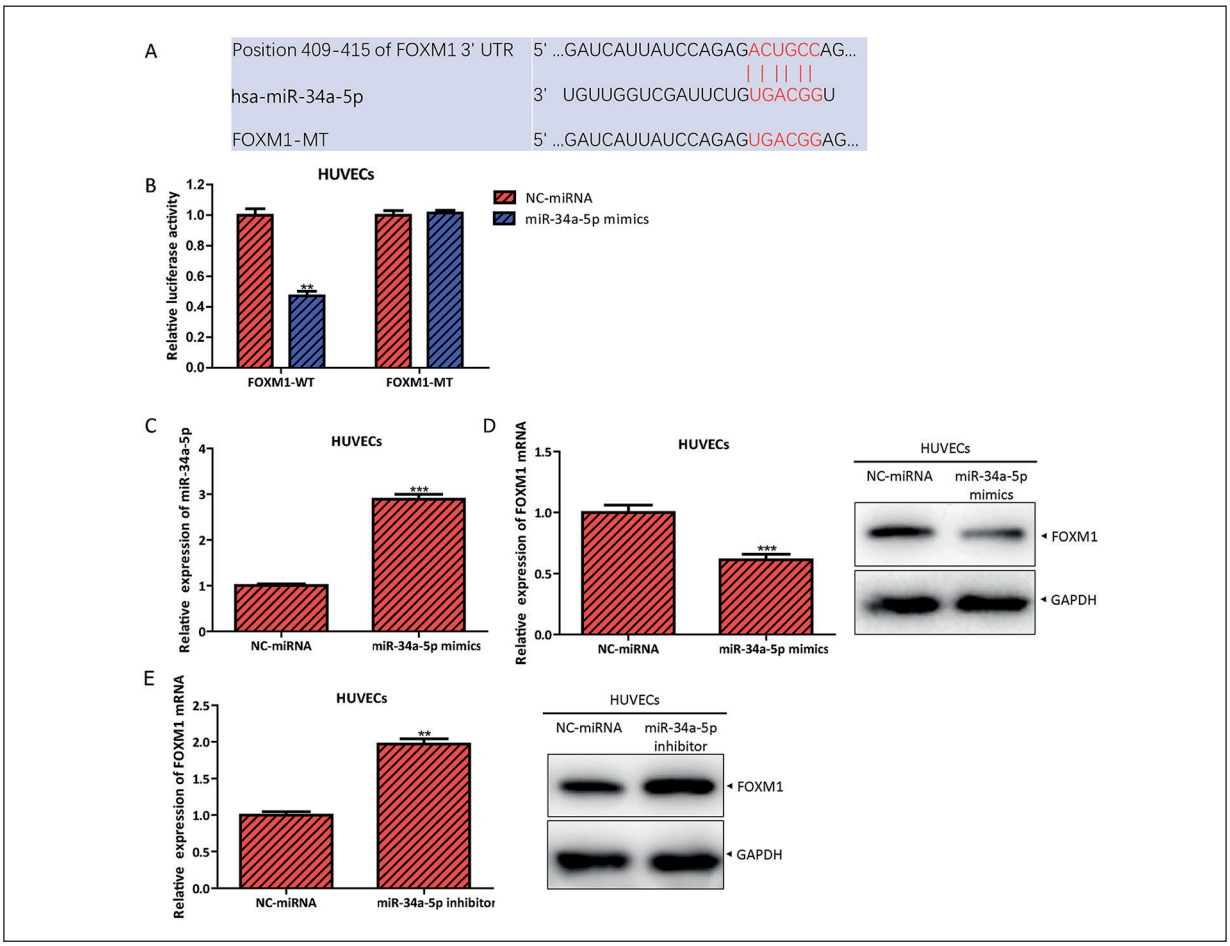

Figure 3. MiR-34a-5p directly targets the 3′UTR of FOXM1 and inhibits its expression in HUVECs. **A,** Predicted binding sites of miR-34a-5p in the 3′UTR of FOXM1 mRNA. **B,** Luciferase assays of HUVECs transfected with pGL3-FOXM1 3′UTR WT or MT reporter together with NC-miRNA or miR-34a-5p mimics. **C,** A qRT-PCR analysis of miR-34a-5p expression in HUVECs transfected with miR-34a-5p mimics or NC-miRNA. Transcript levels were normalized against U6 expression. **D, E,** mRNA and protein level detection of *FOXM1* in HUVECs transfected with miR-34a-5p mimics (**D**) or miR-34a-5p inhibitor (**E**) using qRT-PCR and Western blot. U6 expression and GAPDH accumulation were used as the internal control. Three independent biological repeats were considered. Data are represented as mean ± SD. (**$p < 0.01$, ***$p < 0.001$).

pGL3 vector, downstream of the luciferase open reading frame. The pGL3 vector with FOXM1-WT resulted in a significant decrease in luciferase activity after co-transfection with miR-34a-5p as compared with that of NC-miRNA, which was used as the negative control (Figure 3B). Subsequently, we further examined the effect of miR-34a-5p on *FOXM1* expression in HUVECs. qRT-PCR and Western blot was used to detect the mRNA and protein levels of *FOXM1* in HUVECs transfected with miR-34a-5p mimics or inhibitor. MiR-34a-5p expression was successfully promoted by miR-34a-5p mimic transfection (Figure 3C), and the overexpression of miR-34a-5p drastically inhibited the *FOXM1* mRNA and protein accumulation as compared to the miRNA-NC group (Figure 3D). As expected, the knockdown of miR-34a-5p expression with miR-34a-5p inhibitor in HUVECs remarkably enhanced the expression of *FOXM1* mRNA and protein levels (Figure 3E). These results indicate that FOXM1 is a direct target of miR-34a-5p in HUVECs.

MiR-34a-5p Inhibition Attenuates LPS-Induced HUVEC Injury by Targeting FOXM1

FOXM1 plays a vital role in the restoration of endothelial LPS-induced vascular injury[18]. Our results indicated that miR-34a-5p inhibition effectively alleviated LPS-induced HUVEC injury and that miR-34a-5p could target FOXM1 and inhibit its expression; therefore, we postulated

that miR-34a-5p might regulate LPS-induced HUVEC injury by suppressing the expression of *FOXM1*. The full-length cDNA of *FOXM1* was amplified and ligated in pcDNA (FOXM1-pcDNA) and transfected into HUVECs, with the empty vector acting as a control (pcDNA), to overexpress *FOXM1*. Both the mRNA and protein levels were significantly promoted in these cells (Figure 4A, B). Subsequently, we examined the cell viability and found that the inhibitory effect of LPS on HUVEC viability was significantly reduced by FOXM1-pcDNA transfection, but not in the empty vector (Figure 4C). We further validated the functional role of *FOXM1* in LPS-triggered apoptosis and inflammatory responses in HUVECs. As shown in Figure 4D, FOXM1-pcDNA pre-transfection in HUVECs significantly inhibited LPS-induced cell apoptosis. Additionally, *FOXM1* overexpression remarkably reduced the production of inflammatory molecules (TNF-α, IL-1β, and IL-6) (Figure 4E). Meanwhile, the tube formation assay showed that *FOXM1* could promote the vasculogenic activity of HUVECs (Figure 4F). These results illustrated that *FOXM1* plays a crucial role in LPS-induced EC injury. As expected, concomitant transfection of FOXM1-pcDNA and miR-34a-5p mimics significantly reduced cell viability, enhanced pro-inflammatory cytokine (TNF-α, IL-1β, and IL-6) expression and apoptotic rate, and reduced the tube formation capability of HUVECs as compared to FOXM1-pcDNA alone (Figure 4D-

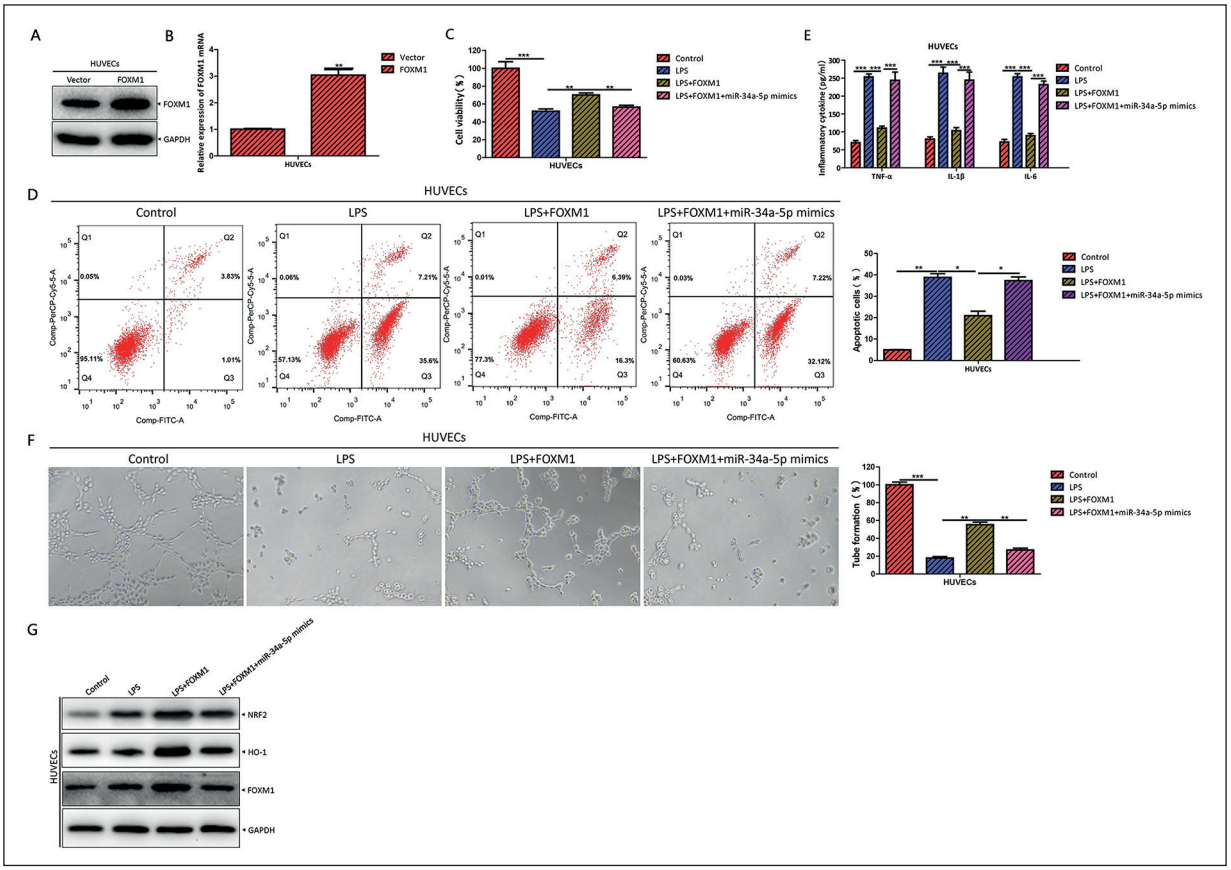

Figure 4. MiR-34a-5p overexpression reversed the effect of FOXM1 in LPS-induced HUVEC injury. **A, B,** protein (**A**) and mRNA (**B**) level analysis of *FOXM1* in HUVECs transfected with pcDNA-FOXM1 or the empty vector (pcDNA) using qRT-PCR and Western blot. U6 expression and GAPDH accumulation were used as the internal control, respectively. **C-F,** Cell viability (**C**), apoptosis rate (**D**), inflammatory factors (TNF-α, IL-1β, and IL-6) accumulation (**E**), vasculogenic activity (**F**) detection following exposure to 10 μg/ml LPS for 24 h with or without pre-transfection of pcDNA-FOXM1 or pcDNA-FOXM1 together with miR-34a-5p mimics for 48 h using MTT, FITC staining flow cytometry assay, ELISA, and Matrigel-based tube formation assays (20×), respectively. **G,** Protein level detection of NRF2, HO-1, and FOXM1 in HUVECs following exposure to 10 μg/ml LPS for 24 h with or without pre-transfection of pcDNA-FOXM1 or pcDNA-FOXM1 together with miR-34a-5p mimics for 48 h; NADPH was used as internal control for protein measurements. All experiments were performed at least three times. Data are represented as mean ± SD. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

F), suggesting that FOXM1-pcDNA-regulated LPS-induced HUVEC injury could be partially reversed by miR-34a-5p mimics. Taken together, our results indicate that miR-34a-5p promotes LPS-induced HUVEC injury by inhibiting *FOXM1*.

FOXM1 has several downstream targets[17]. NRF2 and HO-1 play crucial roles in protecting ECs from injury by eliminating ROS. We noticed enhanced protein accumulation of NRF2 and HO-1 in LPS-treated HUVECs (Figure 4G), which is inconsistent with the previous reports[22]. Surprisingly, FOXM1-pcDNA transfection further increased NRF2 and HO-1 expression in HUVECs as compared with LPS treatment alone, suggesting that NRF2 and HO-1 might be the downstream targets of *FOXM1* in HUVECs. However, co-transfecting HUVECs with FOXM1-pcDNA and miR-34a-5p mimics significantly inhibited FOXM1-induced accumulation of NRF2 and HO-1, indicating that miR-34a-5p inhibition might protect against LPS-induced EC injury by targeting *FOXM1*, which in turn is mediated *via* the NRF2/HO-1 pathway.

Discussion

Endothelial dysfunction is deleterious and leads to tissue damage, multiple organ dysfunction, and is responsible for the high mortality rate seen in sepsis. Endothelial cells (ECs) stimulate the production of pro-inflammatory cytokines and chemokines, facilitating the expression of adhesion molecules, integrins, and immunoglobulin-like adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), thereby playing a crucial role in the inflammatory immune response during systemic infection[23]. These adhesion molecules play a key role in the activation of leukocyte/endothelial cells *via* out-in signaling, which in turn facilitates leukocyte rolling[24], adherence, and transmigration of different immune cells, including monocytes, neutrophils, or T cells[25]. However, the underlying mechanism of LPS-induced HUVEC injury in terms of miRNA remains elusive. The present investigation aimed to elucidate the possible role of miR-34a-5p in LPS-induced HUVEC injury.

LPS triggers the stimulation of EC, leading to the synthesis of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, which ultimately leads to the production of adhesion molecules[26]. ICAM-1 is the most thoroughly studied adhesion molecule and plays a key role in regulating the movement of leukocytes through the endothelium[27]. Dysregulation of *ICAM-1* expression is found to be functionally associated with the pathophysiology of several diseases, such as atherosclerosis and inflammatory disorders (e.g., asthma and autoimmune disorders)[28]. Controlling the production of inflammatory factors has been shown to reduce the production of ICAM-1, thereby reducing the occurrence of these diseases[29,30]. In this study, we found that miR-34a-5p can regulate LPS-induced pro-inflammatory cytokine production through *FOXM1*, therefore, miR-34a-5p may also be involved in the occurrence of these diseases by regulating the expression of *ICAM-1*.

MiRNAs play a pivotal role in physiological and pathological processes, such as cell proliferation, apoptosis, and differentiation[5,31,32]. HUVECs treated with LPS for 24 h exhibited a significant increase in the expression of miR-34a-5p, as shown by miRNA microarray and qRT-PCR, which subsequently led to cell viability inhibition, increased apoptosis, enhanced production of pro-inflammatory cytokines, and inhibition of the vasculogenic activity of HUVECs. The miR-34a-5p inhibitor used in the present study significantly attenuated the inflammatory activation of the LPS-induced HUVECs.

In recent years, several miRNAs have been shown to play a pivotal role in almost all types of vascular disease. MiR-145, miR-221/222, miR-21, and miR-31 are key regulators for vascular smooth muscle cell proliferation and neointimal hyperplasia[33-36], while miRNAs, such as miR-126a-3p, miR-124, and miR-146a play a critical roles in EC injury[37-39]. We demonstrated that miR-34a-5p directly interacted with the 3′UTR of *FOXM1* and suppressed its expression in HUVECs, because overexpression of miR-34a-5p significantly inhibited the mRNA of *FOXM1*, while the knockdown of miR-34a-5p profoundly enhanced the mRNA and protein accumulation of *FOXM1* in HUVECs. Further, we found that overexpression of miR-34a-5p attenuated the protective effect of *FOXM1* on cell viability, apoptosis, pro-inflammatory molecules production, and vasculogenic activity of HUVECs. Previously, it has been suggested[17] that *FOXM1* plays a crucial role in the repair of injured ECs. The mechanism by which *FOXM1* play a protective role in LPS-induced HUVEC injury requires further investigation.

Recently, it has been shown that *FOXM1* can be targeted and regulated by miRNAs in the progression of several cancers. MiRNA-370 mediates the progression of acute myeloid leukemia by targeting *FOXM1*[40]; miRNA-320 suppresses colorectal cancer by inhibiting the expression of *FOXM1*[41]; and miRNA-149 promotes gastric cancer cell sensitivity to cisplatin by directly targeting *FOXM1*[42]. In the present study, we found that miR-34a-5p could directly target *FOXM1* and mediated LPS-induced HUVECs injury. *FOXM1* modulates crucial cell cycle processes, such as G1-S and G2-M transitions, and thus cell cycle progression and proliferation *via* regulating transcription factors, such as *Skp2* and *Cks1*, which are components of the Skp1/cullin1/F-box (SCF) ubiquitin ligase complex[17].

The downstream components of *FOXM1* in the LPS-induced ECs injury process were further explored. We found that the NRF2/HO-1 pathway, which plays a crucial role in protecting ECs from injury by eliminating ROS[22], was involved in miR-34a-5p-inhibited *FOXM1* regulation of LPS-induced HUVECs, because the accumulation of NRF2 and HO-1, core components of the NRF2/HO-1 pathway, was promoted by *FOXM1*, suggesting that the NRF2/HO-1 pathway might be the downstream component of *FOXM1* in LPS-induced EC injury.

Conclusions

Our research investigated the potential role of miR-34a-5p in LPS-induced HUVEC injury. It was found that miR-34a-5p was upregulated in LPS-treated HUVECs, and inhibiting its expression attenuated LPS-induced HUVEC injury by targeting *FOXM1*. Further analysis revealed that the NRF2/HO-1 pathway was activated by *FOXM1* and might be involved in the LPS-induced EC injury process. MiR-34a-5p might act as a potential diagnostic and therapeutic biomarker for the treatment of sepsis-induced EC injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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