miR-374a-5p alleviates sepsis-induced acute lung injury by targeting ZEB1 via the p38 MAPK pathway

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Abstract. The aim of the present study was to investigate the effects of microRNA (miR)-374a-5p on sepsis-induced acute lung injury (ALI) and the associated mechanism. Lipopolysaccharide (LPS)-induced human pulmonary microvascular endothelial cells (HPMVECs) were used to construct the cellular model of sepsis. A luciferase reporter assay was performed to confirm the association between miR-374a-5p and zinc finger E-box binding homeobox 1 (ZEB1). Reverse transcription-quantitative polymerase chain reaction and western blot analysis were performed to assess the relative expression of miR-374a-5p, ZEB1 and apoptosis-related proteins. Cell viability and apoptosis were determined by Cell Counting Kit-8 assay and flow cytometry, respectively. Enzyme-linked immunosorbent assays were used to evaluate inflammatory cytokines. The results revealed that miR-374a-5p was downregulated in sepsis patients and LPS-treated HPMVECs. Upregulation of miR-374a-5p alleviated LPS-triggered cell injury in HPMVECs, as evidenced by restoration of cell viability, and inhibition of apoptosis and the production of proinflammatory cytokines. In addition, ZEB1 was revealed to be a downstream target of miR-374a-5p, and overexpression of ZEB1 could reverse the anti-apoptotic and anti-inflammatory effects of miR-374a-5p on an LPS-induced sepsis cell model. Moreover, miR-374a-5p-induced protective effects involved the p38 MAPK signaling pathway. Collectively, miR-374a-5p exerted a protective role in sepsis-induced ALI by regulating the ZEB1-mediated p38 MAPK signaling pathway, providing a potential target for the diagnosis and treatment of sepsis.

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Introduction

Sepsis is a systemic inflammatory response syndrome (SIRS) caused by infection, which could develop into septic shock and multiple organ dysfunction syndrome (MODS), and has become one of the leading causes of death in critically ill patients, with a mortality rate of 25 to 40% (1,2). Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is one of the most common complications of severe sepsis, involving an inflammatory process (3,4). Therefore, it is of great significance to explore the biomarkers for the early diagnosis, treatment and prognosis evaluation of sepsis-induced ALI. Pulmonary endothelial cell injury plays an important role in the dysfunction of the alveolar-capillary barrier, which is a characteristic feature of ALI (5,6). Lipopolysaccharide (LPS), the bacterial endotoxin, is a primary pathogenic mediator of pulmonary endothelial cell activation, which triggers ALI, involving multiple inflammatory cells, and cytokines (7,8). Alleviating the injury of pulmonary endothelial cells is an important method to prevent sepsis-induced ALI.

MicroRNAs (miRNAs or miRs) are a class of small non-coding RNAs that have been discovered in recent years, which can specifically recognize the 3'-UTR sites of target mRNAs to inhibit protein translation or induce mRNA degradation, and thus regulate gene expression at the post-transcriptional levels (9). MiRNAs play crucial roles in the regulation of multiple physiological and pathological processes such as cell proliferation and apoptosis, differentiation, and inflammatory response (10,11). Previous research has revealed that several miRNAs are implicated in the development of sepsis-induced ALI, such as miR-155 and miR-146a (12). MiR-374a has been demonstrated to inhibit the progression of lung adenocarcinoma and non-small cell lung carcinoma (13-15). MiR-374a was necessary for the inhibition of intestinal inflammation and oxidative injury in rats with colitis (16). MiR-374a-5p was critical for the protective effects on neonatal hypoxic-ischemic encephalopathy (HIE) by inhibiting LPS-induced microglial neuroinflammation (17). A previous study indicated that miR-374a alleviated LPS-induced hyperpermeability of human pulmonary artery endothelial cells (18). However, it remains unclear whether miR-374a-5p is engaged in sepsis-induced ALI.

Zinc finger E-box binding homeobox 1 (ZEB1) is a transcription factor which has critical roles in epithelial-mesenchymal transition (EMT), senescence, and angiogenesis (19-21). It has been reported that ZEB1 induces inflammatory response in periodontal disease and liver fibrosis (22,23). In addition, the p38 MAPK signaling pathway is associated with septic lung injury (24-26). A previous study revealed that the p38 MAPK pathway could be restricted by miR-200s by targeting ZEB1/2 in LPS-induced pulmonary fibrosis (27).

In the present study, human pulmonary microvascular endothelial cells (HPMVECs) induced by LPS were used to investigate whether miR-374a-5p exerted a protective role in sepsis-induced ALI through the p38 MAPK signaling pathway by targeting ZEB1.

Materials and methods

Patients and healthy subjects. In total, 25 patients with sepsis (49.5±10.3 years old; 16 males and 9 females) following intensive care unit (ICU) admission at General Hospital of Ningxia Medical University (Yinchuan, China) from May 2017 to August 2018 were recruited in the present study, according to diagnostic criteria proposed by The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) (28). Exclusion criteria were as follows: i) Patients younger than 18 years old; ii) patients with chronic liver and renal insufficiency; iii) patients with malignant tumors; iv) patients with haematological diseases; v) patients with compromised immune systems; vi) pregnant and lactating patients; and vii) patients with acute cardiovascular and cerebrovascular diseases. In addition, 25 age- and sex-matched healthy individuals (50.6±9.3 years old; 18 males and 7 females) with no history of pulmonary infection or malignancies were enrolled as the controls. Written informed consent was provided from all participants, and the present study was approved by the Ethics Committee of General Hospital of Ningxia Medical University (approval no. IRB2017-GHNMU-38). Clinical characteristics of the study subjects including sex, age, body mass index (BMI), acute physiology and chronic health evaluation (APACHE) II score, sequential organ failure assessment (SOFA) score, C-reactive protein (CRP), procalcitonin (PTC), and blood gas analysis (lactic acid, and PaO_2/FiO_2) are presented in Table I.

Following admission, blood samples (5 ml) were obtained on an empty stomach, and centrifuged at 3,000 x g for 10 min at 4˚C to completely remove cell debris. The collected serum was transferred to RNase/DNase-free sterile tubes and stored at -80˚C until further processing.

Cell culture and LPS treatment. HPMVECs, purchased from American Type Culture Collection (ATCC no. PCS-100-022) were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies; Thermo Fisher Scientific, Inc.) plus 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and 100 μg/ml penicillin/streptomycin (Life Technologies; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. For the cellular model of sepsis, HPMVECs were incubated with 1 μg/ml LPS (Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C. The ethics approval for the use of primary cell lines was received.

Cell transfection. HPMVECs ($1x10^4$ cells/ml) cultured in 96-well plates were transfected with 50 nM miR-374a-5p mimics, miRNA negative control (miR-NC), 4 μg pcDNA3.1 containing the open reading frame of ZEB1 or pcDNA3.1 empty vector (Vector), chemically synthesized by Shanghai GenePharma Co., Ltd. with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C, according to manufacturer's protocol. At 24 h after transfection, HPMVECs were stimulated with 1 μg/ml LPS for 24 h. Sequences of the oligonucleotides are shown in Table II.

Luciferase reporter assay. The binding site between ZEB1 and miR-374a-5p was predicted using StarBase v2.0 (https://starbase.sysu.edu.cn/). The 3'-UTR fragments of ZEB1 containing the predicted wild-type (ZEB1-wt) or corresponding mutated (ZEB1-mut) miR-374a-5p binding sites were amplified by PCR and sub-cloned into a pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega Corporation). When cells reached ~80% confluence, these luciferase reporter plasmids were co-transfected with either miR-374a-5p mimic or miR-NC into HPMVECs using Lipofectamine 2000. After 48 h of transfection, the cells were subjected to further luciferase assays using a Bright-Glo™ LuciferaseAssay System (Promega Corporation). The luciferase activity was normalized to *Renilla* luciferase activity values.

Reverse transfection-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from serum and cultured cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RT was conducted using a Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) for miRNA or a Prime-Script™ One Step RT-qPCR kit (Takara Bio, Inc.) for mRNA strictly according to the manufacturers' instructions. Real-time PCR was conducted using SYBR Premix ExTag™ (Takara Bio, Inc.) on a 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: 95˚C for 10 min, followed by 40 cycles at 94˚C for 45 sec, 55˚C for 30 sec and 72˚C for 1 min. Relative expression levels of miR-374a-5p and ZEB1 were calculated using the $2^{-\Delta\Delta Cq}$ method using U6 snRNA or GAPDH as internal controls (29). The primers used were as follows: miR-374a-5p forward, 5'-GCGCGCTTATAATACAACCTGA-3' and reverse, 5'-AGAGCAGGGTCCGAGGT-3' (universal); ZEB1 forward, 5'-GATGATGAATGCGAGTCAGATGC-3' and reverse, 5'-ACAGCAGTGTCTTGTTGTTGT-3'; U6 forward, 5'-CAGCACATATACTAAAATTGGAACG-3' and reverse, 5'-ACGAATTTGCGTGTCATCC-3'; GAPDH forward, 5'-TGTGGGCATCAATGGATTTGG-3' and reverse, 5'-ACACCATGTATTCCGGGTCAAT-3'.

Cell Counting Kit-8 (CCK-8) assay. A CCK-8 Kit was purchased from Dojindo Laboratories, Inc. to detect the cell viability according to the manufacturer's instructions. In detail, LPS-treated or transfected HPMVECs were seeded in a 96-well plate containing $5x10^3$ cells/well and cultured for 24 h at 37˚C. CCK-8 reagent was added to each well and cells were further incubated at 37˚C for 4 h. The absorbance at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.) was

Table I. Demographic and clinical characteristics of patients with sepsis and healthy controls.

Characteristic	Sepsis (n=25)	Healthy (n=25)	P-value
Sex, male/female	16/9	18/7	0.544
Age, years	49.5±10.3	50.6±9.3	0.694
BMI, kg/m^2	20.5±1.9	20.5±1.8	0.999
APACHE II score	15.9±3.2	-	-
SOFA score	6.1±1.3	-	-
CRP, mg/l	96.7±20.3	6.3±2.6	<0.001
PCT, ng/ml	12.2±5.9	0.7±0.1	<0.001
Lactic acid, mmol/l	2. 8±1.8	1.0±0.4	<0.001
PaO_2/FiO_2, mmHg	187.7±38.1	378.3±43.6	<0.001

Data are presented as n or the mean ± SD. BMI, body mass index; APACHE, acute physiology and chronic health evaluation; SOFA, sequential organ failure assessment; CRP, C-reactive protein; PCT, procalcitonin.

Table II. Sequences of the oligonucleotides.

Oligonucleotides	Sequences (5'-3')
miR-374a-5p	UUAUAAUACAACCUGAUAAGUG
mimics	CUUAUCAGGUUGUAUUAUAAUU
miR-NC	UUCUCCGAACGUGUCACGUTT
	ACGUGACACGUUCGGAGAATT

miR-374a-5p, microRNA-374a-5p; miR-NC, miRNA negative control.

measured to assess the viability of cells. Each experiment was repeated in triplicate.

Apoptosis detection using flow cytometry. The apoptosis of HPMVECs were determined by flow cytometry using an Annexin V-FITC/PI detection kit (Sigma-Aldrich; Merck KGaA) in accordance with a previous study (30). Briefly, cells were washed with PBS, re-suspended in binding buffer and stained with Annexin V/FITC and PI solution for 30 min. The apoptotic cells were detected with a flow cytometer (Beckman Coulter, Inc.) and Expo32 v1.2 software (Beckman Coulter, Inc.).

Western blot analysis. Following lysis in RIPA buffer (Santa Cruz Biotechnology, Inc.) and centrifugation at 12,000 x g for 20 min at 4˚C, proteins were extracted from serum or cells. Bradford method was used to detect the concentration of the proteins using a Bradford assay kit (Thermo Fisher Scientific, Inc.). Sodium dodecyl sulfate polyacrylamide gel electrophoresis on 7.5-10% gels was used to separate the proteins with equal amounts. The proteins (30 μg) were then transferred onto PVDF membranes (MilliporeSigma). Samples were blocked with 5% non-fat milk at room temperature for 1 h, and incubated with the primary antibodies against Bax (product no. 5023), Bcl-2 (product no. 3498), ZEB1 (product no. 3396), phosphorylated (p)-p38 (product no. 4511), p38 (product no. 8690), p-JNK (product no. 4668), JNK (product no. 9252), p-ERK (product no. 4370), ERK (product no. 4695) and GAPDH (product no. 5174) (all 1:1,000 dilution; Cell Signaling Technology, Inc.) overnight at 4˚C, and then the membrane was incubated with the secondary antibodies (anti-rabbit IgG, HRP-conjugated; product no. 7074; 1:2,000 dilution; Cell Signaling Technology, Inc.) at room temperature for 2 h. GAPDH was used as the internal control. A Super Signal West Pico Chemiluminescent Substrate kit (Pierce; Thermo Fisher Scientific, Inc.) was then used to scan the films according to the manufacturer's protocol. Image-Pro Plus software version 6.0 (Media Cybernetics, Inc.) was used to analyze the relative protein expression.

Cytokine assessment. Enzyme-linked immunosorbent assay (ELISA) method was performed to determine the concentration of interleukin (IL)-6 (cat. no. RAB0313), IL-1β (cat. no. RAB0273), and tumor necrosis factor-α (TNF-α) (cat. no. RAB1089) in cell culture supernatant using commercial ELISA kits (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions.

Statistical analysis. All analyses were performed using SPSS version 22.0 (IBM Corp.). Two-sided unpaired Student's *t*-test or analysis of variance (ANOVA) with Tukey's post hoc test was used to compare differences between groups. All results were presented as the mean ± standard deviation (SD) and a P-value <0.05 was considered to indicate a statistically significant difference. Pearson's correlation analysis was employed to analyze the association between serum miR-374a-5p and ZEB1 mRNA levels.

Results

MiR-374a-5p is downregulated in patients with sepsis and LPS-treated HPMVECs. The serum expression levels of miR-374a-5p in patients with sepsis and healthy controls were analyzed by RT-qPCR. A low expression of serum miR-374a-5p was revealed in septic patients compared with healthy controls (Fig. 1A). The expression level of miR-374a-5p was further detected in sepsis-induced ALI *in vitro* using LPS-treated HPMVECs. As revealed in Fig. 1B, miR-374a-5p

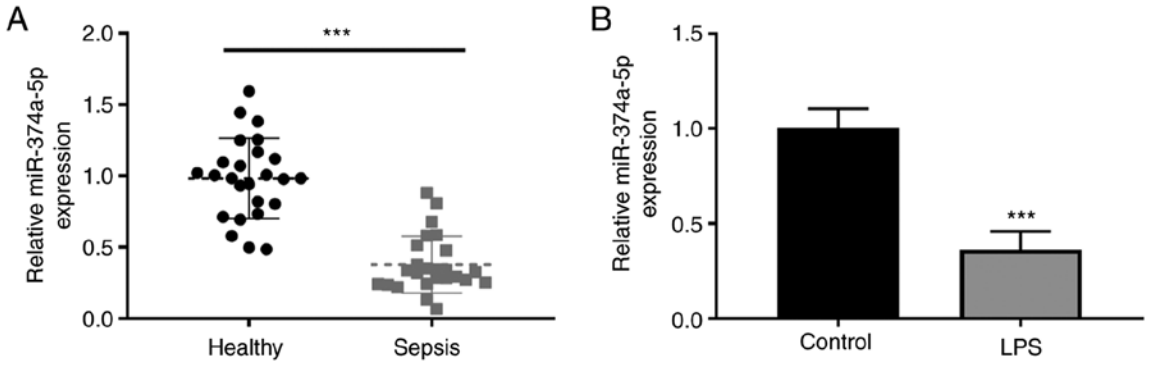

Figure 1. MiR-374a-5p is downregulated in patients with sepsis and LPS-treated HPMVECs. (A) The serum expression of miR-374a-5p in patients with sepsis and healthy controls was assessed by RT-qPCR. ***P<0.001. (B) RT-qPCR was used to detect the expression of miR-374a-5p in HPMVECs after 1 μg/ml LPS treatment for 24 h. ***P<0.001 vs. control. Results represent the means ± SD of 3 independent experiments. MiR-374a-5p, microRNA-374a-5p; LPS, lipopolysaccharide; HPMVECs, human pulmonary microvascular endothelial cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

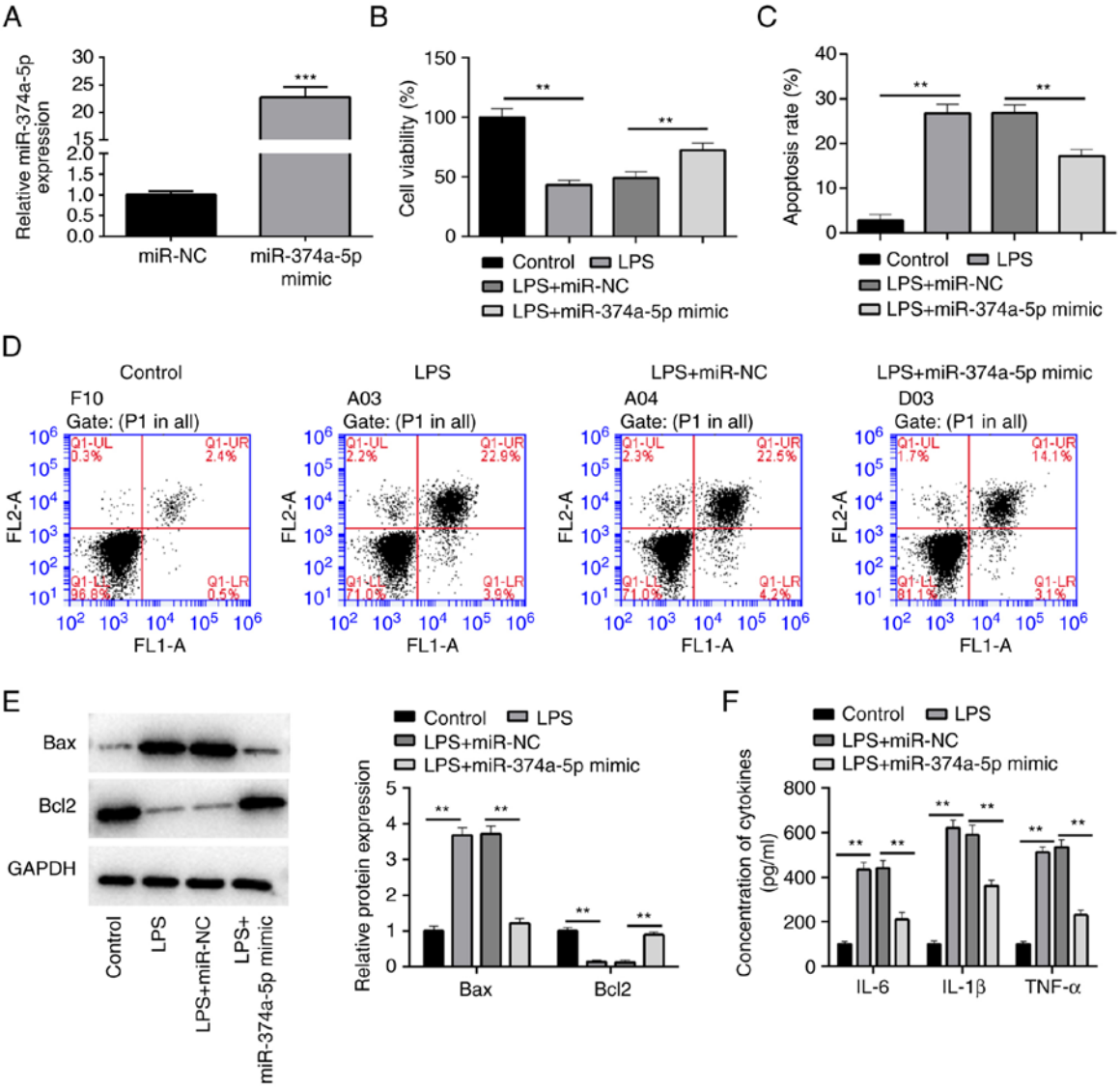

Figure 2. Overexpression of miR-374a-5p alleviates LPS-induced apoptosis and inflammation in HPMVECs. HPMVECs were transfected with miR-NC or miR-374a-5p mimic, and then treated with 1 μg/ml LPS for 24 h. (A) RT-qPCR was used to confirm the transfection efficiency of miR-374a-5p overexpression. ***P<0.001 vs. miR-NC. (B) Cell viability was measured by CCK-8 assay. (C and D) The apoptotic ratio was determined by flow cytometry. (E) The protein expression levels of apoptosis-related genes Bax and Bcl-2 were detected by western blotting. (F) ELISA assay was employed to examine the contents of inflammatory cytokines IL-6, IL-1β and TNF-α in the medium. **P<0.01. Results represent the means ± SD of 3 independent experiments. miR-374a-5p, microRNA-374a-5p; LPS, lipopolysaccharide; HPMVECs, human pulmonary microvascular endothelial cells; miR-NC, miRNA negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CCK-8, Cell Counting Kit-8; ELISA, enzyme-linked immunosorbent assay; IL-, interleukin; TNF-α, tumor necrosis factor-α.

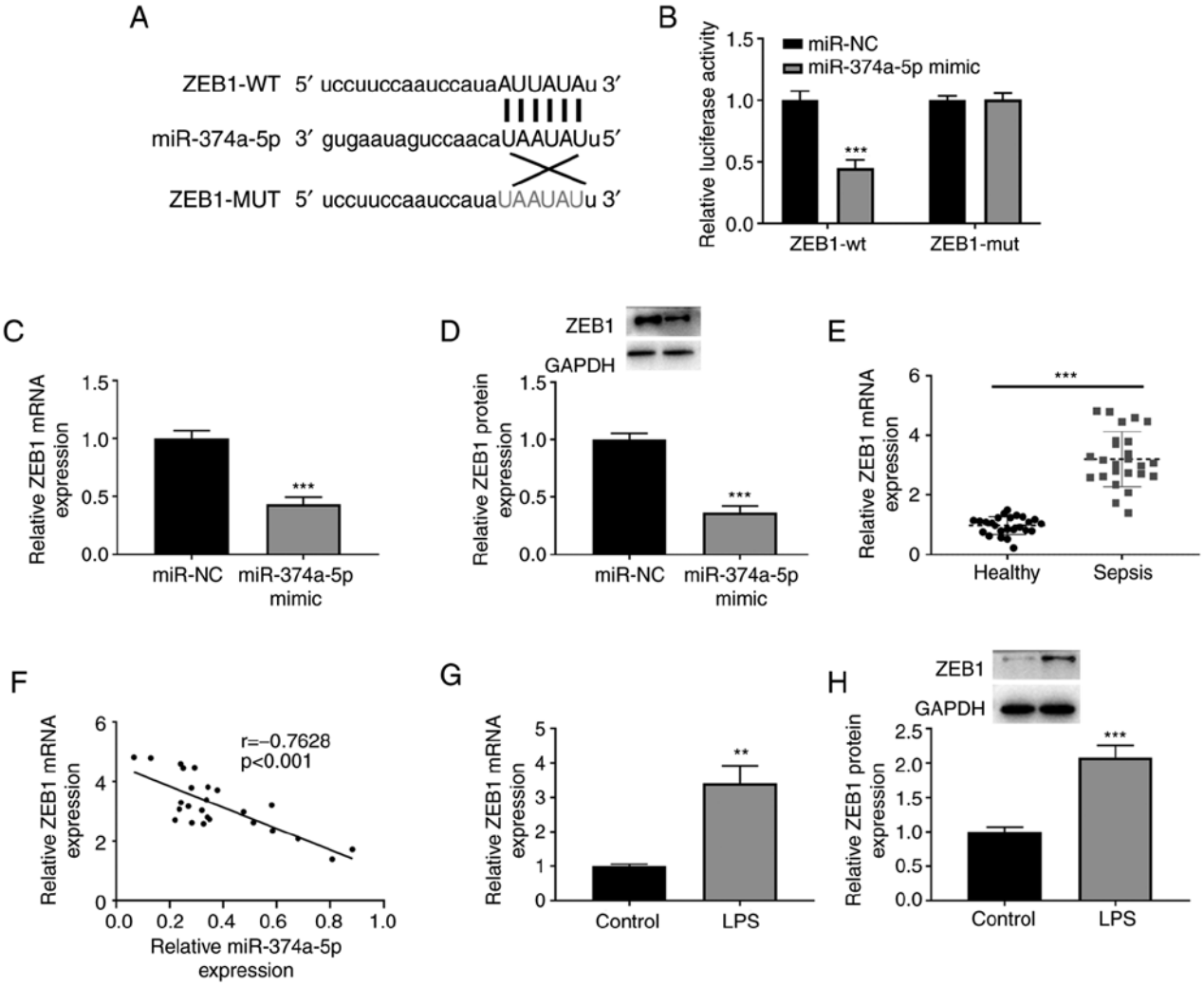

Figure 3. ZEB1 is directly targeted by miR-374a-5p in HPMVECs. (A) The binding sites between miR-374a-5p and ZEB1 are shown. (B) Following transfection with miR-NC or miR-374a-5p mimic, the luciferase activity of ZEB1-wt/mut was assessed by dual-luciferase reporter assay. (C and D) The ZEB1 mRNA and protein levels were determined to assess the effect of miR-374a-5p mimic on ZEB1 expression. ***P<0.001 vs. miR-NC. (E) The serum expression of ZEB1 mRNA in patients with sepsis and healthy controls was assessed by RT-qPCR. ***P<0.001. (F) Pearson's correlation analysis was employed to analyze the association between serum miR-374a-5p and ZEB1 mRNA levels. (G and H) The mRNA and protein expression levels of ZEB1 in HPMVECs after 1 μg/ml LPS treatment were detected by RT-qPCR and western blotting. **P<0.01 and ***P<0.001 vs. control. Results represent the means ± SD of 3 independent experiments. ZEB1, zinc finger E-box binding homeobox 1; miR-374a-5p, microRNA-374a-5p; HPMVECs, human pulmonary microvascular endothelial cells; miR-NC, miRNA negative control; ZEB1-wt, ZEB1 wild-type; ZEB1-mut, ZEB1 mutated; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; LPS, lipopolysaccharide.

was significantly downregulated after LPS treatment in comparison with the untreated cells.

Overexpression of miR-374a-5p alleviates LPS-induced apoptosis and inflammation in HPMVECs. To further explore the effects of miR-374a-5p in LPS-induced HPMVECs, the expression of miR-374a-5p was upregulated in LPS-treated HPMVECs by transfection with miR-374a-5p mimic (Fig. 2A). In addition, upregulation of miR-374a-5p restored cell viability (Fig. 2B) which was decreased by LPS treatment and attenuated the apoptotic rate which was induced by LPS in HPMVECs (Fig. 2C and D). Furthermore, LPS treatment resulted in increased expression of Bax and a significant reduction of Bcl-2 expression, while miR-374a-5p overexpression reversed these effects of apoptosis-related proteins in LPS-treated HPMVECs (Fig. 2E). In addition, LPS-induced secretion of IL-6, IL-1β, and TNF-α in HPMVECs was significantly impeded by miR-374a-5p overexpression (Fig. 2F).

ZEB1 is directly targeted by miR-374a-5p in HPMVECs. Subsequently, the downstream target of miR-374a-5p was searched for using bioinformatics analysis. ZEB1 was predicted as a direct target gene of miR-374a-5p with several putative binding sites using StarBase v2.0 (https://starbase.sysu.edu.cn/) (Fig. 3A). Dual-luciferase reporter assay demonstrated that the luciferase activity of ZEB1-wt was significantly decreased in cells transfected with miR-374a-5p mimic compared with miR-NC transfection (Fig. 3B). The mRNA and protein levels of ZEB1 in miR-374a-5p-overexpressing HPMVECs were then assessed and it was determined that the expression of ZEB1 at the mRNA and protein levels was significantly downregulated by the miR-374a-5p mimic (Fig. 3C and D). There was a higher expression of ZEB1

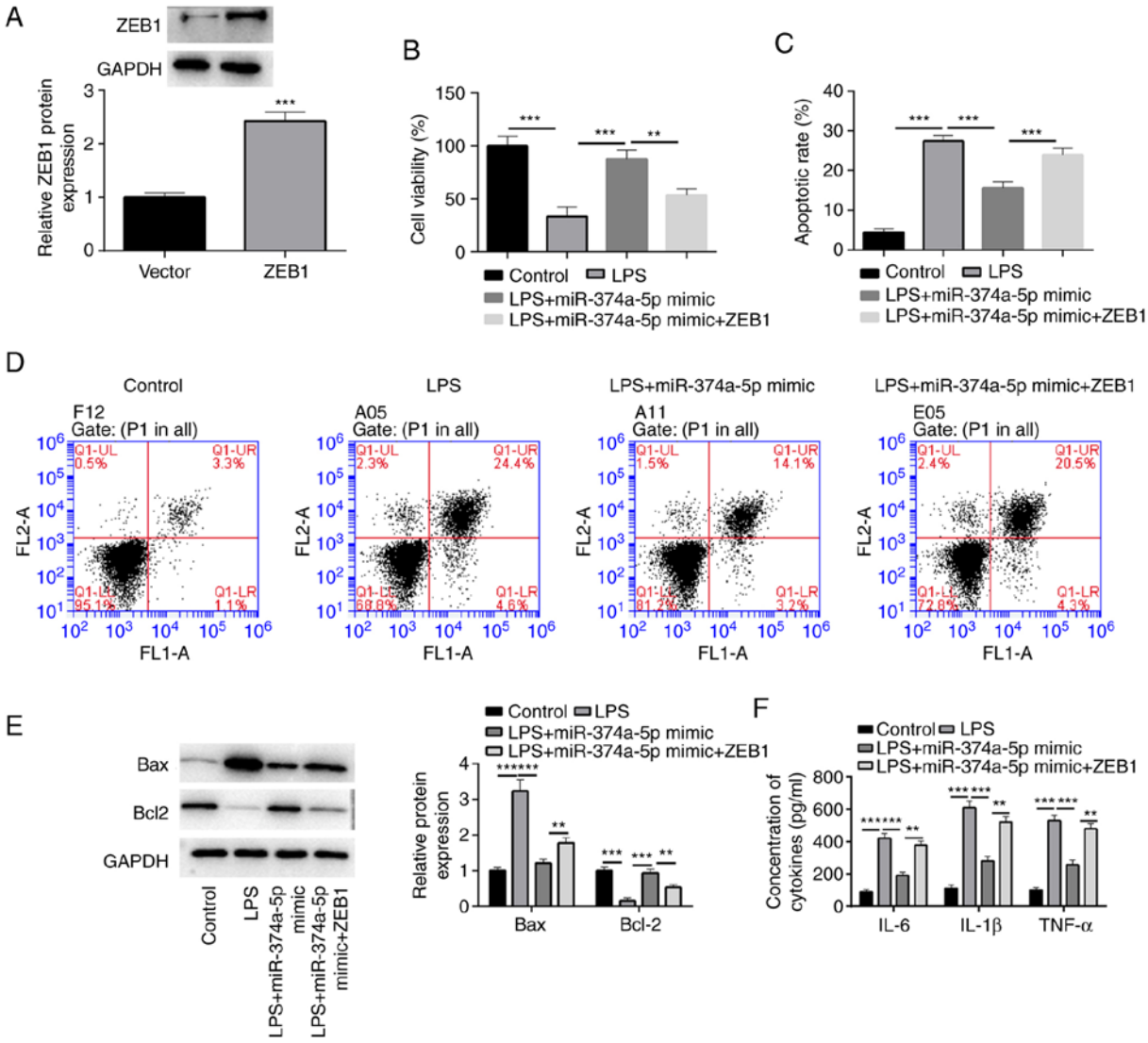

Figure 4. MiR-374a-5p reduces LPS-induced HPMVEC injury by targeting ZEB1. (A) Western blotting confirmation of ZEB1 overexpression. ***P<0.001 vs. vector. (B) Cell viability, (C and D) the apoptotic rate, (E) the protein expression levels of Bax and Bcl-2 and (F) inflammatory cytokine secretion levels, were examined in HPMVECs transfected with miR-374a-5p mimic and ZEB1 following treatment with LPS. **P<0.01 and ***P<0.001. Results represent the means ± SD of 3 independent experiments. MiR-374a-5p, microRNA-374a-5p; LPS, lipopolysaccharide; HPMVEC, human pulmonary microvascular endothelial cell; ZEB1, zinc finger E-box binding homeobox 1.

mRNA in the serum of patients with sepsis than that in healthy controls (Fig. 3E). A negative correlation between miR-374a-5p and ZEB1 mRNA expression was revealed in patients with sepsis (Fig. 3F). As anticipated, LPS stimulation induced the upregulation of ZEB1 mRNA and protein levels in HPMVECs (Fig. 3G and H).

MiR-374a-5p reduces LPS-induced HPMVEC injury by targeting ZEB1. To verify whether miR-374a-5p was involved in sepsis-induced ALI by targeting ZEB1, miR-374a-5p mimic was co-transfected with ZEB1 into HPMVECs, and then the cells were treated with LPS. The expression of ZEB1 in LPS-treated HPMVECs was efficiently increased by transfection of ZEB1 overexpression vector (Fig. 4A). Further results revealed that upregulation of ZEB1 weakened the viability induced by enforced expression of miR-374a-5p in LPS-treated cells (Fig. 4B). As depicted in Fig. 4C-E, ZEB1 overexpression abolished the inhibitory effects of miR-374a-5p on cell apoptosis, as determined by the enhanced apoptotic ratio, the increased Bax level and decreased Bcl-2 level. Introduction of ZEB1 counteracted the suppressive effect of miR-374a-5p on IL-6, IL-1β, and TNF-α production triggered by LPS in HPMVECs (Fig. 4F).

MiR-374a-5p exerts its function in LPS-treated HPMVECs by regulating the ZEB1-mediated p38 MAPK signaling pathway. To investigate the underlying mechanism of miR-374a-5p in sepsis-induced ALI, the expression of the p38 MAPK pathway was determined following overexpression of miR-374a-5p and ZEB1. Western blotting confirmed that the phosphorylation levels of p38, JNK, and ERK were significantly increased in LPS-treated HPMVECs. When miR-374a-5p was overexpressed, the protein expression of p38, JNK, and ERK phosphorylation levels was reduced, whereas ectopic expression of ZEB1 antagonized the down-regulation (Fig. 5).

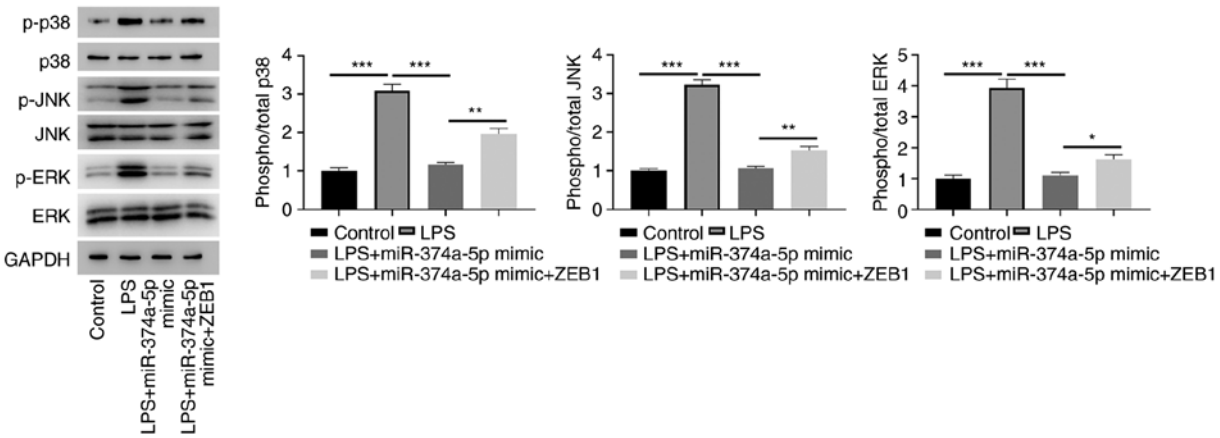

Figure 5. MiR-374a-5p exerts its function in LPS-treated HPMVECs by regulating the ZEB1-mediated p38 MAPK signaling pathway. Expression levels of p-p38 MAPK, p38 MAPK, p-JNK, JNK, p-ERK, ERK were analyzed by western blotting. The representative blots and quantitated results of p-p38/p38 ratio, p-JNK/JNK ratio, and p-ERK/ERK ratio are shown in HPMVECs transfected with miR-374a-5p mimic and ZEB1 after treatment of LPS. Results represent the means ± SD of 3 independent experiments. *P<0.05, **P<0.01 and ***P<0.001. MiR-374a-5p, microRNA-374a-5p; LPS, lipopolysaccharide; HPMVECs, human pulmonary microvascular endothelial cells; ZEB1, zinc finger E-box binding homeobox 1; p-, phosphorylated.

Discussion

Reportedly, the pathogenesis of sepsis-induced ALI is associated with pulmonary endothelial dysfunction, leading to abnormal apoptosis and inflammatory response (31,32). In the present study, for the first time to the best of our knowledge, it is reported that miR-374a-5p ameliorated the progression of sepsis-induced ALI *in vitro* by targeting ZEB1 and thus inactivating the p38 MAPK signaling pathway. This conclusion was supported by the following evidence. First, miR-374a-5p expression was decreased in the serum of patients with sepsis and in HPMVECs treated with LPS. Second, miR-374a-5p overexpression alleviated LPS-induced apoptosis and inflammatory response in HPMVECs. Third, ZEB1 was confirmed as a direct target of miR-374a-5p, and ZEB1 upregulation could attenuate the inhibition of miR-374a-5p on LPS-induced cell injury. Finally, the p38 MAPK signaling axis was essential for LPS-induced injury, and was also involved in the miR-374a-5p/ZEB1 network.

Increasing studies have shown that miR-374a-5p plays its protective roles in the development of neurological dysfunction (17,33) and myocardial injury (34,35). In a study of metabolically healthy obese (MHO), serum miR-374a-5p was highly expressed in MHO, and there was a positive association between miR-374a-5p expression with the downregulation of pro-inflammatory cytokines (36). In the present study, serum miR-374a-5p expression was decreased in septic patients when compared with healthy controls. Similarly, in an *in vitro* cell model of LPS-induced ALI in HPMVECs, miR-374a-5p expression was also downregulated when compared with the untreated cells. MiR-374a-5p mimic was further used to upregulate miR-374a-5p expression in HPMVECs, and it was demonstrated that miR-374a-5p overexpression attenuated the LPS-induced inhibition of cell viability. Bax and Bcl-2, significant members of the Bcl-2 family, are vital regulators of apoptotic cell death (37); and the present research revealed that miR-374a-5p overexpression decreased the proportion of apoptotic cells in LPS-treated HPMVECs, as demonstrated by inhibition of Bax expression and upregulation of Bcl-2. Pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α and inflammatory chemokines appear to trigger the inflammatory cascade, leading to pathological changes (38). In the present study, stimulation of LPS caused increased levels of IL-6, IL-1β and TNF-α in cell supernatants. The gain-of-function assay revealed that miR-374a-5p overexpression mitigated LPS-induced inflammatory response. These results indicated the apoptosis and inflammatory inhibitory effects of miR-374a-5p in HPMVECs.

ZEB1 may serve as a novel therapeutic target for pulmonary inflammation (27), neuroinflammation (39), and inflammatory cancer (40). ZEB1 was confirmed as a direct downstream target of miR-374a-5p and their expression was negatively correlated in the present study. Moreover, ZEB1 overexpression was capable of reversing the protective roles of miR-374a-5p in LPS-induced cell injury of HPMVECs. Extensive evidence indicates that aberrant activation of the p38 MAPK signaling pathway contributes to the pathogenesis of sepsis-induced ALI (24-26). In addition, ZEB1 is increased in LPS-induced pulmonary fibrosis, which is associated with the p38 MAPK signaling pathway (27). JNK and ERK pathways, the other MAPK pathways, also play vital roles in triggering the production of pro-inflammatory cytokines in response to LPS stimulation (41). In the present study, it was determined that p38, JNK, and ERK phosphorylation levels were downregulated when miR-374a-5p was overexpressed in LPS-treated HPMVECs, while ZEB1 overexpression led to the activation of p38 MAPK, JNK and ERK signaling pathways. Collectively, these findings indicated that miR-374a-5p ameliorated sepsis-induced ALI through the ZEB1-mediated p38 MAPK, JNK, and ERK pathways. However, there were some limitations in the present study. First, further validating investigations may have to be performed using a larger cohort to compare cases of sepsis with healthy controls to provide a greater statistical significance. Second, further investigation is required to verify the *in vivo* role of miR-374a-5p in sepsis-induced ALI using animal models. Third, the other signaling pathways involved in the regulatory effect of miR-374a-5p on HPMVEC cell behaviors were not further investigated.

Overall, the present study highlighted miR-374a-5p as a novel target for sepsis therapy and demonstrated that miR-374a-5p attenuated LPS-induced cell injury in HPMVECs, possibly via the ZEB1-mediated p38 MAPK signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JS conducted most of the experiments and wrote the manuscript. XM conducted the experiments and analyzed the data. JS designed the study and revised the manuscript. All authors have read and approved the final manuscript. JS and XM confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of General Hospital of Ningxia Medical University of Science and Technology (Yinchuan, China; approval no. IRB2017-GHNMU-38).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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