

MiR-23b-3p ameliorates sepsis-induced acute lung injury by inhibiting SMAD3-mediated endothelial-mesenchymal transition

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Abstract

Background: Sepsis-associated acute lung injury (ALI) is driven by endothelial barrier dysfunction and endothelial-mesenchymal transition (EndoMT), mediated by TGF- β 1/SMAD3 signaling. Despite the therapeutic potential of SMAD3, current inhibitors face limitations. As endogenous small molecules that are closely related to physiological regulatory processes, microRNAs (miRNAs) have more potential research value for regulating SMAD3. Therefore, this study aimed to investigate the protective effect and molecular mechanism of a key miRNA targeting SMAD3 in sepsis-ALI.

Methods: Screening multiple databases revealed that *miR-23b-3p* was the sole miRNA targeting SMAD3. Lipopolysaccharide (LPS)-stimulated human umbilical vein endothelial cells (HUVECs) and cecal ligation/puncture (CLP) mice were used to model sepsis. Lentivirus was used to construct stable strains. The functional performance and mechanism were verified by key techniques, including dual-luciferase assays, rescue experiments, reverse transcription-quantitative polymerase chain reaction (qPCR)/Western blotting, monocyte adhesion/permeability assays, and histopathology.

Results: In LPS-stimulated HUVECs, *miR-23b-3p* downregulation correlated with TGF- β 1/SMAD3 activation, EndoMT progression, and barrier disruption. *miR-23b-3p* overexpression reversed these effects by restoring the expression of junctional proteins and suppressing the expression of mesenchymal markers. Chromatin isolation by RNA purification-qPCR, RNA pull-down, and dual-luciferase assays confirmed the direct *miR-23b-3p*-SMAD3 3'UTR interaction. Rescue experiments demonstrated that *miR-23b-3p* counteracts TGF- β 1/SMAD3 hyperactivation. In CLP mice, intratracheal agomiR-23b-3p attenuated lung injury, normalized alveolar architecture, and reduced vascular leakage by suppressing endothelial Smad3 upregulation.

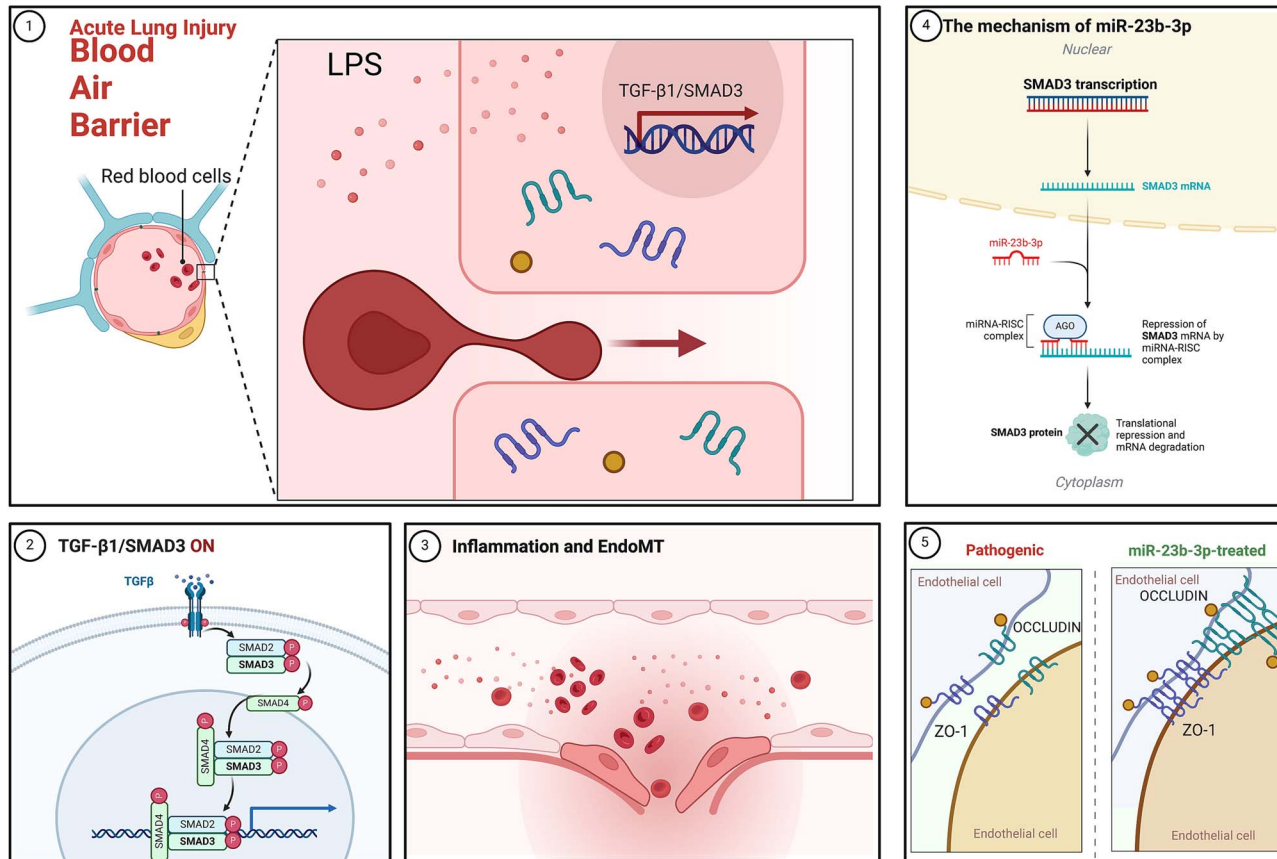
Conclusion: *miR-23b-3p* is a SMAD3-targeting regulator that inhibits EndoMT and repairs endothelial barrier integrity. Mechanistically, *miR-23b-3p* preserves endothelial homeostasis via SMAD3-dependent EndoMT inhibition. This study provides mechanistic insights and a miRNA-based therapeutic strategy for sepsis-induced ALI.

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Graphical Abstract



Keywords: Sepsis; Acute lung injury; Endothelial cells; MiR-23b-3p; TGF- β 1/SMAD3; EndoMT

Highlights

- Mechanistic elucidation of SMAD3-dependent EndoMT suppression and barrier restoration via junctional protein stabilization.
- Preclinical validation of miRNA-based intervention for sepsis-associated ALI across *in vitro* and *in vivo* models.
- Identification of SMAD3 as a translational biomarker for endothelial dysfunction stratification.

Background

Sepsis, defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [1], constitutes a global health emergency. The 2017 Global Burden of Disease Study revealed staggering figures: 48.9 million incident cases and 11 million sepsis-related deaths worldwide, representing nearly 20% of all global mortality [2]. Sepsis can cause systemic reactions and damage to various organs [3–6]. Among sepsis-induced organ injuries, acute lung injury (ALI) predominates (68.2% of cases) [7], and endothelial dysfunction is the central pathophysiological driver of sepsis-induced ALI [8,9]. This clinical reality underscores the urgent need for therapies targeting endothelial preservation.

Vascular endothelial cells (ECs), the sentinels of vascular homeostasis, orchestrate coagulation regulation, growth factor secretion, and dynamic barrier maintenance [8,10]. In sepsis, this delicate equilibrium collapses into an “endothelial catastrophe” characterized by disrupted junctional integrity, pathological leukocyte extravasation, and protein-rich pulmonary edema [8,9]. Among the complex cellular events contributing to this breakdown, endothelial-mesenchymal transition (EndoMT) has emerged as a critical pathogenic

process [11,12]. EndoMT is a pathological process in which endothelial cells lose their characteristic features and acquire a mesenchymal, fibroblast-like phenotype. This transformation leads to the disassembly of cell-cell junctions, increased cell motility, and excessive extracellular matrix deposition, directly contributing to vascular leakage and tissue fibrosis, which are hallmarks of sepsis-induced ALI [13–16].

A crucial driver of this detrimental EndoMT process is the transforming growth factor- β 1 (TGF- β 1)/SMAD3 signaling pathway [17,18]. Significantly, the systemic inflammatory storm characteristic of sepsis, involving the release of various cytokines and damage-associated molecular patterns, is known to robustly activate the TGF- β 1/SMAD3 pathway [19]. This creates a direct and damaging causal link: sepsis triggers the activation of TGF- β 1/SMAD3, which in turn executes the EndoMT program, leading to the dismantling of the endothelial barrier. Therefore, targeting the TGF- β 1/SMAD3 pathway presents a logical therapeutic strategy to interrupt this vicious cycle and preserve endothelial function during sepsis.

While pharmacological SMAD3 inhibitors, such as SIS3, have shown promise in preclinical ALI models [20], their

clinical translation is hampered by significant hurdles. These include a lack of cell-type specificity, leading to systemic disruption of the homeostatic role of SMAD3, and difficulty in achieving sustained, localized drug delivery, particularly for hydrophobic small molecules. This therapeutic gap highlights the need for a more precise regulatory tool. MicroRNAs (miRNAs), ~22 nt noncoding RNAs that act as posttranscriptional regulators [21], offer a promising alternative. Owing to their endogenous nature and compatibility with advanced delivery systems, miRNAs can be engineered for cell-specific targeting, thereby concentrating therapeutic activity in ECs or other cells [22,23].

In this context, our bioinformatic analysis of public databases identified miR-23b-3p as a potential key regulator of SMAD3. This finding is particularly compelling given the complex role of SMAD3 in sepsis; while it can suppress certain inflammatory cytokines, it also promotes pathological responses that reduce survival [24]. The potential ability of miR-23b-3p to specifically downregulate SMAD3 suggests that it can precisely inhibit the profibrotic, EndoMT-driving functions of this pathway without causing widespread systemic disruption. Crucially, prior studies have confirmed that SMAD3 inhibition effectively attenuates EndoMT [18,25]. We therefore hypothesize that miR-23b-3p can protect endothelial barrier integrity in sepsis-induced ALI by directly inhibiting the TGF- β 1/SMAD3 pathway, thereby restraining EndoMT.

To test this hypothesis, we employed complementary *in vitro* and murine cecal ligation and puncture (CLP) models to systematically investigate the protective effects of miR-23b-3p on the endothelium, decipher its SMAD3-dependent molecular mechanisms, and identify novel therapeutic targets for clinical translation. This study aimed to explore the role and mechanism of miR-23b-3p in protecting endothelial cell barrier function, enrich theoretical research on sepsis-associated ALI, and provide new ideas for the clinical treatment of sepsis-associated ALI. This multilevel approach not only advances our understanding of sepsis-associated ALI but also bridges the gap between miRNA biology and critical care therapeutics.

Methods

Analysis of public databases

Candidate miRNAs modulating SMAD3 were identified through intersection analysis of five prediction databases: miRTarBase (<https://miRTarBase.cuhk.edu.cn/>, experimentally validated interactions) [26], DIANA-microT (<https://diana.e-ce.uth.gr/>, miTG score ≥ 0.9) [27], TargetScan (<http://www.targetscan.org>, conserved sites) [28], starBase (<https://starbase.sysu.edu.cn>, CLIP-seq supported) [29], and NPinter (<http://bigdata.ibp.ac.cn/npinter4>) [30]. The Venn intersection [31] yielded one consensus miRNA (Figure S1a). Moreover, the results of the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of miR-23b-3p were obtained from the starBase database (Figure S1b).

Cell culture

Immortalized human umbilical vein endothelial cells (HUVECs) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) to model sepsis-induced pulmonary vascular injury. The cells were maintained

in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 100 U/mL penicillin (Gibco), 100 μ g/mL streptomycin (Gibco), and 10% fetal bovine serum (FBS; Gibco) under standard culture conditions (37°C, 5% CO₂, humidified atmosphere). For experiments, HUVECs (passages 3–15) were seeded into 6-well plates at a density of 2×10^5 cells/well. At 60%–70% confluence, the culture medium was replaced with fresh growth medium containing 10 μ g/mL lipopolysaccharide (LPS; L4391; Sigma–Aldrich; CAS 93572-42-0) to simulate septic conditions [32]. LPS-treated cells were incubated for 24 h prior to downstream processing.

Human pulmonary microvascular endothelial cells (SV40-HPuMVECs) (cat. no. SNL-701) were obtained from Sunncell Biotechnology Co., Ltd (Wuhan, China). Cells were maintained in dedicated culture medium for HPuMVECs (SNLM-701; Sunncell Biotechnology) supplemented with 100 U/mL penicillin (Gibco), 100 μ g/mL streptomycin (Gibco), and 10% FBS (Gibco) under standard culture conditions (37°C, 5% CO₂, humidified atmosphere).

Plasmid construction and production of lentiviral vectors

Hsa-miR-23b was obtained from the cDNA library of Genechem (Shanghai, China) with the following primers: forward: 5'-GGAAAGGACGAAACACCGGTGCTAGCATAA CAGGCGTGA-3' and reverse: 5'-TCACGCCTGTTATGCTA GCACCGGTGTTTCGTCCTTTCC-3'. The lentiviral vector plasmid GV570 (hU6-MCS-CBh-gcGFP-IRES-puromycin) (purchased from Shanghai Genechem Co., Ltd), the vector, and the *hsa-miR-23b* gene sequence were digested by AgeI and EcoRI restriction enzymes, and cloning was completed through the In-Fusion recombination method. The recombinant vector was detected by DNA sequencing. Human SMAD3 (NM_005902.4) was subsequently obtained from the cDNA library of Genechem (Shanghai, China) with the following primers: forward: 5'-TCGACGCGGGCCCCGGG ATCCCCGCCACCATGTGCTCCATCCTGCCTTTC-3' and reverse: 5'-GAAAGGCAGGATGGACGACATGGTGGCGG GATCCCCGGGGCCGCGTCA-3'. The lentiviral vector plasmid GV844 (CMV-MCS-EF1a-mCherry-T2A-neomycin) (purchased from Shanghai Genechem Co., Ltd), the vector, and the SMAD3 gene sequence were digested by BamHI and XbaI restriction enzymes, and cloning was completed through In-Fusion recombination method. The recombinant vector was detected by DNA sequencing.

The viral vectors were transfected into 293T cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) together with two helper plasmids, psPAX2 and pMD2.G. Infectious lentiviruses were harvested 72 h after transfection, rapidly centrifuged to remove cell debris, and then filtered through 0.45- μ m cellulose acetate filters. Viral titers were determined by fluorescence-activated cell sorting analysis of GFP- or mCherry-positive HEK293T cells and were $\sim 1 \times 10^9$ transducing units (TU)/mL (LV-has-miR-23b) and 2×10^9 TU/mL (LV-SMAD3) media. Samples were stored at -80°C until further use.

Lentiviral infection

In brief, 5×10^4 HUVECs per well were first seeded in 6-well plates in complete media in a humidified incubator at 37°C and 5% CO₂ for 24 h. For most cells, it is generally assumed that the number of cells is twice as large as the initial

number after 24 h of culture (1×10^5 cells in our study). Then, according to the multiplicity of infection ($MOI = \frac{\text{Viral titer} \left(\frac{TU}{mL} \right) \times \text{Viral volume (mL)}}{\text{Number of target cells}} \right)$ of HUVECs determined in the preliminary experiment, the calculated viral volume, complete media, and HitransG P solution (40 μ L; Shanghai Genechem) were prepared, and the medium in each well was replaced with 1 mL of the mixed solution. The normal complete medium was first changed after 16 h. Infection efficiency was observed after 1 to 2 days of infection. Infection success was evaluated based on the infection efficiency (threshold >80%), and MOI adjustment was performed when the efficiency fell below this benchmark. After infection, cells from ≥ 3 wells from 6-well plates were pooled into 10-cm dishes and subjected to antibiotic selection (puromycin or G418L) for 14 days to establish stable hsa-miR-23b-3p/SMAD3-expressing cell lines.

Reverse transcription–quantitative polymerase chain reaction

RNA from HUVECs or mouse lung tissue was isolated utilizing the conventional phenol–chloroform RNA extraction and purification technique with TRIzol reagent (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Reverse transcription–quantitative polymerase chain reaction (RT–qPCR) was used to assess the relative expression levels of *IL-1 β* , *IL-6*, *TNF- α* , *TGF- β 1*, *SMAD3*, *CD31*, *vWF*, *CDH2*, *VIM*, *TJP1*, *OCLN*, *CDH5*, and *ICMA1*, which were determined relative to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control gene. The primers were synthesized by Sangon Biotech (Shanghai, China), and the primer sequences are provided in Supplementary Table S1. RT–qPCR analysis was conducted using a T100 Thermal Cycler (Bio-Rad Laboratories, Inc., USA), Evo M-MLV RT Premix for qPCR (Accurate Biology, AG11706, China), StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and SYBR Green Premix Pro Taq HS qPCR Kit III (High Rox Plus) (Accurate Biology, AG11738, China) following the manufacturer's instructions. Data analysis was performed using the $2^{-\Delta\Delta C_t}$ method.

RT–qPCR of miRNAs

Owing to the lack of poly-A tails of miRNAs, the stem–loop method was used to reverse transcribe miRNAs. RT–qPCR of miRNAs was performed to assess the relative expression levels of *has-miR-23b-3p* or *mmu-miR-23b-3p* in HUVECs or mouse lung tissue compared to that of the *U6* control gene. The stem–loop RT primers were synthesized by Accurate Biology (Changsha, China), and the primer sequences are provided in Supplementary Table S1. In accordance with the manufacturers' instructions, RT was conducted using a T100 Thermal Cycler (Bio-Rad Laboratories) and an miRNA 1st strand cDNA synthesis kit (stem–loop) (Accurate Biology, AG11743, China); qPCR was conducted using the StepOnePlus Real-Time PCR System (Applied Biosystems) and SYBR Green Premix Pro Taq HS qPCR Kit III (High Rox Plus) (Accurate Biology, AG11738, China).

Protein extraction and western blot

Total cellular or lung tissue protein was extracted using RIPA buffer (cat. no. WB3100; NCM Biotech Co., Ltd, Suzhou, China) supplemented with protease and phosphatase inhibitors (cat. no. P002; NCM Biotech). The protein

concentration was determined using a bicinchoninic acid (BCA) protein assay kit (cat. no. 23225; Thermo Fisher Scientific, USA). The protein was then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Once the protein sample was obtained, precast gels with a HEPES–Tris buffer system (cat. no. LK201 and cat. no. LK202, Epizyme Biotech Co., Shanghai, China) and three-color prestained protein markers (cat. no. WJ103, Epizyme Biotech) were used for electrophoresis. Using the eBlot™ L1 system (GenScript Biotech Co., Nanjing, China), the protein was electroblotted onto polyvinylidene difluoride membranes, which were subsequently blocked with protein-free rapid blocking solution (cat. no. PS108P; Epizyme Biotech). The primary and secondary antibodies used are listed in Supplementary Table S2. All the primary antibodies were incubated at 4°C overnight, followed by incubation with a horseradish peroxidase–conjugated secondary antibody diluted 1:2000 for 90 min at room temperature. Enhanced chemiluminescence (cat. no. P10300; NCM Biotech) was then conducted to visualize the protein bands using an Amersham Imager 600 (General Electric Company, USA) and were subsequently quantified using ImageJ software.

Construction of plasmids overexpressing SMAD3 and firefly luciferase and overexpressing miR-23b-3p

All plasmids utilized in this study were designed, synthesized, and verified by Genechem Co., Ltd (Shanghai, China). The vector plasmid GV272 (SV40–firefly Luciferase–MCS), the vector, and the *SMAD3* (wild-type (WT) or mutant (MUT))/*has-miR-23b-3p* gene sequence were digested by XbaI restriction enzymes, and cloning was completed through In-Fusion recombination method. The recombinant vector was detected by DNA sequencing. The final products were subsequently transfected into *Escherichia coli* DH5 α followed by extraction with an Endofree plasmid mega kit (Qiagen, Hilden, Germany) to generate a *SMAD3* (WT or MUT)/*has-miR-23b-3p*-overexpressing plasmid. Specifically, partial sequences of the *SMAD3*–3' untranslated region (3'UTR) containing *has-miR-23b-3p* binding sites or its mutated sequence were amplified, cloned, and inserted into the dual-luciferase reporter vector to generate the WT *SMAD3*–3'UTR vector and MUT *SMAD3*–3'UTR vector.

Dual-luciferase reporter assay

Luciferase activity was assessed using the Promega Dual-Luciferase System (Promega Biotech Co., Ltd, Beijing, China). HEK293T cells were cultured in 24-well plates at a density of 0.5 million cells per well and transfected with either the WT or MUT vector (0.1 μ g) in combination with the miR-23b-3p vector or miR-NC vector (0.4 μ g) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., USA). In addition, HEK293T cells were transfected with Renilla vector (20 ng per well). Luciferase activity was observed 48 h after transfection with the plasmids, and Renilla luciferase activity was used as an internal control.

Chromatin isolation by RNA purification–qPCR

To investigate the interaction between *has-miR-23b-3p* and *SMAD3*, as well as the binding affinity with *has-miR-23b-3p* in HUVECs and HPuMVECs, we conducted a chromatin isolation by RNA purification (ChIRP)–qPCR experiment.

Cells were initially cultured overnight to achieve attachment before being counted and harvested at a density not less than 1×10^8 cells. They were then washed twice with prechilled phosphate-buffered saline (PBS), scraped off using PBS containing 10 μ L/mL Halt Cocktail, and collected into 1.5-mL precooled centrifuge tubes. Cell lysis was performed by adding 1 mL of lysis buffer enriched with protease inhibitors, phosphatase inhibitors, and phenylmethylsulfonyl fluorid (PMSF); if the lysate appeared viscous, sonication was applied to ensure complete cell disruption. The lysate was subsequently centrifuged at 12 000 rpm for 15 min, and the supernatant was carefully transferred to a new EP tube for further use. For probe preparation, biotin-labeled DNA probes complementary to the target RNA sequences were synthesized, approximately one probe per 100 nt for linear RNAs (Genechem). These probes were pooled together for immunoprecipitation (IP). In brief, 600 μ L of the prepared cell lysate was mixed with a 2 $\times$ volume of hybridization buffer, 5 μ L of PMSF, 5 μ L of RNase inhibitor, and the appropriate amount of labeled DNA probes, followed by incubation at 37°C for 4 h. Moreover, streptavidin magnetic beads were prepared by washing them with lysis buffer and blocking them with a solution containing 1 mg/mL BSA (15942-050, Invitrogen). After hybridization, the lysate-probe mixture was incubated with the blocked beads at 37°C for 30 min. The beads were then washed 5–6 times with IP Wash Buffer under rotation at 37°C for 5 min each time. RNA was eluted from the beads by incubation with RNA PK buffer and proteinase K at 65°C for 45 min, followed by a brief incubation at 95°C for 5 min to dissociate the DNA probes from the target RNA. RNA was extracted using TRIzol reagent (TaKaRa, Shiga, Japan) for qPCR analysis. For qPCR, RNA samples were reverse transcribed to cDNA, which was then subjected to qPCR using specific primers targeting *SMAD3*, *hsa-miR-23b-3p*, and *GAPDH* as an internal control. Data were analyzed based on Δ Ct values normalized against input, and the percentage of input was calculated to quantify the level of the RNA targets. The probe sequences and sequences of primers used for the experiments are listed in [Supplementary Table S3](#).

RNA pull-down assay

To investigate the interaction between *hsa-miR-23b-3p* and *SMAD3* in HUVECs and HPuMVECs, an RNA pull-down assay was performed with a refined cell lysis procedure. First, biotin-labeled RNA probes specific for *hsa-miR-23b-3p* and an NC probe were prepared by RiboBio. Cells were cultured until they reached optimal growth status before being subjected to a single wash with 5 mL of PBS to remove residual culture medium. The cells were subsequently treated with 1 mL of 0.25% trypsin for digestion; once the digestion was complete, 2 mL of complete culture medium was added to terminate the process. The cell suspension was then centrifuged at 1200 rpm for 1 min, after which the supernatant was discarded.

Following centrifugation, the cells were resuspended in RIPA lysis buffer at a concentration of 1 mL per 1×10^7 cells. For frozen samples, cells were thawed on ice prior to lysis. Each milliliter of RIPA lysis buffer was supplemented with 10 μ L of PMSF as well as phosphatase and protease inhibitors to prevent protein degradation and maintain sample integrity. The mixture was incubated in a rotating device at 4°C for 1–2 h to facilitate thorough lysis. After lysis, the samples were centrifuged at 12 000 rpm for 15 min at 4°C, and the

supernatant, which represented the cell lysate, was carefully collected into new centrifuge tubes. A portion of 10% of the total cell lysate was reserved as the input for subsequent analyses.

For the binding of streptavidin magnetic beads (15942-050, Invitrogen) with biotin-labeled RNA, the beads were washed three times with Wash Buffer I before being resuspended in the same buffer and mixed with denatured RNA probes, which were incubated at 4°C overnight. After the incubation, the mixture was briefly centrifuged at 3000 rpm for 1 min, after which the supernatant was discarded. The bead–RNA complexes were further washed three times with Wash Buffer I to remove any unbound material. Approximately 1 mg of total protein from the cell lysate was subsequently added to the bead–RNA mixture along with RNase inhibitors, and the mixture was incubated at room temperature for 1 h. Following this incubation, the bead–RNA–protein complexes were gently pelleted and washed six times with 1 mL of Wash Buffer II each time.

The RNA was eluted from the complexes through the addition of proteinase K buffer and incubated at 55°C for 30 min. After centrifugation at 3000 rpm for 5 min, the supernatant was transferred to new RNase-free tubes, and RNA was extracted using a phenol:chloroform:isoamyl alcohol (125:24:1) solution. After phase separation by centrifugation at 4°C, the aqueous layer was carefully collected, and RNA was precipitated with ethanol and glycogen at –20°C overnight. Precipitated RNA was recovered by centrifugation, dried briefly at room temperature, and dissolved in DEPC-treated water for storage at –80°C.

Finally, the isolated RNA was reverse transcribed into cDNA using a protocol involving RNA denaturation at 65°C for 5 min, followed by cooling on ice and then the addition of a reaction mixture including random primer, dNTPs, RT buffer, RNase inhibitor, and StarScript III RTase (A231-01, GenStar), which was subjected to multiple temperature steps for optimal reverse transcription. The resulting cDNA was subjected to qPCR analysis to quantify the levels of *SMAD3* mRNA bound to *hsa-miR-23b-3p*. The Δ Ct method was employed for data normalization, where the Ct values of the immunoprecipitated samples were adjusted according to the input dilution factor, and the percentage of input was calculated accordingly. This comprehensive approach enabled the identification of significant interactions between *hsa-miR-23b-3p* and *SMAD3* within the tested cell lines. The probe sequences and sequences of primers used for the experiments are listed in [Supplementary Table S3](#).

Monocyte adhesion assay

HUVECs were seeded in 24-well plates at a density of 2×10^4 cells/well and cultured in complete medium to form a confluent monolayer (>90% confluence). Tohoku Hospital Pediatrics-1 (THP-1) monocytes were centrifuged (300 $\times$ g, 5 min) to remove the original media, resuspended in prewarmed PBS (pH 7.4), and incubated with 1 $\times$ Hoechst 33342 (cat. no. C1028; Beyotime Co., Shanghai, China) at 37°C for 10 min under light-protected conditions for nuclear staining. After centrifugation (300 $\times$ g, 5 min) to remove residual dye, the cells were gently washed three times with PBS to eliminate unbound fluorophores. Stained THP-1 cells (1×10^5 cells/well) were added to HUVEC monolayers in 24-well plates and cocultured in RPMI-1640 medium supplemented with 10% FBS at 37°C under 5% CO₂ for

2 h. Following incubation, the supernatant was aspirated, and nonadherent THP-1 cells were removed by gently rinsing the wells three times with prewarmed PBS (1 mL/well) along the wall of each well. Adherent THP-1 cells were imaged using an inverted fluorescence microscope.

Transendothelial permeability assay

A 24-well Transwell permeable support system (Costar 3413, polycarbonate, pore size 0.4 μm ; Corning, Inc., NY, USA) was used to assess HUVEC permeability under different stimulation conditions. In brief, HUVECs (2×10^5 /well) were seeded on the upper inserts of a Transwell culture plate, and the cells were allowed to grow to full confluence until a monolayer formed. Different groups were pretreated with LPS (10 $\mu\text{g/mL}$) for 24 h. After the indicated treatments, to ensure that the liquid level of the upper inserts was consistent with that of the lower chamber and to exclude the influence of phenol red in the medium in the lower chamber, the medium in the lower chamber was replaced with 600 μL of phenol red-free medium (cat. no. GNM11054-2; GENOM BIO Co., Ltd, Hangzhou, China). Moreover, 150 μL of FITC-dextran (1 mg/mL, cat. no. FD40S-1G; Sigma-Aldrich, USA) was added to the upper inserts of the Transwell culture plates, and 100 μL of the medium in the lower chamber was collected at 60 min for measurement of the fluorescence intensity.

Cell proliferation activity assays

Cell proliferation was assessed using a cell counting kit-8 (CCK-8) assay (cat. no. CK04; Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. HUVECs were plated in 96-well plates at a density of 5×10^3 cells per well in 100 μL of complete DMEM. Multiple groups (0 h, 6 h, 12 h, 24 h, and 48 h) were established according to different time points. Two hours later, nonadherent cells were eliminated by washing twice with PBS, and adherent cells were exposed to LPS (10 $\mu\text{g/mL}$) at 37°C and 5% CO_2 . The cells were subsequently incubated with 100 μL of medium containing 10% CCK-8 reagent at 37°C for 2 h at a preset point in time, after which the absorbance at 450 nm was measured using a multimode microplate reader (SpectraMax M4, CA, USA).

Management and ethical approval of animal protocols

Wild-type (C57BL/6) mice were procured from GemPharmatech Co., Ltd (China) and housed in a pathogen-free environment at a laboratory in Changhai Hospital (China). Six- to eight-week-old mice were utilized in all trials, and sex- and age-matched controls were employed. Mice were maintained in a room with constant humidity ($60\% \pm 5\%$), temperature ($24 \pm 1^\circ\text{C}$), and light cycling (6 a.m. to 6 p.m.) and fed a standard pellet diet *ad libitum*. Approval for the study was obtained from the Shanghai Changhai Hospital Ethics Committee (CHEC (A.E.) 2024-014) in China, and the research was carried out in compliance with the hospital's animal use and care committee regulations. All the protocols were conducted in strict accordance with the principles of laboratory animal welfare and the 3R (replacement, reduction, refinement) guidelines.

Acquisition and administration of miRNA agomirs

An miRNA agomir is a synthetic miRNA agonist designed to mimic endogenous miRNAs through incorporation into

the miRISC complex, thereby modulating the expression of the target mRNA [33]. Compared with conventional miRNA mimics, miRNA agomirs exhibit superior *in vivo* stability and miRNA activity, along with enhanced penetration through cell membranes and interstitial tissues [34]. In this study, all miRNA agomirs were synthesized by Genechem Co., Ltd (Shanghai, China), and the primer sequences are listed in Supplementary Table S1.

In this study, miRNA agomirs were administered by intratracheal instillation. Briefly, mice were anesthetized via inhalation (3% isoflurane for induction, 1.5% for maintenance) and secured in a supine position on a surgical platform with the neck slightly extended to expose the airway. Transoral intratracheal instillation was performed by gently inserting a blunt-tipped catheter (24G) through the oral cavity into the glottis, advancing $\sim 1\text{--}1.5$ cm into the trachea. Proper catheter placement was confirmed by observing respiratory-dependent fluctuations in the syringe fluid meniscus. Each mouse received 20 μL of sterile PBS (pH 7.4) containing 2.5 nmol of each miRNA agomir. Following instillation, the mice were maintained in a head-up position for 30 s to facilitate distribution into the lung parenchyma. The catheter was then carefully removed, and the mice were transferred to a 37°C heating pad for recovery, with continuous monitoring of respiratory activity and locomotor function until full consciousness was regained. To evaluate the administration efficiency of intratracheal instillation, we purchased Cy3-labeled miRNA agomirs from RiboBio. Two hours after administration, lung tissue samples were cryopreserved and sectioned, and immunofluorescence staining was performed (Figure S1d).

Mouse model of septic lung injury

The CLP-induced septic lung injury model was established under strictly aseptic conditions [35]. After surgical plane anesthesia was achieved, with 3% isoflurane for induction and 1.5% for maintenance in 70% O_2 /30% N_2 carrier gas, a 1-cm midline laparotomy was performed to exteriorize the distal cecum. With the use of 6-0 silk sutures, the cecum was ligated at the anatomical location between the distal ileocecal valve and the proximal colonic junction (1 cm from the apex). Two through-and-through perforations were created with a 22-gauge needle, followed by controlled fecal extrusion (<1 mm³ total discharge). The cecum was then repositioned with toothed forceps before abdominal closure using two-layer 6-0 sutures. Sham controls underwent cecal exteriorization and manipulation without ligation/puncture. Each mouse was given subcutaneous saline rehydration (1 mL) postoperatively. They were observed every 6 h, and analgesia was administered to mice exhibiting signs of pain; mice that met the humanitarian endpoint were euthanized. Terminal sample collection at 24 h postprocedure involved bronchoalveolar lavage fluid (BALF) and transcardial perfusion with ice-cold saline with heparin prior to tissue harvesting.

Acquisition of BALF

Bronchoalveolar lavage (BAL) was conducted by inserting a tracheal tube into the trachea and rinsing the lungs eight times with 1.0-mL aliquots of calcium- and magnesium-free PBS (pH 7.4) solution, following established protocols [23]. Protein analysis of BALF obtained from the initial two washes was carried out after centrifugation ($400 \times g$, 10 min, room temperature) and separation of BALF cells. The isolated BAL

cells were subjected to Wright–Giemsa staining. BALF protein concentrations were determined using a BCA protein assay kit (cat. no. 23225; Thermo Fisher Scientific). A Countess 3 automated cell counter (cat. no. AMQAX2000; Invitrogen) was used to count the cells in the BALF.

Hematoxylin and eosin staining and the Smith score

Lung tissues from euthanized mice were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 μ m for hematoxylin–eosin (H&E) staining using standard protocols [36]. Two external pathologists evaluated the lung injury score using images captured according to previously established methods [23]. The evaluation system quantitatively assessed four histopathological parameters, namely, alveolar wall thickness, inflammatory cell infiltration, hemorrhage, and alveolar congestion, with each parameter graded on a scale of 0–4 reflecting the percentage of the affected area per microscopic field (0 = absent; 4 $\geq$ 75% involvement).

Modified Giemsa staining

Modified Giemsa staining was performed according to the protocol provided by Servicebio. For smear staining, slides (cat. no. G6004; Servicebio Co., Wuhan, China) were hydrated in pure water (pH 6.8–7.2) for 2–3 min, followed by the application of freshly prepared Giemsa staining solution (1:9 ratio of Giemsa stock to buffer, cat. no. G1079-100ML, Servicebio) within a pap pen-defined rectangular area for 10 min. Stained slides were gently rinsed with pure water until the runoff became colorless, air-dried, and microscopically inspected (Nikon Eclipse E100 with DS-U3 imaging system) for blue nuclear staining and red-to-blue–purple cytoplasmic coloration. Qualified slides were oven-dried at 65°C for 4 h, cleared in xylene (cat. no. 10023418, China National Pharmaceutical Group), and mounted with neutral gum (cat. no. 10004160, China National Pharmaceutical Group). For paraffin sections, tissues fixed in 4% paraformaldehyde were dewaxed, hydrated, and stained with Giemsa solution (1:9 dilution, 10 min), whereas acetone-fixed frozen sections were subjected to a 1:19 Giemsa-to-buffer ratio. Differentiation was achieved by brief immersion in Giemsa differentiation solution followed by rinses with pure water. All steps were conducted under controlled humidity to prevent crystallization artifacts. Reagents were prepared in strict accordance with the manufacturer's specifications.

Lung wet-to-dry weight ratio

The degree of pulmonary edema was assessed using the wet-to-dry weight ratio method [23], whereby the right lung of each mice was harvested. Blood and water on the surface of the right lung were removed, after which the right lung was weighed immediately (wet). The sample was then dried in an incubator at 60°C for 48 h to determine the dry weight (dry).

Quantitative assessment of pulmonary vascular permeability using Evans Blue extravasation

Pulmonary vascular endothelial permeability was assessed in randomly selected mice ($n \geq 3$ /group) 24 h after model establishment using the Evans Blue (EB) extravasation method. A 1% EB solution (cat. no. E2129, CAS:314-13-6; Sigma–Aldrich, USA) was intravenously injected via the

tail vein at a dose of 25 mg/kg body weight. Following a 2-h circulation period, the mice were euthanized by exsanguination and subjected to right ventricular perfusion with 5 mL of PBS until pulmonary blanching was complete. The right inferior lung lobe was excised, surface moisture was removed by filter paper blotting, and the wet weight was recorded. The tissues were immersed in formamide (cat. no. J410BA0005, CAS:75-12-7; Sangon Biotech Co., Ltd, Shanghai, China) at a ratio of 1 mL per 100 mg tissue weight, followed by 24 h of incubation at 60°C. After centrifugation at 12 000 $\times$ g for 30 min, the supernatants were collected for spectrophotometric analysis. A standard curve was constructed using EB concentrations of 0.275, 0.55, 1.1, 2.2, 4.4, and 8.8 μ g/mL in formamide, with the absorbance measured at 620 nm (200 μ L/well in 96-well plates). The calibration curve demonstrated a linear correlation ($y = 0.034x + 0.0849$, $R^2 = 0.9883$), enabling the quantitative conversion of optical density values to EB concentrations. All reagents were prepared according to the manufacturer's specifications (MedChemExpress).

Multiplex immunofluorescence staining

Following fixation, paraffin-embedded lung sections (4 μ m) were sequentially deparaffinized in xylene and a graded ethanol series. Antigen retrieval was achieved through microwave-mediated heating in sodium citrate buffer (10 mM, pH 6.0, 95°C $\times$ 15 min). The sections were incubated with 3% H₂O₂ in a light-restricted environment for 15 min to quench endogenous peroxidase activity, followed by three 5-min washes with PBS (pH 7.4) on an orbital shaker. Primary antibodies (anti-N-cadherin [rabbit, 1:4000; 22018-1-AP; Proteintech] and anti-vimentin [rabbit, 1:4000; 5741S; Abclonal]; see Supplementary Table S2 for the full list) were applied at ambient temperature. Horseradish peroxidase-conjugated secondary antibodies with corresponding chromogenic substrates (TYR-520 Plus and TYR-570 Plus, cat. no. RC0086-23R; RecordBio) were utilized for signal development. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with antifade medium. Multispectral imaging was performed using a 3D HISTECH panoramic scanner (Hungary), with DAPI visualized at 350 nm excitation/420 nm emission, TYR-570 Plus at 550 nm excitation/570 nm emission, and TYR-520 Plus at 490 nm excitation/520 nm emission. Image analysis was conducted using Case Viewer software (version 2.4.0.119028).

For cell multiplex immunofluorescence, cells cultured on coverslips were fixed with 4% paraformaldehyde (10 min), blocked with 1% BSA (1 h), and incubated with primary antibodies (3 h, room temperature). Alexa Fluor™ 488-conjugated goat anti-rabbit IgG (H + L) secondary antibody (cat. no. A-11034; Invitrogen; 4 μ g/mL in 0.2% BSA-PBS) was applied for 45 min. Hoechst 33342 (cat. no. C1028; Beyotime) was used for nuclear staining. Imaging was performed using an Andor Ultimate confocal microscope (BC43 CF, Oxford Instruments Technology [Shanghai] Co., Ltd) with image acquisition via Imaris Viewer (version 10.2, Oxford Instruments Technology [Shanghai]). All procedures were standardized across biological replicates.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software). The normality of each data

distribution was first assessed using the Shapiro–Wilk test. Parametric comparisons between two groups were made by unpaired *t* tests with Welch's correction (Welch's *t*-test), whereas nonparametric comparisons were made by the Mann–Whitney *U* test. For multigroup comparisons involving a single factor, normally distributed data were evaluated by Brown–Forsythe ANOVA followed by Tukey's *post hoc* test, while nonnormally distributed data were analyzed using the Kruskal–Wallis test with Dunn's multiple comparison adjustment. For experiments analyzing two independent factors, two-way ANOVA was performed to assess the main effects and interactions. A *P* value of $<.05$ was considered to indicate statistical significance (**P* $<.05$, ***P* $<.01$, ****P* $<.001$, *****P* $<.0001$; ns: *P* $\geq .05$). All experiments were performed with at least three biological replicates.

Results

Identification of key miRNAs and construction of the LPS-stimulated HUVEC model

To identify potential key miRNAs influencing sepsis, SMAD3-targeting miRNAs were screened across five databases (miRNATarBase, DIANA-microT, TargetScan, starBase, and NPinter). miR-23b-3p was identified as the only miRNA common to the results of all four database searches (Figure 1a). At an LPS concentration of 10 $\mu\text{g/mL}$, inflammatory cytokine genes (*IL-1 β* , *IL-6*, and *TNF- α*) and intercellular cell adhesion molecule-1 (*ICAM1*) were upregulated, while endothelial junction-related genes (*CDH5*, *TJP1*, and *OCN*) were downregulated (Figure 1b). WB analysis further confirmed the reduced protein levels of ZO-1, OCCLUDIN, and *ICAM1* (an adhesion protein) in the LPS-stimulated HUVECs (Figure 1c). Monocyte adhesion assays revealed increased adhesion between THP-1 cells and LPS-stimulated HUVECs (Figure 1d). Permeability assays revealed impaired barrier function in the LPS-stimulated HUVEC model (Figure 1e), and CCK-8 proliferation assays revealed suppressed cell growth (Figure 1f).

Overexpression of miR-23b-3p inhibits TGF- β 1/SMAD3-mediated EndoMT in an LPS-stimulated HUVEC model

KEGG pathway analysis revealed that miR-23b-3p is involved in cell adhesion, junction regulation, and TGF- β signaling (Figure S1a). In the LPS-stimulated HUVEC model, miR-23b-3p expression was significantly reduced (Figure 2a), and the TGF- β 1/SMAD3 pathway was activated (Figure 2b and c). Downstream EndoMT markers were associated with increased mesenchymal gene (*CDH2* [encoding N-cadherin] and *VIM* [encoding vimentin]) and protein (N-cadherin and vimentin) expression and decreased endothelial marker (*CD31* [encoding PECAM] and *vWF* [encoding VWF]) expression at both the mRNA and protein levels (Figure 2d–e). Lentivirus-mediated overexpression of miR-23b-3p was successfully established in HUVECs (Figures 3a and S1b). RT–qPCR demonstrated that miR-23b-3p overexpression reduced the expression of inflammatory cytokines (*IL-6* and *TNF- α*) but not that of *IL-1 β* (Figure 3b). Additionally, miR-23b-3p suppressed TGF- β 1/SMAD3 pathway activation (Figure 3c and d) and reversed the expression of the following EndoMT markers: mesenchymal genes (*CDH2*

and *VIM*) and proteins (N-cadherin and vimentin) were downregulated, whereas endothelial markers (*CD31* and *vWF*) and proteins (PECAM and VWF) were upregulated (Figure 3e and f).

miR-23b-3p directly targets the 3'UTR of SMAD3 to suppress translation

First, we used TargetScan 8.0 to identify the predicted binding sites of miR-23b-3p on the SMAD3 3'UTR. Two potential binding sites were identified: the SMAD3–1 site (5'-aagucacacgacgcaAAUGUGAu-3') and the SMAD3–2 site (5'-agguaaagaacuuugAAUGUGAu-3'). The efficiency of the designed probes in capturing SMAD3-associated RNAs was assessed through ChIRP–qPCR, and the results are presented in Figure 3g. The data revealed significant enrichment of targeted RNA species compared with the controls, indicating successful and specific isolation of RNAs associated with SMAD3. Notably, SMAD3–2 exhibited a greater capture efficiency (Figure 3g).

Further analysis of the binding between SMAD3 and *hsa-miR-23b-3p* was performed in two cell lines, HUVECs and HPuMVECs (Figure 3h). The qPCR results indicated a detectable interaction between SMAD3 and *hsa-miR-23b-3p* in both cell types. Specifically, the level of *hsa-miR-23b-3p* associated with SMAD3 was greater in HPuMVECs than in HUVECs.

To explore the interaction between *hsa-miR-23b-3p* and SMAD3, an RNA pull-down assay followed by qPCR analysis was conducted in both HUVECs and HPuMVECs. As shown in Figure 3i and 3j, the qPCR results revealed significant enrichment of SMAD3–2 site mRNA in the samples pulled down with *hsa-miR-23b-3p* compared with the nonspecific control probe, indicating a specific interaction between *hsa-miR-23b-3p* and the SMAD3–2 site.

The binding of miRNA to the 3'UTR of its target mRNA leads to the degradation of the target mRNA transcript. To elucidate the specific mechanism by which miR-23b-3p regulates SMAD3 expression, we sought to verify whether miR-23b-3p directly interacts with the 3'UTR of SMAD3. Accordingly, WT and mutant MUT SMAD3 3'UTR plasmids with SMAD3–2 sites, along with an miR-23b-3p eukaryotic expression plasmid, were constructed (Figure 3k). Dual-luciferase assays revealed significantly reduced luciferase activity in cells cotransfected with miR-23b-3p and SMAD3 3'UTR-WT, confirming direct interaction (Figure 3l).

miR-23b-3p restores barrier function and reduces monocyte adhesion in an LPS-stimulated HUVEC model

RT–qPCR revealed restored junctional protein (*CDH5*, *TJP1*, and *OCN*) and adhesion molecule (*ICAM1*) mRNA expression (Figure 4a). Western blotting confirmed elevated ZO-1, PECAM, and occludin protein levels and reduced *ICAM1* expression (Figure 4b). Immunofluorescence (IF) staining revealed weaker *ICAM1* fluorescence intensity and stronger signals for ZO-1, occludin, and VE-cadherin in the miR-23b-3p-overexpressing LPS-stimulated HUVEC model (Figure 4c). Functional assays confirmed reduced monocyte adhesion (Figure 4d), decreased permeability (Figure 4e), and improved cell proliferation (greater OD450 values) (Figure 4f).

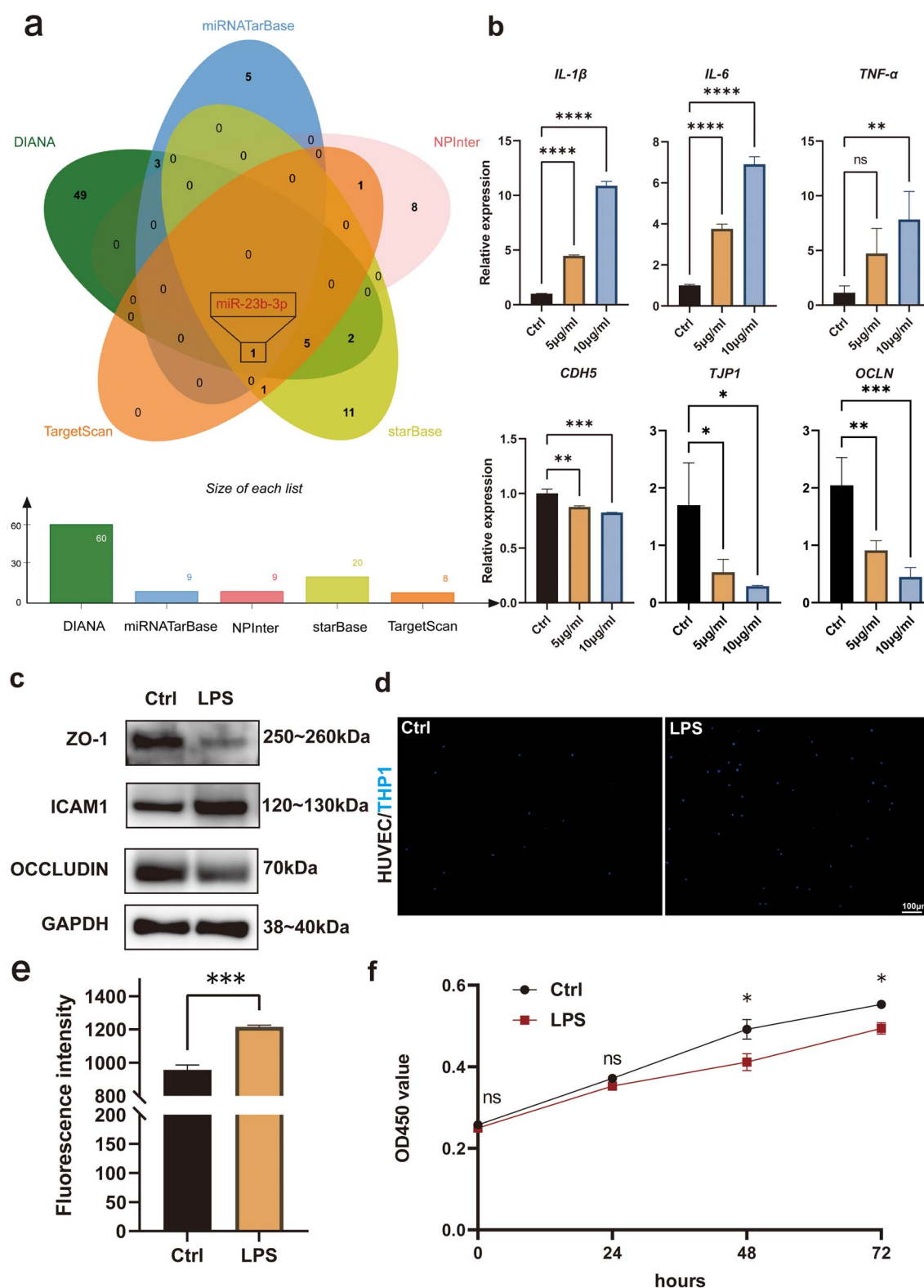


Figure 1. Construction and evaluation of LPS-stimulated HUVECs cell model. **(a)** Venn diagram showing *miR-23b-3p* as the sole miRNA targeting *SMAD3* across four databases (miRNATarBase, DIANA-microT, TargetScan, starBase). **(b)** RT-qPCR analysis of PBS group (Ctrl) and LPS-treated (10 μ g/mL, 24 h) HUVECs group (LPS) showing upregulated inflammatory genes (*IL-1 β* , *IL-6*, *TNF α*) and *ICAM1*, downregulated endothelial junction genes (*CDH5*, *TJP1*, and *OCLN*). **(c)** WB demonstrated reduced ZO-1, OCCLUDIN, and *ICAM1* protein levels in LPS-treated group (LPS). **(d)** Representative images of increased THP-1 monocyte adhesion to LPS-stimulated HUVECs (LPS). Circular dots with fluorescence: the THP-1 monocytes. Scale bar = 100 μ m. **(e)** Elevated endothelial permeability in LPS-stimulated HUVECs (LPS). **(f)** CCK-8 assay revealing suppressed HUVEC proliferation under LPS treatment (LPS). Data are presented as mean $\pm$ SD, $n \geq 3$, *** $P < 0.001$, **** $P < 0.0001$, two-tailed Welch's t -test and two-way ANOVA. *CDH5* VE-cadherin, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *HUVEC* human umbilical vein endothelial cells, *ICAM1* Intercellular Adhesion Molecule 1, *IL* interleukin, *LPS* lipopolysaccharide, *TNF α* tumor necrosis factor alpha, *THP-1* Tohoku Hospital Pediatrics-1, *OCLN* occludin, *ZO-1* Zonula Occludens-1

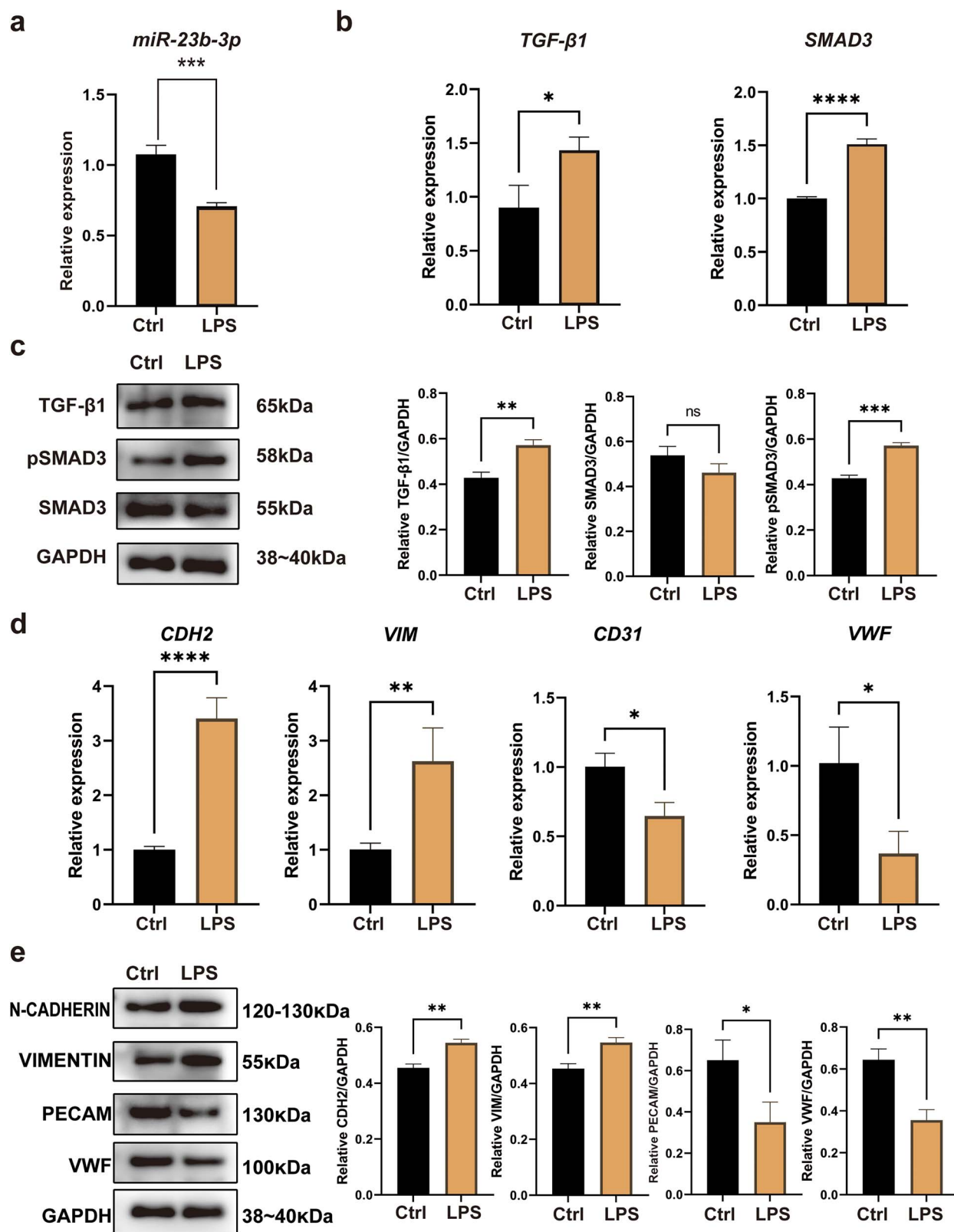


Figure 2. TGF- β 1/SMAD3-mediated EndoMT in LPS-stimulated HUVECs cell model. (a) Reduced miR-23b-3p expression in LPS-stimulated HUVECs (LPS). (b–c) Activation of the TGF- β 1/SMAD3 pathway in LPS-stimulated HUVECs (LPS) in RT-qPCR (b) and WB (c). (d–e) EndoMT marker analysis: upregulated mesenchymal genes (*CDH2*, *VIM*) (d) and proteins (N-CADHERIN, VIMENTIN) (f), and downregulated endothelial markers (*CD31* (PECAM), *vWF* (VWF)) at mRNA (d) and protein levels (e). Data are presented as mean $\pm$ SD, $n \geq 3$, *** $P < 0.001$, **** $P < 0.0001$, two-tailed Welch's t -test. *CD31* Cluster of Differentiation 31 (platelet endothelial cell adhesion molecule 1), *CDH2* N-cadherin, EndoMT endothelial–mesenchymal transition, *PECAM* Platelet Endothelial Cell Adhesion Molecule 1, *SMAD3* Sma and Mad Family Member 3, *TGF-β1* Transforming Growth Factor beta 1, *VIM* vimentin, *VWF* von Willebrand factor

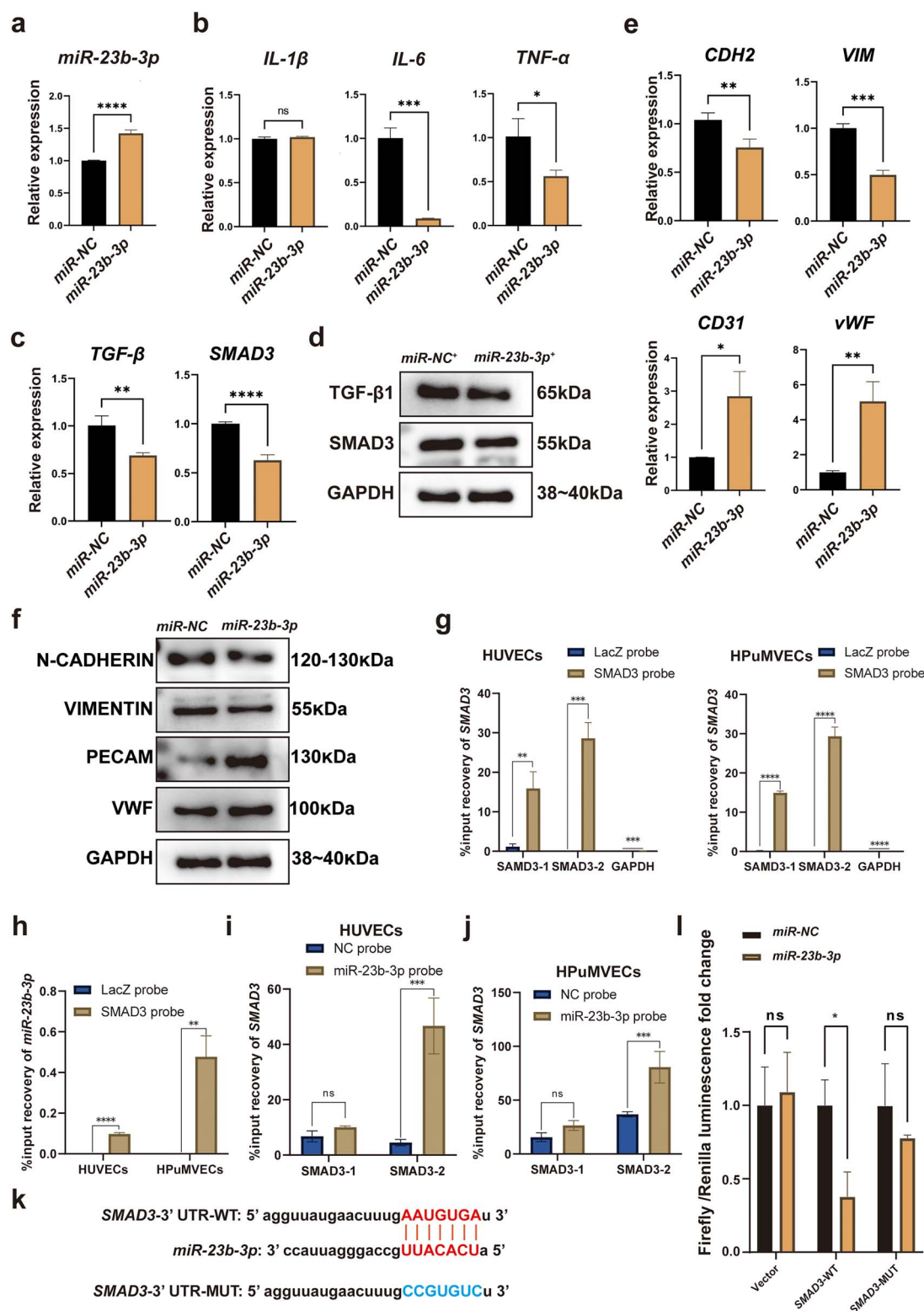


Figure 3. miR-23b-3p directly targeted SMAD3 and mitigated EC dysfunction in LPS-stimulated HUVECs cell model. (a) Lentiviral overexpression of miR-23b-3p in HUVECs (*miR-23b-3p*) compared with the control group (*miR-NC*). (b) The mRNA of inflammatory genes (*IL-6*, *TNF- α*) were downregulated in miR-23b-3p group (*miR-23b-3p*) except *IL-1 β* . (c–d) Suppression of TGF- β 1/SMAD3 pathway activation by miR-23b-3p (RT-qPCR (c) and WB (d)). (e–f) miR-23b-3p reverses EndoMT markers (RT-qPCR (e) and WB (f)). (g) The capture efficiency of SMAD3-1 and SMAD3-2 sites was determined in HUVECs and HPuMVECs, respectively. LacZ probe was used as the external reference, and GAPDH was used as the internal reference. (h) ChIP-qPCR was performed to verify the binding efficiency of *SMAD3* with *hsa-miR-23b-3p* in HUVECs and HPuMVECs cell lines. (i) The binding of *hsa-miR-23b-3p* to SMAD3-1 site or SMAD3-2 site was verified in HUVECs by RNA pull-down assay. (j) The binding of *hsa-miR-23b-3p* to SMAD3-1 site or SMAD3-2 site was verified in HPuMVECs by RNA pull-down assay. (k) Multi-species conserved sequences of wild-type (WT) *SMAD3* are indicated in red and mutant (MUT) 3'UTR *SMAD3* are indicated in lowercase letters blue. (l) Dual-luciferase reporter assays in HEK-293T cells after cotransfection with the control vector (Vector), wild-type (WT), or mutant (MUT) *SMAD3* plasmids and miRNA plasmids. Data are presented as mean $\pm$ SD, $n \geq 3$, *** $P < 0.001$, **** $P < 0.0001$, two-tailed Welch's t -test. HPuMVECs human pulmonary microvascular endothelial cells

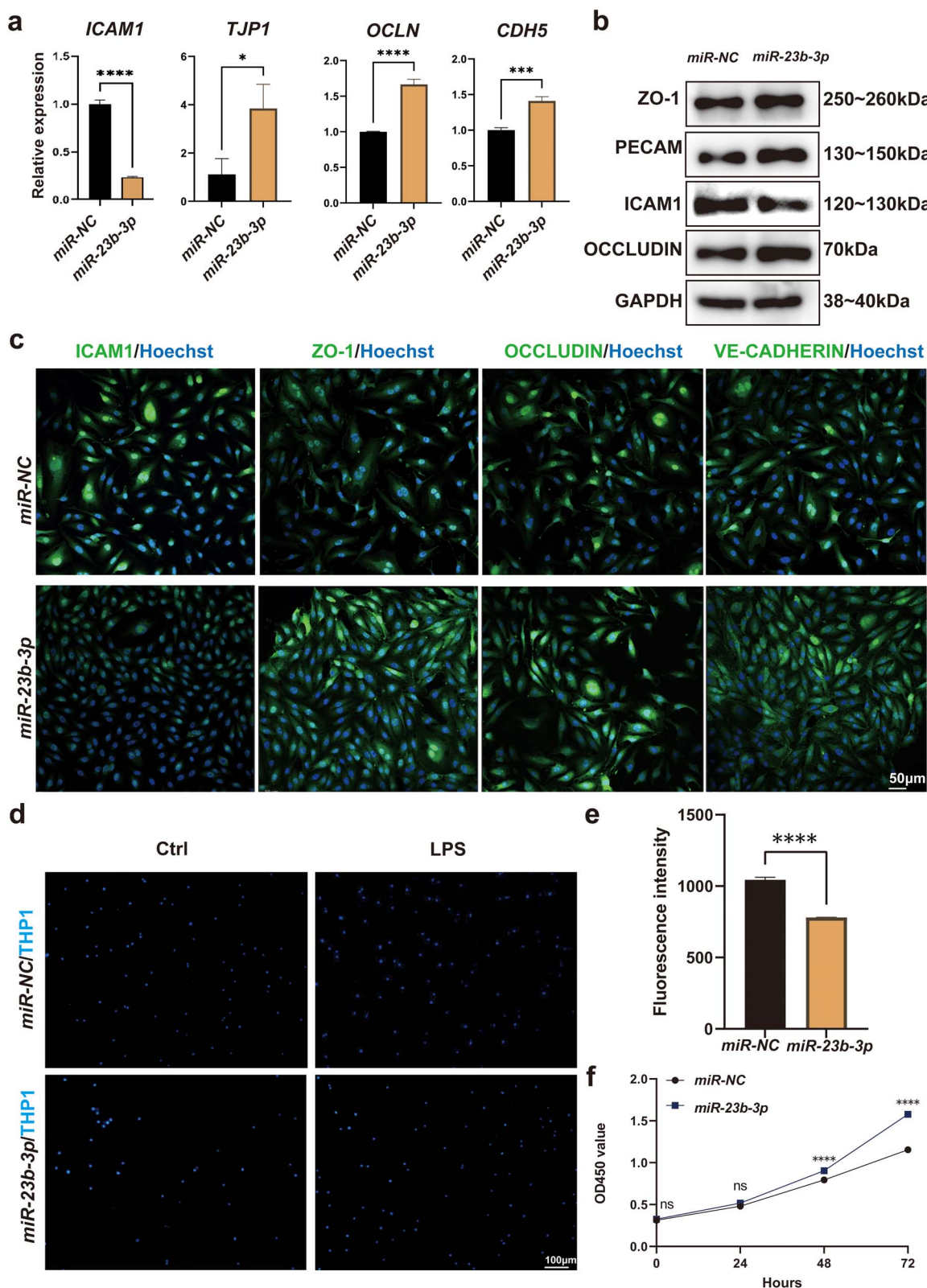


Figure 4. miR-23b-3p restored endothelial barrier integrity and reduced monocyte adhesion. **(a)** RT-qPCR showing rescued junctional protein mRNAs (*CDH5*, *TJP1*, and *OCLN*) and reduced *ICAM1*. **(b)** WB confirming upregulated ZO-1, PECAM, OCCLUDIN, and downregulated ICAM1. **(c)** Representative images of IF staining revealed the enhanced ZO-1/OCCLUDIN/VE-CADHERIN and reduced ICAM1 signals. Green: ZO-1/OCCLUDIN/VE-CADHERIN protein; blue: nuclei. Scale bar = 50 μm. **(d)** Functional assays demonstrated the reduced monocyte adhesion (representative images). Circular dots with fluorescence: the THP-1 monocytes are indicated in blue. Scale bar = 100 μm. **(e)** In overexpression *miR-23b-3p* group (*miR-23b-3p*), EC barrier function was improved. **(f)** CCK-8 assay showed the restored proliferation in overexpression *miR-23b-3p* group (*miR-23b-3p*). Data are presented as mean ± SD, $n \geq 3$, *** $P < 0.001$, **** $P < 0.0001$, two-tailed Welch's *t*-test and two-way ANOVA

miR-23b-3p reverses SMAD3 overexpression in an LPS-stimulated HUVEC model

Rescue experiments using SMAD3-overexpressing HUVECs (Figure S1c) demonstrated that miR-23b-3p counteracted TGF- β 1/SMAD3 pathway activation (Figure 5a and b). It normalized the expression of EndoMT markers (reduced expression of *CDH2/VIM* mRNA and N-cadherin/vimentin proteins and restored expression of *CD31/vWF* mRNA and PECAM/VWF proteins) (Figure 5c and d). miR-23b-3p also reversed the suppression of junctional proteins (*CDH5*, *TJP1*, and *OCN*) and the upregulation of ICAM1 at the mRNA and protein levels (Figure 5e and f). Functional assays confirmed reduced monocyte adhesion (Figure 5g), improved barrier integrity (Figure 5h), and restored proliferation (Figure 5i).

miR-23b-3p attenuates ALI in CLP model mice

In CLP mice, the intratracheal delivery of agomiR-23b-3p ameliorated sepsis-induced lung injury, as evidenced by reduced alveolar congestion, hemorrhage, inflammatory infiltration, and wall thickening on H&E and Giemsa staining (Figure 6a and b). The cell counts in the BALF (Figure 7a), BALF protein levels (Figure 7b), and lung wet/dry ratios (Figure 7c) were normalized in agomiR-23b-3p-treated mice. Evans Blue assays confirmed that vascular leakage was reduced (Figure 7d and e). RT-qPCR revealed decreased *miR-23b-3p* expression and increased *Smad3* mRNA expression in the lungs of the CLP mice, but these changes were reversed by agomiR-23b-3p (Figure 7f–i). Multiplex IF revealed reduced Smad3-Cd31 colocalization and enhanced Z α -1/occludin/VE-cadherin-Cd31 signaling in agomiR-23b-3p-treated lungs (Figure 7j and k).

Discussion

Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection, is pathologically linked to endothelial barrier collapse [37]. This study focused on EndoMT, a central mechanism in sepsis-associated acute lung injury (ALI), and systematically elucidated the molecular mechanism through which miR-23b-3p improves endothelial barrier function by targeting SMAD3 to suppress the TGF- β 1 signaling pathway. These findings not only provide novel theoretical insights into the pathological process of sepsis-induced lung injury but also highlight the therapeutic potential of precision miRNA-based strategies.

Through screening multiple databases, we identified miR-23b-3p as a key upstream regulator of SMAD3. In an LPS-stimulated HUVEC model, miR-23b-3p expression was significantly downregulated, accompanied by TGF- β 1/SMAD3 pathway activation and aberrant EndoMT marker expression, which is consistent with previous reports of the profibrotic role of SMAD3 in inflammatory environments [38]. Notably, ChIRP-qPCR, RNA pull-down, and dual-luciferase reporter assays confirmed that *miR-23b-3p* directly binds to the 3'UTR of *SMAD3* mRNA (the primary specific binding site could be 5'-agguuagaacuuugAAUGUGAu-3') to degrade *SMAD3* and inhibit its translation, establishing a novel molecular interface for miRNA-mediated endothelial homeostasis. Rescue experiments further demonstrated that *miR-23b-3p* overexpression effectively reversed the overactivation of the TGF- β 1/SMAD3 pathway, functionally validating this regulatory cascade.

Mechanistically, our findings offer a profound explanation for how *miR-23b-3p* can reverse the key pathological features of sepsis-associated ALI. The progression of EndoMT, driven by the TGF- β 1/SMAD3 axis, is not merely a molecular event; it is the direct cause of endothelial barrier failure. This transition leads to the loss of tight junction proteins (ZO-1/occludin) and the acquisition of a mesenchymal, hyperpermeable phenotype, which clinically manifests as protein-rich fluid leakage into the alveolar space—the hallmark of noncardiogenic pulmonary edema in ALI. By halting and reversing EndoMT, *miR-23b-3p* counteracts this process, restoring the integrity of the alveolar-capillary barrier. This was evidenced by the upregulation of endothelial markers (PECAM/VWF), restoration of tight junction proteins, and functional improvements in permeability and monocyte adhesion. Furthermore, the role of *miR-23b-3p* is likely multifaceted. Its reported ability to suppress M1 macrophage activation [23] suggests that it simultaneously dampens the proinflammatory cytokine storm and mitigates macrophage-endothelial crosstalk [39], thus addressing both the barrier integrity and hyperinflammatory aspects of septic ALI.

We established a multilevel validation system spanning *in vitro* to *in vivo* models. In mice with CLP-induced sepsis, the intratracheal delivery of agomiR-23b-3p markedly improved the alveolar architecture and reduced vascular leakage. These *in vivo* observations provide strong evidence that miR-23b-3p administration can have tangible physiological benefits, such as alleviating pulmonary edema and preserving the blood-gas barrier, which is essential for improving oxygenation in patients with ALI. Given that endothelial barrier integrity is critical for lung repair [40], the restoration of bronchoalveolar lavage fluid protein levels to sham-operated levels in the intervention group suggests that miR-23b-3p preserves blood-gas barrier integrity. Consistent findings from lung wet/dry ratio determination and EB assays further corroborated the therapeutic efficacy in enhancing microvascular integrity. *SMAD3* expression may serve as a biomarker for endothelial dysfunction, enabling the development of personalized therapeutic strategies. Recent advances using 3D microfluidic fibrosis models have revealed that preexisting myofibroblasts exacerbate vascular abnormalities via TGF- β 1/VEGF crosstalk [41], underscoring the importance of EndoMT modulation in lung injury.

The therapeutic significance of reversing EndoMT via *miR-23b-3p* extends beyond the simple restoration of barrier markers; it represents a fundamental intervention in the vicious cycle that perpetuates septic ALI. In the septic lung, a compromised endothelial barrier does more than just permit fluid leakage; it actively fuels the fire by allowing inflammatory cells, cytokines, and bacterial products to flood the alveolar space, triggering further epithelial injury and amplifying the inflammatory cascade [42]. By reestablishing endothelial integrity, *miR-23b-3p* effectively breaks this deleterious feedback loop. A stabilized barrier not only contains intra-alveolar inflammation but also transforms the endothelium itself from a proinflammatory, prothrombotic surface back to its quiescent, antithrombotic state. The normalization of bronchoalveolar lavage fluid protein levels and lung wet/dry ratios further substantiates its therapeutic efficacy *in vivo*. These results collectively identify *miR-23b-3p* as a potent regulator that protects endothelial barrier function by suppressing TGF- β 1/SMAD3-driven EndoMT. Overall, these findings suggest that miR-23b-3p protects

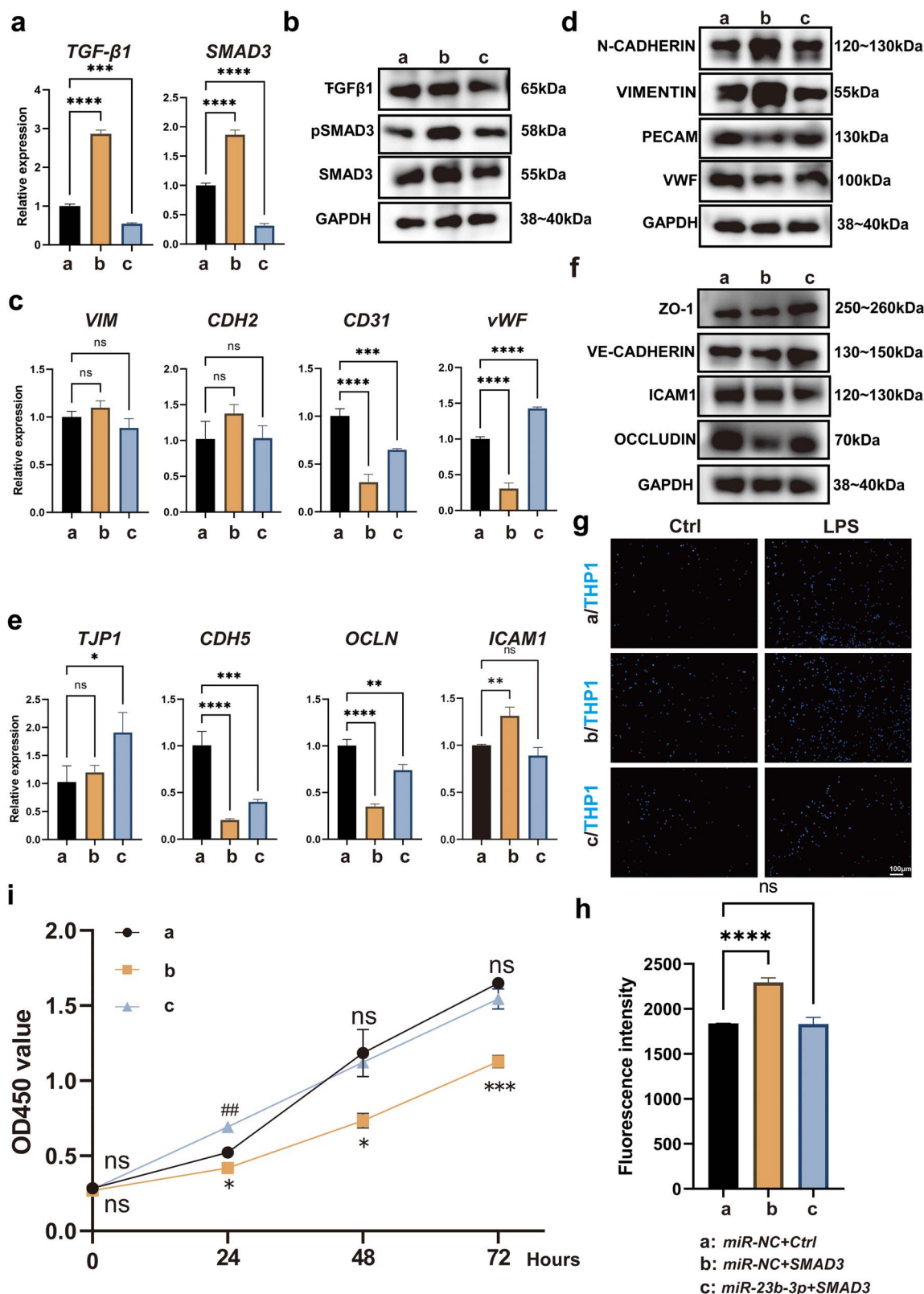


Figure 5. miR-23b-3p counteracted the effect of SMAD3 overexpression to LPS-stimulated HUVECs cell model. (a–b) miR-23b-3p (*miR-23b-3p* + *SMAD3*) rescued the *SMAD3* overexpression induced *TGF-β1*/*SMAD3* pathway activation (RT-qPCR and WB). (c–d) Rescue of EndoMT markers (reduced *CDH2*/*VIM* (N-CADHERIN and VIMENTIN) and restored *CD31*/*vWf* (PECAM and VWF)). (e–f) Normalization of junctional proteins (*CDH5*, *TJP1*, and *OCLN*) and *ICAM1* at mRNA (e) and protein levels (VE-CADHERIN, ZO-1, OCCLUDIN, and *ICAM1*) (f) by miR-23b-3p (*miR-23b-3p* + *SMAD3*). (g–i) Functional recovery in miR-23b-3p group (*miR-23b-3p* + *SMAD3*): reduced monocyte adhesion (Circular dots with fluorescence: the THP-1 monocytes are indicated in blue in representative images; scale bar = 100 μm) (g), improved barrier integrity in transendothelial permeability assay (h), and restored proliferation in CCK-8 assay (i). “a” represents the two kinds of control vector groups (*miR-NC* + *Ctrl*), “b” represents the control vector of miR-23b-3p and the *SMAD3* lentivirus group (*miR-NC* + *SMAD3*), and “c” represents the miR-23b-3p and *SMAD3* lentivirus groups (*miR-23b-3p* + *SMAD3*). Data are presented as mean ± SD, $n \geq 3$, *** $P < 0.001$, **** $P < 0.0001$, one-way ANOVA and two-way ANOVA

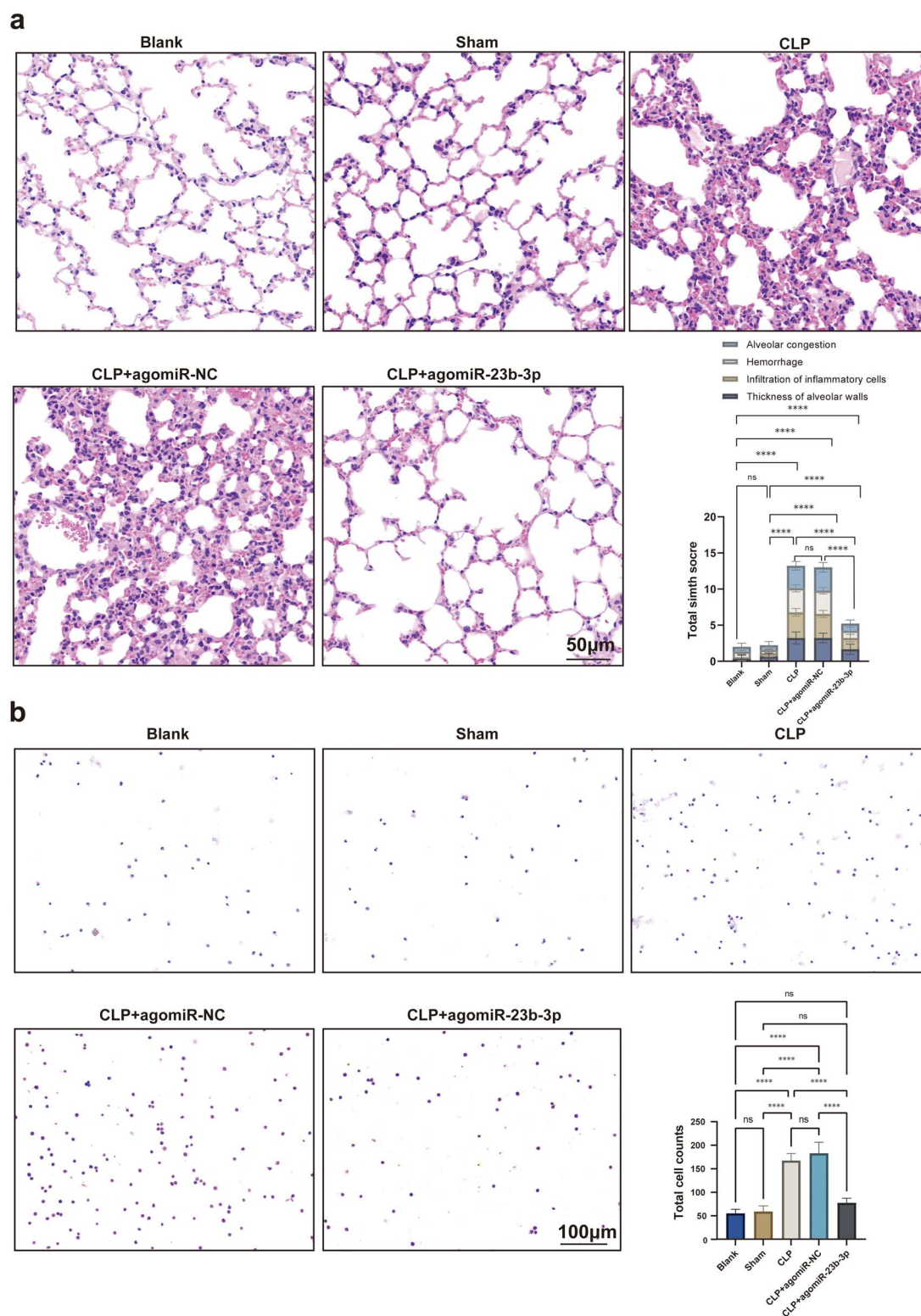


Figure 6. miR-23b-3p alleviated ALI in CLP mice. **(a)** Representative images of H&E staining showed reduced alveolar congestion, hemorrhage, and inflammation in agomiR-23b-3p-treated CLP mice group (CLP + agomiR-23b-3p) compared with normal mice group (Blank), sham mice group (Sham), CLP mice group (CLP), and agomiR-NC-treated mice group (CLP + agomiR-NC). Scale bar = 50 µm. **(b)** Representative images of modified Giemsa staining showed a decrease of inflammatory cells in agomiR-23b-3p-treated CLP mice group (CLP + agomiR-23b-3p). Scale bar = 100 µm. Data are presented as mean ± SD, $n \geq 3$, *** $P < 0.001$, **** $P < 0.0001$, one-way ANOVA



Figure 7. Effects of miR-23b-3p in ALI of CLP mice. **(a)** Reduced total cell counts in BALF of agomiR-23b-3p-treated CLP mice group (CLP + agomiR-23b-3p). **(b)** Normalized BALF of agomiR-23b-3p-treated CLP mice group (CLP + agomiR-23b-3p) protein levels. **(c)** Restored lung wet/dry ratio in agomiR-23b-3p-treated CLP mice group (CLP + agomiR-23b-3p). **(d)** Evans Blue quantification of vascular leakage between normal mice group (Blank), sham mice group (Sham), and CLP mice group (CLP). **(e)** Evans Blue quantification of vascular leakage between agomiR-NC-treated mice group (CLP + agomiR-NC) and agomiR-23b-3p-treated CLP mice group (CLP + agomiR-23b-3p). **(f–i)** RT-qPCR analysis of *mmu-miR-23b-3p* and *Smad3* in lung tissue of mice. **(j)** Representative images of multiplex IF of Smad3 and lung endothelial marker (Cd31). Smad3 is represented by arrows. Scale bar = 50 μ m and 20 μ m. **(k)** Representative images of multiplex IF of tight junction proteins (Zo-1, Occludin, and Ve-cadherin) and lung endothelial marker (Cd31). Zo-1, Occludin, and Ve-cadherin are represented by arrows. Scale bar = 50 μ m and 20 μ m. Data are presented as mean $\pm$ SD, $n \geq 3$, *** $P < 0.001$, **** $P < 0.0001$, two-tailed Welch's *t*-test and one-way ANOVA. ALI acute lung injury, CLP cecal ligation and puncture

endothelial barrier function by suppressing TGF- β 1/SMAD3-driven EndoMT.

Our research paves the way for several promising clinical applications. The most direct translational path for *miR-23b-3p* is its use as a novel therapeutic agent for sepsis-associated ALI. Given its direct protective effect on the lung endothelium, local administration via nebulization or intratracheal delivery, similar to our animal model, could maximize therapeutic efficacy while minimizing systemic side effects. This approach could be particularly valuable within a specific therapeutic window and could be applied early for patients at high risk of developing ALI or as a rescue therapy for established cases. Furthermore, the expression levels of *miR-23b-3p* in circulating exosomes and the levels of *SMAD3* in ECs could serve as valuable biomarkers. These could be used for early diagnosis, patient stratification, or therapeutic response monitoring, thus enabling a more personalized treatment approach. Beyond monotherapy, a combination strategy integrating *miR-23b-3p* therapy with standard-of-care treatments (e.g. antibiotics, vasopressors, and lung-protective ventilation) could be highly synergistic. By ameliorating endothelial dysfunction, *miR-23b-3p* addresses a pathological mechanism that current supportive therapies do not. Critically, by inhibiting the EndoMT process, the therapeutic potential of *miR-23b-3p* may extend beyond the acute phase, potentially mitigating the long-term risk of pulmonary fibrosis, a common and debilitating sequela for survivors of severe ALI/ARDS.

However, our study has several limitations. First, while HUVECs model core endothelial functions, they cannot fully replicate *in vivo* cellular interactions. Second, the CLP model primarily reflects bacterial sepsis; future studies should validate the findings in viral or fungal sepsis. Additionally, long-term safety, endothelial targeting efficiency, and delivery systems for miRNA therapy require further evaluation in large-scale animal studies. Potential solutions include developing endothelial-targeted nanoparticles or exosome-encapsulated *miR-23b-3p* sustained-release systems and exploring synergistic effects with endothelial-protective agents.

Overall, this study delineates the pivotal regulatory role of the *miR-23b-3p*/SMAD3 axis in sepsis-induced endothelial barrier dysfunction by moving beyond a simple description of the mechanism. Additionally, its importance is contextualized in reversing the core pathology of ALI, and a tangible roadmap for its clinical translation is outlined. Future multiomic analyses should elucidate interactions between this pathway and other endothelial injury mechanisms (such as metabolic reprogramming [43] and mitophagy [44]), guiding the evolution of sepsis therapy from single-target anti-inflammatory approaches to multidimensional regulatory strategies.

Conclusions

This study demonstrates that *miR-23b-3p* ameliorates sepsis-associated acute lung injury by directly targeting SMAD3, a key mediator of TGF- β 1-induced endothelial dysfunction. Through experimental validation in LPS-treated endothelial cells and a murine CLP model, we show that the restoration of *miR-23b-3p* expression attenuates endothelial barrier disruption by suppressing SMAD3-driven endothelial-mesenchymal transition (EndoMT), as evidenced by the recovery of junctional proteins (ZO-1 and occludin) and a reduction in mesenchymal markers (N-cadherin and vimentin). ChIRP-qPCR,

RNA pull-down, and dual-luciferase assays confirmed direct binding between *miR-23b-3p* and the *SMAD3* 3'UTR, with SMAD3-2 as the primary specific binding site, and rescue experiments substantiated its role in counteracting overactivation of the TGF- β 1/SMAD3 signaling pathway. *In vivo*, *miR-23b-3p* intervention mitigated lung vascular leakage and alveolar damage, which correlated with suppressed endothelial SMAD3 overexpression. These findings highlight SMAD3 as a critical molecular node in sepsis-induced endothelial injury, with *miR-23b-3p* serving as a potential therapeutic agent to preserve vascular integrity. While further studies are needed to explore clinical translation, this work establishes a foundational link between the miRNA-mediated regulation of SMAD3 and endothelial homeostasis, offering new avenues for developing targeted interventions in critical care settings.

Abbreviations

ALI: acute lung injury
BAL: bronchoalveolar lavage
BALF: bronchoalveolar lavage fluid
ChIRP: chromatin isolation by RNA purification
CLP: cecal ligation and puncture
CD31: Cluster of Differentiation 31 (platelet endothelial cell adhesion molecule 1)
CDH1: E-cadherin
CDH2: N-cadherin
CDH5: VE-cadherin
EB: Evans Blue
ECs: endothelial cells
EndoMT: endothelial-mesenchymal transition
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
H&E: hematoxylin and eosin
HUVECs: human umbilical vein endothelial cells
HPuMVECs: human pulmonary microvascular endothelial cells
ICAM1: Intercellular Adhesion Molecule 1
IF: immunofluorescence
IL: interleukin
LPS: lipopolysaccharide
OCN: occludin
PECAM: Platelet Endothelial Cell Adhesion Molecule 1
RT-qPCR: reverse transcription-quantitative polymerase chain reaction
SMAD3: Sma and Mad Family Member 3
TGF- β 1: Transforming Growth Factor beta 1
THP-1: Tohoku Hospital Pediatrics-1
TNF- α : tumor necrosis factor alpha
TJP1: Tight Junction Protein 1
VIM: vimentin
VWF: von Willebrand factor
WB: Western blot
ZO-1: Zonula Occludens-1

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Author contributions

Luofeng Jiang, Wei Zhang, Heng He, Yongjun Zheng, and Zhaofan Xia conceived the idea. Luofeng Jiang and Wei Zhang did most of

the *in vivo* and *in vitro* experiments and made the visualization of data. Heng He helped in immune blot. Jiezhil Lin, Tianyi Liu, and Lu Yang helped in luciferase assays. Hongchao Huang, Wenzhang Liu, Yingying Liu, and Pengfei Luo guided experimental methods. Xirui Tong facilitated in obtaining the lung tissue samples. Yongjun Zheng and Zhaofan Xia read the manuscript carefully and guided the writing idea. Luofeng Jiang, Wei Zhang, and Heng He prepared the final manuscript. All authors read and approved of the final manuscript. Luofeng Jiang (Conceptualization [equal], Visualization [equal], Writing—original draft [equal], Writing—review & editing [equal]), Wei Zhang (Conceptualization [equal], Validation [equal], Visualization [equal], Writing—original draft [equal], Writing—review & editing [equal]), Heng He (Validation [equal], Writing—original draft [equal], Writing—review & editing [equal]), Xirui Tong (Conceptualization [supporting], Validation [supporting], Visualization [supporting]), Futing Shu (Methodology [supporting], Validation [supporting]), Jiezhil Lin (Methodology [supporting], Validation [supporting]), Lu Yang (Validation [supporting]), Hongchao Huang (Methodology [supporting], Visualization [supporting]), Wenzhang Liu (Methodology [supporting], Resources [supporting]), Tianyi Liu (Methodology [supporting], Validation [supporting]), Yingying Liu (Investigation [supporting], Methodology [supporting]), Pengfei Luo (Methodology [supporting], Resources [supporting]), Yongjun Zheng (Conceptualization [equal], Project administration [equal], Writing—original draft [equal], Writing—review & editing [equal]), and Zhaofan Xia (Conceptualization [equal], Resources [equal], Writing—review & editing [equal])

Supplementary data

Supplementary data is available at *Burns & Trauma Journal* online.

Ethics approval

Male wild-type C57Bl/6J mice (6–8 weeks) were procured from Gem-Pharmatech Co., Ltd (China). The study received approval from the Ethics Committee at Changhai Hospital (CHEC (A.E)2024-014) in Shanghai, China, and was conducted in accordance with the hospital's animal use and care committee.

Conflict of interest

None declared.

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Data availability

The data that support the findings of this study are available from miRNATarBase, DIAN A-microT, TargetScan, starBase, and NPinter Database. The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of generative AI and AI-assisted technologies

During the preparation of this work, the authors used DeepSeek (<https://www.deepseek.com/>) to check grammar, spelling, and logic of the

article. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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