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CircRSU1 alleviates LPS-induced human pulmonary microvascular endothelial cell injury by targeting miR-1224-5p/ITGA5 axis

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Abstract. To investigate the potential functions and regulatory mechanism of circRSU1 on septic acute lung injury (sepsis-ALI) progression. We used lipopolysaccharide (LPS)-stimulated human pulmonary microvascular endothelial cells (HPMECs) to establish the cell model of sepsis-ALI *in vitro*. qRT-PCR and Western blotting were used for the detection of genes and proteins. The migration and tubulogenesis of HPMECs were assessed by transwell, wound healing, and tube formation assays. Inflammatory factors were detected by ELISA analysis. Cell permeability (PA) was determined by transendothelial resistance (TEER) and fluorescein isothiocyanate (FITC) with transwell assay. The interaction between miR-1224-5p and circRSU1 or ITGA5 (Integrin Subunit Alpha 5) was studied by dual-luciferase reporter and RNA pull-down assays. CircRSU1 expression was decreased after LPS treatment in HPMECs. Functionally, re-expression of circRSU1 in HPMECs could alleviate LPS-induced inflammatory response, the inhibition of cell migration and tube formation and enhancement of cell permeability. Mechanistically, circRSU1 acted as a sponge for miR-1224-5p. LPS treatment enhanced miR-1224-5p expression, and inhibition of miR-1224-5p reversed LPS-evoked HPMEC dysfunction mentioned above. Moreover, miR-1224-5p could abolish the protective effects of circRSU1 on HPMECs. In addition, miR-1224-5p directly targeted ITGA5, and circRSU1 was able to regulate ITGA5 expression *via* interacting with miR-1224-5p. CircRSU1 could alleviate LPS-induced HPMEC injury by miR-1224-5p/ITGA5 axis, indicating the potential molecular contribution of circRSU1 in sepsis-ALI.

Key words: circRSU1 — miR-1224-5p — ITGA5 — LPS — Sepsis — ALI

Introduction

Sepsis is the life-threatening organ dysfunction caused by the deregulated response of the host to severe infection, which is the main cause of death of hospitalized patients in the intensive care unit (ICU) (Fleischmann et al. 2016; Singer et al. 2016). Sepsis participation has been recognized as a key event in the prognosis of ICU patients with polytrauma, moreover, 10% of trauma deaths are due to sepsis (Mas-Celis et al. 2021). Sepsis is the main cause of multiple organ failure, among which, the lung is the first and most common organ to fail, and acute lung injury (ALI) is one of the key features of sepsis death (Lagu et al. 2012). Alveolar endothelial cells and tight junctions (TJs) are the main constituent of the alveolar-capillary barrier which is a protective mechanical barrier against inhaled pathogens (Li et al. 2019). The disruption of epithelial-barrier integrity, mediated by inflam-

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matory cascade and apoptotic pathways, loss of the ability to clear alveolar fluid, leading to increased permeability of pulmonary vessels and lung edema, thereby exacerbating symptoms of target organ (Deutschman and Tracey 2014; Li et al. 2018). Accordingly, in-depth investigations on the pathophysiologic mechanism underlying endothelial barrier integrity may have clinical significance for the therapy of sepsis-induced ALI (sepsis-ALI).

CircRNAs are one of endogenous covalently closed-looped noncoding RNAs that lack the 5'cap and 3' poly(A) tail (Chen and Yang 2015), so they show high cellular stability and are resistant to being degraded by exonuclease (Kristensen et al. 2018). Interestingly, emerging proofs revealed that circRNAs are functional molecules rather than splicing by-products and have functions in the regulation of cellular events, like cell apoptosis, angiogenesis and inflammatory response (Liu et al. 2020; Wang et al. 2020; Yan and Chen 2020). Besides that, circRNAs have been revealed to be implicated in regulating inflammatory response and cellular immunity of organisms (Yang et al. 2018; Chen et al. 2019), and it is considered that circRNAs may be important regulators in the initiation and development of sepsis by different mechanisms (Qi et al. 2021). Moreover, a disrupted circRNA expression was associated with sepsis-induced organ dysfunction. For example, He et al. (2021) showed that circ_0114428 expression was higher in septic acute kidney injury (AKI), and circ_0114428 silencing could abolish lipopolysaccharide (LPS), the major pathogenic factors of sepsis, induced apoptotic, inflammatory, oxidative injury and endoplasmic reticulum (ER) stress in sepsis-AKI cell model. Jiang et al. showed that circC3P1 could abolish pulmonary injury, inflammatory response and apoptosis in septic mice models with ALI and LPS-treated murine pulmonary microvascular endothelial cells (MPVECs) (Jiang WY et al. 2020). Besides, circ_0001679 deficiency was demonstrated to reverse LPS-triggered inflammatory and apoptotic injury in mouse lung epithelial cells, and alleviate pulmonary permeability, pulmonary edema and inflammation in the sepsis-ALI mice model (Zhu et al. 2022). CircRSU1 (ID: hsa_circ_0006577) is generated from its host gene RSU1 in chr10: 16794537-16824083, a recent study showed that circRSU1 expression was down-regulated in traumatic lung injury patients (Jiang Y et al. 2020). Herein, we explored the function and regulatory theory of circRSU1 in sepsis-ALI.

LPS is an endotoxin component of Gram-negative bacteria cell wall, and can activate pro-inflammatory mechanisms and induce inappropriate activation of the immune system, it has been recognized as a major pathogenic factor of sepsis, and the LPS injection model has been widely used for sepsis research (Doi et al. 2009; Virzì et al. 2022). Here, this study used LPS-challenged human pulmonary microvascular endothelial cells (HPMECs) to mimic the sepsis-ALI cell model *in vitro*, and then studied the action of circRSU1 on functions. Additionally, it has been well-identified that circRNAs can act as microRNA (miRNA) sponges to regulate the degradation of target mRNAs mediated by miRNAs (Salmena et al. 2011; Hansen et al. 2013). Therefore, the potential miRNA/mRNA axis was explored to elucidate the potential mechanism of circRSU1 on HPMECs.

Table 1. Primers sequences used for qRT-PCR

Name		Primers for qRT-PCR (5'–3')
circRSU1	F	CCCAGAACTAGTTACCTTATCCC
	R	GTTCAGCCTGTTCATGCCAA
miR-1224-5p	F	GGTCGAGTGAGGACTCGGG
	R	TCCGAGGTATTCGCACTGGA
ITGA5	F	GGCTTCAACTTAGACGCGGAG
	R	TGGCTGGTATTAGCCTTGGGT
RSU1	F	AGGGGCTACCTTCCGTGAC
	R	GGTAAAGAGGCCGTTGACATC
GAPDH	F	CAAATTCCATGGCACCGTCA
	R	GACTCCACGACGTACTCAGC
U6	F	CTCGCTTCGGCAGCACA
	R	AACGCTTCACGAATTTGCGT

F, forward; R, reverse.

Materials and Methods

Cell culture

HPMECs were provided by Procell (Wuhan, China) and cultured in Endothelial Cell Medium (EBM-2) plus 2% endothelial growth factor and 10% fetal bovine serum (FBS) (Procell) at 37°C with 5% CO_2. Cells in passages 4 to 7 were used for functional experiments. HPMECs were exposed to 1 μg/ml LPS (Solarbio, Shanghai, China) for 12 h to imitate ALI injury condition caused by sepsis *in vitro*.

Subcellular fractionation and quantitative real-time PCR (qRT-PCR)

The nuclear and cytoplasmic RNAs were separated from HPMECs using the PARIS™ Kit (Life Technologies, Wuhan, China) as *per* the manufacturer's protocol. Total RNAs were isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then quantified using the NanoDrop ND-1000. Then cDNAs were generated using the PrimeScript RT Reagent Kit (Takara, Dalian, China) or miScript II RT kit (Invitrogen), followed by qRT-PCR analysis employing a SYBR kit (Takara) with U6 or GAPDH as the internal reference. The relative RNAs were computed by the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method. The primer sequences are listed in Table 1.

CircRNA identification

About 5 μg of isolated RNAs were treated with RNase R (3 U/μg) for 1 h at 37°C, then abundances of circRSU1 and linear RSU1 were assayed by qRT-PCR.

Random primers or Oligo (dT)18 primers were used in reverse transcription with 2 μg RNAs and then levels of circRSU1 and linear RSU1 were detected by qRT-PCR.

Vector construction and cell transfection

To overexpress circRSU1, the full-length of circRSU1 was cloned into pCD5-ciR plasmids (GenePharma, Shanghai, China) with empty plasmids as the control (vector). miR-1224-5p mimic, inhibitor or the negative control (miR-NC or NC inhibitor) were provided by Sangon Biotech (Shanghai, China). Then 30 nM oligos or 600 ng vectors were transfected into HPMECs using LipofectamineTM 3000 reagent (Invitrogen). 48 h upon transfection, the cells were collected and subjected to LPS treatment for further analysis.

Transwell assay

HPMECs resuspended in serum-free medium were seeded into the top chamber of a 24-well chamber (8 μm pore size; Costar, Corning, NY, USA) with the lower chambers added with 10% FBS-contained culture medium. 24 h later, cells passed through the membrane were stained with 0.5% crystal violet solution (Beyotime, Shanghai, China), the representative images were photographed and migrated cells were counted using a microscope.

Wound healing assay

HPMECs were planted in a 6-well plate and a scratch in the cell monolayer was generated by a sterile 200-μl pipette tip when cells grew to 100% confluence. Cells were incubated with serum-free medium, and the wound closure was taken and observed at 0 and 24 h incubation to assess cell migration.

Enzyme-Linked Immunosorbent Assay (ELISA)

The culture supernatant of HPMECs was collected by centrifugation, then the levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-10 were assayed using the corresponding ELISA kits (Abcam, Cambridge, MA, USA) in line with the instructions.

Tube formation assay

A 96-well plate was coated with 50 μl growth factor-reduced Matrigel for 1 h. HPMECs were pre-starved for 24 h and then seeded on the Matrigel gel with 200 μl complete medium. Finally, the images were taken and the number of tubes was analyzed.

Measurement of HPMEC permeability by transepithelial electrical resistance (TEER) and fluorescein isothiocyanate (FITC)

HPMECs suspended in serum-free medium (1×10^5 cells/well) were seeded into the upper chamber of Transwell filters and cultured to form a monolayer of cells. 600 μl cell serum medium was added into the lower chamber. Then two electrodes were utilized to assay the electrical impedance value across the endothelial cells, and then the TEER value was determined.

FITC-labeled dextran was diluted to 1 mg/ml with serum-free EBM-2, and 200 μl diluent was placed in the apical chamber of the Transwell with 600 μl EBM-2 incomplete medium in the lower chamber. 1 h later, 100 μl solution was taken from the upper chamber and lower chamber, and the fluorescence intensities of each chamber were detected using the fluorospectrophotometer (excitation wavelength 488; emission wavelength 525), and permeability coefficient PA was calculated.

Western blotting

HPMECs were dissolved in RIPA buffer and centrifuged to remove the cell debris. Then about 40 μg of proteins were fractionated by 10% SDS-PAGE gel and then transferred onto PVDF membranes (Solarbio). The membranes were then incubated with primary antibodies against Zonula occludens (ZO)-1 (#13663, 1:1000), Vascular endothelial (VE)-cadherin (#2500, 1:1000), IGTA5 (#98204, 1:1000) (CST, Beverly, MA, USA) and GAPDH (ab9485, 1:2500, Abcam) at 4°C overnight. The blots were then incubated with goat anti-rabbit or anti-mouse secondary antibodies and visualized *via* BeyoECL Plus kit (Beyotime, Shanghai, China).

Dual-luciferase reporter assay

The fragments of circRSU1 or ITGA5 3'UTR possessing presumptive binding sites for miR-1224-5p were introduced into pGL3-basic vectors (Genecreate, Wuhan, China) to generate wild-type (WT) luciferase reporter vectors (WT-circRSU1 or WT- ITGA5 3'UTR). Then mutated (MUT) seed sequences were constructed and MUT-circRSU1 or MUT-ITGA5 3'UTR vectors were established. Thereafter, HPMECs were transduced with constructed pGL3 plasmids, pRL-TK vector and miR-1224-5p or the control for 48 h, and the activities of firefly and Renilla luciferase were assayed by the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA).

RNA pull-down assay

Biotinylated-miR-1224-5p probe (biotin-miR-1224-5p) and the control probe (biotin-NC) were synthesized by RiboBio and then transfected into HPMECs. Then cells were lysed and incubated with streptavidin-coated magnetic beads. Finally, RNA complexes were purified by Trizol and levels of circRSU1 and ITGA5 were detected by qRT-PCR.

Statistical analysis

The results were represented as mean ± standard deviation. GraphPad Prism software (GraphPad, La Jolla, CA, USA) was employed to assess the differences in two groups or multiple groups using Student's *t*-test or analysis of variance (ANOVA). $p < 0.05$ denoted statistically significant.

Results

CircRSU1 expression is decreased in LPS-induced HPMECs

As shown in Figure 1A, LPS treatment significantly up-regulated circRSU1 expression levels. CircRSU1 is generated from exons 2 to 6 of the RSU1 gene in chr10: 16794537-16824083 (Fig. 1B). Thereafter, the circular characteristics of circRSU1 were verified. Random primer or Oligo(dt) primer was used to synthesize cDNAs, the results showed that circRSU1 could only be reversed to cDNA by Random primer, while linear RSU1 could be reversed by either Random primer or Oligo(dt) primer (Fig. 1C). Besides, the 3'-5' exoribonuclease RNase R could markedly digest linear RSU1 but not affect circRSU1 (Fig. 1D). Next, the subcellular localization of circRSU1 was determined and circRSU1 was predominantly located in the cytoplasm of HPMECs (Fig. 1E). Thus, circRSU1 is a relatively stable cytoplasmic transcript that was decreased in LPS-induced HPMECs.

CircRSU1 overexpression reverses LPS-induced HPMEC dysfunction

To explore the potential biological function of circRSU1 in sepsis-ALI, circRSU1 was overexpressed in LPS-induced HPMECs to perform gain-of-function experiments. As expected, the introduction of circRSU1 plasmids significantly elevated circRSU1 expression compared with the control in HPMECs (Fig. 2A). Then transfected cells were treated with 1 μg/ml LPS for 12 h. Functionally, circRSU1 overexpression attenuated LPS-induced arrest on HPMEC migration (Fig. 2B,C) and tubulogenesis (Fig. 2D) abilities. Besides that, ELISA analysis showed that the contents of inflammatory factors IL-6 and TNF-α were elevated, while IL-10 was decreased after LPS treatment, which was reduced by circRSU1 overexpression (Fig. 3A–C). Then cell permeability was as-

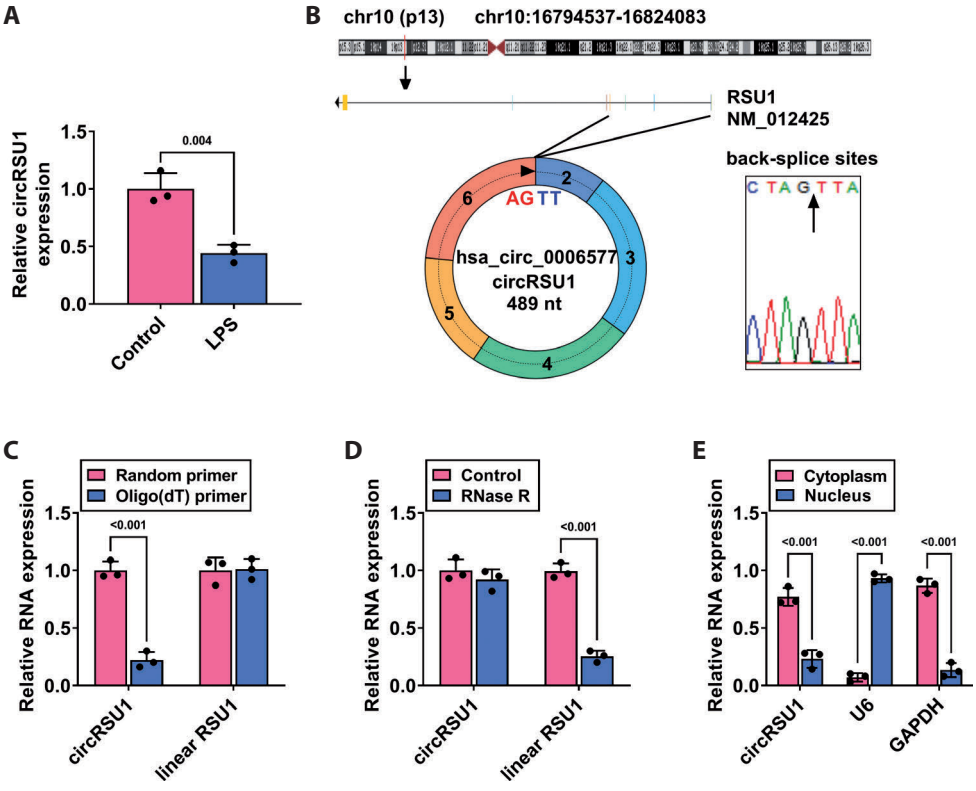

Figure 1. CircRSU1 expression is decreased in LPS-induced HPMECs. **A.** qRT-PCR analysis for circRSU1 expression in HPMECs treated with 1 μg/ml LPS for 12 h. **B.** Schematic illustration showing the formation of circRSU1. **C.** Random primer or Oligo(dt) primer was used to synthesize cDNA and levels of circRSU1 and RSU1 mRNA were detected by qRT-PCR. **D.** qRT-PCR of circRSU1 and RSU1 mRNA expression after RNase R treatment in HPMECs. **E.** Analysis of the subcellular location of circRSU1.

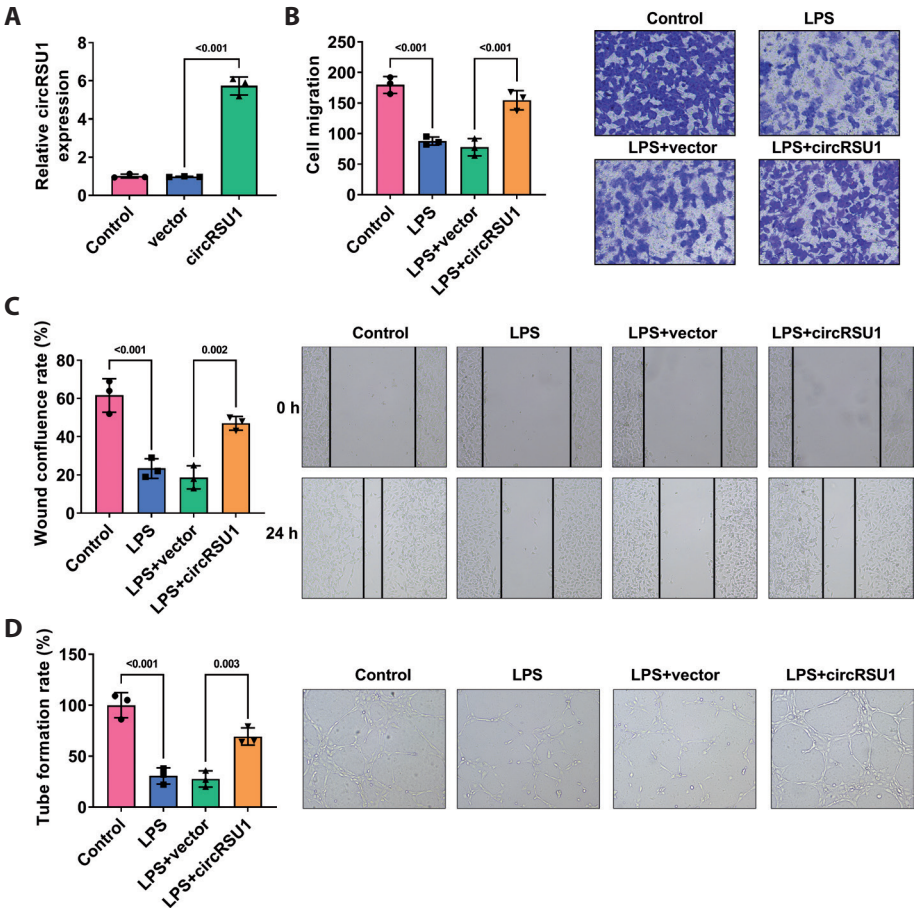

Figure 2. CircRSU1 overexpression reverses LPS-induced HPMEC dysfunction. HPMECs were transfected with circRSU1 or vector, followed by treatment with 1 μg/ml LPS for 12 h. **A.** qRT-PCR analysis for circRSU1 expression. Cell migration analysis using transwell (**B**) and wound healing (**C**) assays. **D.** Tube formation assay for the analysis of cell tubulogenesis ability.

sessed. It was found that the TEER value of HPMECs was decreased after LPS challenge and subsequently increased in response to circRSU1 plasmids (Fig. 3B). Moreover, LPS treatment caused the increase of PA% value, and this condition was rescued by circRSU1 increase in HPMECs (Fig. 3B). Afterwards, Western blotting analysis suggested that the tight junction markers ZO-1 and VE-cadherin were reduced by LPS exposure, while circRSU1 up-regulation could abolish this phenomenon in HPMECs (Fig. 3C). Taken together, circRSU1 overexpression could attenuate LPS-induced inflammation, the inhibition of cell migration and tube formation, as well as the enhancement of cell permeability in HPMECs.

MiR-1224-5p is a target of circRSU1 in HPMECs

Since circRSU1 was distributed predominantly in cell cytoplasm, so we hypothesized that circRSU1 might act as a miRNA sponge to exert its biological functions. Through the prediction of starbase, miR-1224-5p was identified to have complementary sequences on circRSU1 (Fig. 4A). Then dual-luciferase reporter assay was performed to validate our speculation, the result showed that the luciferase activity of WT-circRSU1 plasmid was markedly reduced by miR-1224-5p overexpression, while the luciferase activity of MUT-circRSU1 plasmid was unaffected by the transfection of miR-1224-5p (Fig. 4B). In addition, RNA-pull down indicated that circRSU1 could be captured by biotin-coupled miR-1224-5p probe compared with the control probe (Fig. 4C), further verifying the interaction between circRSU1 and miR-1224-5p (Fig. 4D). Thereafter, we also found LPS treatment could elevate miR-1224-5p expression in HPMECs. Thus, circRSU1 acted as a sponge for miR-1224-5p.

Inhibition of miR-1224-5p attenuates LPS-induced HPMEC dysfunction

Subsequently, the potential action of miR-1224-5p in sepsis-ALI was investigated. MiR-1224-5p inhibitor was designed and transfected into HPMECs. After LPS treatment, it was found that miR-1224-5p inhibition reversed the migration (Fig. 5A,B) and tube formation abilities (Fig. 5C) of HPMECs mediated by LPS. Besides that, the elevation of IL-6 and TNF-α, and decrease of IL-10 induced by LPS were

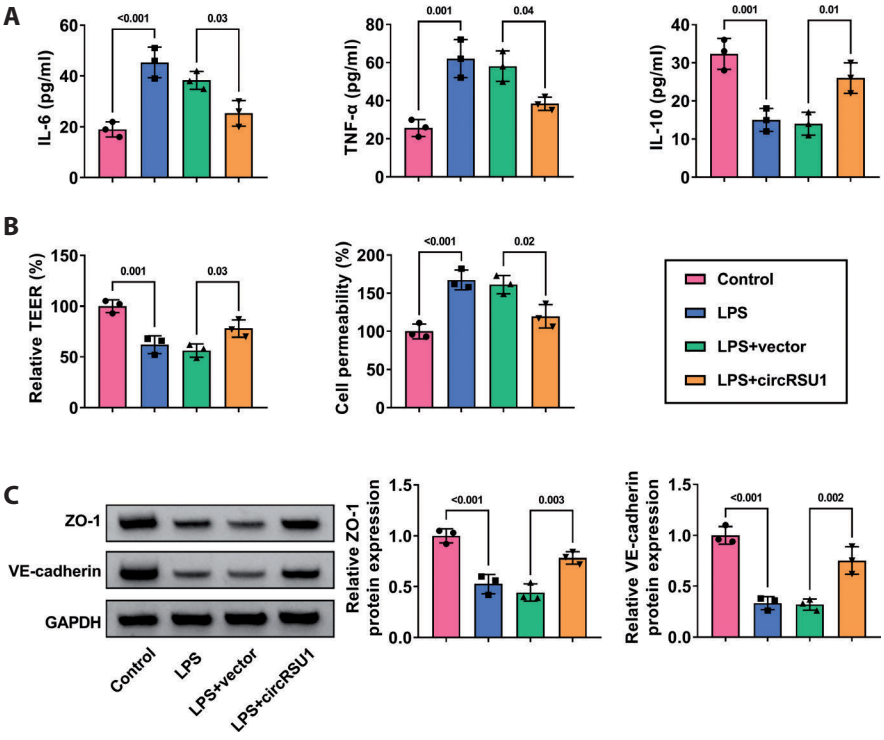

Figure 3. CircRSU1 overexpression reverses LPS-induced HPMEC dysfunction. HPMECs were transfected with circRSU1 or vector, followed by treatment with 1 μg/ml LPS for 12 h. **A.** ELISA analysis for the levels of IL-6, TNF-α and IL-10. **B.** Evaluation of cell permeability by TEER and FITC with transwell assay. **C.** Western blotting analysis for the levels of tight junction markers ZO-1 and VE-cadherin.

also rescued by miR-1224-5p inhibition (Fig. 5D). We also observed that LPS challenge-induced decrease of the TEER value and increase of PA% value in HPMECs were rescued by miR-1224-5p inhibitor (Fig. 5E,F). Moreover, inhibition of miR-1224-5p led to the up-regulation of the tight junction markers ZO-1 and VE-cadherin in HPMECs under LPS treatment (Fig. 5G). Altogether, inhibition of miR-1224-5p could protect HPMECs from LPS-induced injury.

ITGA5 is a target of miR-1224-5p in HPMECs

Next, we explored the underlying targets of miR-1224-5p in HPMECs. Based on the prediction of starbase, miR-1224-5p possesses the binding site on ITGA5 (Fig. 6A). The results of dual-luciferase reporter assay suggested that the luciferase activity in HPMECs co-transfected with miR-1224-5p and WT-ITGA5 reporter was obviously declined, while that

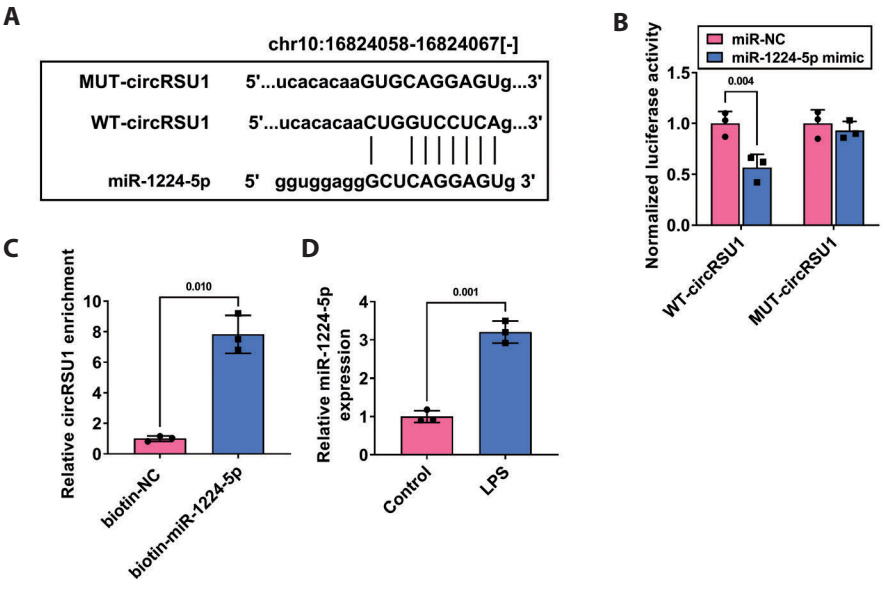

Figure 4. MiR-1224-5p is a target of circRSU1 in HPMECs**. A.** miR-1224-5p has the complementary sequences on circRSU1. The interaction between miR-1224-5p and circRSU1 was verified by dual-luciferase reporter assay (**B**) and RNA pull-down assays (**C**). **D.** qRT-PCR analysis for miR-1224-5p expression in HPMECs treated with 1 μg/ml LPS for 12 h.

of miR-1224-5p and MUT-ITGA5 reporter group did not change (Fig. 6B). RIP pull-down assay showed that ITGA5 was efficiently pulled down by biotin-miR-1224-5p probes compared with the control probes (Fig. 6C), further verifying the interaction between miR-1224-5p and ITGA5. In addition, LPS treatment reduced ITGA5 expression both at mRNA and protein levels, which was rescued by miR-1224-5p inhibition (Fig. 6D,E). Therefore, these data confirmed that miR-1224-5p targeted ITGA5 and suppressed its expression.

miR-1224-5p overexpression partially reverses the protective effects of circRSU1 on LPS-induced HPMECs

To study whether circRSU1 regulated LPS-induced HPMEC dysfunction was mediated by the miR-1224-5p/ITGA5 axis, the rescue assays were conducted. HPMECs were transfected with circRSU1 alone or together with miR-1224-5p mimic. After LPS treatment, we showed that circRSU1 overexpression was accompanied by increased ITGA5 expression in LPS-treated HPMECs, while miR-1224-5p mimic introduction reversed this effect (Fig. 7A,B), indicating the circRSU1/miR-1224-5p/ITGA5 axis. Functionally, miR-1224-5p attenuated the promotion of cell migration (Fig. 7C,D) and tube formation (Fig. 7E) that were caused by circRSU1 up-regulation in LPS-treated HPMECs (Fig. 7C–E). The increase of IL-6 and TNF-α, and decrease of IL-10 mediated by LPS in HPMECs was rescued by circRSU1, while miR-1224-5p mimic abolished these effects induced by circRSU1 in LPS-treated HPMECs (Fig. 7F). Besides, circRSU1-caused enhancement of TEER value and inhibition of PA% value

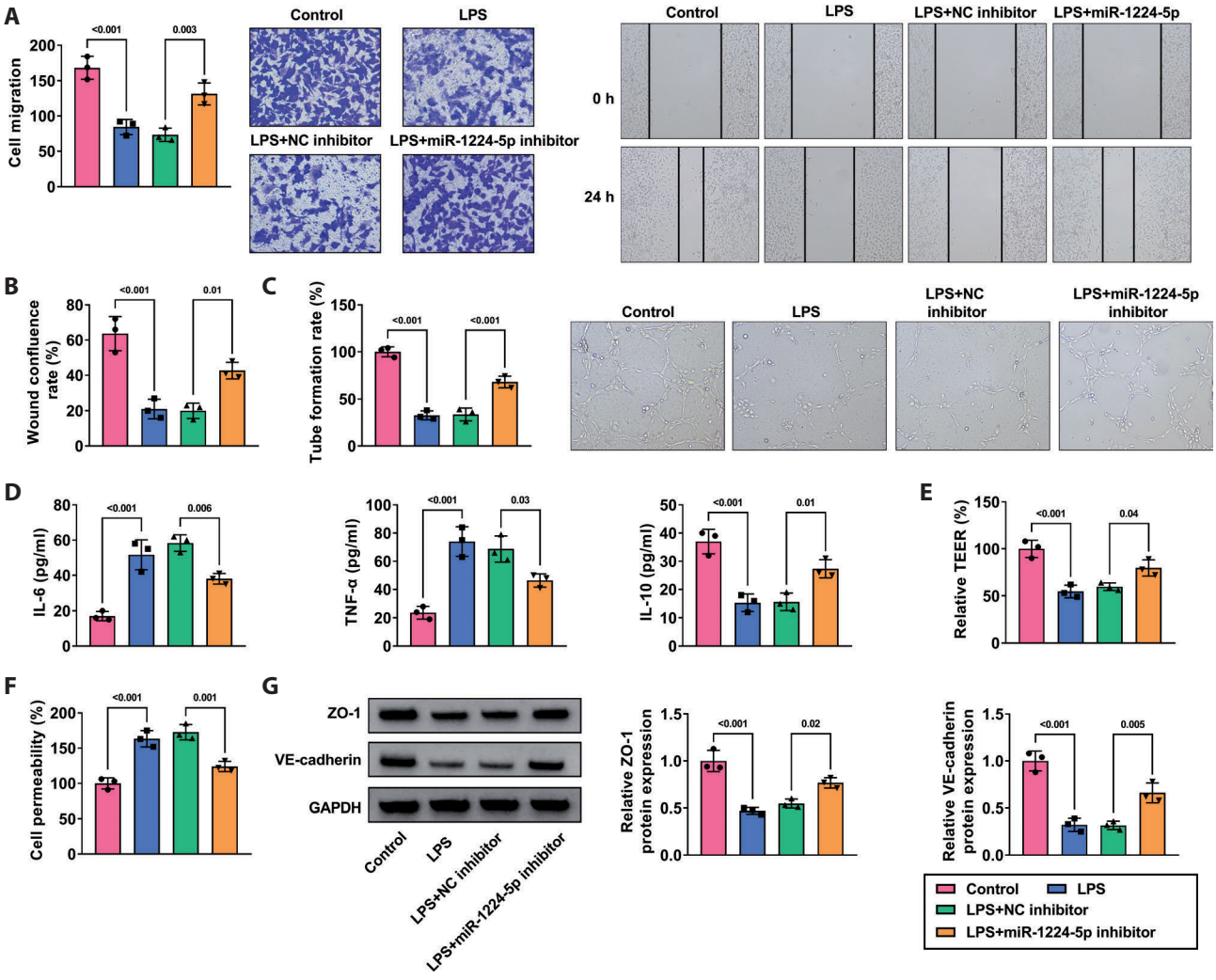

Figure 5. Inhibition of miR-1224-5p attenuates LPS-induced HPMEC dysfunction. HPMECs were transfected with miR-1224-5p inhibitor or NC inhibitor. Cell migration analysis using transwell (**A**) and wound healing assays (**B**). **C.** Tube formation assay for the analysis of cell tubulogenesis ability. **D.** ELISA analysis for the levels of IL-6, TNF-α and IL-10. Evaluation of cell permeability by TEER (**E**) and FITC (**F**) with transwell assay. **G.** Western blotting analysis for the levels of tight junction markers ZO-1 and VE-cadherin.

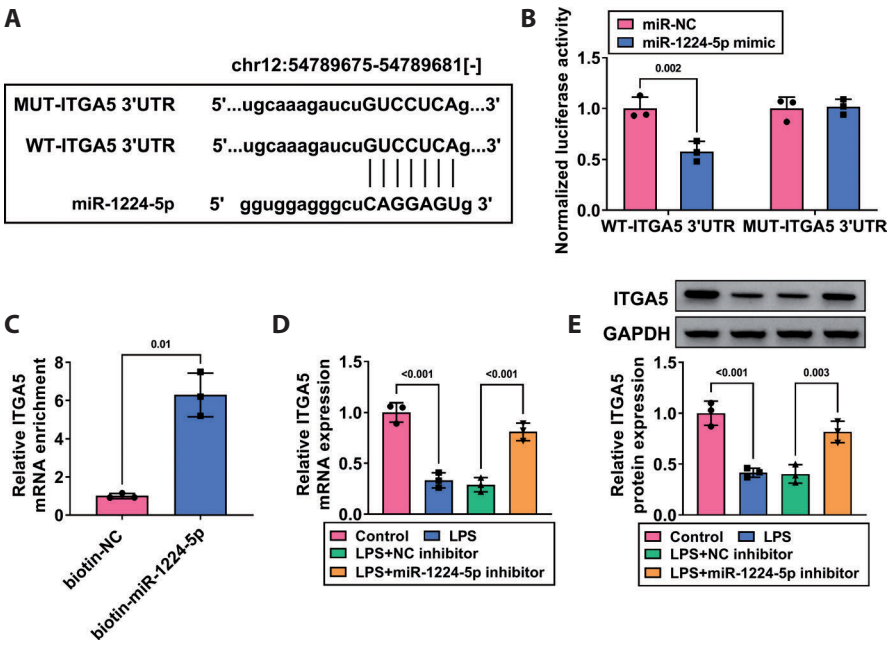

Figure 6. ITGA5 is a target of miR-1224-5p in HPMECs. **A.** miR-1224-5p has the complementary sequences on ITGA5. The interaction between miR-1224-5p and ITGA5 was verified by dual-luciferase reporter assay (**B**) and RNA pull-down assays (**C**). qRT-PCR (**D**) and Western blotting (**E**) analysis for ITGA5 expression in HPMECs treated with LPS, LPS+NC inhibitor, or LPS+miR-1224-5p inhibitor.

in LPS-treated HPMECs were counteracted after the up-regulation of miR-1224-5p in cells (Fig. 7G,H). The levels of the tight junction markers ZO-1 and VE-cadherin in HPMECs treated with LPS were promoted by circRSU1 up-regulation and subsequently reduced in response to miR-1224-5p mimic (Fig. 7I). Collectively, circRSU1 regulated LPS-induced HPMEC dysfunction by miR-1224-5p.

Discussion

Currently, circRNAs have attracted great research interest for their biological characteristics, such as higher expression, structural stability, high conservation and tissue specificity in different species (Barrett and Salzman 2016; Chen et al. 2021). Sepsis is a common complication in patients with serious trauma, burns, or infection that results in life-threatening organ dysfunction, including ALI (Rhodes et al. 2017). Importantly, some circRNAs have been revealed to be closely related to sepsis-induced ALI (Bao et al. 2019; Beltrán-García et al. 2020). For example, circEXOC5 silencing could improve the lung injury of septic mice, and suppress ferroptosis in ALI cell models by the PTBP1/ACSL4 axis (Wang et al. 2022). Hu et al. (2023) showed that circ_0001498 was highly expressed in sepsis-related ALI patients, and circ_0001498 deficiency suppressed LPS-induced apoptosis and inflammation in lung fibroblast cells by decreasing SOX6 *via* miR-574-5p. CircTDRD9 was demonstrated to contribute to LPS-induced inflammatory response, oxidative stress, proliferation arrest, and cell fibrosis in A549 cells *via* up-regulating RAB10 through targeting miR-223-3p (Zhang et al. 2023a). Thus, circRNAs may be potential biomarkers for the diagnosis and treatment of sepsis. CircRSU1 is a functional circRNA, it was found to be elevated in the cartilage of osteoarthritis patients, and circRSU1 overexpression induced oxidative damage and reduced extracellular matrix loss in the mouse model (Yang et al. 2021). In addition, the knockdown of circRSU1 alleviated diabetes mellitus-induced retina vascular dysfunction by suppressing oxidative stress, vascular endothelial growth factor levels, and inflammation (Zhang et al. 2023b). However, the action of circRSU1 on species remains unclear. In our study, we showed a decreased circRSU1 in LPS-induced HPMECs. Functionally, re-expression of circRSU1 reduced the levels of pro-inflammatory cytokines IL-6 and TNF-α and increased the contents of anti-inflammatory cytokine IL-10 in HPMECs under LPS treatment. Besides, circRSU1 overexpression elevated the migration and tubule formation abilities of HPMECs. Moreover, circRSU1 was able to reverse LPS-induced enhancement of HPMEC permeability. VE-cadherin is a strictly endothelial specific adhesion molecule controlling blood vessel formation, vascular permeability and cellular junctions (Vestweber 2008). ZO-1 is an important component of tight junctions, which play key roles in balancing lung permeability through maintaining the formation of normal epithelial barrier (Englert et al. 2015). In this study, we also demonstrated that the reduction of VE-cadherin and ZO-1 proteins mediated by LPS in HPMECs was rescued after circRSU1 overexpression. In all, we have reason to believe that deregulated circRSU1 might be associated with the progression of sepsis-ALI.

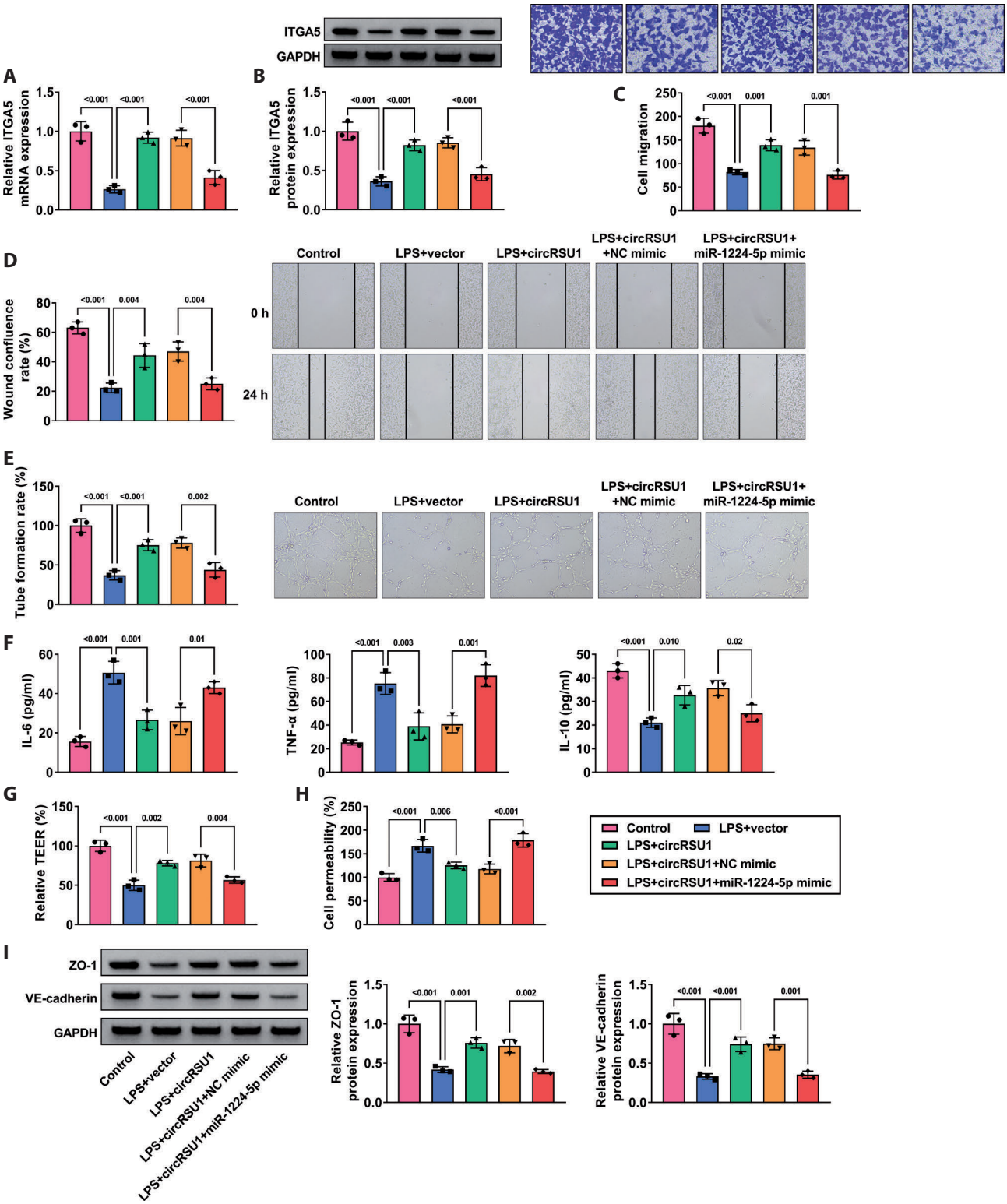

Figure 7. CircRSU1 overexpression reverses LPS-induced HPMEC dysfunction by miR-1224-5p. HPMECs were transfected with circRSU1 alone or together with miR-1224-5p mimic, followed by LPS treatment. qRT-PCR (**A**) and Western blotting (**B**) analysis for ITGA5 expression. Cell migration analysis using transwell (**C**) and wound healing (**D**) assays. **E.** Tube formation assay for the analysis of cell tubulogenesis ability. **F.** ELISA analysis for the levels of IL-6, TNF-α and IL-10. Evaluation of cell permeability by TEER (**G**) and FITC (**H**) with transwell assay. **I.** Western blotting analysis for the levels of tight junction markers ZO-1 and VE-cadherin.

Given the cytoplasmic distribution of circRSU1, we then explored the potential miRNA/mRNA axis of circRSU1 in HPMECs, and the circRSU1/miR-1224-5p/ITGA5 axis in HPMECs was first identified. miRNAs are small noncoding molecules that can modulate diverse biological functions by posttranscriptionally modulating their mRNAs (Chou et al. 2018). Besides, the expression alteration of some miRNAs has been pointed out to be involved in the process of sepsis-ALI (Xie et al. 2018; Ju et al. 2018). miR-1224-5p is a functional miRNA, and was demonstrated to contribute to LPS-evoked inflammatory and oxidative injury and pulmonary dysfunction in sepsis-ALI models (Liu et al. 2022). Consistent with the previous findings, we also confirmed that inhibition of miR-1224-5p counteracted LPS-triggered inflammation, the inhibition of cell migration and tube formation, as well as the enhancement of cell permeability in HPMECs. Moreover, miR-1224-5p overexpression could reverse the protective effects of circRSU1 on HPMEC function. TGA5 is a member of the integrin alpha chain family that serves as cell adhesion receptors to form physical connections between cells and extracellular matrix or cells to cells. Xu et al. indicated that ITGA5 acted as a target of miR-92a to ameliorate miR-92a-induced endothelial barrier dysfunction (Xu and Zhou 2020), indicating the potential implication of ITGA5 in sepsis-ALI. Therefore, we speculated that circRSU1 might regulate the progression of sepsis-ALI *via* miR-1224-5p/ITGA5 axis. Nevertheless, the lack of *in vivo* assays and the functional experiments of ITGA5 in HPMEC dysfunction is a certain limitation in this work, which will be implemented in future experiments.

In conclusion, we first demonstrated that circRSU1 could alleviate LPS-induced HPMEC injury by miR-1224-5p/ITGA5 axis, expanding the molecular contribution and potential clinical application of circRSU1 in sepsis-ALI prevention.

Conflict of interest. The authors have no conflict of interest to declare.

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