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Original Research

MiR-145 Alleviates Sepsis-Induced Inflammatory Responses and Organ Injury by Targeting ADAM17

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Abstract

Background: Current studies have demonstrated that disintegrin and metalloproteinase 17 (ADAM17) plays a critical role in the pathogenesis of sepsis. MicroRNA (miR)-145 is known to control immune responses as an anti-inflammatory modulatory molecule. However, a fundamental understanding of how miR-145 regulates ADAM17 and, more broadly, sepsis-induced inflammatory response remains unknown. **Methods**: We used western blotting and quantitative real-time PCR (qRT-PCR) to measure expression levels of ADAM17 and miR-145. Enzyme-linked immunosorbent assays (ELISA) were performed to measure cytokine production. To determine if ADAM17 is a target gene of miR-145, bioinformatics analyses and luciferase reporter assays were conducted. The impacts of ADAM17 and miR-145 on sepsis-induced inflammatory responses were accessed *in vitro* using human umbilical endothelial cells (HUVECs) treated with lipopolysaccharide (LPS). Sepsis-induced inflammatory response was measured *in vivo* using a polymicrobial septic mouse model induced by cecal ligation and puncture (CLP) with pre-injection of a miR-145 agomir. **Results**: In HUVECs treated with LPS, miR-145 expression was downregulated and miR-145 negatively regulated ADAM17 expression through direct binding to the ADAM17 transcript 3′-UTR. MiR-145 overexpression markedly reduced LPS-induced inflammatory cytokine production by targeting ADAM17 in HUVECs. In comparison to CLP-induced septic mice treated with a control agomir, treatment with a miR-145 agomir significantly reduced the expression of ADAM17, numerous downstream cytokines such as IL-6, TNF-α, IL-1β and MCP-1, and the endothelial injury factors ICAM-1, VCAM-1. The miR-145 agomir also alleviated acute lung and kidney injury and improved the survival rate of septic mice. **Conclusions**: This study showed that miR-145, by specifically targeting ADAM17, negatively regulates sepsis-induced inflammatory responses and vascular endothelial injury, and ultimately improved organ injury and survival during sepsis. The underlying mechanism for the regulation of ADAM17 expression by miR-145 and sepsis-induced inflammatory reactions may offer sepsis patients a novel therapeutic option.

Keywords: ADAM17; miR-145; sepsis; inflammation; vascular endothelial injury; organ injury

1. Introduction

Sepsis-3 describes a sepsis condition of organ dysfunction brought on by a multifaceted host response to an infection, with a complex pathogenesis identified to engage early activation of both pro- and anti-inflammatory responses [1]. Sepsis continues to be the primary cause of mortality in intensive care units (ICU) globally, claiming millions of lives annually and placing a heavy burden on health care systems [2,3].

A disintegrin and metalloproteinase 17 (ADAM17), is one of the major shedding enzymes within the ADAM family, and is widely expressed in endothelial cells, leukocytes, and platelets [4]. ADAM17 is engaged in the shedding of over 80 cellular substrates, including inflammatory cytokines (e.g., TNF-α [5]), cytokine receptor (e.g., IL-6R [6]), and endothelial adhesion proteins (e.g., VCAM-1 [7]). The shedding of these molecules function as a trigger in the activation of several inflammation-associated signaling pathways which play critical roles in the pathogenesis of sepsis [8,9]. ADAM17 is upregulated in sepsis patients and septic animal models which confers susceptibility to organ malfunction and mortality [10–13]. Antagonism or genetic deletion of ADAM17 contributed to bacterial clearance and a significant decrease in inflammatory response, and offered a benefit in survival to septic mice [5,14–16]. These lines of evidence indicated a crucial role of ADAM17 in the pathogenesis of sepsis.

MicroRNAs (miRNAs) are small endogenous RNAs that generally target the 3′-untranslated region (3′-UTR) of mRNAs and regulate gene expression at post-

transcriptional level through the suppression of protein translation [17]. Growing evidence have shown a vital role for miRNAs in modulating activation of immune cells, inflammatory cytokines shedding, and immunological responses [3,17]. It has been observed that certain miRNAs, namely miR-150 and miR-146a, are downregulated in peripheral blood of septic patients, and both miRNAs exerted a significant effect on the pathogenesis of sepsis [3]. With regards to other miRNAs, miR-23b targeted ADAM10 and negatively regulated sepsis-induced inflammatory responses [18]. Similarly, miR-375 ameliorated sepsis by downregulating miR-21 levels via inhibiting JAK2-STAT3 pathway [19].

Recently, the effects of miR-145 on sepsis and other inflammatory responses *in vivo* and *in vitro* have been investigated. Cao *et al.* [20] identified a significant decrease of miR-145 in exosomes from blood samples of septic patients and in lung tissues from LPS-treated mice. Additionally, miR-145 was found to attenuate sepsis-induced acute lung injury. Huang *et al*. [21] found that miR-145 regulated macrophage polarization by targeting IL-16 mRNA, while the function of miR-145 in improving macrophage-mediated inflammation through Arf6 was observed in other studies [22].

Bioinformatic analysis predicted an extraordinarily well-conserved binding between 3′-UTR of the ADAM17 mRNA and miR-145. In the present study, we sought to establish a role for miR-145 in the regulation of ADAM17, sepsis-associated inflammation, and vascular endothelial injury. Functional experiments in HUVECs and a polymicrobial sepsis mouse model were used to investigate the role of miR-145 in regulating inflammatory responses by targeting ADAM17 during sepsis both *in vitro* and *in vivo*, respectively. The fundamental mechanism in which miR-145 regulates ADAM17 production and sepsis-induced inflammatory injury may provide critical insight for new pharmacologic targets and novel strategies for sepsis therapy.

2. Materials and Methods

2.1 Cell Culture, Cell Engineering, and LPS Treatment

American Type Culture Collection (ATCC; Manassas, VA, USA) provided human umbilical vein endothelial cells (HUVECs) and the human embryonic kidney cell line 293T. All cell lines were validated by STR profiling and tested negative for mycoplasma. Cells were all cultured in a humidified incubator at 37 °C and 5% CO_2. RPMI 1640 or DMEM high-glucose medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA), containing 1% streptomycin/penicillin (Sigma-Aldrich, ST. LOUIS, MO, USA) and 10% fetal bovine serum (Invitrogen, Waltham, MA, USA) were used to culture the HUVECs and 293T cells, respectively.

GenePharma, Inc. (Shanghai, China) designed and constructed the LV-miR-145 plasmid consisting of the green fluorescence protein (GFP) reporter gene that overexpressed miR-145, and the LV-negative control (LV-NC) plasmid which does not target any recognized human genes. To establish cell lines that stably overexpress miR-145, HUVECs were infected with LV-miR-145 and control cells were infected with LV-NC. The related sequences were as followed: miR-145, 5′-GTCCAGTTTTCCCAGGAATCCCT-3′; NC, 5′-TTCTCCGAACGTGTCACGT-3′. Then the cells were observed and imaged using an inverted fluorescent microscope (Nikon ts2-fl) to initially evaluate the transfection efficiency. qRT-PCR analysis was performed to verify miR-145 overexpression in HUVECs infected with LV-miR-145. Cells were stimulated with 500 ng/mL LPS from Escherichia coli O55:B5 (L2880, Sigma–Aldrich, USA; dissolved in PBS) for 6 hours in a 5% CO_2 incubator at 37 °C. And the control cells underwent a treatment with equal volume PBS.

2.2 Laboratory Animals

C57BL/6 male mice aged 5–8 weeks were utilized in this study. The experimental animal ethics committee of Guangdong Medical University reviewed and approved the animal experimentation protocols outlined in this manuscript. Per a previous study [23], the cecal ligation and puncture (CLP) operation was performed on mice to construct the polymicrobial sepsis model. Briefly, the mice were anesthetized using ketamine (100 mg/kg) and xylazine (5 mg/kg) by intraperitoneal injections. A surgical incision was subsequently made in the middle of abdomen, cecum was uncovered, ligated beneath the ileocaecal valve, punctured twice using a 20-gauge needle, and a tiny amount of feces from both sides of the perforation site was gently extruded. After the surgery, the cecum was placed back into the abdominal cavity and the incision was sutured. In sham-operation mice, the same surgical operation was carried out without conducting the CLP operation. All mice were given a subcutaneous injection of normal saline (50 mL/kg) immediately after the procedure to sustain hemodynamic conditions. Throughout the experiments, mice were provided water and food *ad libitum*.

The miR-145-5p agomir (5′-GUCCAGUUUUCCCAGGAAUCCCU-3′) and miR-145 agomir normal control (NC) agomir (5′-UUCUCCGAACGUGUCACGUTT-3′) were synthesized by GenePharma, Inc. (Shanghai, China). Mice were allocated into three groups at random: Sham, CLP+NC and CLP+miR-145 agomir. Mice in the CLP+miR-145 agomir group were intraperitoneally injected with miR-145 agomir (30 mg/kg) 12-h before operation, while mice in CLP+NC group were injected with the miR-145 NC agomir the same time. Twelve hours after the procedure, blood was taken from mice and the serum concentrations of IL-6, IL-1β, TNF-α, VCAM-1, ICAM-1 and MCP-1 were measured.

2.3 RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Following the manufacturer's protocol, total RNA was extracted from cells using TRIzol reagent (Beyotime Biotechnology, Shanghai, China). The complementary DNA (cDNA) was synthesized for qRT-PCR analysis of ADAM17 by using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Shiga, Japan). An Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used for qRT-PCR using a SYBR Green RT-PCR Kit (Takara). Hairpin-it™ microRNA RT-PCR Quantitation Kit (GenePharma, Inc., Shanghai, China) was used for qRT-PCR analysis of miR-145. Sangon Biotech (Shanghai, China) Co., Ltd. designed the primers of qRT-PCR according to the Primer3Plus software, available online, version 3.3.0 (http://www.bioinformatics.nl/primer3plus), as followed: ADAM17, 5′-CGACTGCACGTTGAAGGAAG-3′ (forward) and 5′-TTAATCGCCTCCTGGCACTT-3′ (reverse); GAPDH 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward) and 5′-TGGTGAAGACGCCAGTGGA-3′ (reverse); miR-145 5′-TGCCGAGTCCAGTTTTCCC-3′ (forward) and 5′-TATGGTTGTTCACGAGTCCTTCAC-3′ (reverse); and U6 5′-CGCTTCGGCAGCACATATAC-3′ (forward), and 5′-TTCACGAATTTGCGTGTCATC-3′ (reverse). Relative levels of miR-145 and ADAM17 mRNA were calculated using the $2^{-\Delta\Delta CT}$ method, with U6 and GAPDH transcripts used as the internal standards, respectively [24].

2.4 Western Blot Analysis

RIPA lysis buffer (Beyotime Biotechnology) was used to solubilize cells or tissue homogenates. Total protein concentrations were measured using a BCA assay (Beyotime Biotechnology). A total of 30 µg Proteins were electrophoresed on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel (NCM Biotech, Suzhou, China). And 1.5 µL of Prestained Color Protein Ladder samples (P0076, Beyotime Biotechnology) was also loaded into each lane. The SDS polyacrylamide gels were subsequently transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with antibodies against ADAM17 (sc-390859, mouse monoclonal antibody, 1:800, Santa Cruz Biotechnology, Santa Cruz, CA, USA; A00604, rabbit polyclonal antibody, 1:800, Boster Biological Technology, China) or β-actin (sc-47778, mouse monoclonal antibody, 1:4000, Santa Cruz Biotechnology, USA) at 4 °C overnight. Subsequently, HRP-conjugated secondary antibodies were applied and a chemiluminescence imaging system (Tanon 5200 Multi, China) was used to visualize protein bands. Bands were quantified using Image J software, version 1.5.3 (NIH, Bethesda, MD, USA).

2.5 Bioinformatic Prediction and Dual-Luciferase Reporter Assay

We used several bioinformatic tools in this investigation, including Starbase (https://starbase.sysu.edu.cn) and TargetScan (http://www.targetscan.org), to predict the regulatory mechanism between ADAM17 and miR-145. The wild-type 3′-UTR of ADAM17 contained a target site predicted to bind miR-145, whereas an ADAM17 mutant did not contain this target site. Both wild-type and mutant sequences were cloned into pmirGLO luciferase reporter (Promega, Madison, WI, USA), and were designated ADAM17-3′UTR-Wt and ADAM17-3′UTR-Mut, respectively. Longqian Biotech (Shanghai, China) designed and established the reporter plasmids pmirGLO-ADAM17-3′UTR-Wt and pmirGLO-ADAM17-3′UTR-Mut. To create cell lines that stably overexpress miR-145 and NC sequences, 293T cells were infected with LV-miR-145 or LV-NC, respectively. Subsequently, pmirGLO-ADAM17-3′UTR-Mut or pmirGLO-ADAM17-3′UTR-Wt were utilized to co-transfect the cells using Lipofectamine 2000 (Invitrogen) based on the guidelines provided by the manufacturer. A dual-luciferase assay kit (Beyotime Biotechnology) was used in accordance with the manufacturer's instructions. Cells were lysed after a 48 hr incubation period to measure both Firefly and Renilla luciferase activity. Firefly luciferase is employed as a reporter gene, while Renilla luciferase is used as a standardized endogenous control in each individual analysis.

2.6 Cytokine Measurement by Enzyme-Linked Immunosorbent Assay (ELISA)

With the use of commercial ELISA kits from Boster Biological Technology (Wuhan, China), the expression levels of IL-6, TNF-α, IL-1β, MCP-1, ICAM-1, and VCAM-1 in the serum of mice and the culture supernatants of HUVECs were measured following the manufacturer's guidelines. Absorbance was measured at 450 nm using a multiscan spectrum instrument (H1M, Aglient, Santa Clara, CA, USA). A standard curve was used to interpolate specific cytokine concentrations.

2.7 Tissue Section Preparation and Immunohistochemical Staining

Kidney and lung tissues were removed from sacrificed mice challenged with either sham or CLP surgery and subsequently fixed in formaldehyde solution for 48 hours. Per conventional methodology, the fixed tissues were embedded in paraffin and sectioned (4.5 µm thickness) for immunohistochemical (IHC) staining, immunofluorescence (IF) staining and hematoxylin-eosin (H&E) staining.

To permeabilize the sections for IHC, immunostaining permeabilization solution with Triton X-100 (Beyotime Biotechnology) was used following deparaffinization and hydration. The slices were then subjected to steam in improved citrate antigen retrieval solution (Beyotime Biotech-

nology) for 30 minutes and cooled at room temperature for more than 30 minutes. Endogenous peroxidase activity was quenched with blocking buffer. Then, an anti-ADAM17 antibody (A00604, rabbit polyclonal antibody, 1:200, Boster Biological Technology) diluted to 1:300 was added overnight at 4 °C. After that, sections were incubated with polymer helper and polyperoxidase-anti-rabbit IgG (Boster Biological Technology, Wuhan, China) for 1 h at 37 °C. Diaminobenzidine (DAB; Boster Biological Technology) was used as the chromogen and following counterstaining with hematoxylin, specimens were dehydrated, mounted, and observed under a microscope. The staining intensity of ADAM17 was quantified using Image J software with the estimation of average optical density obtained.

2.8 Immunofluorescence Staining

The lung and kidney sections were permeabilized as described above and incubated with QuickBlock™ blocking buffer (Beyotime Biotechnology). After this, tissue slices were incubated with anti-ADAM17 antibody (A00604, rabbit polyclonal antibody, 1:300, Boster Biological Technology). Following a PBS rinses, the sections were stained in the dark with Alexa Fluor 488-labeled Goat Anti-Rabbit IgG (Beyotime Biotechnology). Subsequently, tissue slices were stained using DAPI in the dark and were observed under a fluorescence microscope. Captured images were then processed and analyzed using Image J software.

2.9 Hematoxylin-Eosin Staining and Histopathological Analysis

To evaluate the pathological change in lung and kidney tissues, the sections were stained with a hematoxylin-eosin (H&E) staining kit (Beyotime Biotechnology) according to the manufacturer's guidelines. As previously described [23,25,26], the pathological changes, specifically inflammatory cell infiltration, alveoli wall edema, pulmonary alveoli congestion, and hemorrhage in lung tissues, were graded using a scoring scheme from 0 to 3 and used to estimate sepsis-related lung damage. Histological scoring of kidney injury was scored as 0, no injury; 1, <25%; 2, 25–50%; 3, 50–75%; 4, >75% of affected areas per optical field [27]. Histologic evaluation of kidney injury was based on different manifestations of tubular injury, including tubular cell necrosis, brush border loss, vacuolization, tubule dilation, or cast formation.

2.10 Statistical Analyses

GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA) and SPSS software version 25.0 (SPSS Inc., Chicago, IL, USA) were used for statistical analyses. The difference between two independent samples was evaluated using either Student's *t* test or a non-parametric Mann-Whitney U test. For comparison of multiple groups, a one-way ANOVA followed by Tukey's multiple comparison post-hoc test was used. The log-rank test was used to assess the difference of Kaplan-Meier survival analyses. $p < 0.05$ was considered statistically significant.

3. Results

3.1 miR-145 Downregulated ADAM17 Expression in HUVECs

After stimulating with LPS (500 ng/mL) for 6 h, miR-145 expression in HUVECs was detected by using qRT-PCR. As shown in Fig. 1A, miR-145 expression in LPS-stimulated HUVECs was significantly decreased compared with HUVECs treated with PBS as control. For the purpose of assessing the role of miR-145 in regulation of ADAM17 expression in HUVECs, we used LV-miR-145 to infect HUVECs and establish stably miR-145 overexpressed cell lines. Results of qRT-PCR validated overexpression of miR-145 in HUVECs infected with LV-miR-145 (Fig. 1B). As presented in Fig. 1C,D, both qRT-PCR and western blot analysis indicated that miR-145 overexpression significantly decreased ADAM17 mRNA expression and protein production by 62.3% and 32.6%, respectively.

3.2 MiR-145 Regulated ADAM17 Expression via Targeting the 3′-UTR of ADAM17

Bioinformatic analyses predicted a binding site for miR-145 within the 3′-UTR of the ADAM17 mRNA. A dual-luciferase reporter assay was employed to verify direct binding between ADAM17 and miR-145. 293T cells were infected with LV-NC or LV-miR-145. GFP fluorescence was observed to track transfection efficiency, and qRT-PCR analysis validated overexpression of miR-145 in 293T cells infected with LV-miR-145 (Fig. 1E,F). Dual-luciferase reporter plasmids of pmirGLO-ADAM17-3′UTR-Wt and pmirGLO-ADAM17-3′UTR-Mut were engineered (Fig. 1G). Subsequently, the 293T cells overexpressing miR-145 or NC sequences were transfected with either pmirGLO-ADAM17-3′UTR-Mut or pmirGLO-ADAM17-3′UTR-Wt. As shown in Fig. 1H, pmirGLO-ADAM17-3′UTR-Wt reporter luciferase activity was significantly inhibited by miR-145 overexpression, whereas no apparent change was observed in the reporter luciferase activity of pmirGLO-ADAM17-3′UTR-Mut.

3.3 miR-145 Alleviated LPS-Induced Endothelial Inflammatory Injury by Targeting ADAM17 in HUVECs

To produce stable cell lines and test the impact of miR-145 on LPS-stimulated ADAM17 expression as well as inflammatory reactions in HUVECs, we used lentivirus engineered from the LV-ADAM17-siRNA and LV-miR-145 plasmid to transfect HUVECs and GFP fluorescence was observed in an inverted fluorescent microscope (Fig. 2A). As presented in Fig. 2B, the protein production of ADAM17 was significantly decreased by 33.3% in HUVECs trans-

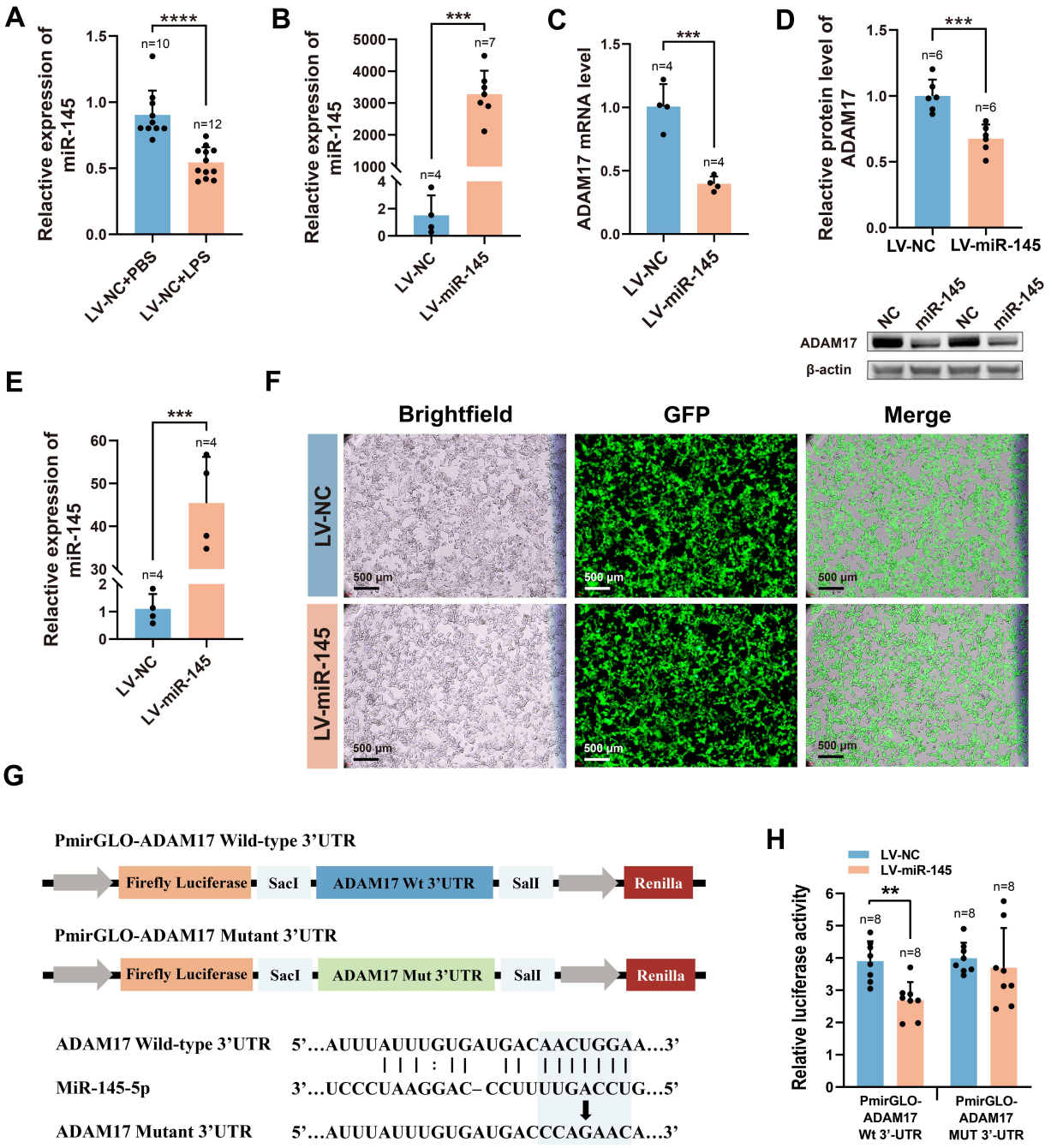

Fig. 1. Lipopolysaccharide (LPS) reduces miR-145 expression, overexpression of miR-145 downregulated ADAM17 expression by directly binding to the 3′UTR of ADAM17. (A) The expression of miR-145 in human umbilical vein endothelial cells (HUVECs) stimulated with 500 ng/mL LPS for 6 h was detected by quantitative real-time polymerase chain reaction (qRT-PCR). (B) qRT-PCR analysis was performed to verify the transfection efficiency of LV-miR-145 in HUVECs. (C,D) qRT-PCR and western blot analysis was performed to evaluate the effect of miR-145 overexpression on ADAM17 gene expression and protein production in HUVECs, respectively. (E,F) Green fluorescence protein (GFP) fluorescence was observed in an inverted fluorescent microscope to track transfection efficiency of LV-miR-145 and LV-NC (both plasmids consisting of the GFP reporter gene) in 293T cells, and the expression levels of miR-145 in 293T cells were detected by qRT-PCR. (G) Bioinformatics analysis of miR-145 predicted binding site in the 3′-UTR of ADAM17 and the mutations introduced into the 3′-UTR. (H) The luciferase activities of reporter vectors carrying wild-type (Wt) or mutant (Mut) 3′-UTR of ADAM17 were measured by dual-luciferase report assay in 293T cells infected with LV-miR-145 or LV-NC. Data represent one or two independent experiments with at least three technical replicates per experiment. Error bar represent standard deviation of the mean (SD). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. NC, negative control.

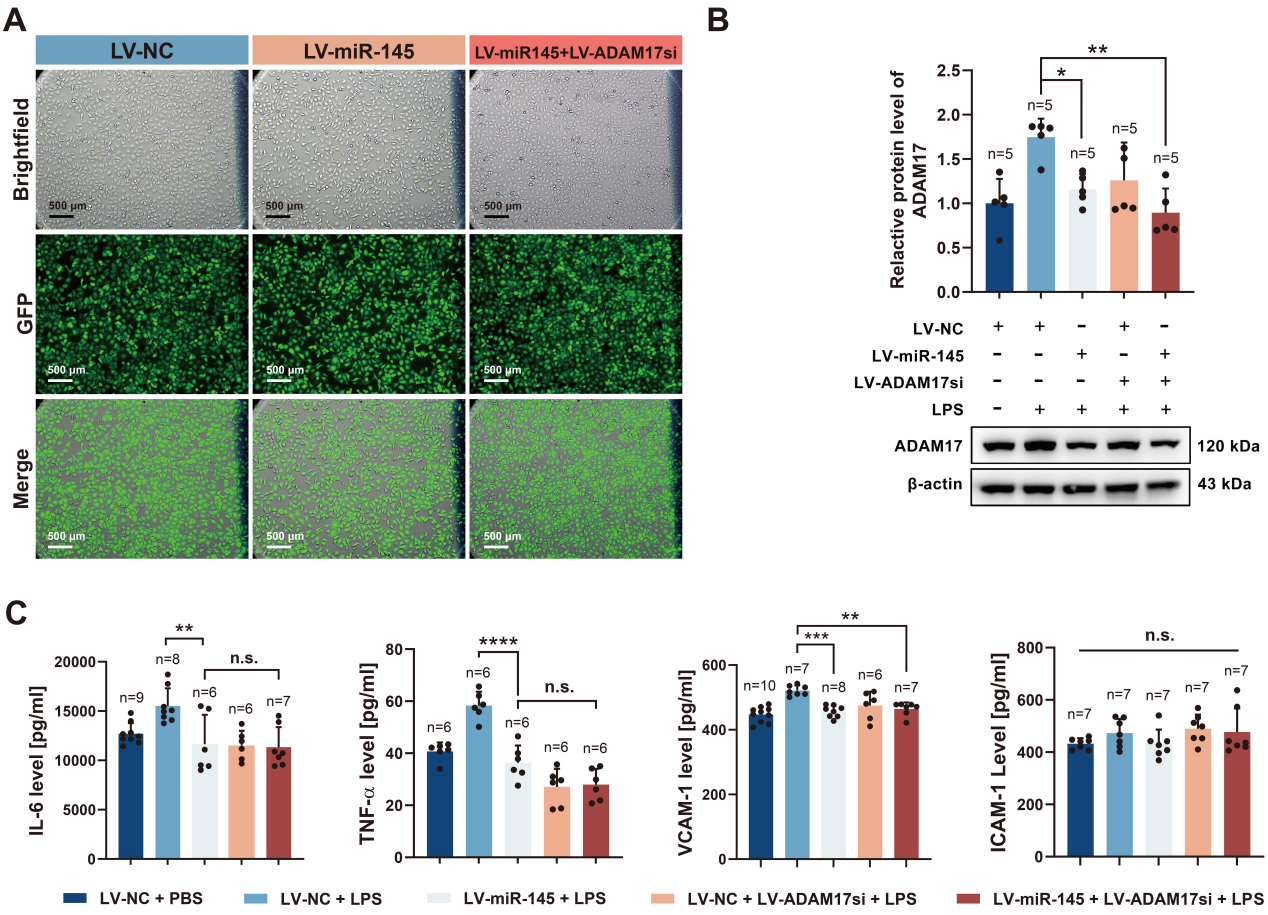

Fig. 2. miR-145 alleviated LPS-induced endothelial inflammation by targeting ADAM17 in HUVECs. HUVECs were infected by LV-NC, LV-miR-145, LV-ADAM17-siRNA or the combined of LV-miR-145 and LV-ADAM17-siRNA. (A) Green fluorescence protein (GFP) fluorescence was observed in an inverted fluorescent microscope to track transfection efficiency of LV-NC, LV-miR-145 and LV-ADAM17-siRNA. (B) After 6 hours of LPS (500 ng/mL) stimulation, the expression of ADAM17 in HUVECs was detected by western blot. (C) After 6 hours of LPS (500 ng/mL) stimulation, ELISA assay was performed to detect the expression levels of IL-6, TNF-α, VCAM-1 and ICAM-1 in cell supernatant. Data represent one or two independent experiments with at least three technical replicates per experiment. Error bar represent standard deviation of the mean (SD). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n.s., no significance.

fected with LV-miR-145 compared with cells transfected with LV-NC following LPS stimulation. Similar results were observed in HUVECs transfected with LV-ADAM17-siRNA. These results indicated that overexpression of miR-145 produced a similar effect to siRNA-induced knockdown of ADAM17 expression. In addition, ADAM17 knockdown significantly downregulated the expression levels of IL-6, TNF-α, and VCAM-1, and that these results were consistent with findings in cells that overexpressed miR-145 (Fig. 2C). The combined transfection of HUVECs with LV-miR-145 and LV-ADAM17-siRNA did not markedly reduce ADAM17 and the LPS-induced expression of IL-6, TNF-α and VCAM-1 in HUVECs when compared with cells transfected with LV-ADAM17-siRNA or LV-miR-145 individually, indicating that miR-145 alleviated LPS-induced endothelial inflammation in response to LPS challenge by targeting ADAM17.

3.4 Overexpression of miR-145 Protects against Inflammatory Response and Acute Lung Injury during Polymicrobial Sepsis by Regulating ADAM17 Expression

Given the role of miR-145 in the downregulation of ADAM17 expression *in vitro*, we next investigated whether miR-145 overexpression is associated with diminished inflammatory response *in vivo*. Mice were injected with a miR-145 agomir or control (NC) agomir 12 h prior to CLP or sham surgery. Subsequently, mice were sacrificed and serum, kidney, and lung tissues obtained 12 h after surgery (Fig. 3A). Western blotting indicated that ADAM17 expression was significantly decreased by 58.5% in lung tissues taken from polymicrobial septic animals treated with miR-145 agomir compared to those treated with the NC agomir (Fig. 3B).

Next, we conducted IHC and IF staining with an antibody against ADAM17 to locate the protein in lung tis-

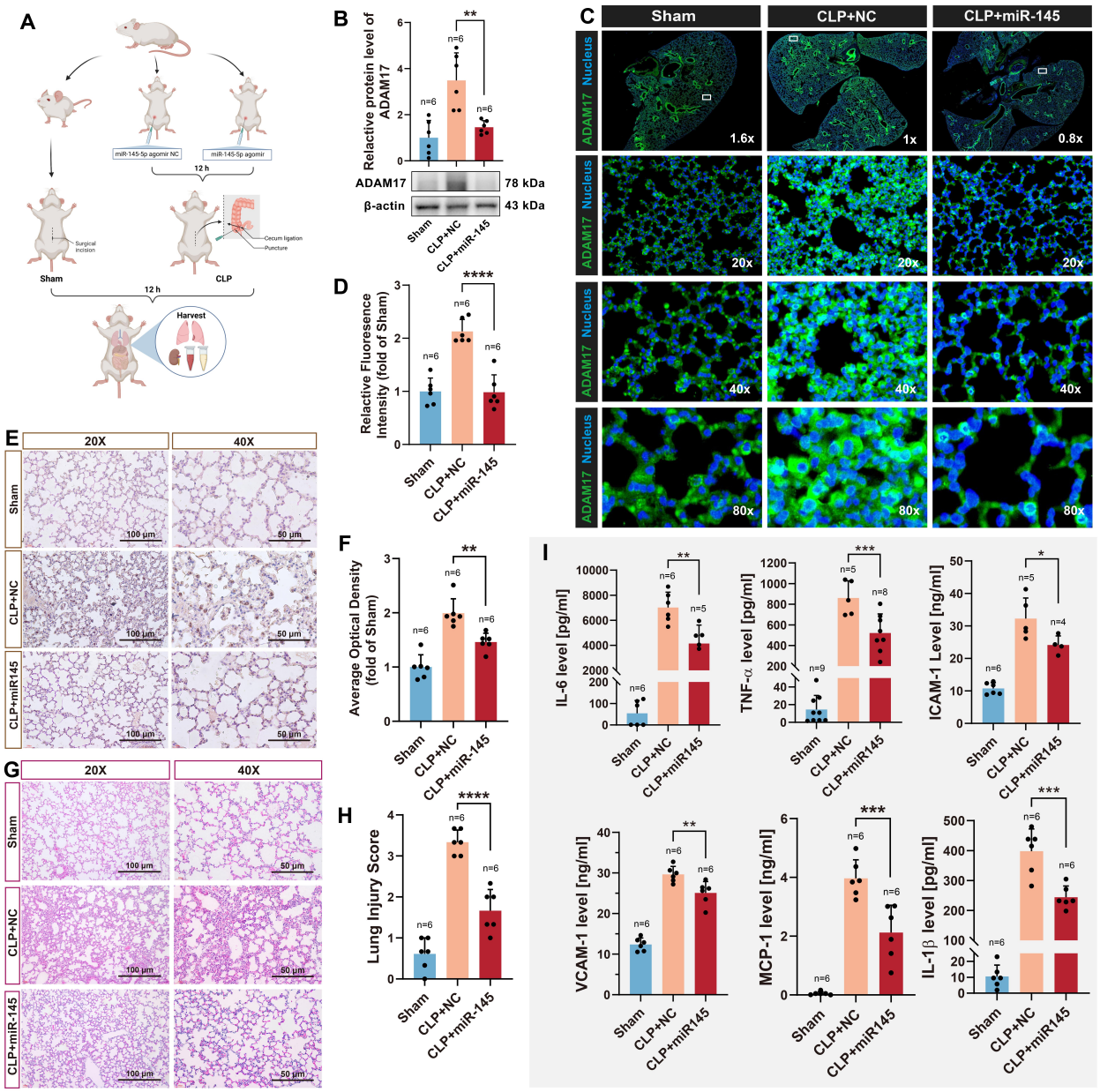

Fig. 3. Overexpression of miR-145 reduces expression of ADAM17, attenuates sepsis-induced inflammatory responses and acute lung injury. (A) Schematic of experimental design and time line. Polymicrobial sepsis model of mice was induced in C57BL/6 mice by cecal ligation and puncture (CLP) operation, and miR-145 agomir or miR-145 agomir NC (30 mg/kg) were delivered into the mice by intraperitoneal injection 12 h before CLP. The same surgical procedure but without CLP was performed in sham-treated mice. Lung tissues, kidney tissues and blood were harvested 12 h after the operation. (B) Expression of ADAM17 protein in lung tissues was detected by western blot. (C,D) Immunofluorescence (IF) staining with antibody against ADAM17 (green) was performed to observe ADAM17 localization in lung tissue sections, and cell nucleus was stained by DAPI (blue). Results of relative fluorescence intensity reflecting the level of ADAM17 expression. (E,F) Representative images showing ADAM17 immunohistochemical (IHC) staining in lung tissue sections. Scale bars, 100 μm and 50 μm. Results of average optical density reflecting the level of ADAM17 expression. (G,H) Representative images of H&E staining of lung tissue sections. Scale bars, 100 μm, 50 μm. The severity of lung injury was evaluated by lung injury score. (I) Cytokines (IL-1β, IL-6, TNF-α, ICAM-1, VCAM-1 and MCP-1) in the serum of mice were measured by ELISA assays. Error bar represent standard deviation of the mean (SD). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

sues. As shown in Fig. 3C,D, results of immunofluorescence staining showed that ADAM17 expression in lung tissues of mice following CLP was increased relative to sham mice, while significant downregulation of ADAM17

was observed in mice treated with the miR-145 agomir when compared to controls. Results of IHC analysis also indicated ADAM17 production in lung tissues of septic mice treated with miR-145 agomir was significantly reduced (Fig. 3E,F).

To further explore the severity of lung injury, we performed histopathological analysis of lung tissues. The results of H&E staining (Fig. 3G,H) showed obvious alveolar tissue damage and neutrophil accumulation induced by CLP within the lungs. Furthermore, evaluation of lung injury score indicated a significant reduction in mice injected with the miR-145 agomir compared to controls. These findings suggest that miR-145 protects against sepsis-induced inflammation and acute lung injury by suppressing ADAM17 expression. The serum levels of ICAM-1, VCAM-1, IL-1β, IL-6, TNF-α and MCP-1 in miR-145 agomir-pretreated mice were significantly lowered relative to those in mice treated with NC agomir following CLP-induced sepsis (Fig. 3I), indicating the anti-inflammatory function of miR-145.

3.5 miR-145 Agomir Reduces ADAM17 Expression in the Kidney and Alleviates Sepsis-Induced Acute Injury

As shown in Fig. 4A, ADAM17 protein production was significantly decreased by 44.8% in kidney tissues of mice treated with miR-145 agomir when compared to NC agomir-treated mice upon CLP operation. The results of IF analysis indicated that ADAM17 abundance was more concentrated in the renal cortex than in the medulla, and overall ADAM17 abundance was slightly reduced in kidney sections from miR-145 agomir-treated septic mice compared with the NC agomir-treated mice upon CLP operation (Fig. 4B,C). As presented in Fig. 4D,E, IHC indicated decreased production of ADAM17 in the kidney tissue from miR-145 agomir treated mice relative to NC agomir treated mice upon CLP operation. H&E staining indicated that miR-145 agomir significantly alleviated intracellular vacuolization, tubular cell necrosis, tubule dilation, and improved kidney histological scores compared to septic mice treated with control agomir (Fig. 4F,G). Taken together, these results suggest a protective function of miR-145 against sepsis-induced acute kidney injury by reducing the expression of ADAM17.

3.6 MiR-145 Agomir Improves Survival of CLP-Induced Septic Mice

As shown in Fig. 4H, Kaplan-Meier survival analysis demonstrated that miR-145 agomir injection significantly ($p = 0.0195$) improved septic mouse survival during a four-day window relative to septic mice injected with NC agomir. These findings clearly indicate a protective impact of miR-145 agomir against sepsis-induced mortality.

4. Discussion

Sepsis is a detrimental response to pathogenic microorganism infection inducing systemic inflammation. It has been demonstrated that dysregulated inflammatory and immunological responses, as well as dysfunction within vascular endothelium play crucial roles in the pathogenesis of sepsis [3]. LPS and other pathogen-associated molecular patterns (PAMPs) activate both immune cells and endothelium cells, which can generate a variety of inflammatory cytokines that seriously harm cells and disrupt microcirculation, ultimately leading to organ dysfunction. In the current study, we examined the molecular functions of the miR-145/ADAM17 axis on acute organ damage and inflammatory responses induced by sepsis. These results provide evidence for a mechanism by which miR-145 limits sepsis-induced organ injury and inflammation by downregulating ADAM17.

miRNAs, as posttranscriptional regulators by targeting 3′-UTR of their target gene through imperfect sequence complementarity, play an essential role in sepsis by modulating immunological and vascular responses. Bioinformatics analysis predicted a miR-145-binding site in the 3′-UTR of ADAM17. The present study attempted to investigate the potential regulatory effect of miR-145 on ADAM17 expression in endothelial cells. miR-145 overexpression significantly decreased ADAM17 mRNA expression and protein production in HUVECs by 62.3% and 32.6% respectively, indicating a regulatory effect of miR-145 on ADAM17 expression in endothelial cells. Furthermore, pmirGLO-ADAM17-3′UTR-Wt reporter luciferase activity was significantly inhibited by miR-145 overexpression, whereas no apparent change was observed in the reporter luciferase activity of pmirGLO-ADAM17-3′UTR-Mut, suggesting that miR-145 downregulated ADAM17 expression via targeting the 3′-UTR of ADAM17. Two recent studies have identified miR-145 as a microRNA targeting ADAM17 in nasopharyngeal carcinoma cells and liver cancer cells [28,29], which further supported our results of endothelial cells. Besides, using LPS-stimulated HUVEC model, we investigated the regulatory effects of miR-145 and ADAM17 in sepsis-induced endothelial inflammatory injury. We measured decreased expression of miR-145 in LPS-stimulated HUVECs, and attenuated inflammatory cytokine (VCAM-1, IL-6, and TNF-α) production in LPS-treated HUVECs overexpressing miR-145. These findings are consistent with the findings outlined by Ma *et al.* [30]. More importantly, the reduced inflammatory response was accompanied by reduced of ADAM17 expression. These findings offer solid evidence that miR-145 possesses anti-inflammatory properties in endothelial cells during sepsis-induced inflammatory response, and that this function is likely linked to decreased expression of ADAM17.

ADAM17, also known as tumor necrosis factor alpha-converting enzyme (TACE), mediates inflammatory reactions by cleaving the extracellular domains of proinflammatory substates at specific transmembrane sites [8,9]. The shedding of numerous substrates, such as VCAM-1, ICAM-1, IL-6R, and TNF-α, are induced by ADAM17, and in-

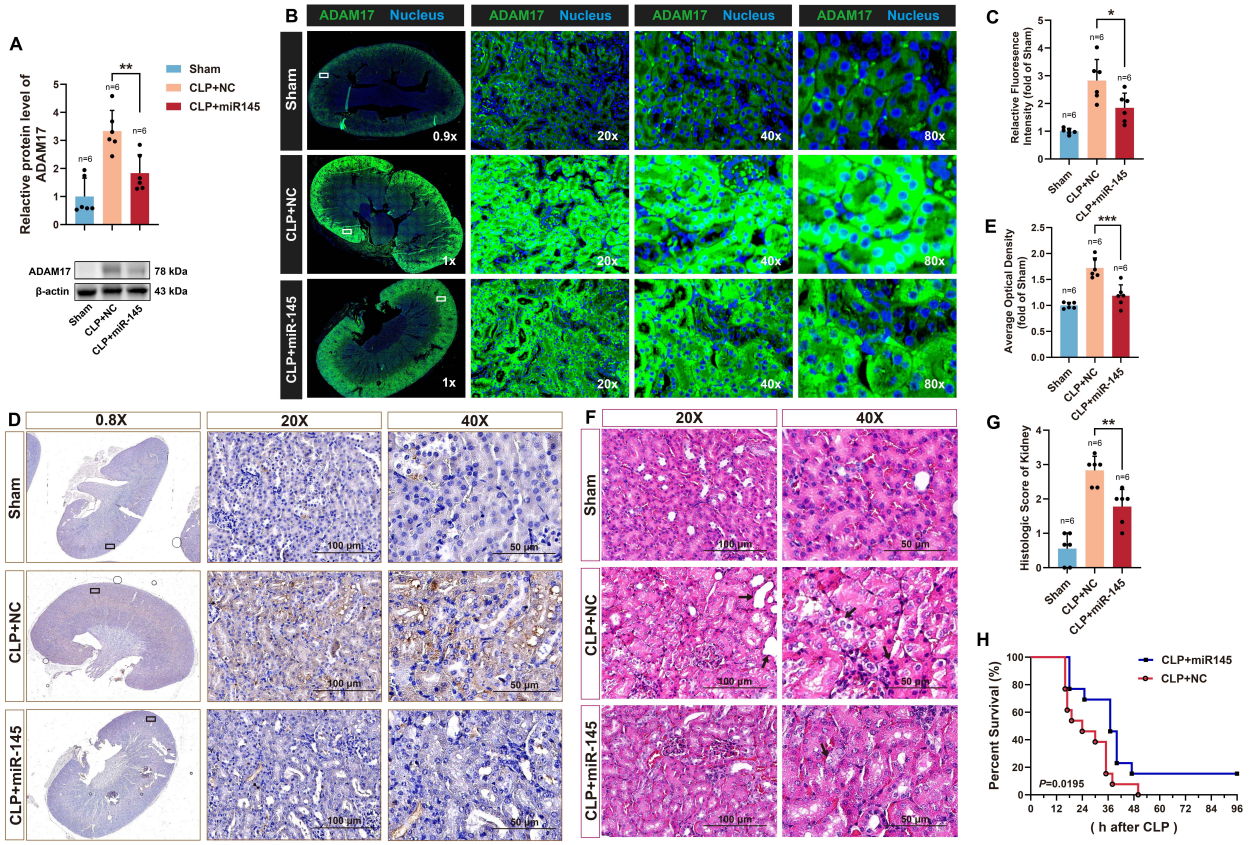

Fig. 4. MiR-145 agomir reduces expression of ADAM17, attenuates sepsis-induced acute kidney injury and offers survival benefit. (A) Expression of ADAM17 protein in kidney tissues was detected by western blot. (B,C) IF staining with antibody against ADAM17 (green) was performed to observe ADAM17 localization in kidney tissue sections, and cell nucleus was stained by DAPI (blue). Results of relative fluorescence intensity reflecting the level of ADAM17 expression. (D,E) Representative images showing ADAM17 IHC staining in kidney tissue sections. Scale bars, 100 μm and 50 μm. Results of average optical density reflecting the level of ADAM17 expression. (F,G) Representative images of H&E staining of kidney tissue sections. Scale bars, 100 μm and 50 μm. Semiquantitative analysis of tubular injury: tubular cell necrosis, loss of the brush border, vacuolization, tubule dilation, or cast formation. (H) The survival curves of septic mice injected with miR-145 agomir and miR-145 agomir NC respectively (n = 13 per group). Data were analyzed by log-rank test. Error bar represent standard deviation of the mean (SD). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

volved in regulating various cellular processes to actuate inflammatory response. Upregulated ADAM17 expression in septic patients and mice was found to be closely related to disease severity and mortality [10–13]. The results obtained in our study indicated a significant elevation of ADAM17 expression in LPS-stimulated HUVECs. The production of inflammatory cytokines IL-6, TNF-α and VCAM-1 in HUVECs upon LPS induction was significantly decreased by miR-145 overexpression or ADAM17 knockdown. These results indicated a role for ADAM17 in promoting vascular inflammation during septic response, and corroborate the findings of our previous study showing a relationship between ADAM17 and vascular inflammation in endothelial cells [10]. When endothelial cells interact with pathogens through pathogen recognition receptors (PRRs), they actuate a proinflammatory state and release cytokines [2]. ADAM17 (TNF-α Converting Enzyme) is the main proteases responsible for the release of TNF-α, which interacts with receptor TNF receptor 1 to induce the expression of various pro-inflammatory cytokines (e.g., IL-1β and IL-6), pro-proliferative/antiapoptotic proteins, and numerous chemokines (e.g., MCP-1) via NF-κB activation [31]. In addition, Notch signaling activated by ADAM17 results in direct upregulation of interleukin-6 expression [32,33]. Thus, we speculate that the reduction of IL-6 in HUVECs after miR-145 treatment might be due to the inhibition of NF-κB and Notch signaling following ADAM17 downregulation. Further experimental results showed that the combination of miR-145 overexpression and ADAM17 knockdown did not further alleviate ADAM17 and downstream cytokine production, which was similar to those induced by either miR-145 overexpression or ADAM17 knockdown alone. Hence, these results demonstrated that ADAM17 acted downstream of miR-145 to regulate sepsis-indued endothelial inflammatory injury.

To further elucidate the *in vivo* pathological mechanisms of miR-145 and ADAM17 we used a septic mouse model. CLP, a widely used model for establishing polymicrobial sepsis, markedly increased ADAM17 expression in lung and kidney tissue. Although substantial residual amount of ADAM17 remained, miR-145 treatment dramatically decreased ADAM17 expressions in CLP mice to a similar level as those in mice without CLP operation, indicating an antagonistic effect of miR-145 against upregulation of ADAM17 following sepsis. Evidence indicates that ADAM17-dependent shedding/modulation events are involved in the development and progression of inflammation via activation of several key inflammatory signaling including NF-κB and Notch, and various cellular processes [31–33]. Consistent with the results of the cell experiments, the expression levels of ADAM17 substrates (TNF-α, VCAM-1 and ICAM-1), as well as other pro-inflammatory cytokines (IL-6, IL-1β and MCP-1) were observed to be significantly decreased in septic mice with miR-145 agomir treatment compared with those treated with NC agomir. Actually, the inflammatory system *in vivo* is complex and vast, and the downregulation of ADAM17 by miR-145 treatment might exert a wider, more complicated role in regulation of body's inflammatory immune system response according to different substrates of ADAM17. Significantly, previous study demonstrated that mice deficient in ADAM17 showed significant downregulation in expression of inflammatory mediators such as IL-6 and IL-1β, and had a survival benefit following sepsis [34]. In addition, treatment with specific ADAM17 antagonist or inhibition also inhibited TNF-α and IL-6 expression and protected mice against sepsis [35,36]. All these evidences indicated a potential therapeutic target of ADAM17 for sepsis. We also found that miR-145 agomir benefitted overall survival of CLP-induced septic mice, a finding that is consistent with a previous study using LPS-treated mice [20,30]. Thus, we conclude that miR-145 functions in a protective capacity during vascular inflammatory reaction and serves to limit lung injury and improve survival rates in septic mice.

Sepsis is a life-threatening organ dysfunction due to dysregulated host response to infection, leading to inflammatory damage to nearly every organ system. Thus, we further evaluate the role of miR-145 agomir in sepsis-induced acute lung injury (ALI) and acute kidney injury (AKI). The expression of ADAM17 in lung tissue as judged by IHC and IF staining was decreased in septic mice treated with miR-145 agomir when compared to controls. Moreover, the histopathological analysis of lung tissues showed that miR-145 agomir improved lung histology and limited leukocyte infiltration. In accordance with these findings, previous studies demonstrated a role of miR-145 in mitigating LPS-induced acute lung injury [37,38]. In related studies, COVID-19-related lung inflammation and damage in a preclinical mouse model were observed to be exacerbated by ADAM17 activation and ameliorated by its silencing [39]. Hence, it is reasonable to hypothesize that miR-145 limits CLP-induced acute lung injury by reducing ADAM17 expression. Similar results obtained in the analysis of kidney tissues further validated a proinflammatory role for ADAM17 in acute kidney injury, and this is consistent with other studies [40,41].

Overall, miRNA-145, as an upstream mediator of ADAM17, functions in limiting LPS-induced endothelium inflammatory response in HUVECs, and constrains organ injury in mice during response to sepsis. ADAM17 is not the only target gene of miR-145. The miRNAs-associated off-target adverse effects due to the pleiotropic nature of miRNAs are causing concern. Targeting miR-145 also potentially knockdown the expression of other irrelevant genes in different tissues and organs, which may be challenging and cause unexpected side effects, as shown in the recent studies of the unexpected miR-145's toxicity to podocytes [42,43]. Our results provided evidence that miR-145 was potentially effective in anti-inflammatory therapy for sepsis. However, the network of miR-145 target genes and cellular signaling still lacks a full understanding. And optimal delivery of miR-145 specifically to damaged vascular endothelial cells still remains challenge.

5. Conclusions

In summary, we have found that miR-145 directly binds to the 3′-UTR of the ADAM17 mRNA. This downregulates ADAM17 expression and suppresses endothelial inflammatory response following LPS challenge in HUVECs. miR-145 significantly alleviated CLP-induced inflammation as well as lung and kidney damage in mice. Our findings provide novel evidence of miR-145 as a pivotal regulator of ADAM17 expression important in protecting against sepsis-associated inflammation and acute organ injury. miR-145 ultimately provides a survival benefit for septic mice, and may offer a novel tactic for the future development of therapeutic approaches to treat sepsis.

Availability of Data and Materials

The authors declare that all data supporting the findings of this study are available within the paper and its Additional file.

Author Contributions

QL, JH and MX conceived and designed the experiments, and participated in its design and coordination and helped to draft the manuscript and revise the manuscript. YYL and LL performed the experiment and statistical analysis, and drafted and revised the manuscript. YL, RY and SL helped to perform the experiments, statistical analysis and draft the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the experimental animal ethics committee of Guangdong Medical University (GDY2202700).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2901044.

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