Online Supplemental Materials

MicroRNA-574-5p Attenuates Acute Respiratory Distress Syndrome by Targeting

HMGB1

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Online Supplemental Methods
Human subjects

Whole blood samples were collected in tubes containing anticoagulant (EDTA) from healthy controls and patients suffering from pneumonia–related ARDS. Then, the samples were centrifuged within 2 hours at 1,600 g for 15 min for plasma collection. All specimens were stored in a -80°C freezer. All ARDS subjects met the Berlin diagnostic definition. To measure the levels of microRNAs, plasma samples were collected on the 1st day of diagnosis with ARDS. Patients younger than 18 years old and those with diffused alveolar hemorrhage or chronic lung diseases, which may mimic ARDS, were excluded. Patients with neutropenia, immunosuppression secondary to medication or diseases such as HIV infection and those undergoing treatment with granulocyte colony-stimulating factor or inhibitors of tumor necrosis factor were excluded. The study protocol was approved by the ethics committees of Jinling Hospital. Informed consent was obtained from all participants and/or their legal guardians.

microRNA isolation and quantitative RT-PCR

RNA was extracted from plasma using the Qiagen miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Cel-miR-39 was spiked-in prior to RNA isolation as an additional quality control. Reverse transcription and quantitative PCR (qRT-PCR) were conducted with Bulge-Loop miRNA qRT-PCR Starter Kit (RiboBio, Guangzhou, China). The primers for miRNA reverse transcription and quantification were synthesized and purified by RiboBio (Guangzhou, China). qPCR was performed on an Applied Biosystems Viia 7 Real-Time PCR machine (Life Technologies, CA, USA). The
PCR cycling program included 95°C for 10 min and 40 cycles of 95°C for 2 s, 60°C for 30 s, and melting curve analysis was carried out at the end of the cycling program. Mean absolute CT values and standard deviations are displayed on the graphs.

**Transfection with siRNA, miRNA mimics/agomir and inhibitor/antagomir.**

HMGB1 siRNA, negative control siRNA (NC-siRNA), miR-NC mimics, miR-574-5p mimics, miR-NC inhibitor, miR-574-5p inhibitor, miR-NC agomir, miR-574-5p agomir, miR-NC antagonir, and miR-574-5p antagonir were obtained from RiboBio (Guangzhou, China). HPMECs were transfected with miRNA or siRNA along with Lipofectamine 3000 reagent (Invitrogen, MA, USA) for 24 hours following the manufacturer’s instruction. Mice were administered miR-NC agomir or miR-574-5p agomir (10 nmol per mouse) i.v. 24 hours before LPS challenge following the manufacturer’s instruction.

**Actinomycin D cell treatment.**

Twenty-four hours after transfection of HPMECs with miR-574-5p mimics and NC mimics, 5 µg/mL actinomycin D (MedChem Express, USA) was added into the plates. Cells were subsequently harvested at different time points (0, 2, 4, 6, 8 h). HMGB1 mRNA levels were detected by qRT–PCR.

**Measurement of mRNA expression**

Total RNA was extracted using TRIZol (Invitrogen, MA, USA) according to the manufacturer’s instructions. RNA was eluted in RNase-free water and stored at -80°C. Total RNA was reverse transcribed to cDNA using a PrimeScript™ RT Master Mix (Perfect Real
Time) cDNA synthesis kit (Takara, Dalian, China) according to the manufacturer's protocol.

qPCR was performed on an Applied Biosystems Viia 7 real-time PCR machine (Life
Technologies, CA, USA) using SYBR Premix Ex Taq (Takara, Dalian, China). The PCR
cycling conditions were conducted as follows: 95°C for 30 sec followed by 40 cycles of 95°C
for 5 sec and 60°C for 34 sec. The following primer sequences were used: h-β-actin forward
primer 5′-CTCTTCCAGCCTTCCTTCTTCT-3′ and h-β-actin reverse primer 5′-
AGCACTGTGTGCGTGTTGACGACT-3′, h-HMGB1 forward primer 5′-
GCTCCATAGAGACAGCGCCGGG-3′ and h-HMGB1 reverse primer 5′-
CCTCAGCGAGGCACAGAGTCGC-3′, h-IL-6 forward primer 5′-
AACCTGAACCTTCCAAAGATGG-3′ and h-IL-6 reverse primer 5′-
TCTGGCTTGTTCCTCAGTACACT-3′, h-IL-1β forward primer 5′-
ATGATGCTTATTACAGTGGCAA-3′ and h-IL-1β reverse primer 5′-
GTCGGAGATTCGTAGCTGGA-3′, h-TNF-α forward primer 5′-
GAGGCAAGCCTTGGTGATGCA-3′ and h-TNF-α reverse primer 5′-
CGGGGCATTGATCTCAGC-3′, m-β-actin forward primer 5′-
GTGACGTTGACATCCGTAAGA-3′ and m-β-actin reverse primer 5′-
GCCGGACTCATCCTGACTCC-3′, m-HMGB1 forward primer 5′-
GGCGGCTTGTCCTGTGACAT-3′ and m-HMGB1 reverse primer 5′-
ACCCAAAATGGGCAAAAGCA-3′, m-IL-6 forward primer 5′-
TACCACCTCACAAGTACGGAGGC-3′ and m-IL-6 reverse primer 5′-
CTGCAAGTGCATCTCAGTTC-3′, m-IL-1β forward primer 5′-
TGGACCTTCAGGATTGAGGACA-3′ and m-IL-1β reverse primer 5′-
GTTCATCTCGGAGCCTGTAGTG-3’, m-TNF-α forward primer 5’-
GGTGCCCTATGTCTCAGCCTTT-3’ and m-TNF-α reverse primer 5’-
GCCATAGAACTGATGAGGGAG-3’. The 2^ΔΔCt cycle threshold method was used to
determine relative quantitative levels of individual mRNAs in each well, and these were
expressed as the fold difference to the β-actin levels.

**Western blot analysis.**

The cells were lysed in RIPA buffer, and the protein samples were separated by
electrophoresis on 10% SDS–PAGE gels and transferred to polyvinylidene fluoride
membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim
milk (Sigma-Aldrich Co.) at room temperature for 2 hours and incubated overnight at 4°C
with rabbit monoclonal antibodies specific for HMGB1 (Abcam, USA) (1:10000 dilution),
NLRP3 (Abcam, USA) (1:1000 dilution), caspase-1 (Abcam, USA) (1:1000 dilution),
histone-3 (Servicebio, Wuhan, China) (1:1000 dilution), T-IκBα (Abcam, USA) (1:10000
dilution), p-IκB (Cell Signaling Technology, USA) (1:2000 dilution), p65 (Abcam, USA)
(1:2000 dilution), p-p65 (Millipore, Billerica, MA, USA) (1:10000 dilution), β-actin
(Servicebio, Wuhan, China) (1:3000 dilution). Labeling by the primary antibody was
detected using relevant secondary antibody conjugated to HRP (Cell Signaling Technology,
USA) (1:2000 dilution) and detected using ECL reagents (Millipore, Billerica, MA, USA).

**ELISA**

IL-1β levels were determined by ELISA (MultiSciences Biotech, Co., Ltd) according to the
manufacturer’s instructions.
Chromatin immunoprecipitation (ChIP) analysis

ChIP analysis was performed according to the manufacturer’s instructions (Millipore, CA, USA) to test the binding of NF-κB p65 to the miR-574-5p promoter. Immunoprecipitation was performed with anti-NF-κB p65 antibody and isotype matched IgG as a control. Immunoprecipitated DNA was further subjected to PCR amplification. The primers for ChIP were as follows (amplicon size: 163 bp): 5′- AGCGACCTCTTCCCAGTGAC -3′ (forward) and 5′- TCTCCTGGAAACTTGCTTGTA -3′ (reverse) for the binding site. PCR amplification profiles were as follows: 95°C for 30 s; 35 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s; 72°C for 5 min. The PCR products were analyzed on 1.5% agarose gel and visualized under UV (Center for Ubiquitous Communication by Light) light.

Dual-luciferase reporter assay

The 3’ UTR fragment of human HMGB1 mRNA contains four putative miR-574-5p binding sites (3’ UTR: 47-69, 1088-1107, 1222-1243, 2620-2645) according to RNA22 software (http://www.mybiosoftware.com/rna22-v2-microrna-target-detection.html). Five luciferase reporter vectors were constructed. The first was the full sequence of the HMGB1 3’ UTR, then the four binding sites were mutated respectively on the background of the full sequence of the HMGB1 3’ UTR. These five sequences were cloned at the XhoI and NotI sites into the pmiR-RB-REPORT luciferase reporter vector (RiboBio Co. Ltd., Guangzhou, China), and the resulting vectors were named pmiR-HMGB1-WT, pmiR-HMGB1-MUT1, pmiR-HMGB1-MUT2, pmiR-HMGB1-MUT3 and pmiR-HMGB1-MUT4. All the constructs were further confirmed by sequencing.
For luciferase activity assay, each construct was cotransfected with miR-574-5p mimics or miR-NC (RiboBio) in a 96-well plate using Lipofectamine 3000 (Invitrogen, MA, USA) for 48 h. Luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer’s instructions. Luminescent signal was quantified by a luminometer (Glomax; Promega), and luciferase activity was presented as relative hRlu/hLuc ratio. The tests were repeated in triplicate.
SUPPLEMENTAL FIGURE LEGENDS

Figure E1 miR-574-5p reduced the expression of HMGB1 and suppressed the transcription of inflammatory factors in LPS-induced RAW 264.7

RAW 264.7 were transfected with miR-574-5p mimics followed by stimulation with LPS. Western blot analysis of HMGB1 in RAW 264.7 was measured by western blot. IL-6, IL-1β, TNF-α mRNA levels were measured by qRT-PCR. The values presented are the mean ± SD. Comparisons were made by t-test. *P < 0.05. **P < 0.01.
Figure E1 miR-574-5p reduced the expression of HMGB1 and suppressed the transcription of inflammatory factors in LPS-induced RAW 264.7. RAW 264.7 were transfected with miR-574-5p mimics followed by stimulation with LPS. Western blot analysis of HMGB1 in RAW 264.7 was measured by western blot. IL-6, IL-1β, TNF-α mRNA levels were measured by qRT-PCR. The values presented are the mean ± SD. Comparisons were made by t-test. *P < 0.05. **P < 0.01.