European Review for Medical and Pharmacological Sciences 2018; 22: 4625-4633

Overexpressed microRNA-615-3p promotes progression of neonatal acute respiratory distress syndrome by inhibiting differentiation of mesenchymal stem cells to alveolar type II epithelial cells

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Abstract. – OBJECTIVE: To explore whether microRNA-615-3p participates in the progression of neonatal acute respiratory distress syndrome (ARDS) by inhibiting differentiation of mesenchymal stem cells (MSCs) to alveolar type II epithelial cells (ATII) via Wnt/β-catenin pathway.

PATIENTS AND METHODS: Expression levels of microRNA-615-3p and inflammatory factors (IL-1, IL-6, IL-8, and TNF-α) in peripheral blood of 24 neonatal ARDS patients and 14 healthy newborns were detected by qRT-PCR (quantitative Real-Time Polymerase Chain Reaction). MSCs were isolated from bone marrow of mice and identified by flow cytometry. The effect of microRNA-615-3p on regulating the differentiation of MSCs to ATII was analyzed. After altering expressions of microRNA-615-3p and DKK1 by plasmids transfection, Wnt/β-catenin pathway-related genes were detected by Western blot.

RESULTS: Higher expression levels of microRNA-615-3p and inflammatory factors (IL-1, IL-6, IL-8, and TNF-α) were observed in peripheral blood of neonatal ARDS patients than those of healthy newborns. ATII-specific genes were upregulated, and inflammatory factors were downregulated after the microRNA-615-3p knockdown in MSCs. Moreover, expressions of Wnt/β-catenin pathway-related genes were downregulated after the microRNA-615-3p overexpression, which was partially reserved by the DKK1 knockdown.

CONCLUSIONS: Overexpressed microRNA-615-3p promoted ARDS development through inhibiting differentiation of MSCs to ATII via Wnt/β-catenin pathway.

Key Words:
MicroRNA-615-3p, Mesenchymal stem cells, Alveolar type II epithelial cells, Wnt/β-catenin pathway.

Introduction

Acute respiratory distress syndrome (ARDS) is a progressive hypoxemic respiratory failure that occurs in various primary diseases. ARDS is manifested as the diffuse alveolar epithelium and microvascular endothelium injuries caused by pulmonary parenchyma inflammatory response. Current studies have demonstrated that ARDS is the consequence of the interaction of various inflammatory factors and cytokines. The dynamic balance between pro-inflammatory cytokines and anti-inflammatory cytokines is an essential factor that remarkably affects ARDS prognosis[1]. Therefore, it is of great significance to explore the specific pathogenesis of ARDS, so as to better improve the clinical treatment.

MicroRNAs are a group of endogenous single-stranded, non-coding small RNAs with 18-24 nt in length. Functionally, microRNAs interfere with protein synthesis by directly degrading or inhibiting translation of target genes at the post-transcriptional level. Stable microRNAs have been detected in serum and plasma, that are, circulating microRNAs[2,3]. Circulating microRNAs are differentially expressed under different physiological and pathological conditions, which are closely related to human health[4-6]. As an expression regulator, microRNAs can target approximately 60% of protein-coding genes[7], which exert a crucial role in biological processes, such as cell proliferation, differentiation, apoptosis, immunity, and inflammation[8]. Studies have found that circulating microRNAs secreted by tumor cells possess

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their own regulatory effects[9]. For example, microRNA-21 and microRNA-29 secreted by tumor cells can activate TLR-mediated inflammatory responses by binding to Toll-like receptors (TLRs), thereby regulating tumor growth and metastasis[10]. MicroRNA-615-3p not only participates in the development of many tumors, but also is related to the immune and inflammatory responses[11-13]. The role of microRNA-615-3p in neonatal ARDS, however, has not been reported yet.

Bone marrow mesenchymal stem cells are the origin of osteoblasts, which present the potential of multi-directional differentiation and strong proliferation. Under some circumstances, MSCs are capable of differentiating to ATII[14]. In the present study, we detected the differentiation ability of MSCs regulated by microRNA-615-3p, which provides new directions in treating neonatal ARDS.

Patients and Methods

Patients and Sample Collection

24 neonatal ARDS patients and 14 healthy newborns in our hospital from June 2016 to December 2017 were selected. No significant differences in age, gender and body weight were found between neonatal ARDS patients and healthy newborns. The investigation was approved by the Hospital Ethics Committee and patients' families signed the informed consent. 1 ml of peripheral blood samples of all enrolled subjects were collected under fasting state in the morning and preserved at -80°C.

RNA Extraction From Peripheral Blood

0.25 mL of peripheral blood was added to 0.75 mL of TRI Reagent LS and 0.2 ml of chloroform. The mixture was gently shaken, incubated for 2-5 min, and centrifuged at 4°C, 12,000 g for 15 min. The aqueous complex was transferred to another tube and isodose isopropanol was added for RNA extraction. After centrifugation, RNA was washed and air dried for 5 min. RNA was then dissolved in diethyl pyrocarbonate (DEPC) water without RNase, followed by preservation at -80°C for the following experiments.

Enzyme-Linked Immunosorbent Assay (ELISA)

According to the instructions of ELISA kit (EBiosciences, San Diego, CA, USA), corresponding antibodies were diluted at a density of 1-10 μg/mL with the coating buffer. Briefly, cells were blocked in 5% bovine serum for 40 min. After washing for three times, the sample and enzyme-labeled antibody were added to each well. The substrate solution was used to terminate the reaction and ELISA results were determined within 20 min. Absorbance at the wavelength of 450 nm was detected using ELISA detector.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

The mRNAs were reversely transcribed to cDNAs using PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan), followed by the qRT-PCR reaction according to the instructions of miScript SYBR Green PCR kit. The reaction conditions were as follows: denaturation at 94°C for 30 s, followed by annealing at 55°C for 30 s, and extension at 72°C for 90 s, for a total of 40 cycles. Each sample was performed for 3 times. Primers used in this experiment were as the follows: MicroRNA-615-3p, F: 5'-ACACTCCAGCTGGGTCCGAGCCTGGGTCTC-3', R: 5'-TGGTGTCGTGGAGTCG-3'; IL-1, F: 5'-CTCAGCAACACTCCTAT-3', R: 5'-TCCTGGTCTGCAGGTAA-3'; IL-6, F: 5'-TCCAATCTGGGTTCAATCAGGCGA-3', R: 5'-TTCCCTCATACTCGTTCTGGAGGT-3'; IL-8, F: 5'-CTTGTCATTGCCAGCTGTGT-3', R: 5'-TGACTGTGGAGTTTTGGCTG-3'; TNF-α, F: 5'-CACGTTGTAGCCGACATCAACTCT-3', R: 5'-GTTGTCTTCCAGCTTCACACCGTT-3';Occludin, F: 5'-TGAAAGTCCACCTCCTTACAGA-3', R: 5'-CCGGATAAAAAGAGTACGCTGG-3'; KGF, F: 5'-TGGGCACTATATCTCTAGCTTGC-3', R: 5'-GGGTGCGACAGAACAGTCT-3'; CK18, F: 5'-CAGCCAGCGTCTATGCAGG-3', R: 5'-CCTTCTCGGTCTGGATTCCAC-3'; SpA, F: 5'-CTTGGCCTTTGGTGGCTACTT -3', R: 5'-GAGAGGTCTTAGGGAATGTCACT-3'; SpB, F: 5'-CAGCGGGTAGGAAGCAGTTTC-3', R: 5'-CCCTGCACCTCATCCCTGA-3'; SpC, F: 5'-GGTGAGCAACTGGCTACTGAG-3', R:5'-CCCTGGCGCGTACATTCTTT-3'; Wnt3a, F: 5'- CTCCTCTCGGATACCTCTTAGTG-3', R: 5'-CCAAGGACCACCAGATCGG-3'; β-catenin, F: 5'-ATGGAGCCGGACAGAAAAGC-3', R: 5'-TGGGAGGTGTCAACATCTTCTT-3'; DKK1, F: 5'- CAGTGCCACCTTGAACTCAGT-3', R: 5'-CCGCCCTCATAGAGAACTCC-3'; GAPDH, F: 5'-ACCCACTCCTCCACCTTTGA-3', R: 5'-CTGTTGCTGTAGCCAAATTCGT-3'; U6, F: 5'-CTCGCTTCGGCAGCACATATA-3', R: 5'- AAATATGGAACGCTTCACGA-3'.

Isolation and Culture of MSCs

Sprague Dawley rats (Model Animal Research Center of Nanjing University, Nanjing, China) were executed with dislocation of the cervical vertebra. The femur and tibia were collected under aseptic condition. The marrow cavity was washed with L-DMEM (L-Dulbecco's Modified Eagle Medium). After centrifugation at 1000 r/min for 5 min, MSCs were re-suspended in L-DMEM containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). MSCs were then seeded in 6-well plates at a density of 2.5×10^6/L. Culture medium was replaced every 3-4 days. After cell culture for 10 days, MSCs were digested with trypsin and centrifuged at 4°C, 1500 rpm for 5 min. First-passage MSCs (P1 MSCs) were seeded in 6-well plates at a density of 5×10^5/L.

MSCs Identification

Phenotype identification of MSCs was carried out by flow cytometry. MSCs were re-suspended with phosphate buffered saline (PBS) at a density of 1×10^6/mL. 500 μL of cell suspension was incubated with 5 μL of CD34 and CD44 without light for 30 min. Cells were washed and re-suspended with PBS, followed by flow cytometry detection of MSCs phenotypes.

Cell Culture of ATII

ATII cells were obtained from ATCC, Manassas, VA, USA. Briefly, ATII cells were cultured in DMEM (Gibco, Grand Island, NY, USA) containing 5% FBS and incubated in a 5% CO_2 incubator at 37°C. Cell passage was performed with trypsin when cell confluence was up to 80-90%.

Co-Culture of MSCs and ATII

The cell density of ATII was adjusted to 1×10^4/mL with DMEM containing 10% FBS. 1.5 mL of cell suspension was added to the transwell chamber. The cell density of MSCs was adjusted to 8×10^4/mL with DMEM containing 10% FBS and third-passage MSCs were seeded into the 6-well plates. After culturing for 24 h, transwell chamber was put into the 6-well plate with the culture medium containing 10% FBS, 1% non-essential amino-acid, and DMEM with 1% L-glucose.

Western Blot

Total protein was extracted from treated cells by radioimmunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China). The protein sample was separated by electrophoresis on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA). After membranes were blocked with skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with TBS-T (Tris-buffered Saline with Tween 20) and followed by the incubation of secondary antibody. The protein blot on the membrane was exposed by chemiluminescence.

Cell Transfection

Cells in good growth condition were selected and seeded in the 6-well plates. Cell transfection was performed when the cell confluence was up to 60%-70% according to the instructions of Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Transfection plasmids (microRNA-615-3p mimic, microRNA-615-3p inhibitor, si-NCDKK1-1, and si-NCDKK1-2) were purchased from GenePharma (Shanghai, China).

Statistical Analysis

We used Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x} \pm s$). The *t*-test and Chi-square analysis were used for comparing measurement and categorical data, respectively. $p<0.05$ was considered statistically significant.

Results

MicroRNA-615-3p Was Overexpressed in Neonatal ARDS Patients

We detected expression levels of microRNA-615-3p and inflammatory factors (IL-1, IL-6, IL-8, and TNF-α) in peripheral blood of 24 neonatal ARDS patients and 14 healthy newborns by qRT-PCR. The results indicated higher expressions of microRNA-615-3p and inflammatory factors in neonatal ARDS patients than those of healthy newborns (Figure 1A and 1B). Besides, no significant differences in age, gender, and body weight were found between the two groups. Routine examinations showed that PRISM-III score, Modified Murray's score, and oxygenation index (OI) were remarkably elevated, whereas PaO_2/FiO_2 (partial pressure of artery/fraction of inspired oxygen) and Crs (compliance of the re-

Table I. Basic characteristics of study population.

	ARDS group (n=24)	Control group (n=14)	*p*
Age (days)	16 (4-28)	17.5 (3-25)	0.523
Weight (kg)	1.8 (1.3-3.8)	2.5 (1.5-4.2)	0.774
Male sex	10 (41.7%)	7 (50%)	0.468
PRISM-III24	14.3 (8.2-20.1)	2.9 (2-4.8)	<0.001
Modified Murray's score	5 (4.4-5.9)	0.75 (0.5-1.2)	<0.001
PaO_2/FiO_2	121 (80-160)	480 (342-687)	<0.001
OI	12.3 (9.3-17.2)	1.8 (1.3-2.4)	0.032
Crs (mL/cm H_2O/kg)	0.36 (0.22-0.43)	0.89 (0.8-0.9)	<0.001
Deaths	4 (16.7%)	1 (7.1%)	
Co-morbidities	Severe sepsis (3) RSV infection (6) H1N1 flu (4) Aspiration (4) Trauma (3) Late onset GBS infection (2) Malignancy (2)	Metabolic disease (1) SAH (2) Status epilepticus (2) General anesthesia (9)	

IQR, interquartile range; HCT, hematopoietic cellular transplantation; ARDS, acute respiratory distress syndrome; PRISM-3, Pediatric Risk of Mortality III score; OI, oxygenation index.

spiratory system, mL/H_2O/kg) were reduced in neonatal ARDS patients (Table I).

Phenotype Identification of MSCs

MSCs exhibited fibroid-like and elongated morphology after cell culture for 3 and 5 days, respectively (Figure 2A). Third-passage MSCs were collected for identification using flow cytometry. The data showed negative-antigen CD34 (3.71%) and positive-antigen CD44 (99.54%), suggesting the successful isolation of MSCs with high purity (Figure 2B).

MSCs Directly Differentiated to ATII

Co-culture of MSCs and ATII was established by transwell assay to induce the differentiation of MSCs to ATII. After differentiation induction for 1, 3, 5, 7, and 14 days, ATII-specific genes (Occludin, KGF, CK18, SpA, SpB, and SpC) were detected, respectively. Elevated expressions of

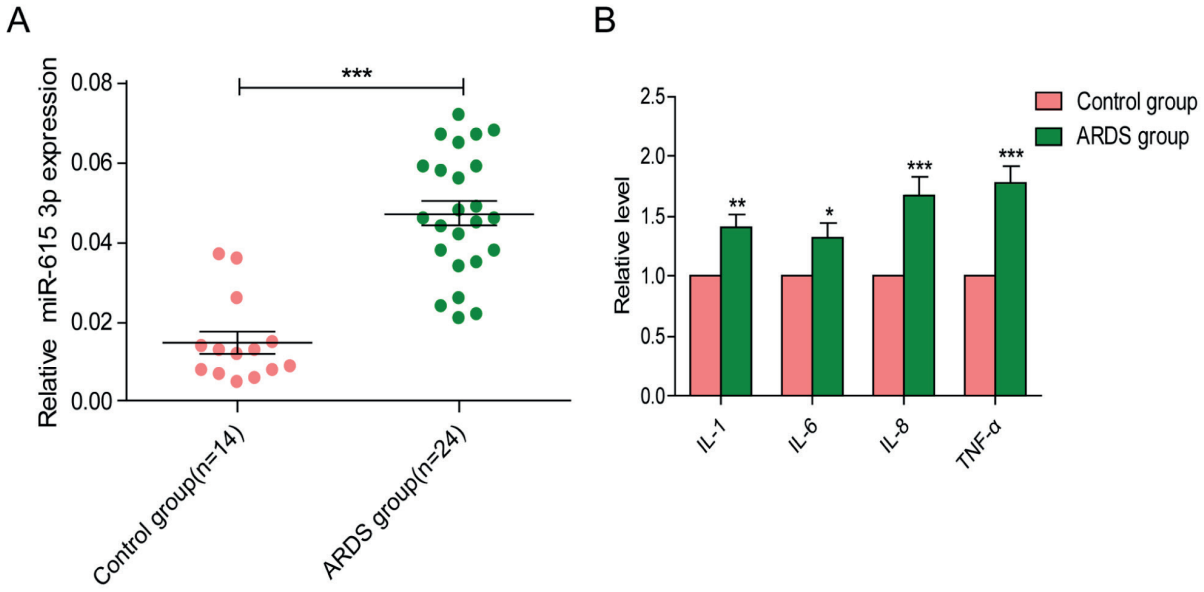

Figure 1. MicroRNA-615-3p was overexpressed in neonatal ARDS patients. ***A,*** MicroRNA-615-3p expression was higher in neonatal ARDS patients than those of healthy newborns. ***B,*** Expression levels of IL-1, IL-6, IL-8, and TNF-α were higher in peripheral blood of neonatal ARDS patients than those of healthy newborns.

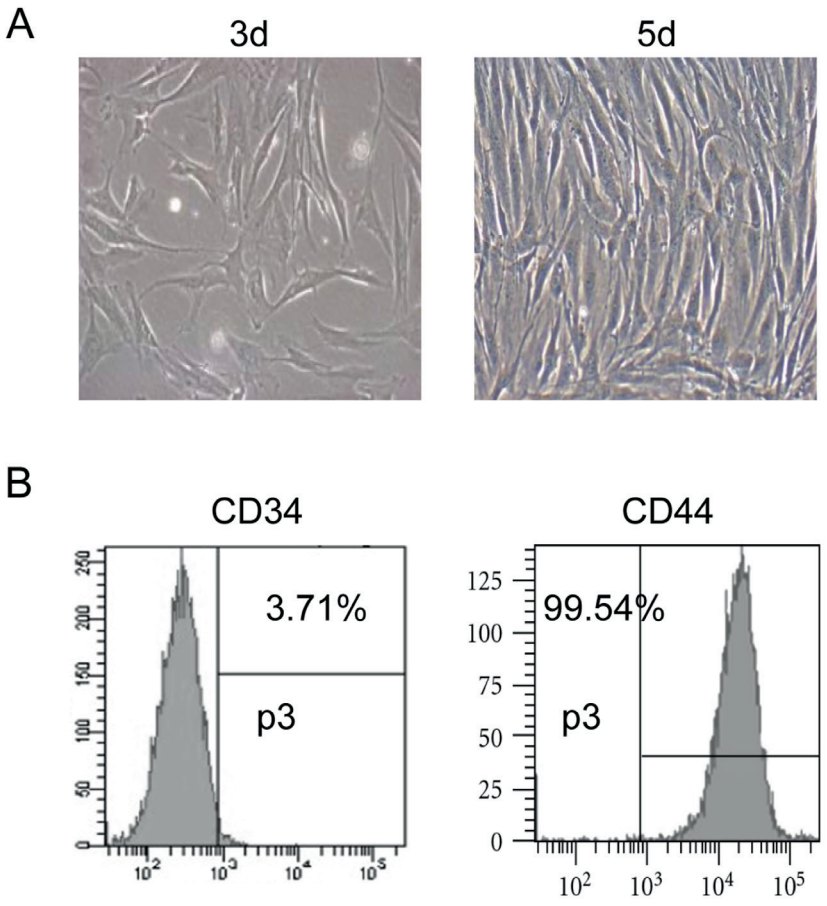

Figure 2. Phenotype identification of MSCs. ***A,*** MSCs exhibited elongated morphology after culturing for 3 and 5 days. ***B,*** Specific surface antigens of MSCs were detected by flow cytometry.

these genes were found from the 3rd day, and were increased in a time-dependent manner (Figure 3A). We next detected expressions of Occludin and CK18 at the different time points of differentiation. The data indicated gradual upregulation of Occludin and CK18 from the 3rd day (Figure 3B). Meanwhile, microRNA-615-3p expression in MSCs was decreased in a time-dependent manner (Figure 3C).

MicroRNA-615-3p Inhibited Wnt/β-Catenin Pathway

MicroRNA-615-3p mimic and microRNA-615-3p inhibitor were constructed and their transfection efficacies were verified by qRT-PCR (Figure 4A). We then transfected si-NCDKK1-1 or si-NCDKK1-2 in MSCs and found si-NCDKK1-1 presented a higher transfection efficacy (Figure 4B). Therefore, si-NCDKK1-1 was selected for the following experiments. To explore whether microRNA-615-3p participated in the inflammatory response, we detected expressions of IL-1, IL-6, IL-8, and TNF-α after altering microRNA-615-3p expression in MSCs. Our results demonstrated that overexpression of microRNA-615-3p resulted in elevated expressions of inflammatory factors (Figure 4C).

Wnt/β-catenin pathway has been reported to participate in regulating the inflammatory response. We therefore speculated whether Wnt/β-catenin pathway was involved in the regulation of MSCs by microRNA-615-3p. The results demonstrated downregulated Wnt3a and β-catenin, as well as upregulated DKK1 after microRNA-615-3p overexpression both in mRNA and protein levels (Figure 4D and 4E), indicating microRNA-615-3p may regulate MSCs *via* Wnt/β-catenin pathway.

MicroRNA-615-3p Promoted ARDS development via Inhibiting Wnt/β-Catenin Pathway

MicroRNA-615-3p has been proved to exert its specific role *via* Wnt/β-catenin pathway. Whether microRNA-615-3p promoted ARDS *via* Wnt/β-catenin pathway, however, still needed to

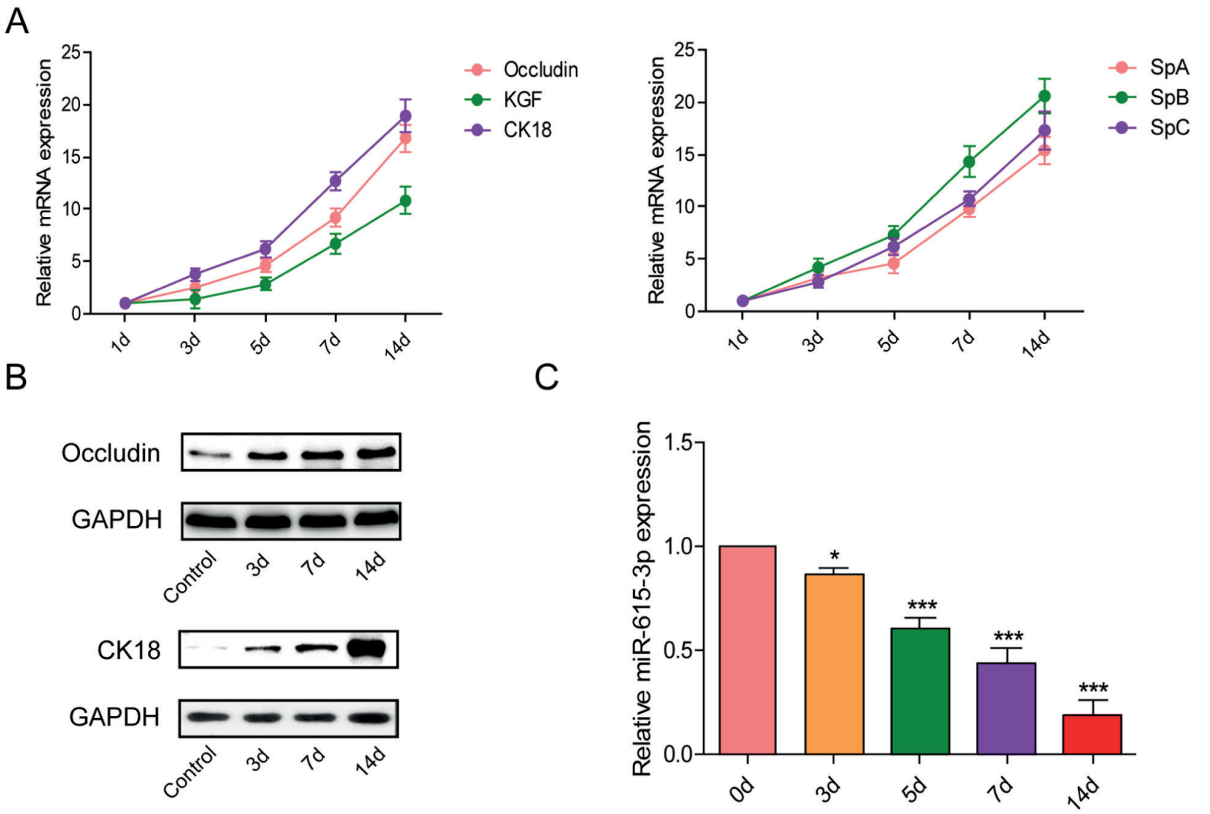

Figure 3. MSCs directly differentiated to ATII. ***A,*** Expression levels of ATII-specific genes (Occludin, KGF, CK18, SpA, SpB, SpC) were elevated in a time-dependent manner. ***B,*** Expressions of Occludin and CK18 were increased in a time-dependent manner. ***C,*** MicroRNA-615-3p was downregulated in a time-dependent manner.

be further explored. Reduced differentiation ability of MSCs to ATII and decreased expressions of Occludin and CK18 resulted from microRNA-615-3p overexpression were reversed after si-DKK1-1 transfection (Figure 4F and 4G). Similarly, increased expressions of IL-1, IL-6, IL-8, and TNF-α resulted from microRNA-615-3p overexpression were also reversed after inhibiting Wnt/β-catenin pathway by si-DKK1-1 transfection (Figure 4H). The above data demonstrated that microRNA-615-3p promoted ARDS *via* inhibiting Wnt/β-catenin pathway.

Discussion

ARDS is an acute, progressive hypoxemic respiratory failure characterized by alveolar-capillary inflammation caused by extra-pulmonary and extra-pathogenic factors[15,16]. ARDS is often complicated by multiple organ failures, therefore resulting in a high mortality rate[17-19]. Damaged ATII and inflammatory exudates in the alveoli lead to decreased production and abilities of pulmonary surfactant, which further aggravate pulmonary edema and atelectasis. Finally, decreased lung compliance and imbalanced ventilatory blood flow severely deteriorate lung function, which is manifested as ARDS[16].

MicroRNA has significant effects on gene expression, cell differentiation, apoptosis, etc. It is reported that differentially expressed microRNAs are involved in inflammatory responses and many diseases. For example, Cai et al[20] showed that multiple microRNAs in the lungs of lipopolysaccharide-induced ALI (acute lung injury) mice are dynamically altered. Among them, microRNA-214 and microRNA-415 were remarkably upregulated, whereas microRNA-181a/b, microRNA-199a and microRNA-16 were downregulated. Further study found that overexpressed microRNA-16 inhibits pulmonary inflammatory response *via* targeting to TNF-α. Other studies have shown that microRNA-615-3p is involved

in the tumor development, such as prostate cancer, pancreatic cancer, and cutaneous carcinoma[21,22]. The specific role of microRNA-615-3p in neonatal ARDS, however, has not been fully elucidated. In this study, we found that microRNA-615-3p is overexpressed in neonatal ARDS, which is capable of inhibiting the differentiation of MSCs to ATII cells and promoting secretion of inflammatory factors.

Wnt/β-catenin pathway is a highly conserved signaling pathway that controls cell growth, differentiation, apoptosis, and self-renewal. In tumor development, Wnt/β-catenin pathway is often abnormally activated and antagonizes the proliferation, migration, and invasion of tumor cells. Studies have confirmed that Wnt/β-catenin pathway can promote the differentiation of MSCs to ATII, thereafter resisting pulmonary oxidative stress[23].

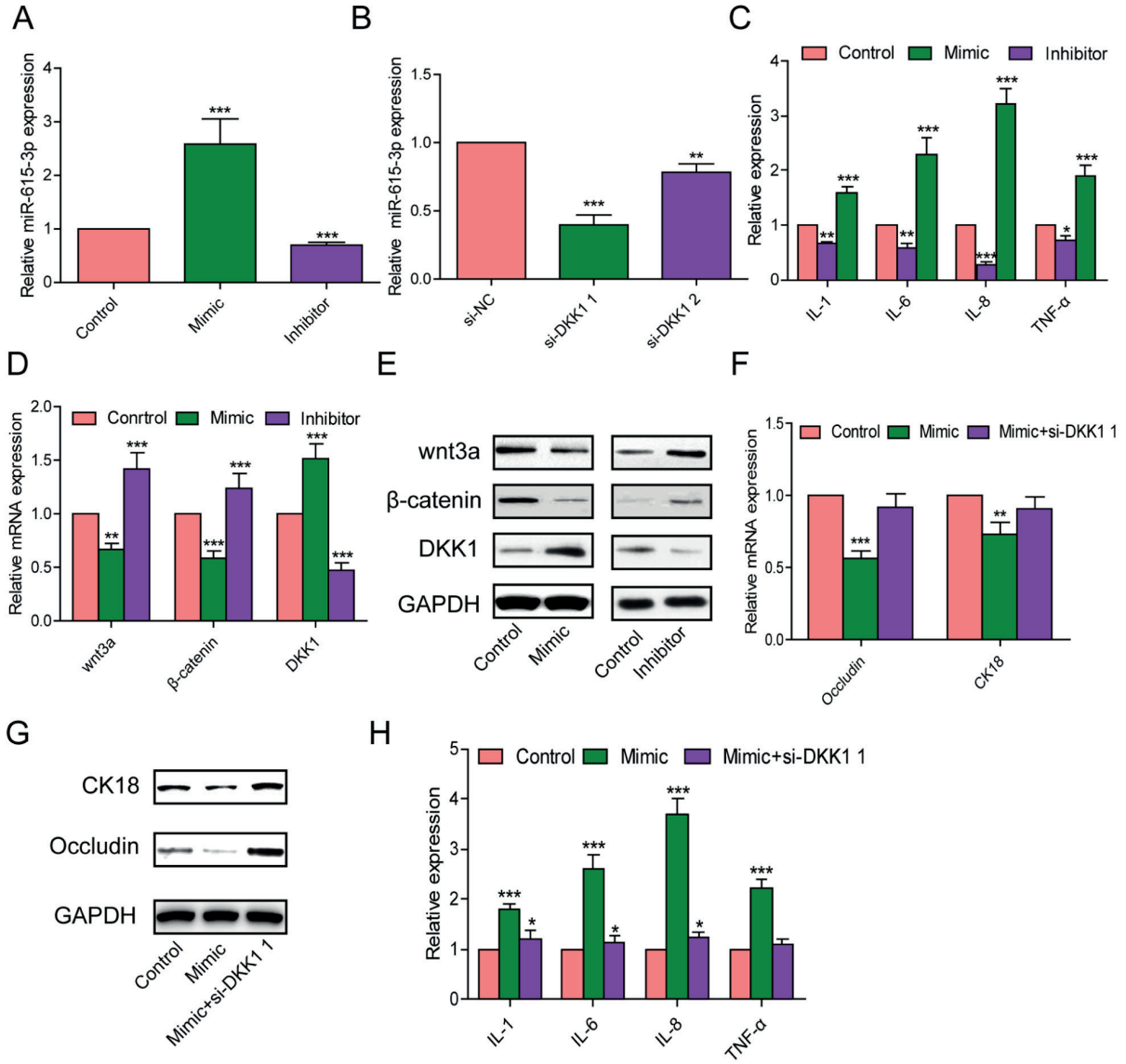

Figure 4. MicroRNA-615-3p promoted ARDS development *via* inhibiting Wnt/β-catenin pathway. ***A,*** MicroRNA-615-3p expression after knockdown or overexpression of microRNA-615-3p. ***B,*** Transfection efficacy of si-DKK1. ***C,*** Expression levels of IL-1, IL-6, IL-8, and TNF-α were increased after microRNA-615-3p overexpression. ***D,*** Expression levels of Wnt3a, β-catenin, and DKK1 after microRNA-615-3p knockdown. ***E,*** Expression levels of Wnt3a, β-catenin, and DKK1 after microRNA-615-3p overexpression. ***F, G,*** Decreased mRNA (***F***) and protein (***G***) expressions of Occludin and CK18 after microRNA-615-3p overexpression were reversed by DKK1 knockdown. ***H,*** Increased expressions of IL-1, IL-6, IL-8, and TNF-α after microRNA-615-3p overexpression were reversed by DKK1 knockdown.

Similarly, overexpressed catenin in MSCs could activate Wnt/β-catenin pathway, so as to improve the pulmonary epithelial permeability and attenuate pulmonary edema. Overexpressed catenin could also improve pulmonary fibrosis, thereby repairing the lung damage resulted from ARDS[24]. In the present study, expression levels of Wnt/β-catenin pathway-related genes were detected after altering microRNA-615-3p expression. The results elucidated that microRNA-615-3p inhibits the differentiation of MSCs to ATII by inhibiting Wnt/β-catenin pathway, so as to improve ARDS development.

Conclusions

We demonstrated that overexpressed microRNA-615-3p promoted ARDS development through inhibiting differentiation of MSCs to ATII *via* Wnt/β-catenin pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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