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miR-155-5p Promotes Progression of Acute Respiratory Distress Syndrome by Inhibiting Differentiation of Bone Marrow Mesenchymal Stem Cells to Alveolar Type II Epithelial Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: We investigated whether microRNA-155-5p is involved in the differentiation of bone marrow mesenchymal stem cells (BMSCs) into alveolar type II epithelial (AT II) cells by regulating the Wnt signaling pathway, thus participating in the development of acute respiratory distress syndrome (ARDS).

Material/Methods: Serum levels of microRNA-155-5p in 50 ARDS patients and 50 normal controls were detected by quantitative real-time PCR (qRT-PCR). Marrow mesenchymal stem cells (MCSs) were isolated from mouse bone marrow and identified by flow cytometry. Subsequently, the effect of microRNA-155-5p on differentiation of BMSCs into AT II cells was evaluated by detecting the expression levels of AT II-specific genes. The expression levels of proteins in the Wnt signaling pathway after overexpression or knockdown of microRNA-155-5p were detected by Western blot.

Results: Serum levels of microRNA-155-5p in ARDS patients were significantly higher than that in normal controls. Expression levels of AT II-specific genes were enhanced after downregulating microRNA-155-5p in BMSCs. MicroRNA-155-5p overexpression showed the opposite result. Furthermore, microRNA-155-5p inhibited the expression levels of proteins in the Wnt signaling pathway.

Conclusions: MicroRNA-155-5p can attenuate the differentiation of BMSCs into AT II cells by inhibiting the Wnt signaling pathway, thus promoting the progression of ARDS.

MeSH Keywords: Mesenchymal Stromal Cells • MicroRNAs • Wnt Signaling Pathway

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Background

Acute respiratory distress syndrome (ARDS) is an acute inflammatory lung injury caused by a variety of extrapulmonary and pathogenic factors [1]. The hallmarks of ARDS are membrane permeability alteration, surfactant dysfunction, diffuse interstitial and alveolar edema, the formation of a transparent membrane, and alveolar collapse. Previous studies have shown that multiple organ dysfunction syndrome (MODS), acute physiology and chronic health evaluation score II (APACHE II score) [2], and shock [3] are related to the ARDS prognosis. Inflammatory dysfunction and cell apoptosis are involved in ARDS development. Previous studies have highlighted the value of proinflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in the ARDS prognosis. However, the treatment of ARDS is still insufficient. Therefore, studying the mechanism of the development and progression of ARDS is of great significance for its prevention and treatment.

miRNAs are small non-coding RNAs that regulate gene expressions at the post-transcriptional level. MiRNAs can be paired with mRNAs of the target genes to form miRNA-induced silencing complexes (miRISCs), thus degrading or inhibiting the translation of the target genes. Multiple studies have found that the differentially expressed miRNAs are closely related to the inflammatory response and exert a crucial role in various acute and chronic inflammatory diseases [4,5]. miRNAs are present not only in tissues and cells, but also in the circulatory system. Lawrie et al. [6] demonstrated the presence of miRNAs in the circulatory system. Subsequent studies have also shown that circulating miRNAs are differentially expressed in a variety of diseases, including ARDS. It is recognized that circulating miRNAs are mainly secreted by living cells through membrane vesicles, which are highly stable under the protection of vesicular structures and protein complexes. Some studies have found that circulating miRNAs are upstream regulators of many genes related to tumors. One study revealed that miRNAs influence the development and progression of hepatocellular carcinoma by regulating cell proliferation and apoptosis. Fabbri et al. [7] found that tumor-secreted miR-21 and miR-29 can regulate the tumor microenvironment by activating Toll-like receptors (TLRs)-induced inflammatory responses. Inhibition of mircoRNA-34a enhances survival of human bone marrow mesenchymal stromal/stem cells under oxidative stress [8]. Fewer microRNAs have been identified in the biological process of ARDS [8]. MicroRNA-155-5p is considered to participate in the regulation of multiple physiological and pathological processes, including immune and inflammatory responses, differentiation of hematopoietic cells, and activation of immune cells, which are crucial in the development of ARDS. However, the role of microRNA-155-5p in ARDS has not been reported yet.

Bone marrow mesenchymal stem cells (BMSCs) are the origin of osteoblasts. BMSCs have the potential of multi-directional differentiation, which can differentiate into a variety of cells, including alveolar epithelial type II cells (AT II) [9]. Bone marrow mesenchymal stem cells exhibit anti-inflammatory effects in several diseases, including myocardial infarction, ARDS, sepsis, hepatic failure, and acute renal failure. The compelling benefits of BMSCs for lung injury have also generated a great deal of interest [10], but the mechanism is still unknown. In this study, we measured the serum levels of microRNA-155-5p in ARDS patients and explored the effects of microRNA-155-5p on AT II cells differentiated from BMSCs. Our study provides a novel insight into investigating the mechanism involved in the development and progression of ARDS.

Material and Methods

Isolation and culture of BMSCs

Specific-pathogen-free (SPF) mice were sacrificed by dislocations of cervical vertebrae and then immersed in 75% ethanol for 2 min. The femurs and tibias of mice were taken and placed in Dulbecco's modified Eagle's medium (DMEM) containing penicillin and streptomycin. The femur and tibia were cut open at the bone marrow cavity with sharp scissors. DMEM was used to wash the bone marrow cavity and the BMSCs were then collected. After the cell density was adjusted to 2.5×10^6/mL, cells were then maintained in a 5% CO_2 incubator at 37°C. Fresh culture medium was replaced 3 h later to discard non-adherent cells. Phosphate-buffered saline (PBS) was used for washing the adherent cells and culture medium was changed every 3–4 days. After culturing for 10 days, large cloning masses were digested and passaged with trypsin. Cloning masses were then centrifuged at 1500 rpm for 5 min. Cells were inoculated into a 100-mm culture dish at a dose of 5×10^5/mL and recorded as P1.

Identification of BMSCs by flow cytometry

The fifth-passage BMSCs were collected and the expression levels of CD29, CD34, and CD45 in BMSCs were detected by flow cytometry. We incubated 1×10^6/mL cells with their corresponding antibodies for 30 min at 37°C in the dark. After washing with PBS 3 times, treated cells were detected by FACS Aria I and data were analyzed using FLOWJO software.

AT II cell culture

The AT II cell line was purchased from ATCC. AT II cells were resuspended with high-glucose DMEM medium containing 5% fetal bovine serum was seeded into 6-well plates at a density of 2×10^5/mL. Cells were placed in a 5% CO_2 incubator at 37°C.

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Culture medium was replaced every other day. AT II cells were digested and passaged with 0.25% trypsin until the cell confluence was 80–90%.

Co-culture of BMSCs and ATII cells by Transwell assay

AT II cells were resuspended in high-glucose DMEM containing 10% FBS at a dose of 1×10^4/mL. We added 1.5 mL of the cell suspension into the upper chamber, and 2.5 mL of high-glucose DMEM containing 10% FBS was added in the lower chamber for 24-h incubation. The second-passage BMSCs were resuspended in low-glucose DMEM containing 10% MSC qualified FBS at a dose of 0.8×10^5/mL. We added 2.5 mL of the cell suspension in 6-well plates and cultured it for 24 h. AT II cells in the chamber and the culture medium of BMSCs in the 6-well plate were aspirated after cells grew by static adherence. Cell chambers were placed in a 6-well plate supplemented with low-glucose DMEM medium containing 10% fetal bovine serum (FBS), 1% non-essential amino acids, and 1% glutamine amine.

RNA extraction and qRT-PCR

Total RNA was extracted by TRIzol method for reverse transcription. Briefly, the RNA was reversely transcribed into cDNA according to the manufacturer's instructions and then amplified using the miScript II RT kit. QRT-PCR was then performed based on the instructions of the miScript SYBR Green PCR kit. The expression level of U6 was taken as an internal reference for microRNA and GAPDH for mRNA. Primers used in this study were as follows:
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'),
wnt5a (F: 5'-CAACTGGCAGGACTTTCTCAA-3',
R: 5'-CCTTCTCCAATGTACTGCATGTG-3'),
CyclinD (F: 5'-CCCCCAATCGCAAGGATTCTT-3',
R: 5'-CTTGGTTCGGTGGGTCTGTC-3'),
C-myc (F: 5'-GCTGGACACGCTGACGAAA-3',
R: 5'-TCTAGGCGAAGCAGCTCTATTT-3'),
β-catenin (F: 5'-ATGGAGCCGGACAGAAAAGC-3',
R: 5'-TGGGAGGTGTCAACATCTTCTT-3'),
DKK1 (F: 5'-CAGTGCCACCTTGAACTCAGT-3',
R: 5'-CCGCCCTCATAGAGAACTCC-3').

Western blotting

Total proteins were extracted from cells by RIPA method and then quantified using bicinchoninic acid (BCA) based on the instructions. Proteins were separated in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of primary antibody (purchased from Abcam) overnight (wnt3a, wnt5a, Cyclin D, C-myc, β-catenin, DKK1, and GAPDH). Membranes were then incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method.

Subjects and sample collection

We selected 50 ARDS patients and 50 normal controls treated from June 2013 to December 2016. No significant difference in age between the 2 groups was observed. The inclusion criteria of ARDS were based on the Berlin definition [1]. Exclusion criteria were pregnancy, adolescence, and patients with interstitial lung disease. This study was approved by the Ethics Committee of Yantai Hospital of Traditional Chinese Medicine. Signed written informed consents were obtained from all participants before the study began We drew 5 mL of venous blood from ARDS patients and normal controls while fasting and in a quiet state. Blood samples were placed at room temperature for 30 min, and then centrifuged at 3000 g/min for 10 min. The upper layer of serum was harvested and centrifuged again at 13 500 g/min for 15 min. Finally, the upper layer of serum was harvested and placed into EP tubes and stored at –80°C.

Cell transfection

BMSCs in logarithmic growth phase were transfected with microRNA-155-5p mimics, microRNA-155-5p inhibitor, and negative control following the instructions of the Lipofectamine 2000 (Invitrogen, Shanghai, China) kit when the cell density was about 80% of the 6-well plate. The culture medium was replaced 6 h after transfection. Transfected primers in this study were as follows: microRNA-155-5p mimics (5'-UUAAUGCUAAUCGUGAUAGGGGU-3', R: 5'-CCCUAUCACGAUUAGCAUUAAUU-3'), microRNA-155-5p inhibitor (5'-ACCCCUAUCACGAUUAGCAUUAA-3').

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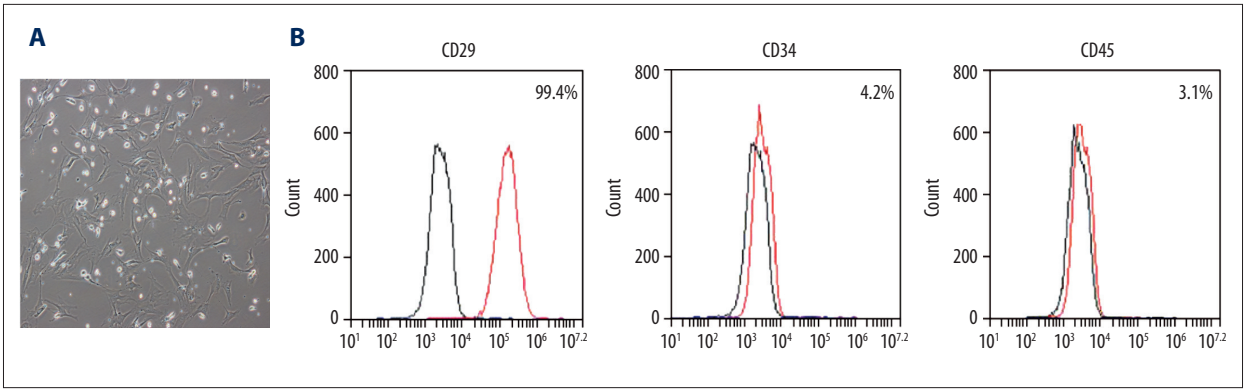

Figure 1. Phenotype identification of BMSCs. (**A**) The morphology of BMSCs on the 4th day. (**B**) The BMSCs specific surface antigens were identified by flow cytometry, including CD29 (99.4%), CD34 (4.2%), and CD45 (3.1%).

ELISA detection of inflammatory cytokines

Antibodies were diluted with coating buffer to a dose of 1–10 μg/mL and incubated at 4°C overnight. Solution was discarded the next day and washed with wash buffer 3 times for 3 min each. We added 0.1 mL of protein sample into the coated well and incubated it at 37°C for 1 h. We then added 0.1 mL of enzyme-labeled antibody into each reaction well for 1-h incubation at 37°C. Subsequently, 0.1 mL of TMB substrate solution was added into each reaction well and samples were incubated at 37°C for 10–30 min. We added 0.05 mL of sulfuric acid to terminate the reaction. OD value at the wavelength of 450 nm was measured on the ELISA detector.

Statistical analysis

SPSS19.0 (Armonk, NY, USA) statistical software was utilized for analyzing data. All data are expressed as mean ± standard deviation. Comparison of measurement data was conducted using the *t* test. $P<0.05$ was considered statistically significant.

Results

Phenotype identification of BMSCs

BMSCs grew by static adherence after 20-h inoculation. It was observed that cells grew into a long fusiform and were arranged with polarity. BMSCs were digested when cell confluence was up to 80% and passaged for 9 generations. Cell morphology on the 4th day of inoculation is shown in Figure 1A. In this study, the seventh-passage BMSCs were harvested for flow cytometry detection. Results indicated that the cell surface antigens of BMSCs were CD29-positive (99.4%), CD34-negative (4. 2%), and CD45-negative (3.1%), which were compliant with the purity requirements of BMSCs (Figure 1B).

Differentiation of BMSCs into AT II cells

Differentiation of BMSCs into AT II cells was achieved by *in vitro* co-culture of BMSCs and AT II cells. Expression levels of genes related to AT II cells, including Occludin, KGF, CK18, SpA, SpB, and SpC, were detected on the 1st, 3rd, 7th, and 14th days after induction. Increased expressions of the above genes were observed 3 days after incubation, which were gradually elevated in a time-dependent manner (Figure 2A). Immunofluorescence staining of surface antigens showed that BMSCs were mainly differentiated into AT II cells (95.31%) after co-culture of BMSCs and AT II cells (Figure 2B). Subsequently, protein expressions of Occludin and CK18 were detected, which were also increased in a time-dependent manner (Figure 2C). The expression level of microRNA-155-5p was significantly downregulated as BMSC differentiated (Figure 2D).

MicroRNA-155-5p inhibited the Wnt signaling pathway

The expression levels of microRNA-155-5p in 50 ARDS patients and 50 normal controls were detected by RT-PCR. No significant differences in age, sex, or body mass index between the 2 groups were observed (Table 1). The results showed that the serum levels of microRNA-155-5p in ARDS patients were remarkably higher than that of normal controls ($P<0.001$, Figure 3A). These results suggest that microRNA-155-5p may be involved in the ARDS development. The transfection efficacy of microRNA-155-5p mimics and microRNA-155-5p inhibitor was confirmed by qRT-PCR (Figure 3B). Studies have shown that microRNA-155-5p participates in the regulation of inflammatory responses. Therefore, we speculated that BMSCs participates in regulation of the Wnt signaling pathway. The expressions of genes related to the Wnt signaling pathway were detected, including wnt3a, wnt5a, CyclinD, C-myc, β-catenin, and DKK1. Our results illustrated that overexpressed microRNA-155-5p significantly reduces the mRNA expression levels of wnt3a, wnt5a, CyclinD, C-myc, β-catenin, and DKK1 in BMSCs. MicroRNA-155-5p knockdown showed the opposite

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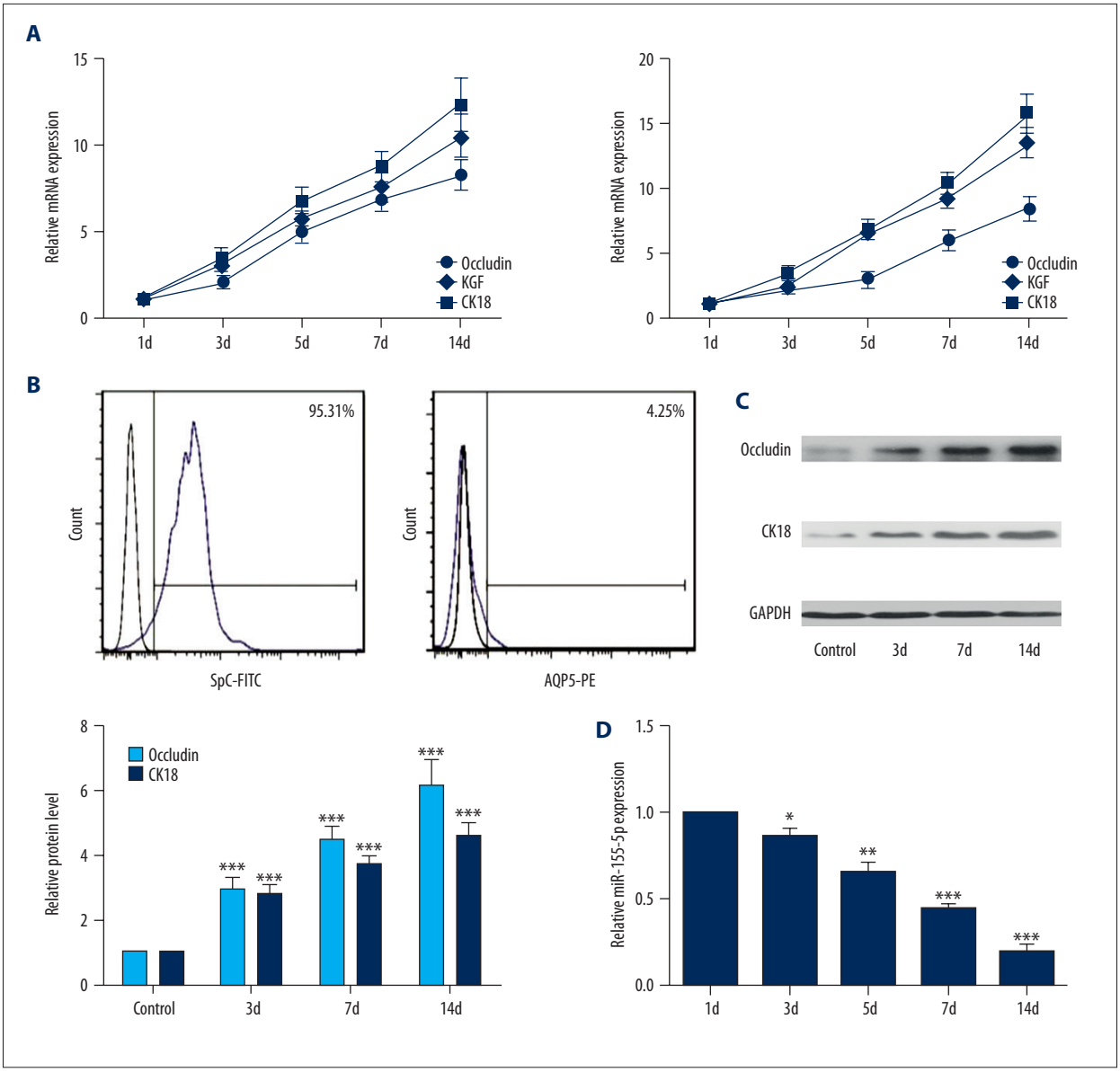

Figure 2. Differentiation of BMSCs into AT II cells. (**A**) Expression levels of genes related to AT II cells (Occludin, KGF, CK18, SpA, SpB, SpC). (**B**) Surface antigens of AT I cells (4.25%) and AT II cells (95.31%) detected by flow cytometry. (**C**) Expression levels of Occludin and CK18 were increased in a time-dependent manner. (**D**). The expression level of microRNA-155-5p was significantly down-regulated as BMSC differentiated. *** *P*<0.001, compared with Control.

Table 1. Baseline characterisics.

	Control	ARDS	*P*
n	50	50	
Age (years)	63±11	62±9	0.62
Female	46%	52%	0.55
BMI (kg/m²)	21.1±3.8	21.4±3.1	0.67
Smoker (n)	3	2	1.000
Alcohol (n)	1	2	1.000

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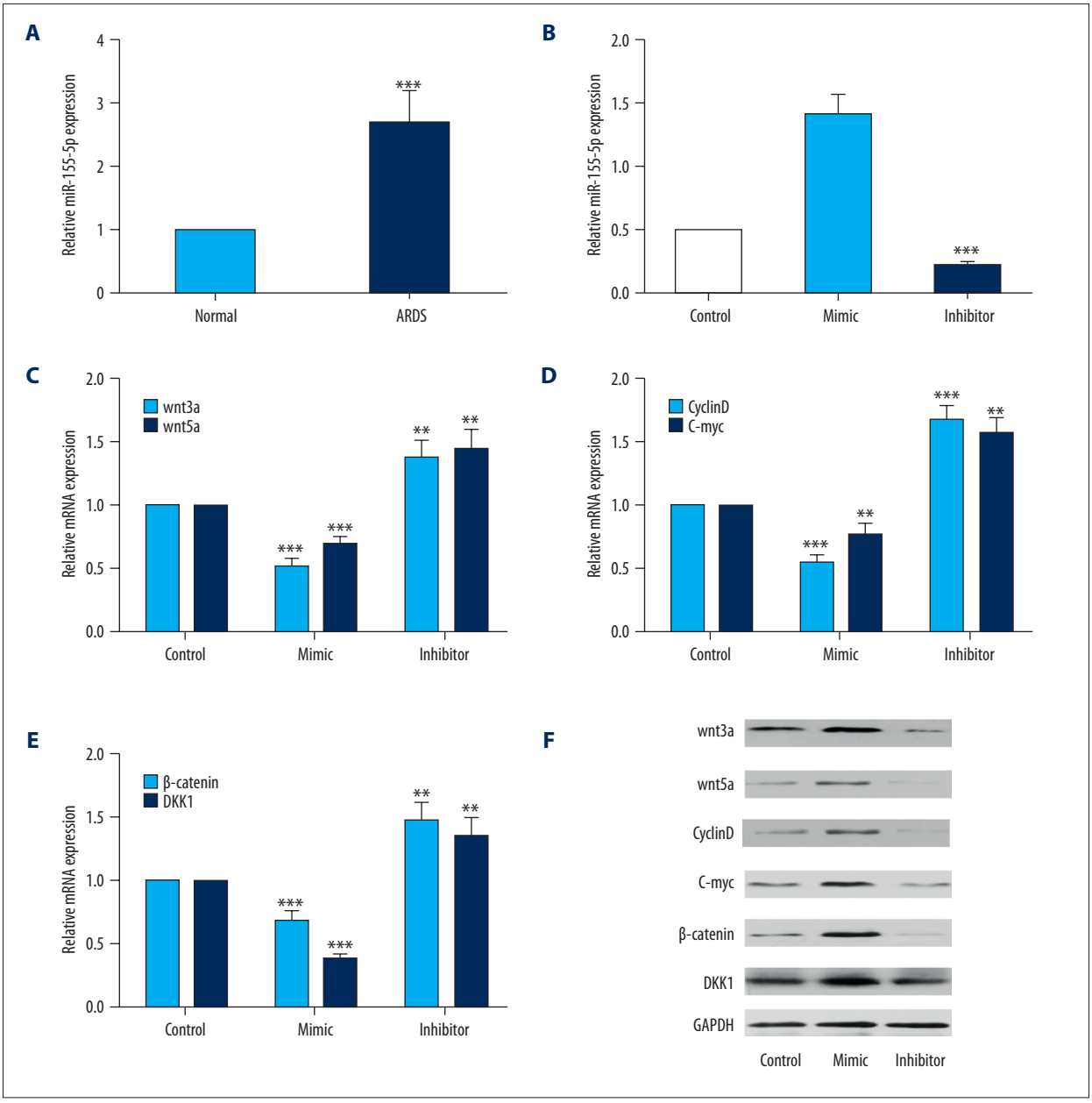

Figure 3. MicroRNA-155-5p inhibited the Wnt signaling pathway. (**A**) The serum expression of microRNA-155-5p in 50 ARDS patients was significantly higher than that of 50 normal controls. (**B**) Transfection efficacy of microRNA-155-5p mimics and microRNA-155-5p inhibitor. (**C–E**) After overexpression of microRNA-155-5p, the mRNA expressions of wnt3a, wnt5a, CyclinD, C-myc, β-catenin, and DKK1 in BMSCs were decreased. MicroRNA-155-5p knockdown obtained the opposite results. (**F**) After overexpression of microRNA-155-5p, the protein expressions of wnt3a, wnt5a, CyclinD, C-myc, β-catenin, and DKK1 in BMSCs were decreased. MicroRNA-155-5p knockdown obtained the opposite results. ** $P<0.01$, *** $P<0.001$, compared with Control.

results (Figures 3C–3E). The protein expressions of wnt3a, wnt5a, CyclinD, C-myc, β-catenin, and DKK1 were consistent with their mRNA expressions (Figure 3F).

MicroRNA-155-5p promoted ARDS progression by inhibiting the Wnt signaling pathway

Although microRNA-155-5p has been considered to be involved in the Wnt signaling pathway, whether it promotes ARDS progression by inhibiting the Wnt signaling pathway remains to be verified. After overexpression or inhibition of

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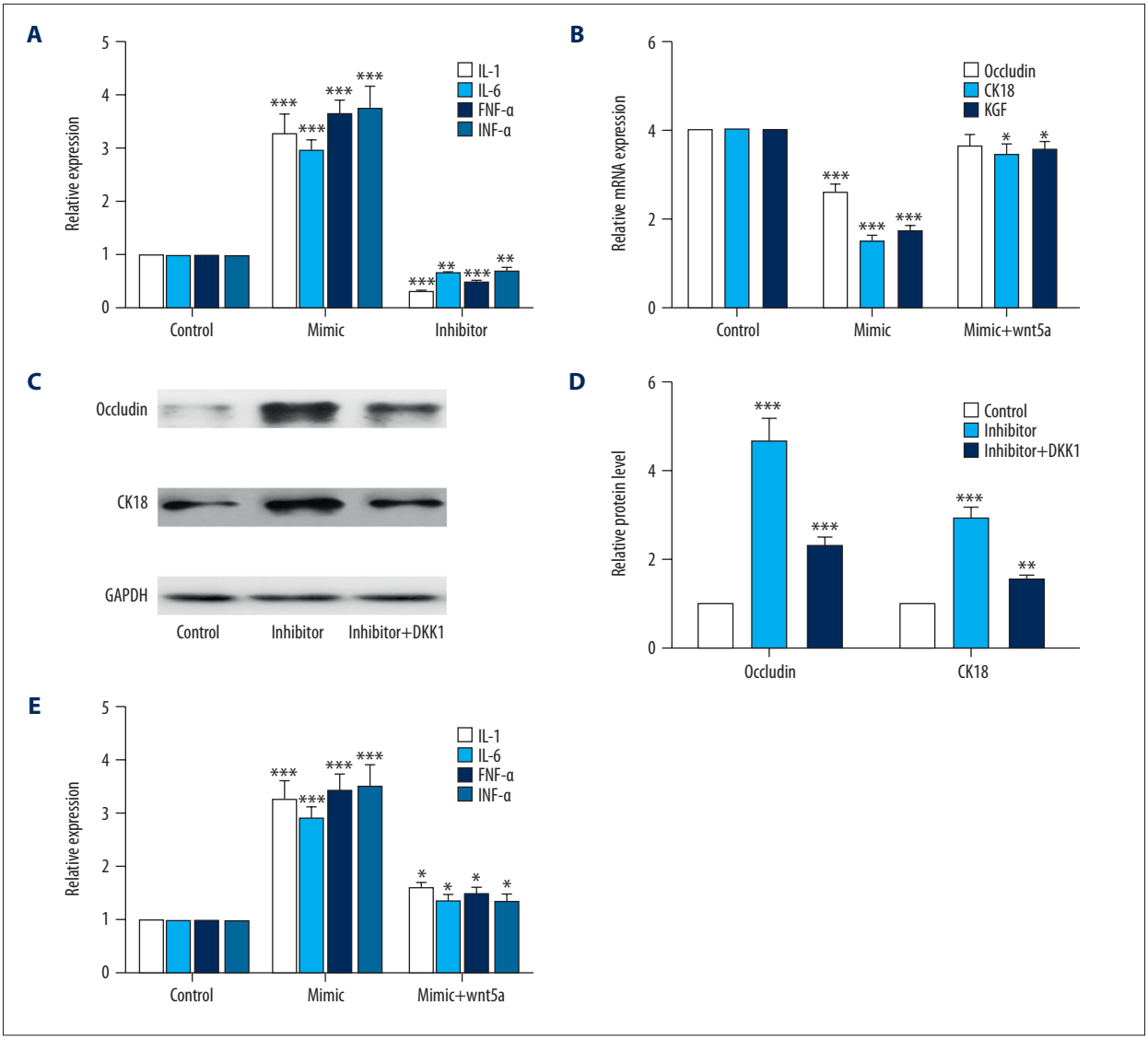

Figure 4. MicroRNA-155-5p promoted ARDS progression by inhibiting the Wnt signaling pathway. (**A**) The expression levels of IL-1, IL-6, TNF-α, and INF-α were significantly increased after overexpression of microRNA-155-5p. (**B**) The expression levels of Occludin, KGF, and CK18 in AT II cells were decreased after overexpression of microRNA-155-5p, which were reversed by wnt5a. (**C, D**). The expression levels of Occludin and CK18 were significantly increased after microRNA-155-5p knockdown, which were rescued by DKK1. (**E**) Transfection of microRNA-155-5p mimics significantly increased the expression levels of IL-1, IL-6, TNF-α, and INF-α, which were rescued by wnt5a. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$, compared with Control.

microRNA-155-5p, the expression levels of IL-1, IL-6, TNF-α, and INF-α were detected. Results demonstrated that upregulated microRNA-155-5p remarkably increased the expression levels of IL-1, IL-6, TNF-α, and INF-α, while microRNA-155-5p knockdown showed the opposite results (Figure 4A). Moreover, the differentiation ability of BMSCs into AT II cells decreased after the overexpression of microRNA-155-5p. The expression levels of Occludin, KGF, and CK18 were also decreased after transfection with microRNA-155-5p mimics, but were rescued by wnt5a, the Wnt signaling pathway promoter (Figure 4B). The differentiation ability of BMSCs into AT II cells was increased after inhibition of microRNA-155-5p. The expression levels of Occludin and CK18 were also significantly increased, which were rescued by DKK1, the Wnt signaling pathway inhibitor (Figure 4C, 4D). Furthermore, transfection of microRNA-155-5p mimics significantly increased the expression levels of IL-1, IL-6, TNF-α, and INF-α, which were rescued by wnt5a (Figure 4E). These results indicate that microRNA-155-5p promotes ARDS progression by inhibiting the Wnt signaling pathway.

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Discussion

Acute respiratory distress syndrome (ARDS) is the consequence of the development of acute lung injury [11–13]. Acute lung injury (ALI) is characterized by alveolar epithelial cells and pulmonary capillary endothelial damage, which lead to damaged alveolar permeability, destroyed alveolar surfactant, alveolar edema, hyaline membrane formation, and alveolar collapse [14,15]. According to the Berlin definition, ALI may progress into ARDS. Current studies have suggested that the inflammatory reaction is the main pathogenesis of ALI/ARDS [16,17], and systemic inflammatory response syndrome (SIRS) is the leading cause of ARDS [18]. The possible mechanisms of ARDS include activation of inflammatory cells, synthesis and release of inflammatory mediators, and disorders of proinflammatory/anti-inflammatory mediators.

MiRNAs are small non-coding RNAs that can negatively regulate target genes by recognizing and binding to the 3 'UTR of mRNA at the post-transcriptional level [19]. Studies have shown that miRNA is closely related to diverse diseases [20]. In addition, studies have shown that miRNA is present in the whole blood, serum, plasma, and urine, which is called circulating miRNA [21]. In addition, miRNAs possess great stability and tissue specificity and can serve as biological markers [6].

Previous studies have shown that microRNA-155-5p participates in diverse processes. Baba et al. [22] confirmed that microRNA-155-5p is upregulated in oral squamous cell carcinoma and is correlated with tumor malignancy. It promotes epithelial mesenchymal transformation (EMT) by increasing SOCS1 and decreasing STAT3 expressions, which in turn promote tumor metastasis. In addition, Qu et al. [23] found that microRNA-155-5p is correlated with the invasion depth and TNM stage of colon cancer. The role of microRNA-155-5p has also been explored in [24] hepatoma-associated mesenchymal stem cells and regulating the tumor microenvironment [25–27]. However, the role microRNA-155-5p has not been identified in ARDS. BMSCs have gained researcher attention because of its diverse differentiation, which will be widely used in treating disease such as ARDS. Many studies have proven that microRNA can mediate the development of ARDS. For example, NF-κB-induced microRNA-211 inhibits IL-10 in macrophages of rats with LPS-induced ARDS [28] [29402831]. In the present study, microRNA-155-5p expression was significantly elevated in ARDS patients. This indicates that micorRNA-155-5p participates in the development of ARDS. In addition, the expression level of microRNA-155-5p was down-regulated as the BMSC differentiated. These previous results further pointed out that microRNA-155-5p is an important favorable factor in ARDS. However, the mechanism of microRNA-155-5p in regulating ARDS remains unclear.

Wnt activates intracellular signaling pathways by binding with receptors located on the cell membrane, which exerts a crucial role in cell proliferation, differentiation, migration, polarization, and apoptosis. Recent research indicates that the activation of the Wnt signaling pathway can promote the differentiation of BMSCs into AT II cells, thus resisting the oxidative stress of lung tissues [29]. Similarly, Cai et al. [30] found that activation of the Wnt signaling pathway by catenin overexpression in BMSCs can alleviate epithelial damage in respiratory distress syndrome. Thus, in the present study, the expression levels of key proteins related to the Wnt signaling pathway were detected after overexpression or knockdown of microRNA-155-5p. As expected, the Wnt signaling pathway was silenced, while up-regulation of microRNA-155-5p and the opposite results were observed with inference of microRNA-155-5p. In addition, the rescue assay also proved that microRNA-155-5p regulates activation of the Wnt pathway. The previous study showed that microRNA-155-5p suppresses the differentiation of BMSCs into AT II cells by inhibiting the Wnt signaling pathway.

To the best of our knowledge, this is the first study to report the role and the mechanism of microRNA-155-5p in ARDS. However, the experiments were performed *in vitro,* and *in vivo* experiments are needed to confirm our findings. MicroRNA-155-5p may be a new target for treatment of ARDS in the future.

Conclusions

In summary, the present study found that microRNA-155-5p is upregulated in ARDS patients and inhibits the differentiation of BMSCs into AT II cells by inhibiting the Wnt signaling pathway, thus promoting ARDS progression.

Conflict of interest

None.

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