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MicroRNA-26b-5p promotes development of neonatal respiratory distress syndrome by inhibiting differentiation of mesenchymal stem cells to type II of alveolar epithelial cells *via* regulating Wnt5a

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Abstract. – OBJECTIVE: The aim was to investigate the role of microRNA-26b-5p in regulating mesenchymal stem cells (MSCs) differentiation to type II of alveolar epithelial cells (AECII) in the disease course of neonatal respiratory distress syndrome (NRDS).

MATERIALS AND METHODS: MSCs were first derived from rat bone marrow. In vitro induction of MSCs differentiation to AECII was conducted by SAGM. The mRNA levels of microRNA-26b-5p, Wnt5a, and AECII-related genes (Occludin, KGF, CK18, SpA, SpB, and SpC) during the process of cell differentiation were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Enzyme-linked immunosorbent assay (ELISA) was conducted for detecting levels of inflammatory factors tumor necrosis factor-α (TNF-α), interferon-α (INF-α), and interleukin-1 (IL-1) in cell supernatant. Dual-luciferase reporter gene assay was then carried out to verify the regulatory effect of microRNA-26b-5p on Wnt5a. MicroRNA-26b-5p expression in serum samples of NRDS neonates and healthy neonates was detected by qRT-PCR as well.

RESULTS: MicroRNA-26b-5p was overexpressed in NRDS neonates than those of healthy neonates. Besides, microRNA-26b-5p was highly expressed in the process of MSCs differentiation to AECII. MicroRNA-26b-5p overexpression remarkably inhibited AECII differentiation and Wnt5a expression. Levels of TNF-α, INF-α, and IL-1 in cell supernatant during differentiation induction were elevated. The regulatory effects of microRNA-26b-5p on AECII differentiation, Wnt5a expression, and inflammatory response were reversed by Wnt5a overexpression.

CONCLUSIONS: MicroRNA-26b-5p inhibits MSCs differentiation to AECII via inhibiting Wnt5a expression through the Wnt pathway.

Key Words
MSCs, MicroRNA-26b-5p, ACE II cells, NRDS.

Introduction

Neonatal respiratory distress syndrome (NRDS) is a common pulmonary complication in preterm infants, which is the leading cause of death in preterm infants. NRDS is also known as neonatal hyaline membrane disease, resulting from lung parenchymal damage induced by excessive inflammatory response due to the lack of pulmonary surfactant. Damage to alveolar epithelial cells and pulmonary microvascular endothelial cells that constitute blood gas barrier eventually leads to NRDS[1-3]. Hence, updating and repairing alveolar epithelial cells may be an effective strategy for the treatment of NRDS. In recent years, stem cells with self-renewal and multi-directional differentiation potentials have been well recognized, which may serve broad application prospects in treating NRDS.

Mesenchymal stem cells (MSCs) exert the multi-directional differentiation and self-renewal abilities for promoting organizational remodeling, which are widely distributed in tissues and organs[4]. MSCs have a wide range of sources and are easy to be isolated. They also present immunomodulatory effects that are capable of alleviating inflammatory response. In recent studies, differentiation of MSCs to type II of alveolar epithelial cells (AECII) have been well investigated in treating diseases, including NRDS[5]. It is reported that homing of MSCs to impaired lung tissues contributes to cell differentiation into AEC or pulmonary microvascular endothelial cells, directly repairing the blood gas barrier and reducing the inflammatory response. However, few researches have been conducted on exploring the potential mechanism of MSCs differentiation.

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The Wingless proteins (Wnt) family is a type of cysteine-rich glycoprotein that is responsible for regulating embryonic development, cell proliferation, migration, differentiation, and apoptosis[6,7]. Wnt pathway is greatly involved in the lung development[8]. It also participates in regulating proliferation and differentiation of MSCs. Some genes in the Wnt family have already been proved to be related to stem cell characteristics of MSCs, including Wnt3a, Wnt5a, Wnt6, Wnt10a, and Wnt10b. Wnt/JNK and Wnt/PKC pathways are confirmed to regulate MSCs differentiation to AECII[9-11].

MiRNA is an endogenous, non-coding, small RNA consisting of 18-25 nucleotides. MiRNAs can be divided into three types according to the location of miRNA genes in the genome, namely intergenic miRNA, intron miRNA, and exon miRNA. Intergenic miRNAs account for the majority of miRNAs. To date, more than 1,000 miRNAs have been identified in the human genome. Although these, miRNAs account for only 1% to 3% of the human genome, they regulate more than 30% of human genes *via* degrading or inhibiting the translation of target mRNAs[12]. MiRNAs are capable of regulating multiple biological processes, thus participating in disease progression. Differentially expressed miRNAs may affect the normal functions of MSCs[13].

This study aims to investigate the regulatory effect of microRNA-26b-5p on the Wnt pathway in the differentiation of MSCs into AECII. Our results provide new suggestions for clinical treatment of NRDS.

Patients and Methods

Sample Collection

30 neonates diagnosed as NRDS and 30 healthy neonates in Yantai Affiliated Hospital of Binzhou Medical University from June 2015 to March 2018 were enrolled. Peripheral blood samples of NRDS neonates and healthy neonates were harvested. The informed consent was obtained from the neonates' families. This study was approved by the Yantai Affiliated Hospital of Binzhou Medical University Ethics Committee.

MSCs Isolation and Cell Culture

Rat femur and tibia were harvested from 4-week-old Sprague Dawley rats under aseptic condition. Epiphysis of femur and tibia were cut off. The marrow cavity was washed with Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA), followed by cell collection with centrifugation. Cells were resuspended for adjusting cell density. Subsequently, cells were seeded in the culture bottle at a density of 1×10^6 cells/cm. Culture medium was replaced every 3 days. Cell passage was performed until 80%-90% of cell confluence using 0.5% trypsin-EDTA (Ethylene Diamine Tetraacetic Acid).

Differentiation of MSCs to AECII

Third-passage MSCs were seeded in the 6-well plates. After cell confluence was up to 80%, SAGM (small airway growth media) was replaced for inducing differentiation to AECII. SAGM was replaced every 2 days for consecutive 2 weeks. Cells were collected on the 1st, 5th, and 10th day for the following experiments, respectively.

Cell Transfection

One day prior to cell transfection, cells were seeded in the 6-well plates at a density of 4×10^4 cells/cm. Serum-free DMEM was replaced, followed by cell transfection based on the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced 6 hours later.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-RCR)

Cells after differentiation for 1, 5 and 10 days, as well as peripheral blood, were harvested for RNA extraction using TRIzol method (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using EasyScript First-Strand complementary Deoxyribose Nucleic Acid (cDNA) Synthesis SuperMix (TaKaRa, Otsu, Shiga, Japan). After RNA concentration was determined, PCR was performed at 95°C for 1 min, 95°C for 15 s, and 60°C for 1 min, for a total of 40 cycles. The relative gene expression was calculated using the $2^{-\Delta Ct}$ method. Primers used in the study were as follows. SPA-forward: 5'-TGTCACTGTGTTCTTTGG-3'; SPA-reverse: 5'-GTTGTTGTACTTCTTC-GC-3'. SPB-forward: 5'-CTGCTTCCTAC-CCTCTGCTG-3'; SPB-reverse: 5'-CTTGG-CACAGGTCATTAGCTC-3'. SPC-forward: 5'-CAGACACCATCGCTACCTT-3'; SPC-reverse: 5'-TAGCCAAAGCCTCAAGACT-3'. Occludin-forward 5'-CCTCCAATGG-CAAAGTGAAT-3'; Occludin-reverse: 5'-CTC-

CCCACCTGTCGTGTAGT-3'. KGF-forward: 5'-CAATCTAGAATTCACAGATAGGAGGAGGC-3'; KGF-reverse: 5'-AGAATTCCAACTGCCACAGTCATGATTTC-3'. CK18-forward: 5'-CGCATCGTCTTGCAGATCGAC-3'; CK18-reverse: 5'-GCTGAGACCAGTACTTGTCCAG-3'. wnt5a-forward: 5'-ATTGGAATATTAAGCCCG-3'; wnt5a-reverse: 5'-GTGACCATAGTCGATGTT-3'. GAPDH-forward: 5'-GTTCAACGGCACAGTCAAG-3'; GAPDH-reverse: 5'-GCCAGTAGACTCCACGACAT-3'

Western Blot

Cells were lysed with RIPA (radioimmunoprecipitation assay) lysis buffer (Beyotime, Shanghai, China) containing a protease inhibitor on ice. After centrifugation at 12 000 r/min for 20 min, the supernatant was collected for detecting protein concentration using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The protein sample was separated by gel electrophoresis, transferred on polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and blocked with skim milk. Membranes were incubated with primary and secondary antibodies, followed by enhanced chemiluminescence (ECL).

Enzyme-Linked Immunosorbent Assay (ELISA)

Cell supernatant was collected and incubated with primary antibodies at 37°C for 30 min. After phosphate-buffered saline (PBS) wash, samples were incubated with corresponding secondary antibodies, followed by color development in the dark. Finally, the TMB solution was added for development termination. Optical density was detected at a wavelength of 450 nm using a microplate reader.

Dual-Luciferase Reporter Gene Assay

Cells were seeded in the 96-well plates and co-transfection was performed until 70% of cell confluence. Luciferase activity was detected using the Dual-Glo™ Luciferase Assay System (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Measurement data were analyzed by the *t*-test. $p<0.05$ considered the difference was statistically significant.

Results

Differentiation Identification of MSCs to AECII

MSCs were first derived from rat bone marrow for *in vitro* differentiation induction to AECII by SAGM. With the prolongation of SAGM induction, the mRNA levels of AECII-related genes (Occludin, KGF, and CK18) were gradually elevated (Figure 1A). The mRNA levels of relative genes to the surface activity of AECII (SpA, SpB, and SpC) were elevated in a time-dependent manner as well (Figure 1A). Protein expressions of Occludin and CK18 were upregulated as well (Figure 1B). The above data indicated the successful induction of cell differentiation.

MicroRNA-26b-5p Was Highly Expressed in NRDS

Previous studies[14,15] have suggested that circulating miRNAs can function as intercellular communication to regulate cellular biological functions. We detected microRNA-26b-5p expression in peripheral blood samples of NRDS neonates and healthy neonates by qRT-PCR. The results showed higher expression of microRNA-26b-5p in NRDS neonates than those of controls (Figure 2A). Similarly, *in vitro* results indicated that microRNA-26b-5p expression is gradually decreased during the process of MSCs differentiation to AECII (Figure 2B). Wnt5a expression was upregulated alongside the prolongation of SAGM induction (Figure 2C). After overexpression of microRNA-26a-5p, levels of inflammatory factors tumor necrosis factor-α (TNF-α), interferon-α (INF-α), and interleukin-1 (IL-1) in the culture medium were significantly increased (Figure 2D). Knockdown of microRNA-26b-5p obtained the opposite results.

Wnt5a Was Specifically Bound to and Regulated by MicroRNA-26a-5p

Further experiments showed that Wnt5a specifically binds to microRNA-26b-5p (Figure 3A). Dual-luciferase reporter gene assay demonstrated that transfection of wild-type Wnt5a partially quenched the fluorescence in MSCs overexpressing microRNA-26b-5p, whereas no significant difference in luciferase activity was found by mutant-type Wnt5a transfection (Figure 3B). Subsequently, qRT-PCR data revealed that the mRNA level of Wnt5a is negatively regulated by microRNA-26b-5p (Figure 3C). Western blot results also indicated that the protein level of Wnt5a was negatively regulated by microRNA-26b-5p (Figure 3D).

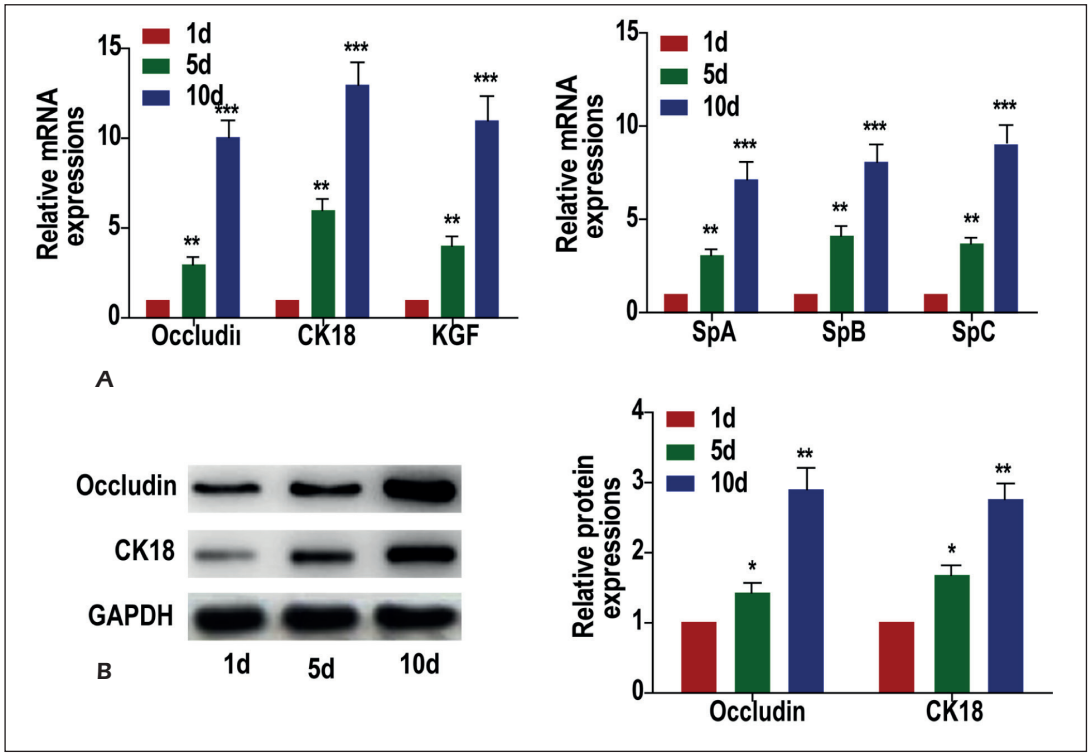

Figure 1. Differentiation identification of MSCs to AECII. **A**, The mRNA levels of Occludin, KGF, CK18, SpA, SpB and SpC on the 1st, 5th, and 10th day of cell differentiation. **B**, Protein expressions of Occludin and CK18 in the process of cell differentiation.

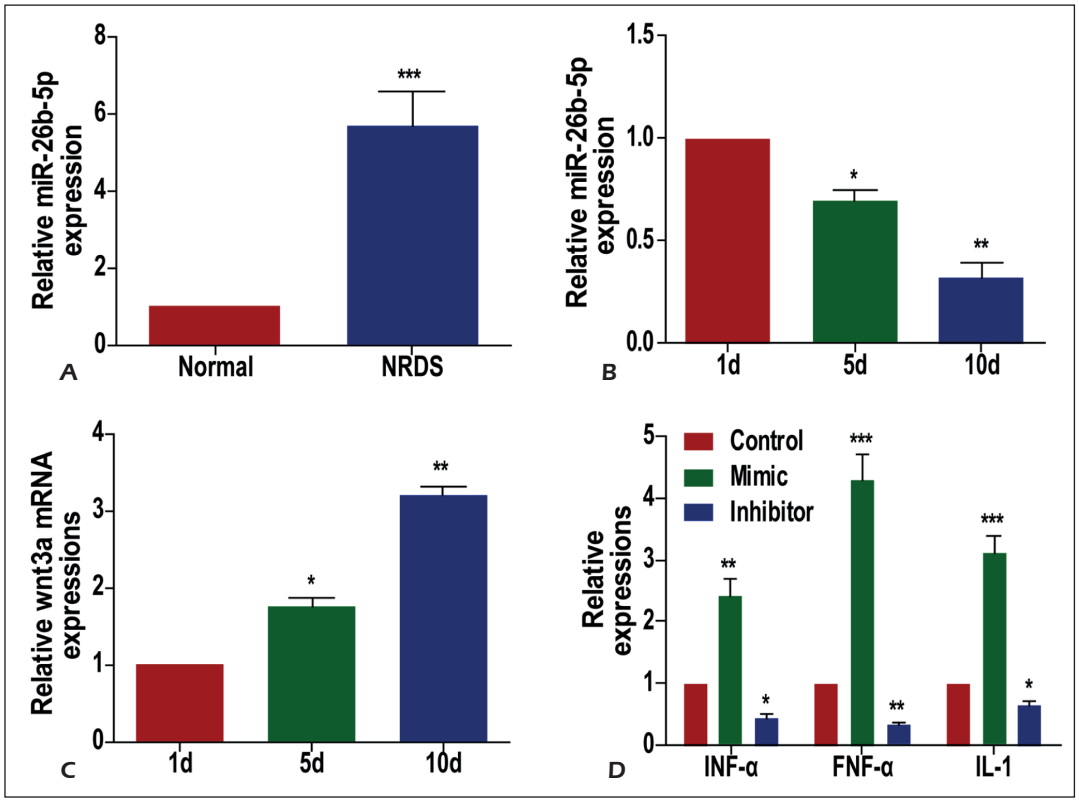

Figure 2. MicroRNA-26b-5p was highly expressed in NRDS. **A**, MicroRNA-26b-5p expression in peripheral blood of NRDS and healthy neonates. **B**, MicroRNA-26b-5p expression on the 1st, 5th, and 10th day of cell differentiation. **C**, Wnt5a expression on the 1st, 5th, and 10th day of cell differentiation. **D**, Levels of TNF-α, INF-α and IL-1 in cell supernatant detected by ELISA.

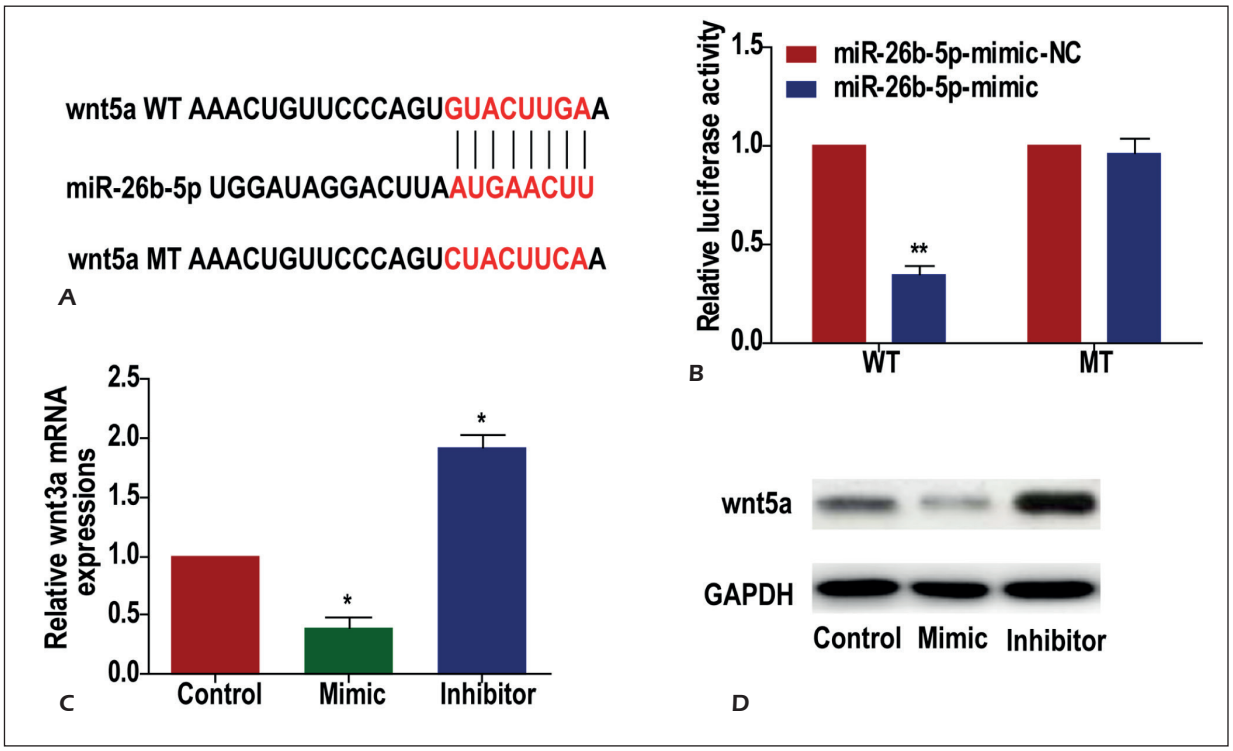

Figure 3. Wnt5a was the target gene of microRNA-26a-5p. **A**, The binding site of Wnt5a and microRNA-26b-5p. **B**, Luciferase activity after co-transfection of microRNA-26a-5p and wild-type or mutant-type Wnt5a. **C**, The mRNA level of Wnt5a after microRNA-26b-5p overexpression or knockdown. **D**, Protein level of Wnt5a after microRNA-26b-5p overexpression or knockdown.

MicroRNA-26a-5p Inhibited Differentiation of MSCs to AECII Via Inhibiting Wnt/β-Catenin Pathway

After overexpression of microRNA-26b-5p, mRNA levels of Occludin, KGF, CK18, SpA, SpB, and SpC were remarkably downregulated (Figure 4A). Besides, protein expressions of Occludin and CK18 were downregulated by microRNA-26b-5p overexpression as well, which were reversed by Wnt5a overexpression (Figure 4B). Additionally, the elevated levels of TNF-α, INF-α, and IL-1 induced by microRNA-26b-5p overexpression were also reversed by Wnt5a overexpression (Figure 4C). The above results indicated that the regulatory effect of microRNA-26b-5p on MSCs differentiation to AECII depends on inhibition of the Wnt pathway.

Discussion

MSCs that are currently used for researches mainly include bone marrow mesenchymal stem cells, embryonic mesenchymal stem cells, umbilical mesenchymal stem cells, and adipose-derived mesenchymal stem cells[16,17]. Here we utilized MSCs extracted from bone marrow due to its simple collection, small trauma, sufficient sources, and immunomodulatory ability[18,19].

MSCs exert their therapeutic effects on tissue damage mainly through two pathways, which are cell replacement for tissue regeneration and enhancement of cellular function. Accumulating evidence has confirmed that exogenously transplanted MSCs can be differentiated into lung epithelial cells by immunofluorescence labeling[20]. At present, inducible approaches for stem cell differentiation to AECII mainly include co-culture method, SAGM induction, AECII extract stimulation, and liquid-gas interface induction method, among which the co-culture method and SAGM induction are mostly applied[9,20,21]. In this study, 2-week SAGM induction was performed for cell differentiation. Cell differentiation is the result of the selective expression. We found that AECII-related genes were upregulated during the process of cell differentiation, including Occludin, KGF, CK18, SpA, SpB, and SpC, indicating the successful cell differentiation induction.

As a complex network of protein interactions, Wnt pathway exerts a crucial role in lung development. It is suggested that the Wnt pathway activators Wnt3a and LiCl, as well as β-catenin

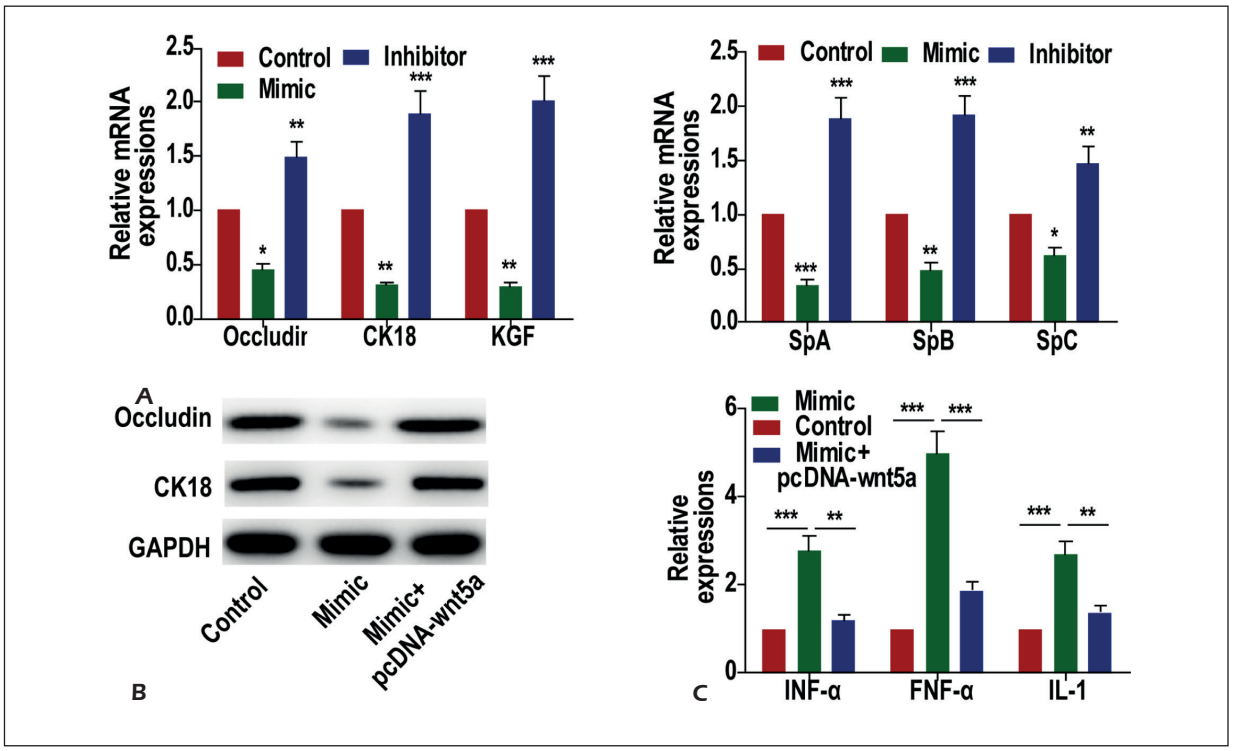

Figure 4. MicroRNA-26a-5p inhibited differentiation of MSCs to AECII via inhibiting Wnt/β-catenin pathway. **A**, The mRNA levels of Occludin, KGF, CK18, SpA, SpB, and SpC after microRNA-26b-5p overexpression or knockdown. **B**, Protein expressions of Occludin and CK18 after overexpression of microRNA-26b-5p and Wnt5a. **C**, Levels of TNF-α, INF-α, and IL-1 in cell supernatant after overexpression of microRNA-26b-5p and Wnt5a detected by ELISA.

overexpression, could activate the Wnt pathway, which in turn promotes MSCs differentiation to AECII. Activation of Wnt/JNK and Wnt/PKC pathways through Wnt5a treatment obtains the similar effects[11]. Differentiation of rat adipose-derived MSCs is also promoted by the Wnt pathway. Hence, in-depth researches on Wnt pathway contribute to better understanding of MSCs differentiation, so as to improve clinical outcomes of NRDS and other pulmonary diseases.

We found that microRNA-26b-5p is highly expressed in NRDS. Further experiments confirmed that microRNA-26b-5p expression is up-regulated during MSCs differentiation to AECII. Overexpression of microRNA-26b-5p inhibited directional differentiation of AECII and inflammatory response *via* targeting Wnt5a.

Conclusions

We found that microRNA-26b-5p inhibits MSCs differentiation to AECII *via* inhibiting Wnt5a expression, which provides novel directions in developing therapeutic strategies for NRDS.

Conflict of Interests

The authors declare that they have no conflict of interest.

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