miR-206 inhibits FN1 expression and proliferation and promotes apoptosis of rat type II alveolar epithelial cells

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Abstract. Bronchopulmonary dysplasia (BPD) is a syndrome of respiratory distress caused by chronic lung injury, primarily in preterm infants. miR-206 and fibronectin 1 (FN1) are associated with the development of BPD. The present study used rat type II alveolar epithelial cells (AECII) to investigate the underlying mechanisms of BPD. AECII were isolated using a primary cell culture prior to alkaline phosphatase staining and immunofluorescence of surfactant protein C (SP-C). These were used to verify the presence of AECII. AECII were then divided into four groups, which were transfected with four different plasmids. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to determine the relative expression of miR-206 in the each group. The gene and protein expression level of FN1 was detected by RT-qPCR and immunofluorescence. The proliferation of AECII in each of the four groups was evaluated using an MTT assay 48 h following transfection. The percentage of apoptotic cells was determined by flow cytometric analysis. The present study demonstrated that upregulation of miR-206 decreased the expression of FN1 ($P<0.05$) and low levels of miR-206 led to increased expression of FN1 ($P<0.05$) in AECII. Furthermore, the forced expression of miR-206 suppressed proliferation and promoted apoptosis of AECII while downregulation of miR-206 had the opposite effect ($P<0.05$). The results of the current study provide valuable insights into the prevention of BPD and suggest that miR-206 may be used as a potential molecular target for BPD therapy in the future.

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Abbreviations: AECII, type II alveolar epithelial cells; BPD, bronchopulmonary dysplasia; ECM, extracellular matrix; miRNAs, microRNAs; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TGF-β1, transforming growth factor-β1; FN1, fibronectin 1

Key words: bronchopulmonary dysplasia, fibronectin 1, lung injury, rat type II alveolar epithelial cells, microRNA-206

Introduction

Bronchopulmonary dysplasia (BPD) is a common chronic lung disease in preterm infants who require assisted ventilation (1,2). BPD is characterized by impaired alveolarization, a primary cause of mortality in premature infants (3). A number of of genetic and environmental factors have been identified to be responsible for development of BPD (4-6). However, the complete mechanism of BPD remains unknown and further studies are required to identify novel mechanisms involved in the process.

Fibronectin 1 (FN1), a component of the extracellular matrix (ECM), is widely distributed in blood vessel and smooth-muscle cell layers (7). Abnormal expression of FN1 has been identified in a number of human diseases, including cancers (8). FN1 may also interact with other ECM proteins, which may in turn serve important roles in the development of BPD (9,10).

microRNAs (miRNAs or miRs) were first identified in the early 1990s (11,12). miRNAs regulate gene expression post-transcriptionally and serve essential roles in a wide range of biological functions, including cell proliferation, apoptosis and differentiation (13-16). A number of previous investigations indicated that miRNAs are associated with the process of fetal lung maturation and metabolism of pulmonary surfactant (17,18). Previous studies have demonstrated the important role of miRNAs in the development of BPD (19,20). It has also been indicated that miR-206 is downregulated in mice modeling BPD and BPD in human patients (21).

BPD is a chronic disease characterized by disruption of the alveolar and microvascular development of the peripheral lung. The regeneration or stimulation of type II alveolar epithelial (AECII) cell growth has been the focus of studies aiming to regenerate the lung (1,3,7). Therefore, AECII is critical for development and thus the treatment of BPD (21,22). Due to the importance of AECII, it was selected as the experimental model to elucidate the mechanism of miR-206 affecting BPD in the present study. Using a primary cell culture of AECII allows the possible effects of miR-206 on AECII to be studied. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

and immunofluorescence were performed to study the effect of miR-206 on FN1. Different levels of miR-206 expression indicated proliferation and apoptosis of AECII. The present study provides insight into the function of AECII and improves the understanding of its role in the development of BPD.

Materials and methods

Cell isolation and culture. Four two-month old Sprague-Dawley female rats (200-250 g) were obtained from Beijing Military General Hospital of Southern Medical University (Beijing, China). Rats were housed in cages at the temperature of 22±2˚C and 40±5% humidity under a 12-h light/dark cycle and received a standard diet and water *ad libitum*. Following anesthetization with an injection of pentobarbital (0.2 ml/100 g; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), rat lungs were obtained by surgical resection and digested by trypsin (Gibco; Thermo Fisher Scientific, Inc., Grand Island, NY, USA) through the trachea (18). Lung cells were transferred to dishes coated with rat immunoglobulin (Ig) G (500 μg/ml; SP032; Beijing Solarbio Science & Technology Co., Ltd.) on the basis of the different adherence abilities of cells. AECII in the supernatant was collected by pipette and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in an incubator at 37˚C with 5% CO_2 for 24 h.

Identification of AECII. For the alkaline phosphatase (BCIP/NBT) staining, cells were fixed in 4% formaldehyde (Beijing Solarbio Science & Technology, Co., Ltd.) at room temperature for 10 min. Following washing with phosphate-buffered saline (PBS) three times, cells were incubated with 3 ml BCIP/NBT solution (Beyotime Institute of Biotechnology, Shanghai, China) for 10 min at room temperature. Cells were then rinsed with water, blotted dry and imaged using a C1si inverted microscope (Nikon Corporation, Tokyo, Japan).

Immunofluorescence of surfactant protein C (SP-C) was used to verify AECII. SP-C rabbit anti-mouse polyclonal antibody (1:100; sc-13979; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was incubated at 4˚C for 15 h, then TRITC-conjugated goat anti-rabbit polyclonal antibody (1:200; 85851; Jackson ImmunoResearch Laboratories, Inc., West Grove, BA, USA) was added and incubated at 37˚C for 1 h. Hoechst 33258 (Beyotime Institute of Biotechnology) was added to label the nuclei. A confocal microscope (Nikon Corporation) was used to observe the cells. The present study was approved by the Ethics Committee of Beijing Military General Hospital (Beijing, China) and conformed to the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (23).

Plasmid synthesis. All plasmids were synthesized by Shanghai GenePharma, Co., Ltd. (Shanghai, China), including the overexpression plasmid for miR-206 (pcmv-206), the negative control (pcmv-nc), miRNA inhibitors for miR-206 (pgpu6-206) and inhibitor negative control (pgpu6-nc).

Transient transfection. AECII were randomly divided into four groups. Group 206 were transfected with pcmv-206, group nc were transfected with pcmv-nc, group 206-i were transfected with pgpu6-206 and group nc-i were transfected with pgpu6-nc. Cells were transfected by Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer protocol. When grown to 80-90% confluence 24 h later, AECII were transfected with the four plasmids. AECII were harvested at 48 h following transfection for subsequent studies. Transfection experiments were completed three times.

RNA extraction and RT-qPCR. RT-qPCR was performed to detect the gene expression of miR-206 and FN1. Total RNA of AECII was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to a standard protocol (7). Then cDNA was synthesized by reverse-transcription reaction. RT-qPCR was conducted using THUNDERBIRD® SYBR® qPCR mix (Toyobo, Co., Ltd., Osaka, Japan). The ABI Prism® 7000 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to analyze the product. The PCR primers were designed using Primer Premier 5.0 (PREMIER Biosoft, Inc., Paolo Alto, CA, USA) complementary to rat miR-206 and FN1 cDNA sequences (Sangon Biotech Co., Ltd., Shanghai, China). The primer sequences for RT-qPCR were as follows: miR-206 forward, 5'-GGGTGGA ATGTAAGGAAGT-3' and reverse, 5'-TGCGTGTCGTGGA GTC-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAA AAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGT CAT-3; FN1 forward, 5'-CTCGCTTTGACTTCACCACCA-3' and reverse, 5'-TCTCCTTCCTCGCTCAGTTCGTACT-3'; β-actin forward, 5'-TGGAATCCTGTGGCATCCATGAAAC-3' and reverse, 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'. The ABI Prism® 7000 Sequence Detection system program was as follows: 95˚C initial denaturation for 5 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 59˚C for 15 sec and elongation at 72˚C for 33 sec. Relative expressions of miR-206 and FN1 were analyzed with normalization against U6 and β-actin values respectively using the $2^{-\Delta\Delta Cq}$ method described by Livak and Schmittgen (24). All reactions were performed three times.

Immunofluorescence of FN1. Four slides of AECII were incubated with a polyclonal rabbit anti-mouse FN1 antibody (1:100; 15613-1-AP; Proteintech, Rosemont, IL, USA) at 4˚C overnight. A goat anti-rabbit polyclonal IgG conjugated with TRITC (85851; Jackson ImmunoResearch Laboratories, Inc.) was added as the secondary antibody (1:200) at 37˚C for 1 h. The cellular nuclei were stained with Hoechst 33258 (Beyotime Institute of Biotechnology). A confocal microscope (Nikon Corporation) was used to image the immunofluorescence.

Proliferation assays. To study the effect of miR-206 on the proliferation of AECII, $4x10^4$ cells were seeded into 96-well culture plate and transfected with one of the four plasmids. The cell viability was analyzed using an MTT assay at 48 h after transfection. First, 20 μl MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to the 96-well plate and incubated at 37˚C for 4 h in the dark. Then, the supernatant was carefully discarded and 150 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to the plate at room temperature for 20 min. The MTT optical density value was measured at 492 nm using a microplate reader (Bio-Rad

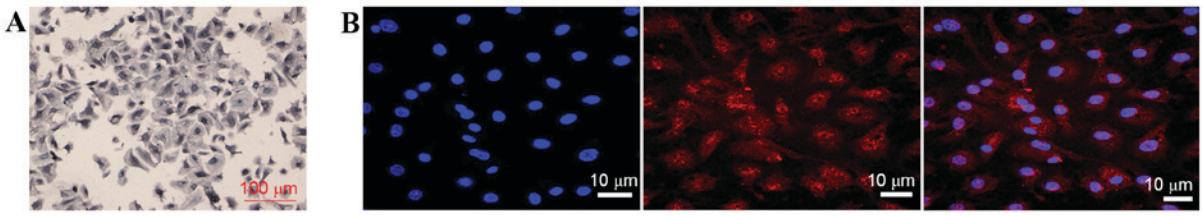

Figure 1. Identification of AECII following alkaline phosphatase staining and immunofluorescence of SP-C. (A) Alkaline phosphatase staining of AECII. Positive staining indicated by grayish staining in cells. (B) Immunofluorescence of surfactant protein SP-C in AECII. A strong red fluorescence signal indicated by SP-C. AECII, type II alveolar epithelial cells; SP-C, surfactant protein C.

Laboratories, Inc., Hercules, CA, USA). The experiment was repeated three times.

Apoptosis assay. A total of $1x10^6$ AECII were plated into six-well plates and transfected with four plasmids using Lipofectamine® 2000. Apoptosis inducer, cisplatin (Beyotime Institute of Biotechnology) was also added to the culture. Cells were collected 48 h following transfection and stained with Annexin V and propidium iodide (APT750; Merck Millipore, Billerica, MA, USA) according to manufacturer protocol. The apoptotic percentage was determined by flow cytometric analysis (Beckman Coulter, Inc., Brea, CA, USA). All these procedures were repeated in triplicate.

Statistical analysis. Experiments were repeated three times and data were presented as the mean ± standard deviation. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Differences between two groups were calculated with the two-tailed *t*-test. P<0.05 was considered to represent statistically significant differences.

Results

Identification of AECII. AECII have alkaline phosphatase activity however, other lung cells including alveolar macrophages lack phosphatase activity. Therefore, alkaline phosphatase activity was assessed to distinguish AECII from other cells. Positive staining was indicated by grey staining in the cells (Fig. 1A).

AECII secretes pulmonary surfactants. SP-C, the only AECII-specific surfactant, was also used to identify AECII (Fig. 1B). The red fluorescence in Fig. 1B indicates SP-C, which was located in the cytoplasm. The blue fluorescence indicates the nuclei. The results indicated that the isolation of AECII was successful.

Expression of miR-206 in AECII. To investigate the function of miR-206, exogenous miR-206 plasmids, inhibitors and control plasmids were assessed. To increase the expression of miR-206, pcmv-206 was transfected into AECII. To maintain the stable expression of miR-206 in AECII, pcmv-nc was transfected into AECII. AECII were harvested 48 h following transfection and the expression of miR-206 was determined using RT-qPCR. The level of miR-206 in group 206 significantly increased by ~30-fold compared with group nc (P<0.05; Fig. 2A).

To downregulate the expression of miR-206, pgpu6-206 was transfected into AECII and pgpu6-nc was transfected into AECII as controls. In group 206-i, the expression of miR-206

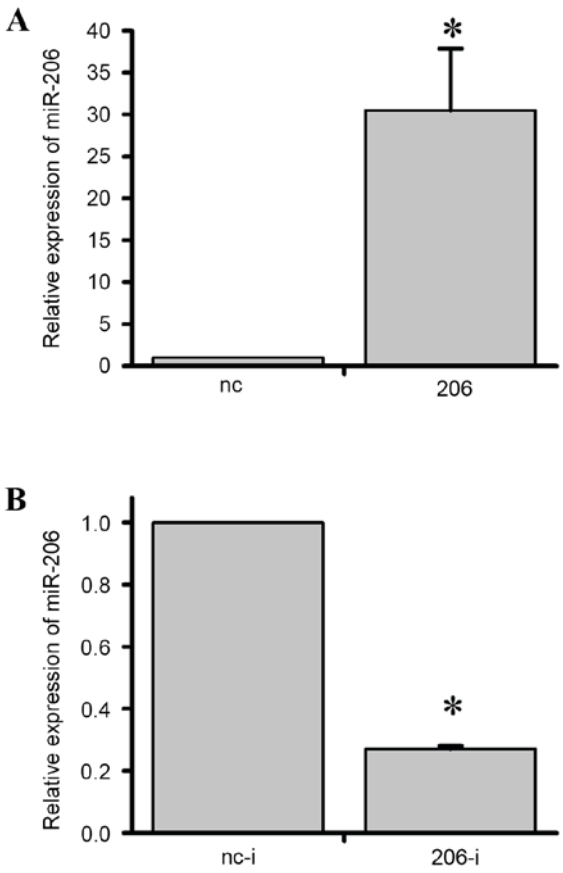

Figure 2. Relative expression of miR-206 in four groups. (A) Relative expression of miR-206 in group 206 increased compared with nc group. (B) Relative expression of miR-206 in group 206-i decreased compared with group nc-i. *P<0.05. miR-206, micro RNA 206; nc, group transfected with pcmv-nc; 206-i, group transfected with pgpu6-206; nc-i, group transfected with pgpu6-nc.

significantly reduced compared with group nc-i (P<0.05; Fig. 2B). These data indicated that the four different plasmids transfected AECII successfully, which provides important basis for further study.

MiR-206 inhibited the expression of FN1 in AECII. To determine whether the synthesis of FN1 is affected by miR-206 in AECII, RT-qPCR was completed to detect the relative expression of FN1 at a gene level. As presented in Fig. 3A, FN1 mRNA expression in group 206 decreased by 80% compared with the control group (P<0.05). By contrast, low expression of miR-206 caused an increase in FN1 expression by ~seven-fold compared with control group (P<0.05; Fig. 3A).

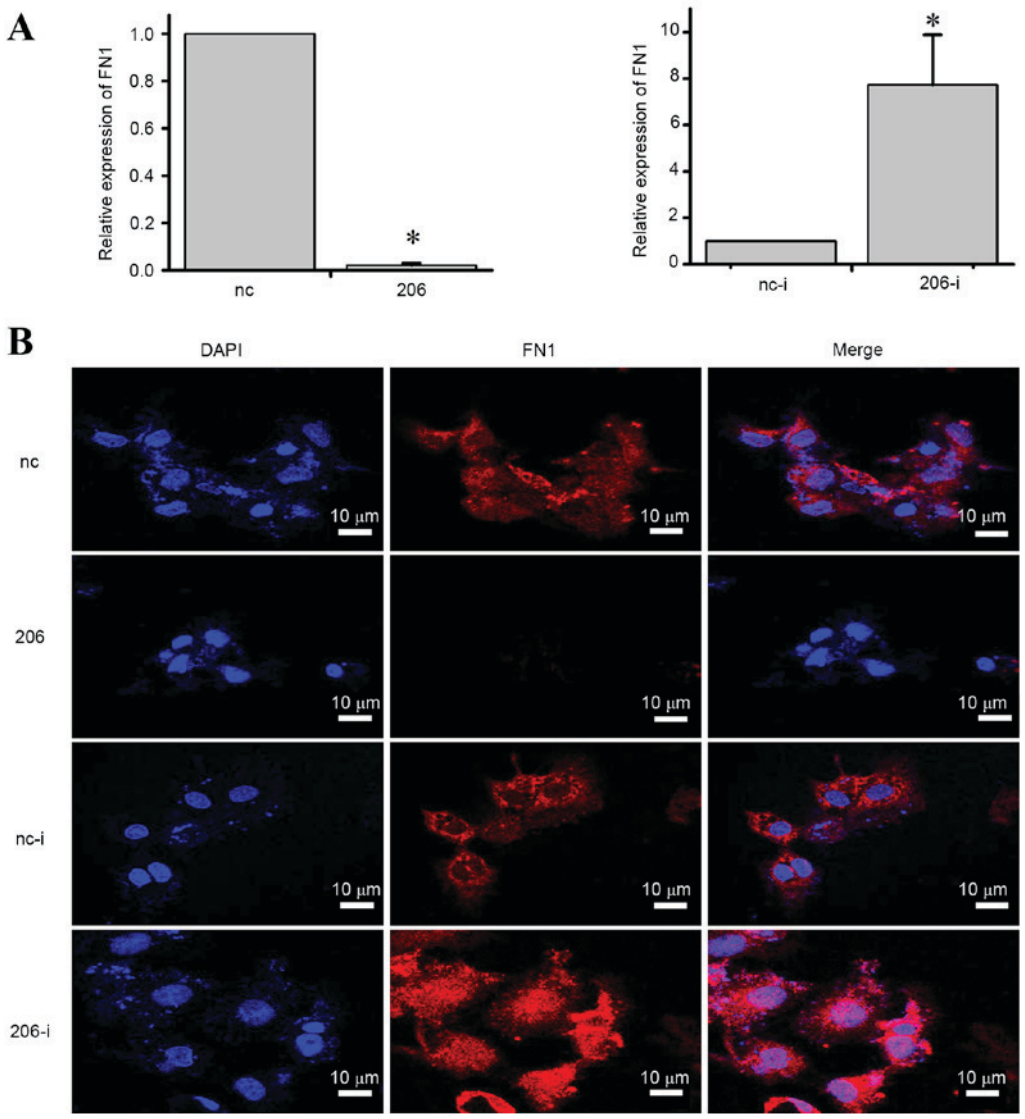

Figure 3. Relative expression of FN1 in four groups. (A) Detection of FN1 mRNA expression in AECII was performed by reverse transcription-quantitative polymerase chain reaction (*P<0.05). (B) Detection of FN1 protein expression in AECII performed by immunofluorescence. N1, fibronectin 1; AECII, type II alveolar epithelial cells; nc, group transfected with pcmv-nc; nc-i, group transfected with pgpu6-206; 206, group transfected with pcmv-206; 206-i, group transfected with pgpu6-206.

To confirm the effect of miR-206 on protein level, immunofluorescence was used to detect the level of FN1 protein in AECII. Group 206 demonstrated a weak signal of red fluorescence of FN1 and 206-i group demonstrated a strong signal of red fluorescence of FN1 (Fig. 3B). This was consistent with the effect of miR-206 on the expression of genes.

In the present study, four different plasmids were transfected into AECII to investigate the effect of miR-206 on FN1 in AECII. The results indicated that FN1 may be negatively regulated by miR-206 in AECII thus, suggesting that FN1 may be involved in the process of BPD.

MiR-206 inhibits proliferation and promotes apoptosis of AECII. MTT assay was performed to assess whether miR-206 had an effect on the proliferation of AECII. Following transfection with pcmv-206 (group 206), the proliferation capability of AECII decreased by 19% compared with control group (P<0.05). When transfected with pgpu6-206 (group 206-i), the proliferation capability of cells increased compared with cells transfected with pgpu6-nc by 23% (P<0.05; Fig. 4A). The flow cytometry assay results indicated that the apoptotic rate increased in the 206 group and reduced in group 206-i compared with the respective control groups (P<0.05; Fig. 4B). The findings suggest that miR-206 inhibited the proliferation and promoted the apoptosis of AECII.

Discussion

Although much remains to be fully studied, previous investigations suggest that miRNAs serve an important role by controlling the function of various genes involved in cell functions (13-15). miRNAs regulate gene expression post-transcriptionally and inhibit the expression of the target genes (13,25). As a member of the miRNA family, miR-206 regulates the translation of target mRNAs based on sequence complementarity (12,26). Previous studies have suggested that miR-206 is associated with a variety of diseases (26-29). Lewis *et al* (30) reported that miR-206 was associated with

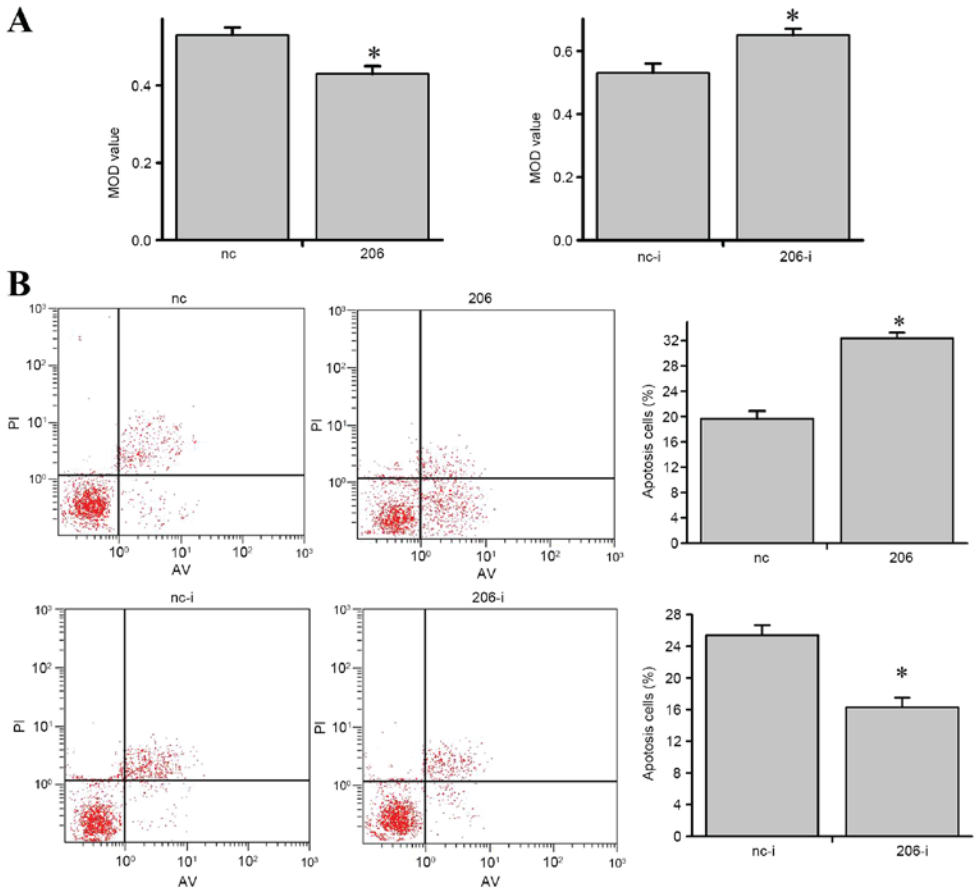

Figure 4. Effect of miR-206 on cell proliferation and apoptosis. (A) Cell proliferation in the four groups (nc, nc-I, 206 and 206-i). OD value was recorded to evaluate cell proliferation. (B) Cell apoptosis rate in the four groups. Compared with control group (nc, nc-i, respectively). *P<0.05. OD, optical density; AV, Annexin V; PI, propidium iodide. miR-206, microRNA 206; nc, group transfected with pcmv-nc; nc-i, group transfected with pgpu6-206; 206, group transfected with pcmv-206; 206-i, group transfected with pgpu6-206. MOD, mean optical density.

chronic obstructive pulmonary disease. miR-206 is involved in the formation of smooth muscle in the airway (31) and hypoxia-induced pulmonary hypertension due to an interaction with the hypoxia inducible factor 1a/four and a half limb domains 1 pathway (32).

In previous studies, the expression of miRNAs in the lungs of BPD mice was demonstrated to be different from non-BPD mice (17,19). It was also reported that expression of miR-206 was lower in the plasma of BPD patients and BPD mice compared with control group (7). These results indicated that miR-206 was closely associated with BPD.

It has also been indicated that abnormal remodeling of the ECM is a hallmark feature of BPD (3,7). The ECM proteins form intricate fibrillar networks, which regulate cell adhesion, migration and growth. Genes associated with ECM remodeling, including transforming growth factor-β1 (TGF-β1), tissue inhibitor of metalloproteinases 1 and collagen 1α, were deregulated in the development of BPD (7).

FN1, as a component of the ECM, is involved in mediating a number of biological processes including cell adhesion, migration, apoptosis and signal transduction. The glycoprotein is widely distributed in blood vessel and smooth-muscle cell layers. Abnormal regulation of FN1 has been reported in several human diseases, including cancers. FN1 is able to interact with other ECM proteins, including collagen, vascular endothelial growth factor and TGF-β1, which serve important roles in the development of BPD (8,9). Furthermore, FN1 is induced by inflammation, which has been associated with causing BPD (33,34). The expression of FN1 was demonstrated to increase in BPD mice and patients (7). Therefore, implying that FN1 is involved in the development of BPD.

An miRNA may regulate a variety of genes. In the present study, the gene and protein expression of FN1 was detected following the transfection of different plasmids into AECII. The results indicated that upregulation of miR-206 may inhibit the expression of FN1 in AECII. While FN1 serves a vital role in BPD, the current study may help improve the understanding of the underlying mechanism of BPD.

AECII are able to synthesize and secrete alveolar surfactants to reduce surface tension. AECII are able to differentiate into the type I alveolar epithelial cells, which are progenitor cells for the re-epithelization of impaired alveoli (3,16,21,22). However, excessive proliferation and abnormal differentiation of AECII is closely associated with BPD (3,7). Data from the current study indicated that miR-206 inhibited proliferation and promoted apoptosis of AECII. Therefore indicating that miR-206 may partly prevent the progression of BPD.

In conclusion, the present *in vitro* study suggests that miR-206 modulates numerous aspects of biology in AECII. Offering novel insights into the prevention of BPD and indicating that miR-206 may be used as a promising molecular target for BPD therapy in the future.

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