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Mutual regulation between miR-21 and the TGF β /Smad signaling pathway in human bronchial fibroblasts promotes airway remodeling

Zhi-Wei Yu, MD, Ya-Qin Xu, MMed, Xiao-Juan Zhang, MMed, Jian-Rong Pan, MMed, Hong-Xia Xiang, MMed, Xiao-Hong Gu, MD, Shan-Bao Ji, MMed, and Jun Qian, MMed

Department of Pediatrics, Wuxi Children's Hospital Affiliated to Nanjing Medical University, Wuxi, China

ABSTRACT

Objective: Airway remodeling is an important pathological feature of asthma. Excessive deposition of extracellular matrix (e.g., collagen) secreted from fibroblasts is a major factor contributing to airway remodeling. Currently, the mechanism by which collagen continues to be oversynthesized in the airway remains unclear. In this study, we investigated the role of the microRNA-21 (miR-21) and TGF β /Smad signaling pathway in human bronchial fibroblasts (HBFs), and explored the regulatory mechanism of airway remodeling. **Methods:** HBFs were cultured *in vitro* and treated with the transforming growth factor β (TGF β), receptor inhibitor (SB431542), and TGF β 1. miR-21 and Smad7 overexpressing lentiviruses, as well as an miR-21 interfering lentivirus were constructed and transfected into HBFs. Western blotting was used to determine the expression of airway remodeling-related proteins and proteins in the TGF β /Smad signaling pathway. miR-21 expression was measured by quantitative real-time PCR. **Results:** The high expression of miR-21 induced by TGF β 1 was reduced following the treatment with the SB431542 in HBFs. Smad7 overexpression inhibited the elevated expression of the COL I protein induced by miR-21 overexpression in HBFs. Inhibiting miR-21 expression upregulated the level of Smad7 protein, thus reducing the expression of airway remodeling-related proteins induced by TGF β 1 stimulation in HBFs. **Conclusions:** TGF β 1 can induce miR-21 expression in HBFs through the TGF β /Smad signaling pathway to promote airway remodeling. miR-21 downregulates Smad7, activates the TGF β /Smad signaling pathway, and promotes airway remodeling. Mutual regulation between miR-21 and the TGF β /Smad signaling pathway in HBFs promotes airway remodeling.

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MicroRNA-21; transforming growth factor β ; TGF β signaling; airway remodeling; human bronchial fibroblasts

Introduction

The expression of TGF β 1 is significantly increased in the airway mucosa of asthmatic lungs, and is related to thickening of the basement membrane, an increased number of fibroblasts, and disease severity. In addition, TGF β 1 can promote the proliferation, inhibit the apoptosis, promote collagen synthesis (i.e., collagen type I [COL I]), and induce the expression of α -smooth muscle actin (α -SMA), fibronectin (FN), and other airway remodeling-related proteins in fibroblasts; therefore, TGF β 1 facilitates the formation of fibrosis, stimulates airway remodeling, and induces pathological changes in the structure of the airways (1,2).

The TGF β /Smad signaling pathway is important for the biological function of TGF β 1. Two TGF β receptors are essential for the signal transduction of TGF β 1: TGF β type I receptor (TGF β RI) and TGF β type II receptor (TGF β RII). TGF β 1 first binds to TGF β RII, which has serine/threonine-specific protein kinase activities, then

TGF β RI binds to TGF β 1; their binding induces the phosphorylation of TGF β RI in the glycine/serine-enriched region. The activated TGF β RI causes the phosphorylation of the pathway-restricted Smads (Smad2 and Smad3), which bind to Smad4. The Smad4 and phosphorylated Smad2/Smad3 complex is translocated into the nucleus and binds to the specific DNA element of the target gene, thus activating transcription. The inhibitory Smad protein, Smad7, also binds to TGF β RI to prevent the phosphorylation of Smad2/Smad3 induced by TGF β RI and attenuate TGF β receptor signal transduction. Therefore, any factor that affects the synthesis, activation, or signaling of TGF β 1 could contribute to the regulation of TGF β 1-mediated activities (3–5).

Currently, the mechanism by which collagen is continuously oversynthesized in the airway remains unclear. Moreover, there is no effective approach that has been established for the prevention and treatment of airway remodeling in a clinical setting. miRNA can bind to the

3' untranslated region of target genes and induce mRNA degradation or suppress the protein translation of target genes. As a key regulator of physiological and pathological events in the human body, miRNA is involved in many biological processes, including cellular proliferation and differentiation, the repair of tissue damage, and organ fibrosis. Increased TGF β 1 can activate the TGF β /Smad signaling pathway, elevate miR-21 expression, and promote fibrosis (6). Moreover, it has been reported that inhibiting miR-21 expression can reduce the severity of bleomycin-induced pulmonary fibrosis in rats (7). However, the role of miR-21 in airway remodeling and whether its mechanism is similar to that of pulmonary fibrosis require further clarification.

In this study, we used a TGF β 1 receptor inhibitor and TGF β 1 to stimulate HBFs, constructed miR-21 over-expressing and interfering lentivirus vectors, and transfected HBFs with such lentiviruses *in vitro* to explore the roles of miR-21 and the TGF β /Smad signaling pathway in airway remodeling.

Methods

Reagents

Anti-COL I, anti- α -SMA, Anti-FN, Anti-TGF β RII, Anti-Smad7, anti-phosphorylated Smad2 (pSmad2), and anti-phosphorylated Smad3 (pSmad3) antibodies were purchased from Abcam Inc. (Cambridge, MA, USA).

Cell culture

Human bronchial fibroblasts (HBFs) were purchased from CHI Scientific Co., Ltd. (Maynard, MA, USA). The

cell passage, cryopreservation, resuscitation, and culture were performed according to the manufacturer's instructions. Cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin and incubated in a 5% CO₂ atmosphere at 37°C.

Optimal concentration of TGF β 1 and the time of stimulation for HBFs

The HBFs were seeded into six well plates and cultured until the number of cells reached 5E5. When the cells grew to 50% to 70% confluence, 0, 1, 2, 5, and 10 ng/mL TGF β 1 (Abcam, Cambridge, MA, USA) was applied to stimulate the cells for 48 h separately. The expression of COL I protein was determined by Western blot to identify the optimal stimulating concentration of TGF β 1 (5 ng/mL) (Figure 1A and Figure 1B).

A concentration of 5 ng/mL TGF β 1 was used to stimulate cells for different durations (0, 6, 12, 24, 48, and 72 h). The expression of COL I protein was determined by Western blot to identify the optimal duration of TGF β 1 stimulation (48 h) (Figure 1C and Figure 1D).

Effect of the TGF β receptor inhibitor on miR-21 expression in HBFs

HBFs were cultured until the number of cells reached 5E5. When the cells reached 50% to 70% confluence, they were divided into four groups and treated with: 1) 5 ng/mL TGF β 1 for 48 h in group A; 2) 10 μ M (8,9) TGF β receptor inhibitor, SB431542 (Abcam, Cambridge, MA, USA), for 48 h in group B; 3) both TGF β 1 and SB431542 for

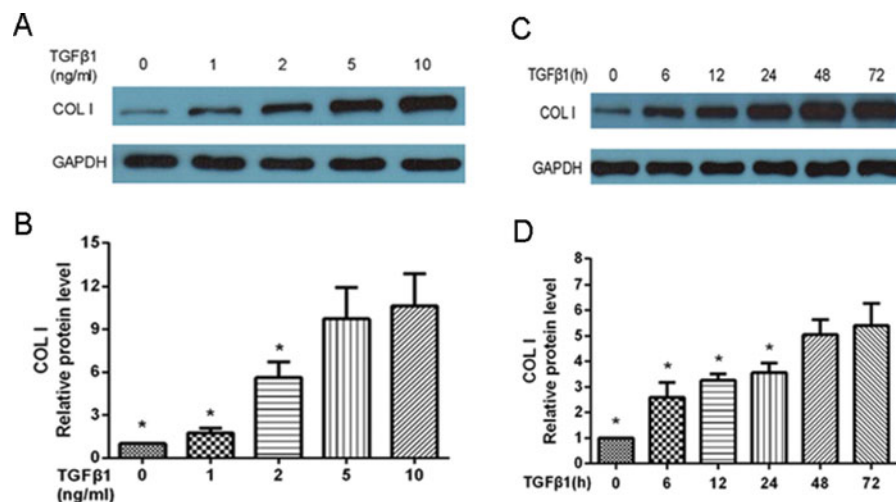


Figure 1. Time and concentration of TGF β 1 used for human bronchial fibroblasts (HBFs) stimulation. (A, B) Western blot results for determining COL I expression in HBFs treated with different concentrations of TGF β 1 for 48 h (*P < 0.05 vs. treatment with 5 ng/ml TGF β 1). (C, D) Western blot results for determining COL I expression in HBFs treated with 5 ng/ml TGF β 1 for different durations (*P < 0.05 vs. treatment with TGF β 1 for 48 h). The results are expressed as mean $\pm$ SD. All data are representative of three independent experiments.

48 h in group C; and no treatment as a control in group D. The expression of miR-21 was measured by qRT-PCR following RNA extraction. The protein expression of COL I, α -SMA, FN, pSmad2, and pSmad3 was determined by Western blot.

HBFs were infected with an miR-21 overexpressing lentivirus

According to the sequence of miR-21, the pre-miRNA of miR-21 was synthesized and constructed into pLVX-shRNA2-ZsGreen vector to obtain an miR-21-overexpressing lentivirus (Gencreate Biological Engineering Co., Ltd., Wuhan, China). HBFs were cultured until the number of cells reached 5×10^5 . Determining the optimal Multiplicity of infection (MOI) value: HBFs were infected with the virus at an MOI value of 1, 5, 10, 15, 20, 25, or 30. After a 48-h incubation, the optimal MOI value of the infected HBFs was determined by fluorescence microscopy (MOI = 20). When the cells grew to 50% to 70% confluence, the HBFs were infected with the lentivirus at an MOI value of 20, and the cells treated with the empty vector served as the control. The HBFs were stimulated with 5 ng/mL TGF β 1 for 48 h. After 48 h of lentiviral infection, a fluorescence microscope was used to ensure a successful infection. The cell morphology was observed using white fluorescence, and the cell fluorescence was observed by green fluorescence. The expression of miR-21, Smad7 mRNA, and TGF β RII mRNA was measured by qRT-PCR. The protein expression of COL I, α -SMA, FN, TGF β RII, pSmad2, pSmad3, and Smad7 was determined by Western blot.

HBFs were infected with a Smad7 overexpressing lentivirus

The Smad7 gene sequence was synthesized and constructed into pCDH-CMV-MCS-EF1-copGFP vector to obtain a Smad7 overexpressing lentivirus (Gencreate Biological Engineering Co., Ltd., Wuhan, China). Cells overexpressing miR-21 were infected with the Smad7 overexpressing lentivirus and co-cultured for 48 h. The expression of Smad7 and COL I was determined by Western blot after collecting the cells and extracting the protein.

HBFs were infected with an miR-21 interfering lentivirus

The interfering gene was designed according to the mature miR-21 sequence and constructed into pLVX-shRNA2-ZsGreen vector to obtain an miR-21 interfering lentivirus (Gencreate Biological Engineering Co., Ltd.,

Wuhan, China). HBFs were cultured until the number of cells reached 5×10^5 . When the cells reached 50% to 70% confluence, the HBFs were infected with the lentivirus at an MOI of 20, and the cells treated with the empty vector served as the control. After 48 h of lentiviral infection, the HBFs were stimulated with 5 ng/mL TGF β 1 for 48 h. After 48 h of cell culture, fluorescence microscopy was used to ensure that the cells were successfully infected. After cell collection and RNA extraction, the expression of miR-21 was determined by qRT-PCR. After cell collection and protein extraction, the expression of COL I, α -SMA, FN, TGF β RII, pSmad2, pSmad3, and Smad7 was measured by Western blot.

Western blot

The cells were solubilized in lysis buffer and the protein concentration was determined by using BCA Protein Assay (Sigma-Aldrich, St. Louis, MO, USA). Protein samples were resolved on 4–12% BisTris gel and transferred to PVDF membranes. The membranes were incubated in PBS containing specific antibodies, 5% dry milk, and 0.1% Tween 20 at 4°C overnight. Subsequently, membranes were washed in PBS, 0.1% Tween 20, incubated for 1 h at room temperature with HRP-labeled secondary antibodies. Immunoreactive bands were visualized using an Enhanced Chemiluminescence kit (Vigorous Biotechnology Co., Ltd., Beijing, China). Developed X-ray films were scanned and densitometry of the bands was quantified with the Image J software. The intensity of each protein band was normalized against GAPDH in the same samples blotted by anti-GAPDH antibody.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA). Briefly, total RNA was reverse-transcribed to cDNA using ReverTra Ace qPCR RT Kit (TOYOBO Co., Ltd., Osaka, Japan) and a stem-loop reverse transcriptase primer according to the manufacturer's instructions. Real-time PCR was performed using SYBR[®] Premix Ex Taq[™] II kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) on an ABI PRISM 7900-HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. All reactions were run in triplicate and analyzed by the $2^{-\Delta\Delta Ct}$ method. In the experiments presented in this paper, miRNA expression in cells was normalized to U6 small noncoding RNA. For mRNA quantification, total RNA was reverse-transcribed to cDNA using oligo(dT). Real-time qPCR was performed using SYBR Green normalized to β -actin. The primers used for qRT-PCR in this study were summarized in Table 1.

Table 1. The primers used for qRT-PCR.

Gene	Primer sequence
mir-21 F	5'-ACACTCCAGCTGGGTAGCTTATCAGACTGA-3'
mir-21 R	5'-TGGTGTCTGGAGTCG-3'
U6 F	5'-CTCGCTTCGGCAGCACA-3'
U6 R	5'-AACGCTTCACGAATTTGCGT-3'
TGF β RII F	5'-GCCAACAACATCAACCACAACAC-3'
TGF β RII R	5'-GCCACTGTCTCAAAGTCTCTG-3'
Smad7 F	5'-AAGAGGCTGTGTTGCTGTGAATC-3'
Smad7 R	5'-TAGTTCGACAGTCGGCTAAGG-3'
β -actin F	5'-TGGACTTCGAGCAAGAGATG-3'
β -actin R	5'-GAAGGAAGCTGGAAGAGTG-3'

Note: F, forward primer; R, reverse primer.

Statistical analysis

Data are expressed as mean $\pm$ SD. Results were tested for variance homogeneity using Levene's test and normal distribution using Shapiro–Wilk test. Normally distributed and homogeneous data were analyzed using analysis of variance (ANOVA). All post hoc comparisons were carried out using Tukey test for significant effects. Square root transformation was done on variables that were not normally distributed and homogeneous, producing variables that were normally distributed and homogeneous to be analyzed by ANOVA. If the square-root-transformed data were not normally distributed and homogeneous, groups were compared by nonparametric Kruskal–Wallis test with Nemenyi procedure posthoc analysis. $P < 0.05$ was considered to be significant.

Results

Time and concentration of TGF β 1 used for HBF stimulation

The different concentrations of TGF β 1 (0, 1, 2, 5, and 10 ng/mL) were used to stimulate HBFs for 48 h. TGF β 1 increased the expression of COL I protein in a concentration-dependent manner. COL I expression was significantly increased when 5 ng/mL TGF β 1 was applied (Figure 1A and Figure 1B, $P < 0.05$).

HBFs were treated with 5 ng/mL TGF β 1 for different durations (0, 6, 12, 24, 48, and 72 h). TGF β 1 increased the expression of COL I protein in a time-dependent manner. COL I expression was significantly increased after 48-h stimulation (Figure 1C and Figure 1D, $P < 0.05$).

Stimulation of HBFs with the TGF β receptor inhibitor (SB431542) and TGF β 1

Compared with the control group, miR-21 expression was significantly increased in HBFs after TGF β 1 treatment and decreased following the addition of SB431542 (Figure 2A, $P < 0.05$).

Compared with the control group, the expression of COL I, α -SMA, FN, pSmad2, and pSmad3 was increased in HBFs after TGF β 1 treatment and decreased following the addition of SB431542 (Figure 2B and Figure 2C, $P < 0.05$).

HBF infection with an miR-21-overexpressing lentivirus

Immunofluorescence was used to determine the success rate of miR-21 overexpressing lentivirus infection in HBFs. The cell morphology was observed by white fluorescence, and the green fluorescent protein carried by the miR-21-overexpressing lentiviral vector was observed by green fluorescence. The success rate of lentivirus infection in the cells was more than 80% (Figure 3A).

The level of miR-21 expression in the miR-21-overexpressing group was significantly higher than that in the control group (Figure 3B, $P < 0.05$). The level of TGF β RII mRNA in the miR-21-overexpressing group was significantly lower than that in the control group (Figure 3C, $P < 0.05$). The level of Smad7 mRNA was not significantly different between the miR-21-overexpressing group and the control group (Figure 3D, $P > 0.05$).

Compared with the control group, the expression of COL I, α -SMA, FN, TGF β RII, pSmad2, and pSmad3 in TGF β 1-stimulated HBFs was increased, while the expression of Smad7 protein was decreased. Compared with the control group, the expression of COL I, α -SMA, FN, pSmad2, and pSmad3 in the miR-21-overexpressing group was increased, while the expression of TGF β RII and Smad7 was decreased (Figure 3E and Figure 3F, $P < 0.05$).

Smad7-overexpressing lentivirus infection in HBFs

In Smad7-overexpressing lentivirus-infected HBFs, Smad7 protein expression was significantly higher than that in the control group (Figure 4A and Figure 4B, $P < 0.05$). Smad7-overexpressing lentivirus infection in the miR-21-overexpression group reduced the previously increased COL I expression induced by miR-21 overexpression (Figure 4C and Figure 4D, $P < 0.05$).

HBFs infected with an miR-21-interfering lentivirus

Immunofluorescence was used to determine the success rate of the miR-21 interfering lentivirus infection in HBFs. The cellular morphology was observed by white fluorescence, and the presence of the green fluorescent protein carried by the miR-21-interfering lentiviral vector was observed by green fluorescence. The success rate

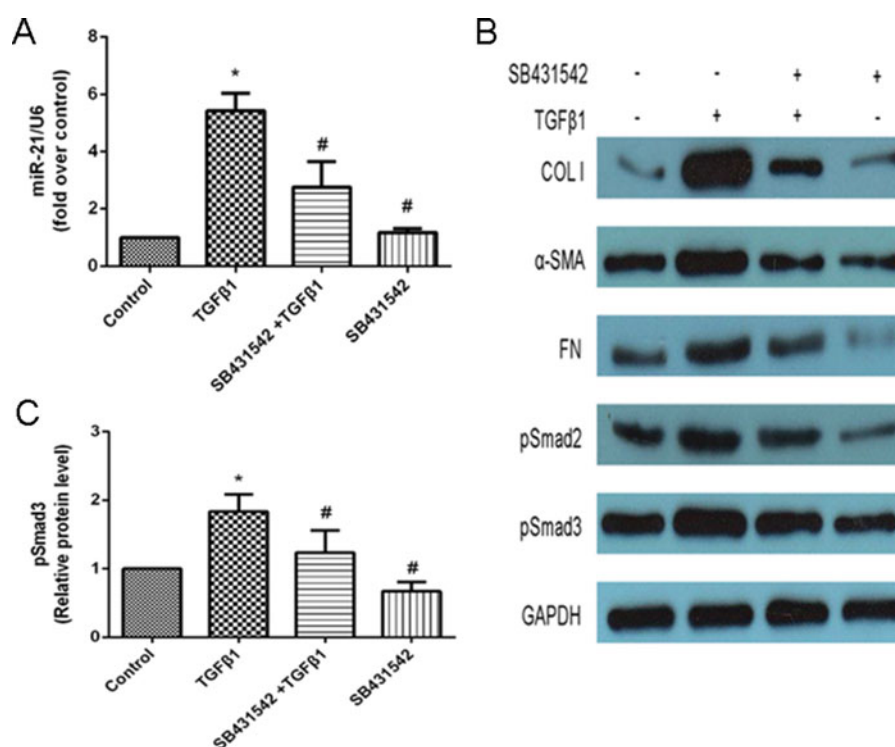


Figure 2. Stimulation of human bronchial fibroblasts (HBFs) with the TGF β receptor inhibitor (SB431542) and TGF β 1. (A) qRT-PCR results for the level of miR-21 expression after the total RNA extracted from HBFs, with U6 being used as the internal reference. (B, C) Western blot analysis after the total proteins extracted from HBFs, with GAPDH being used as the internal reference. The results are expressed as mean $\pm$ SD. All data are representative of three independent experiments (* P < 0.05 vs. control group, # P < 0.05 vs. TGF β 1-stimulated group).

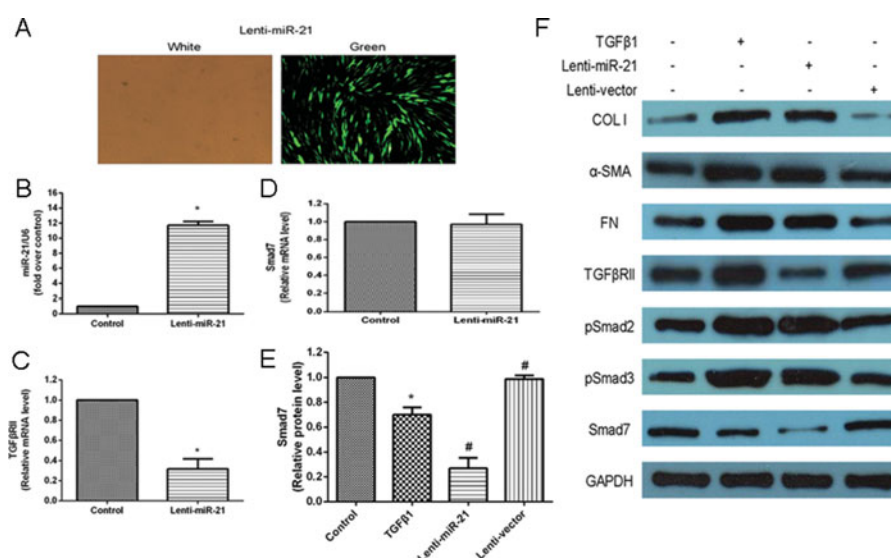


Figure 3. Human bronchial fibroblasts (HBFs) infection with an miR-21-overexpressing lentivirus. (A) Immunofluorescence was used to determine the success rate of miR-21 overexpressing lentivirus infection in HBFs (White: white light as the excitation light, Green: blue light as the excitation light). (B) qRT-PCR results for the level of miR-21 expression after the total RNA extracted from HBFs, with U6 used as the internal reference. (C) qRT-PCR results for determining the level of TGF β RII mRNA expression after the total RNA extracted from HBFs, with β -actin used as the internal reference. (D) qRT-PCR results for determining the level of Smad7 mRNA expression after the total RNA extracted from HBFs, with β -actin used as the internal reference. (E, F) Western blot analysis after the total proteins extracted from HBFs, with GAPDH used as the internal reference. The results are expressed as mean $\pm$ SD. All data are representative of three independent experiments (* P < 0.05 vs. control group, # P < 0.05 vs. TGF β 1-stimulated group).

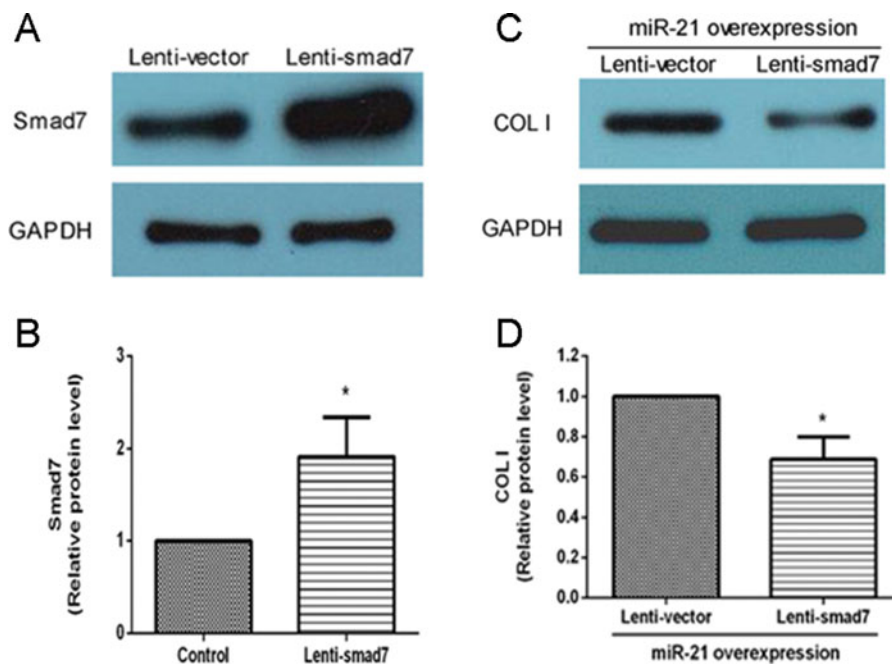


Figure 4. Smad7-overexpressing lentivirus infection in human bronchial fibroblasts (HBFs). (A, B) Western blot results of the level of Smad7 protein expression in HBFs infected with the Smad7-overexpressing lentivirus (* $P < 0.05$ vs. control group). (C, D) The level of COL I protein expression in miR-21-overexpressing HBFs infected with the Smad7-overexpressing lentivirus as determined by Western blot (* $P < 0.05$ vs. Lenti-vector group). The results are expressed as mean $\pm$ SD. All data are representative of three independent experiments.

of lentivirus infection in fibroblasts was more than 80% (Figure 5A). The level of miR-21 expression in the miR-21 interference group was significantly lower than that in the control group (Figure 5B, $P < 0.05$).

Compared with the control group, the expression of COL I, α -SMA, FN, TGF β RII, pSmad2, and pSmad3 in the TGF- β 1-stimulated HBFs was increased, while the expression of Smad7 protein was significantly decreased. miR-21 interference reduced the previously elevated expression of COL I, α -SMA, FN, pSmad2, and pSmad3 induced by TGF β 1 in HBFs, increased the previously low expression of the Smad7 protein, and further increased the previously elevated expression of the TGF β RII protein (Figure 5C and Figure 5D, $P < 0.05$).

Discussion

Bronchial asthma is a chronic inflammatory disease of the airway, which involves airway inflammatory cells, airway structural cells, and various cellular components. Airway remodeling is an important pathological feature of asthma, characterized by airway wall thickening, airway smooth muscle hyperplasia, and airway subepithelial fibrosis. The proliferation and activation of airway fibroblasts are closely associated with airway subepithelial fibrosis, which is regarded as a key factor in the induction of airway remodeling (10–14).

The excessive deposition of extracellular matrix (e.g., collagen) secreted by fibroblasts is a major factor leading to airway remodeling in asthma. Moreover, the overactivation of the TGF β /Smad signaling pathway is associated with fibrotic diseases (2,15). For example, hepatic and renal fibrosis is related to the downregulation of Smad7 expression. In addition, the upregulation of Smad7 expression can inhibit the activation of the TGF β /Smad signaling pathway by suppressing Smad2 and Smad3 phosphorylation, thus inhibiting fibrosis (16–18). TGF β 1 can promote the proliferation and inhibit the apoptosis of fibroblasts, promote the synthesis of collagen, and induce fibroblasts to express α -SMA, FN, and other airway remodeling-related proteins, finally promoting the induction of fibrosis and airway remodeling (1,19).

The dysregulation of miR-21 expression is associated with fibrotic disease and the TGF β /Smad signaling pathway (20,21). The miR-21 sequence is highly conserved in the chromosomes of humans, rats, mice, and other species. miR-21 is located on chromosome 17 in the human genome, and can inhibit the expression of target genes by degrading mRNA or suppressing the translation of mRNA (22). Our study showed that TGF β 1 can promote airway remodeling by inducing the expression of miR-21 in HBFs via the TGF β /Smad signaling pathway.

A bioinformatics analysis and dual-luciferase reporter assay revealed that Smad7 and TGF β RII are the target genes of miR-21 (23). The elevation of TGF β 1 can

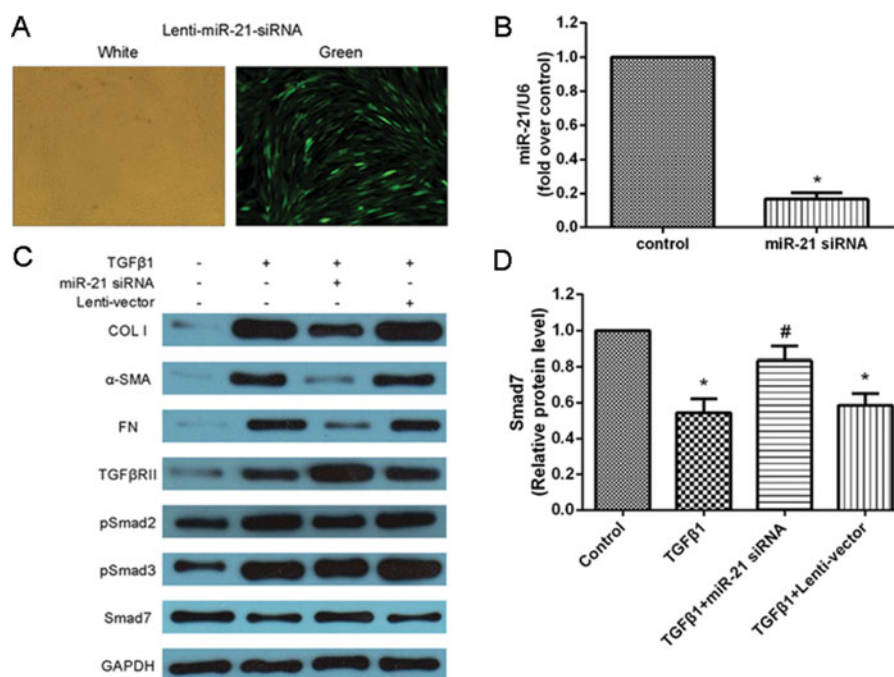


Figure 5. Human bronchial fibroblasts (HBFs) infected with an miR-21-interfering lentivirus. (A) Immunofluorescence was used to determine the success rate of the miR-21 interfering lentivirus infection in HBFs (White: white light as the excitation light, Green: blue light as the excitation light). (B) qRT-PCR results for the level of miR-21 expression after the total RNA extracted from HBFs, with U6 used as the internal reference. (C, D) Western blot analysis after the total proteins extracted from HBFs, with GAPDH used as the internal reference. The results are expressed as mean $\pm$ SD. All data are representative of three independent experiments (* $P < 0.05$ vs. control group, # $P < 0.05$ vs. TGF β 1-stimulated group).

increase the level of TGF β RII expression, which activates the TGF β /Smad signaling pathway and promotes fibrosis. Moreover, the elevation of TGF β 1 can also increase the expression of miR-21, which downregulates the expression of TGF β RII mRNA and protein, thereby inhibiting the TGF β /Smad signaling pathway and suppressing fibrosis. Therefore, miR-21 plays a protective role during fibrosis (24,25). In this study, the expression of airway remodeling-related proteins (COL I, α -SMA, and FN), miR-21, and TGF β RII was increased in TGF β 1-stimulated HBFs, indicating that miR-21 exerts an anti-fibrotic effect by downregulating TGF β RII expression. In addition, TGF β 1 exhibits a profibrotic effect by increasing TGF β RII expression in HBFs. The effect is stronger than the former, manifested as profibrotic events, including the enhanced expression of airway remodeling-related proteins.

Under normal physiological conditions, TGF β 1 can induce Smad7 expression, with the negative regulation of the TGF β /Smad signaling pathway considered to be an anti-fibrotic effect. In contrast, during pathological conditions, TGF β 1 is overexpressed, which increases miR-21 expression, decreases the protein expression of target gene Smad7, and attenuates the negative regulation of the TGF β /Smad signaling pathway by Smad7, resulting in fibrosis (6,26,27). This study found that Smad7

overexpression can inhibit airway remodeling caused by miR-21 overexpression in HBFs. Moreover, inhibiting miR-21 expression in HBFs can upregulate Smad7 protein expression to inhibit airway remodeling. Thus, there is mutual regulation between miR-21 and the TGF β /Smad signaling pathway. In the development of asthma, one miRNA has multiple target genes, and miRNA can have a prominent phenotypic effect on a certain target gene (28). Both TGF β 1 and miR-21 have dual effects during fibrosis and play different regulatory roles in different stages of asthma (23,29).

It has been reported that some miRNAs are involved in the metabolic process of extracellular matrix in fibrotic diseases. miRNA interference therapy has been applied to fibrotic diseases using antisense strands of miRNA to antagonize highly expressed endogenous miRNA (29). Since miRNA plays a key role in fibrotic diseases, gene therapy targeted at miRNA has attracted growing attention (30).

Conclusions

This study showed that the mutual regulation between miR-21 and the TGF β /Smad signaling pathway can promote airway remodeling in HBFs. The pathogenesis of asthma is highly complex, involving multiple miRNAs,

several signaling pathways, and the various interactions between them. Since our study only focused on the roles of miR-21 and the TGF β /Smad signaling pathway in airway remodeling, the mechanism by which other miRNAs and signaling pathways regulate airway remodeling requires further research.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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