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The anti-fibrotic effects of microRNA-153 by targeting TGFBR-2 in pulmonary fibrosis

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial fibrotic lung disease with an undefined etiology and no effective treatments. By binding to cell surface receptors, transforming growth factor-β (TGF-β) plays a pivotal role in lung fibrosis. Therefore, the screening of microRNAs (miRNAs), especially those interrupting the effects of TGF-β, may provide information not only on the pathomechanism, but also on the treatment of this disease. In the present study, we found that miR-153 expression was dysregulated in the lungs of mice with experimental pulmonary fibrosis and TGF-β1 decreased miR-153 expression in pulmonary fibroblasts. Moreover, increased miR-153 levels attenuated, whereas the knock down of miR-153 promoted the pro-fibrogenic activity of TGF-β1, and miR-153 reduced the contractile and migratory activities of fibroblasts. In addition, TGFBR2, a transmembrane serine/threonine kinase receptor for TGF-β, was identified as a direct target of miR-153. Furthermore, by post-transcriptional regulation of the expression of TGFBR2, phosphorylation of SMAD2/3 was also influenced by miR-153. These data suggest that miR-153 disturbs TGF-β1 signal transduction and its effects on fibroblast activation, acting as an anti-fibrotic element in the development of pulmonary fibrosis.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive and lethal fibrotic lung disease of undefined etiology, which is characterized by alveolar epithelial cell injury, excessive proliferation of fibroblasts and significant deposition of interstitial extracellular matrix (ECM), leading to disruption of normal lung architecture and respiratory failure (Verma and Slutsky, 2007; Wynn, 2011).

Transforming growth factor-β (TGF-β) has been shown to play a pivotal role in pulmonary fibrosis, not only by stimulating the proliferation of fibroblasts and the transformation of fibroblasts to myofibroblasts, but also by induction of epithelial to mesenchymal transition (EMT) in alveolar epithelial cells. TGF-β mediates its effects by interacting with transmembrane receptors. There are three types of receptors for TGF-β, including type I (TGFBR1, 50–60 kDa), type II (TGFBR2, 75–85 kDa) and type III (TGFBR3, a 280 kDa proteoglycan with a 120 kDa core protein), in which only TGFBR2, which is autophosphorylated, can bind TGF-β. Upon binding with TGF-β, TGFBR2 recruits TGFBR1 and phosphorylates TGFBR1 which in turn initiates signal transduction mediated by the downstream SMAD proteins (Massague, 1996). Khalil et al. (2001) reported increased expression of TGFBR1 and TGFBR2 on interstitial fibroblasts in rats 14 days after bleomycin injury. To further explore the role of TGF-β receptors on TGF-β-induced extracellular matrix (ECM) production in fibroblasts, Zhao (1999) overexpressed the kinase-deleted truncation of TGFBR2 in rat lung fibroblasts. They found that the induction of tenascin and fibronectin (Fn) by TGF-β in these cells was abolished and the truncated TGFBR2 mutant was a dominant inhibitor of TGF-β signal transduction. Collectively, these data suggest that, in pulmonary fibrosis, the expression of TGFBRs by interstitial fibroblasts is critical for TGF-β-mediated connective tissue synthesis, thereby contributing to the fibrotic response in IPF.

Bleomycin-induced fibrotic lung injury is the most frequently used animal model for investigating the pathobiology and treatment of IPF. Following bleomycin challenge, a series of pathological changes can be observed, including initial epithelial cell apoptosis, inflammatory cell infiltration, and subsequent fibroblast activation and ECM deposition. Studies have showed that the "switch" between the inflammatory and fibrotic phase is around Day 9 (Chaudhary et al., 2006).

MicroRNAs (miRNAs) are a class of evolution conserved, 19–22 nt in length, non-coding small RNAs, which bind to the 3′-UTR of targets and inhibit the translation or degradation of target mRNAs. Increasing evidence suggests that miRNAs play important roles in the development of many diseases. Recently, a number of studies have proved that miRNAs are also involved in the initial stage and progression of pulmonary fibrosis and may be new therapeutic targets for fibrogenic lung

Abbreviations: miRNA, Micro-RNA; BLM, Bleomycin; IPF, Idiopathic pulmonary fibrosis; TGFBR2, Transforming growth factor-β type II receptor; Fn, Fibronectin; SMA-α, Smooth muscle actin-α; Smad2/3, Mothers against DPP homolog 2/3; TGF-β, Transformation growth factor β; EMT, Epithelial to mesenchymal transition; ECM, Extracellular matrix.

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diseases, including IPF (Berschneider et al., 2014; Liang et al., 2014a; Xiao et al., 2012; Yang et al., 2012, 2013). However, the screening and study of miRNAs in pulmonary fibrosis is still in the early stages. Neurodegenerative diseaseneurodegenerative disease.

miR-153 is an intragenic microRNA which is embedded in genes encoding islet-associated protein. It has been shown to participate in the pathogenesis of neurodegenerative diseases, such as Parkinson's and Alzheimer's disease (Liang et al., 2012). Moreover, it was reported to inhibit the proliferation and invasion of many types of malignant cells, for example osteosarcoma and lung cancer cells (Niu et al., 2015). Thus, it is viewed as a tumor suppressor. More recently, miR-153 was shown to be dysregulated in mice with experimental lung injury (Xie et al., 2011). However, the role of miR-153 in the pathogenesis of pulmonary fibrosis has not been studied in detail.

In the present study, we confirmed that miR-153 was down-regulated in the lungs of a bleomycin-induced pulmonary fibrosis mouse model. miR-153 inhibited the fibrogenic activity of TGF-β1 and diminished the contractile and migratory activities of lung fibroblasts by directly targeting TGFBR2-3'UTR. Moreover, we demonstrated that miR-153 was decreased by TGF-β1 and functioned in an amplifying circuit to enhance TGF-β1 signal transduction in human fibroblasts. Our data suggest that miR-153 is a suppressor of pulmonary fibrosis and may be a potential treatment for IPF.

2. Experimental procedures

2.1. Mouse model of lung fibrosis

8-week-old C57BL/6 J mice, 20 ± 2 g, were used in this study and procedures were performed in accordance with the Institutional Animal Care Guidelines which followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Maryland, USA), authorized animal number: SCXK-2012–0002. To induce pulmonary fibrosis, the mice were anesthetized with 10% chloral hydrate. Bleomycin was injected intratracheally at a dose of 2.5 U/kg body weight on Day 0, and the control group received the same volume of sterile saline. The mice were sacrificed at days 7, 14, 21 and 28 after instillation and the lungs were removed. Half of the samples were used for RNA and protein analysis and the remaining samples were processed for histopathological evaluation.

2.2. Histopathology

Lung tissue fixed in 4% formalin and 1% glutaraldehyde in 0.1 M cacodylate buffer was embedded into paraffin. 4 μm sections were cut and placed on poly-L-lysine-coated slides. Histological changes were analyzed by hematoxylin-eosin (HE) staining and collagen deposition was assessed using Masson's trichrome stain as previously described (Yi et al., 1996).

2.3. Cell culture

The human pulmonary fibroblast line MRC-5 and HEK-293 T cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in medium, MEM for MRC-5 cells and DMEM for HEK-293 T cells (Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C.

2.4. Transfection procedures

2×10^5 MRC5 cells were seeded in 6-well plates. The next day, the cells were transfected with miR-153, miR-153 inhibitor or their corresponding negative controls (Kima Gene Co., Ltd. Shanghai, China), using Lipofectamine 2000 according to the manufacturer's instructions.

2.5. Luciferase reporter assays

The sequence of human TGFBR2 3'UTR was amplified with the primer (restriction sites were included): 5′- CAGTTCTCGAG ACGGCTCCCTAA ACACTACC-3′ (Forward)/5′- GTCAAGCGGCCGC AGCTACTAGGAATGGG AACAG-3′ (Reverse). The PCR product was introduced downstream of the Renilla luciferase stop codon at the XhoI/NotI cloning sites of the psiCHECK vector (Promega, Madison, WI, USA). Mutation of the miR-153 binding site in TGFBR2 3'UTR was generated using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA). One day before transfection, 1×10^5 293 T cells were cultured in 24-well plates, the miR-153 mimic or negative control was transfected along with wild- or mutant-type TGFBR2-3'UTR psiCHECK vector, respectively. Luciferase activity was measured as previously described (Liang et al., 2012).

2.6. Real-time RT-PCR

Total RNAs were isolated from the lungs of mice or cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed using the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantification was performed using the IQ SYBR Green Supermix (Bio-Rad, CA, USA). The primers were designed as follows: miR-153: 5′-TTCCTTTGATTGCATAGTCACAA-3′ (Forward)/5′-CCTCCA CACACTCACCTCATC-3′ (Reverse); U6snRNA: 5′-ATTGGAACGATACAGA GAAGATT-3′ (Forward)/5′-GGAACGCTTCACGAATTTG-3′ (Reverse). Primers used to detect TGFBR2 were as follows: 5′-GACCAGAAATTCCC AGCTTCT-3′ (Forward)/5′- CAACGTCTCACACACCATCTG-3′ (Reverse); GAPDH: 5′-TGCTGAGTATGTCGTGGAGTCTA-3′ (Forward)/5′-AGTGGG AGTTGCTGTTGAAATC-3′ (Reverse). Each sample was analyzed in triplicate and the data were quantified using the △△Ct method. GAPDH was chosen as the internal control for mRNA and U6 snRNA for miRNA.

2.7. Western-blot analysis

Western blot analysis was performed as described previously (Liang et al., 2012). Rabbit anti-Fn and rabbit anti-SMA-α antibodies were from Proteintech (Chicago, IL, USA). Rabbit anti-TGFBR2 antibody was from Abgent (San Diego, CA, USA). Rabbit anti-SMAD2/3 and Phospho-SMAD2/3 monoclonal antibodies were from Cell Signaling (Danvers, MA, USA). Rabbit anti-GAPDH antibody was used as a loading control.

2.8. Wound-healing (migration) assay

MRC-5 cells were transfected with control mimics or miR-153 mimics as described above. Forty eight hours after transfection, cells were starved for 3 h in 0.1% FBS. Wounds were created by mechanically scratching the cell monolayer. The cells were then cultured in the medium and 10 ng/mL TGF-β1 was added. The motility of the cells was assessed by evaluating the degree of confluence from one side of the scratch to the other at different time points using Image Pro Plus Software.

2.9. Fibroblast contraction assay

Lung fibroblasts were transfected with control mimics or miR-153 mimics. Forty eight hours after transfection, the cells were mixed with 1.5 mg/mL rat tail collagen I (Shengyou Biotechnology, Hangzhou, China) diluted in medium 199 and then seeded into a 24-well plate at $1–2 \times 10^5$ cells/well. Following incubation at 37 °C for 30 min, 500 μl MEM containing 10% FBS was added to each well. The attached collagen gels were then freed from the wells using a pipette tip. The mixture of collagen and cells was incubated at 37 °C for 1 d, and the diameters of the collagen gels were measured.