Int J Clin Exp Med 2017;10(1):347-356
www.ijcem.com /ISSN:1940-5901/IJCEM0037676

Original Article

MicroRNA-155 inhibits the pro-fibrogenic activities of TGF-β1 in human lung fibroblasts

Li Chu[1,4*], Ye Li[2,4*], Xiaofei Zhang[1,4], Xiaolong Fu[3], Zhengfei Zhu[1,4]

Departments of [1]Radiation Oncology, [2]Integrative Oncology, Fudan University Shanghai Cancer Center, Shanghai, China; [3]Department of Radiation Oncology, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China; [4]Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China. [*]Equal contributors.

Received August 10, 2016; Accepted November 3, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Transforming growth factor-β1 (TGF-β1) stimulates extracellular matrix production, and is considered a master regulator of tissue fibrosis, including idiopathic pulmonary fibrosis and radiation-induced fibrosis. A recent study identified dysregulated miR-155 expression during pulmonary fibrosis. However, the role of miR-155 in TGF-β1-induced pulmonary fibrosis remains unclear. In this study, we demonstrated that TGF-β1 downregulates miR-155 expression in human lung fibroblast cell lines. MiR-155 inhibits the pro-fibrogenic activities of TGF-β1 by decreasing the expression offibronectin, collagen type I, alpha 1, and α-smooth muscle actin. We also identified transforming growth factor-β receptor-2 (TGFBR2) as a direct target of miR-155. Knocking down TGFBR2 in lung fibroblasts significantly decreased TGFBR2 expression and inhibited the pro-fibrogenic activities of TGF-β1. Expression of a miR-155-insensitive TGFBR2 construct restored fibrogenesis induced by TGF-β1 stimulation in miR-155 expressing cells. Therefore, our study suggests an important role for miR-155 in fibrotic lung diseases, and indicates that miR-155 is a potent therapeutic microRNA for treating pulmonary fibrosis.

Keywords: miR-155, lung fibrosis, TGF-β1, TGFBR2

Introduction

Fibrosis is the excessive accumulation of collagen and other extracellular matrix (ECM) components following a breakdown in the normal balance of ECM synthesis and degradation [1]. Uncontrolled ECM production can lead to clinically important fibrotic diseases, including idiopathic pulmonary fibrosis (IPF) and radiation-induced fibrosis (RIF) [2, 3]. Pathological hallmarks include alveolar epithelial injury and hyperplasia, aberrant wound healing, formation of fibroblast foci, as well as excessive matrix deposition resulting in disrupted lung architecture and respiratory insufficiencies [4]. However, the fundamental molecular drivers and control points that coordinate this aberrant process are poorly understood. Aberrant growth factor signaling within the epithelial-mesenchymal unit has been shown to contribute to the pathobiology of pulmonary fibrosis [5, 6]. Among the secreted factors driving fibrosis, transforming growth factor-β1 (TGF-β1), which is produced by a wide range of inflammatory, mesenchymal, and epithelial cells, can convert fibroblasts and other cells into matrix-producing myofibroblasts [7].

MicroRNAs (miRNAs) are a class of small (~22-nt), non-coding RNA molecules that function in the post-transcriptional regulation of gene expression. These molecules downregulate protein expression by binding to partially complementary sequences in mRNAs and degrading and/or inhibiting mRNA translation [8]. There is growing evidence that dysregulated miRNA expression may contribute to many types of human diseases, including pulmonary fibrosis. The expression of approximately 10% of microRNAs is significantly changed in IPF lungs [9]. miRNAs are involved in regulating lung inflammation, TGF-β1-mediated epithelial to mesenchymal transition (EMT) and fibroblast differentiation, ECM gene expression, and in the pro-

gression of lung fibrosis [10]. They can also serve as potential targets for progressive pulmonary fibrosis [9]. Recent studies also identified dysregulated miR-155 expression during pulmonary fibrosis [11]. However, the role of miR-155 in TGF-β1-induced pulmonary fibrosis remains unclear.

We evaluated miR-155 expression after TGF-β1 stimulation and studied the potential role of miR-155 in TGF-β1-induced pro-fibrogenic activities. TGF-β1 treatment downregulatedmiR-155 expression in human lung fibroblasts, and overexpression of miR-155 inhibited the pro-fibrogenic activities. In addition, we identified transforming growth factor-β receptor-2 (TGF-BR2) as a direct target of miR-155, mediating the suppression of TGF-β1-induced fibrogenesis. Therefore, our study suggests an important role for miR-155 in fibrotic lung diseases, and that miR-155 may serve as a potential target for developing novel therapeutics to treat pulmonary fibrosis.

Methods

Cell lines

Normal human lung primary fibroblasts, named WI-38, and human embryonic lung fibroblasts, named HLF1, were obtained from the Cell bank of The Chinese Academy of Sciences (Shanghai, China), and were grown in complete growth medium as recommended by the manufacturer. The cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. All cell lines were regularly authenticated by checking their morphology and confirming the absence of mycoplasma contamination by MycoAlert (Lonza, Rockland, ME, USA).

RNA isolation

Total RNA was isolated from freshly frozen tissue samples and cell lines using TRIzol® Reagent (Invitrogen, San Diego, CA, USA). Small RNA enrichment was conducted using a mirVanamiRNA isolation kit (Ambion, Inc.), according to the manufacturer's instructions and as described previously [12].

qRT-PCR assays

Real-time PCR was performed to evaluate the level of miR-155 using specific TaqMan primers through the determination and comparison of mean C_T values. Briefly, a 2-μL aliquot of enriched small RNAs from cells was reverse transcribed to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, San Diego, CA, USA) according to the manufacturer's protocol. Then, 2 μL of cDNA was used as a template for the quantitative PCR amplification. The quantitative PCR reaction was performed using 10 μL TaqMan Universal Master Mix (2×) (Applied Biosystems), 1 μL gene-specific primers/probe, and nuclease-free H_2O to a final volume of 20 μL. Template-free controls were included in both steps to ensure target-specific amplification. Quantitative PCR was run on a 7500 HT quantitative PCR machine (Applied Biosystems). The C_T values of different samples were compared using the $\Delta\Delta C_T$ method as previously described [13]. Semi-quantitative real-time RT-PCR using SYBR Green I was performed to compare the relative expression levels of specific mRNAs as previously described (Lekanne Deprez *et al*, 2002).

Oligonucleotide transfection

For transfection experiments, miRNA duplexes corresponding to the indicated miRNAs were designed as previously described [14]. The negative control (NC) RNA duplexes for the miRNA mimics and the small-interfering RNAs (siRNAs) were not homologous to any known human gene sequences. siRNAs were designed to target the human TGFBR2 transcript (GenBank Access No: NM_001024847). All RNA oligoribonucleotides were purchased from Genepharma (Shanghai, China). The sequences were as follows: miR-155 mimics, 5'-UUAAUGCUAAUCGUGAUAGGGGU-3' and 5'-ACCCCUAUCACGAUUAGCAUUAA-3'; NC, 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'; TGFBR2 siRNA, 5'-CCCATCCACTGAGACATATTAATAA-3' (sense); Anti-miR-155 (an inhibitor of miR-155), 5'-ACCCCUAUCACGAUUAGCAUUAA-3'; Anti-miR-C (used as a negative control for anti-miR-367 in the antagonism experiment), 5'-GUGGAUAUUGUUGCCAUCA-3'. The oligonucleotide transfection was performed using Lipofectamine 2000 (Invitrogen). For each transfection, 50 nm/L of RNA duplex were used.

Vector constructs

To generate the TGFBR2 expression vector, the open reading frame of human TGFBR2 was

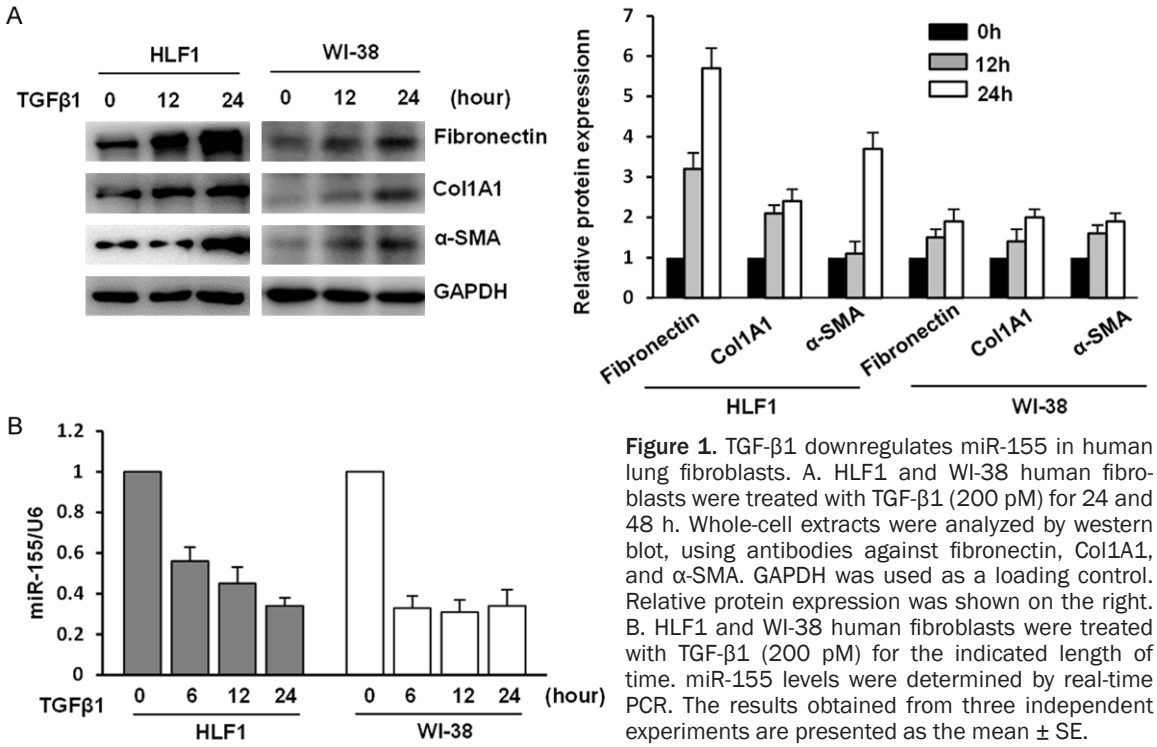


Figure 1. TGF-β1 downregulates miR-155 in human lung fibroblasts. A. HLF1 and WI-38 human fibroblasts were treated with TGF-β1 (200 pM) for 24 and 48 h. Whole-cell extracts were analyzed by western blot, using antibodies against fibronectin, Col1A1, and α-SMA. GAPDH was used as a loading control. Relative protein expression was shown on the right. B. HLF1 and WI-38 human fibroblasts were treated with TGF-β1 (200 pM) for the indicated length of time. miR-155 levels were determined by real-time PCR. The results obtained from three independent experiments are presented as the mean ± SE.

amplified and cloned into the pcDNA3.0 vector. Luciferase constructs were generated by ligating oligonucleotides containing the wild-type or mutated putative target site from the TGFBR2 3'-UTR into the Psi-CHECK2 vector (Promega, Madison, WI, USA) downstream of the luciferase gene.

Luciferase assay

HEK293 and HLF1 cells were co-transfected with 80 ng of the luciferase reporter plasmid, 40 ng of pRL-TK-Renilla-luciferase plasmid (Promega), and the indicated RNAs (final concentration: 20 nM). Twenty-four hours after transfection, firefly and Renilla luciferase activities were measured using a Dual-Luciferase assay kit (Promega). Each transfection was performed in triplicate and repeated twice.

Western blot analysis

Western blot analysis was performed as described in our previous report [15]. Briefly, protein was extracted from cultured cells and quantified using the bicinchoninic acid assay kit (Pierce, Rockford, IL, USA) with bovine serum albuminas a standard. Equal amounts of protein from different samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% non-fat milk and incubated with primary antibodies. The target proteins were detected using an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The following primary antibodies were used: anti-fibronectin, anti-Col1A1, anti-α-SMA, anti-Smad2, anti-TGFBR2 (Epitomics), anti-phosphorylated Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-GAPDH (ProteinTech Group Inc., Chicago, IL, USA).

Target-site inhibition assays

The miScript Target Protector for the miR-155 binding site in the 3'-UTR of TGFBR2 mRNA was obtained from Qiagen (target binding site sequence: 5'-AGGAAGGGACCCAUGACAGCAUUAGCAUUUGACAAUCACAC-3'). The miScript Target Protector is a single-stranded modified RNA that specifically interferes with the interaction of a miRNA with a single target, leaving the regulation of other targets of the same miRNA unaffected. miR-155 mimics and miScript Target Protector were co-transfected into primary microglial cells according to the manufacturer's protocol. miR-155 mimic and NCmiScript Target Protector designed not to bind the mRNA of

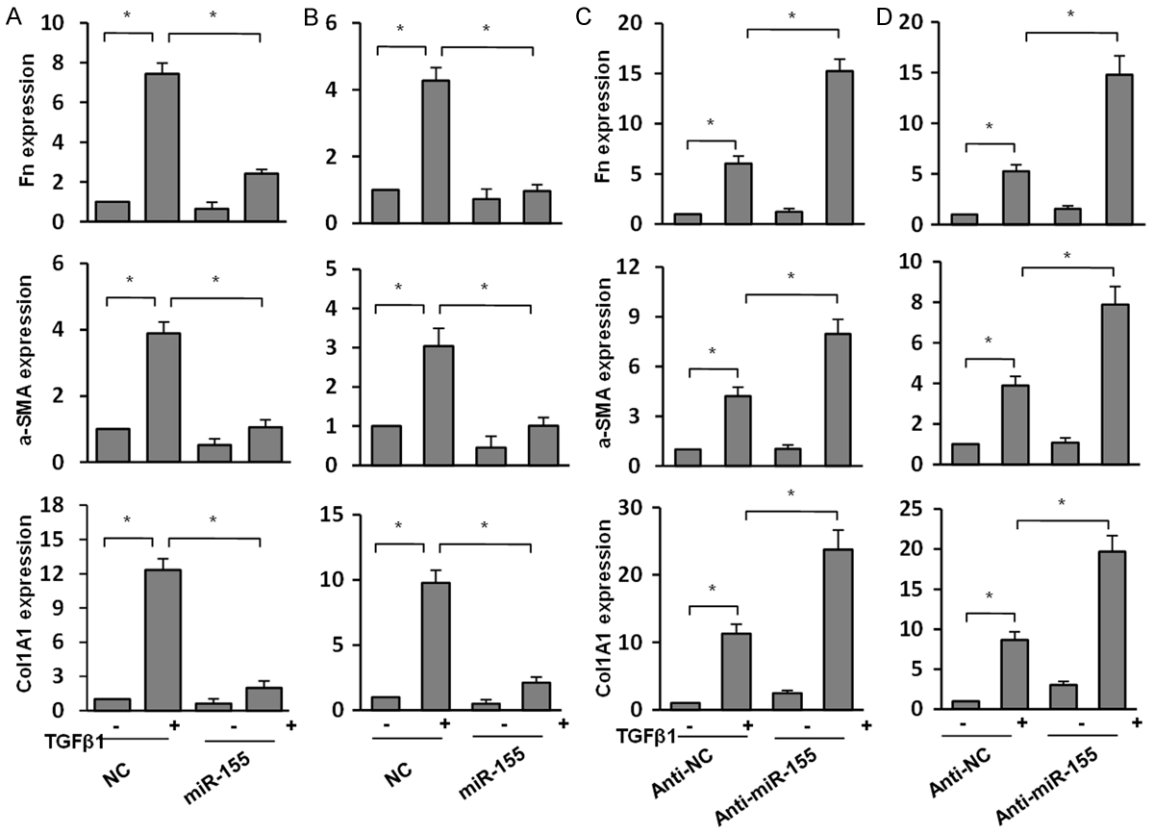


Figure 2. miR-155 inhibits the pro-fibrogenic activities of TGF-β1. A, B. Fibroblasts were transfected with 50 nM miR-155 mimics or control mimics. Forty-eight hours after transfection, the fibroblasts were treated with TGF-β1 (200 pM) for 24 h. The levels of fibronectin, α-SMA, and Col1A1 mRNA were determined by real-time PCR. C, D. Fibroblasts were transfected with 50 nM miR-155 inhibitors or control inhibitors. Forty-eight hours after transfection, the fibroblasts were treated with TGF-β1 (200 pM) for 24 h. The levels of fibronectin, α-SMA, and Col1A1 mRNA were determined by real-time PCR. The results obtained from three independent experiments were presented as the mean ± SE. Statistical significance was evaluated using analysis of variance. *P<0.05.

mammals were co-transfected into HLF1 cells as a negative control. After 48 h of transfection, total RNA was isolated, and TGFBR2 mRNA levels were measured by quantitative real-time PCR.

Statistical analysis

All results were expressed as the mean ± SD. Analysis of variance and Student's *t*-tests were used to analyze significant differences between samples. A *P*-value less than 0.05 was considered to be statistically significant.

Results

TGF-β1 downregulates miR-155 expression in human lung fibroblasts

TGF-β1 is one of the most potent pro-fibrotic cytokines and is involved in myofibroblast differentiation [16]. The human fibroblast cell lines HLF1 and WI-38 were treated with TGF-β1 (200 pM) for 24 and 48 h. After treatment, we foundincreased expression of fibronectin, collagen type I, alpha 1 (Col1A1), and α-smooth muscle actin (α-SMA) (**Figure 1A**), which suggested that TGF-β1 treatment mimicked a profibrotic environment *in vitro*. Next, we treated HLF1 and WI-38 cells with TGF-β1 (200 pM) and evaluated miR-155 expression at different time points, observing that miR-155 expression decreased following TGF-β1 treatment (**Figure 1B**). These results confirmed that TGF-β1 downregulate smiR-155 expression in human lung fibroblasts.

miR-155 inhibits the pro-fibrogenic activities of TGF-β1

TGF-β1 induces fibroblast differentiation into more fibrogenicmy of ibroblasts [17]. We have shown above that TGF-β1 up-regulated fibro-

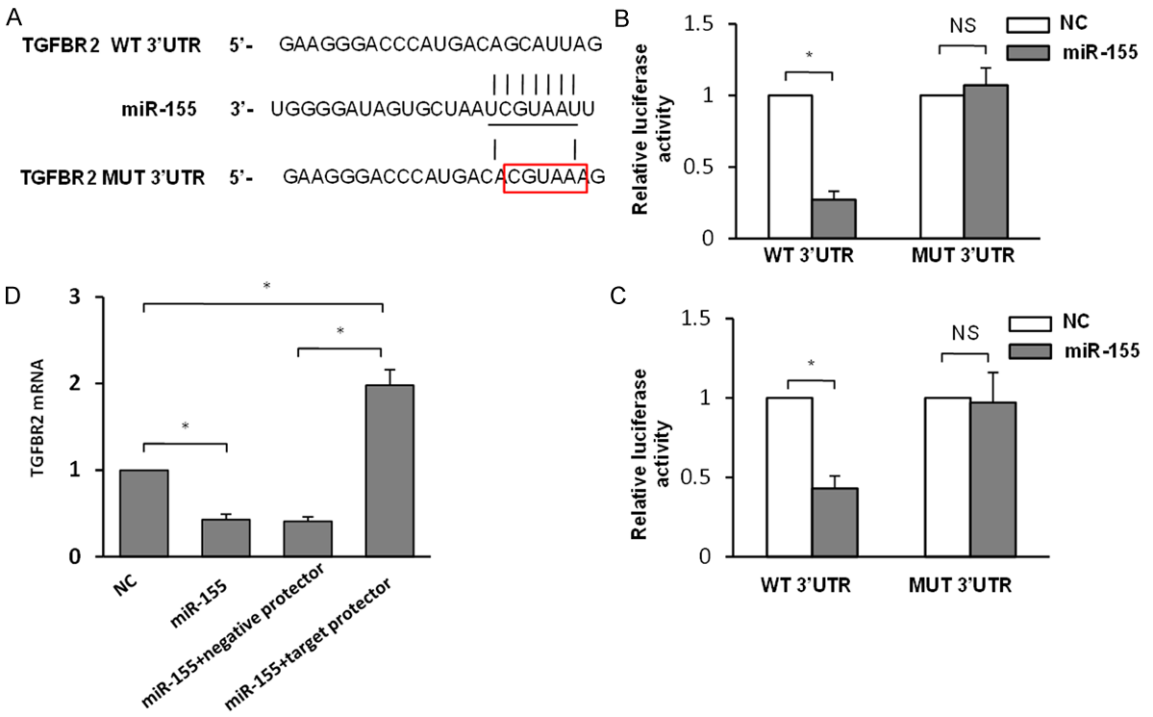


Figure 3. TGFBR2 is a direct target of miR-155 in human lung fibroblasts. (A) Predicted miRNA binding sites in the 3'UTR of TGFBR2 mRNA. A wild-type or mutatedhuman TGFBR2 3'UTR fragment was cloned downstream of the luciferase reporter gene. Mutations complementary to predicted miR-155 binding sites were generated in the TGFBR2 3'UTR sequences as indicated. (B, C) Luciferase activity assays using wild-type or mutated human TGFBR2 3-UTR reporters were performed after co-transfection with miR-155 or NC mimics in HEK293 (B) or HLF human lung fibroblasts (C). Luciferase activity of the NC transfection in each experiment was used to normalize the data. (D) HLF1 cells were co-transfected with miR-155 mimics and target protector or negative controloligos. After 48 h of transfection, total RNA was isolated and the levels of TGFBR2 mRNA were measured by quantitative real-time PCR. The data are expressed as mean ± SE of three independent experiments. *P<0.05.

nectin, Col1A1 and α-SMA, but down-regulated miR-155 expression in human lung fibroblasts. We hypothesized that the pro-fibrogenic activities of TGF-β1 might be the result of the down-regulating miR-155. To test this, fibroblasts were transfected with 50 nM miR-155 mimics or control mimics. We found that transfection of miR-155 mimics increased miR-155 levels and decreased TGF-β1-induced transcription of fibronectin, Col1A1, and α-SMA in human pulmonary fibroblasts (**Figure 2A**, **2B**, Supplementary Figure 1A, 1B). Conversely, miR-155 knockdown enhanced TGF-β1-induced transcription of fibronectin, Col1A1, and α-SMA (**Figure 2C**, **2D**, Supplementary Figure 1C, 1D). Collectively, these results suggest that miR-155 inhibits the pro-fibrogenic activities of TGF-β1.

TGFBR2 is a direct target of miR-155 in human lung fibroblasts

Next, we sought to identify an mRNA target of miR-155 that might play a role in antagonizing the pro-fibrogenic activities of TGF-β1. Putative miR-155 targets were predicted using the target prediction programs TargetScan and miRbase. We focused on TGFBR2 because the 3'-UTR of TGFBR2 mRNA contains a putative target sequence for miR-155 (**Figure 3A**), and TGFBR2 is involved in TGFβ signaling. To demonstrate that miR-155 directly regulates TGFBR2, we employed a dual-luciferase reporter system. We found that co-expression of miR-155 significantly suppressed the firefly luciferase activity of the wild-type TGFBR2 3'-UTR but not amutated 3'-UTR (**Figure 3B**, **3C**), indicating that miR-155 suppresses TGFBR2 expression through binding them RNA in its 3'-UTR. To further demonstrate that TGFBR2 is a target of miR155, we performed target-site inhibition assays. We found that the levels of TGFBR2 mRNA were significantly lower in cells transfected with miR-155 than in control cells. TGFBR2 mRNA levels were significantly higher in cells co-transfected with miR-155 mimics and target protector oligos than in cells

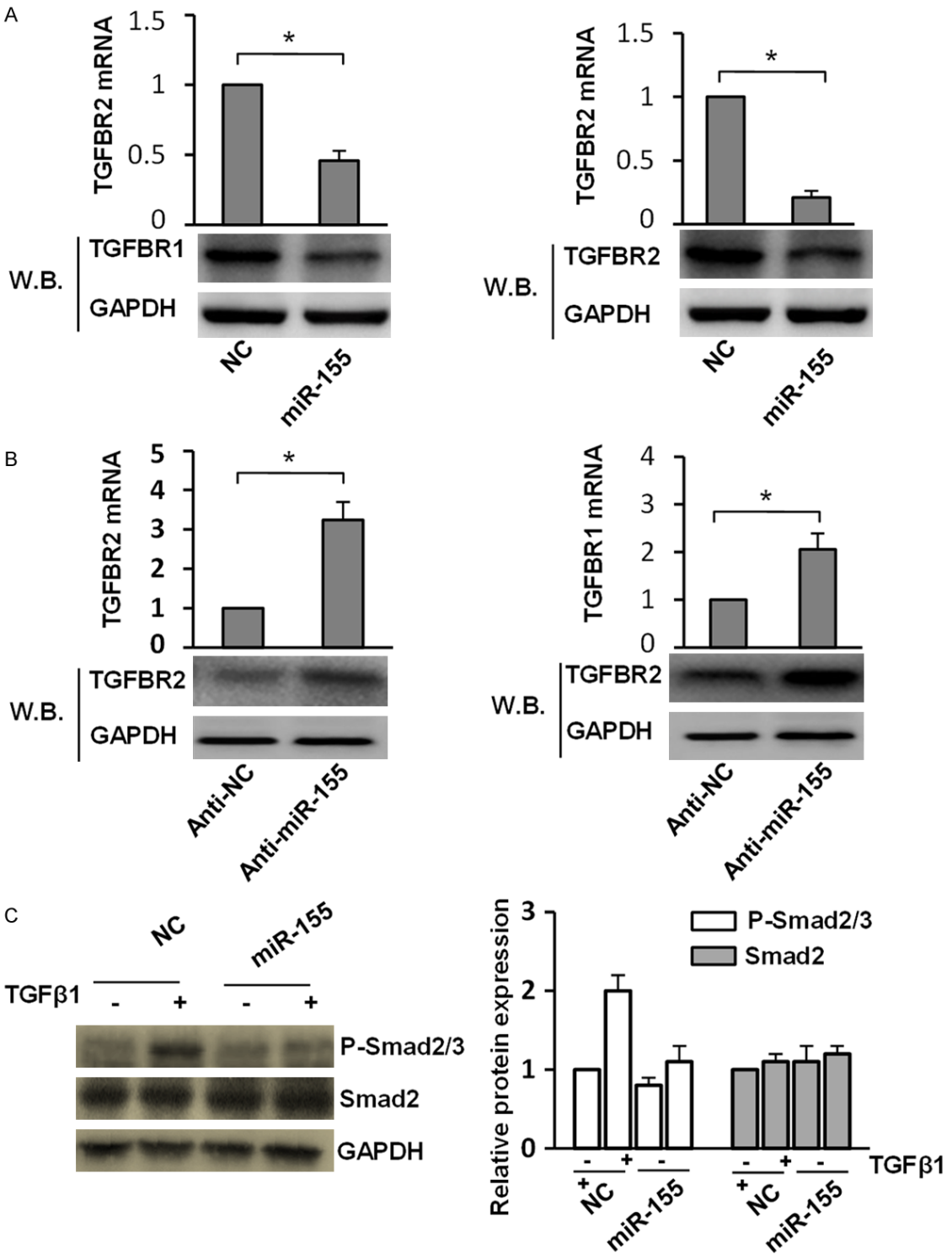


Figure 4. miR-155 regulates TGFBR2 expression in human lung fibroblasts. A. HLF1 (left) and WI-38 (right) cells were transfected with miR-155 mimics or negative control (NC). After 48 h, cells were harvested, and TGFBR2 mRNA and protein expression were evaluated by RT-PCR and western blot. The data are expressed as mean ± SE of three independent experiments. *P<0.05. B. HLF1 (left) and WI-38 (right) cells were transfected with anti-miR-155 or anti-miR-C inhibitors. After 48 h, cells were harvested, and TGFBR2 mRNA and protein expression were evaluated by RT-PCR and western blot. The data are expressed as mean ± SE of three independent experiments. *P<0.05. C. HLF1 cells transfected with miR-155 mimics or negative control (NC) were cultured with and without TGF-β1 (200 pM) for 1 h. Whole cell extracts were analyzed by western blot using antibodies against Smad2 and phosphorylated Smad2/3. GAPDH was used as a loading control. Relative protein expression was shown on the right.

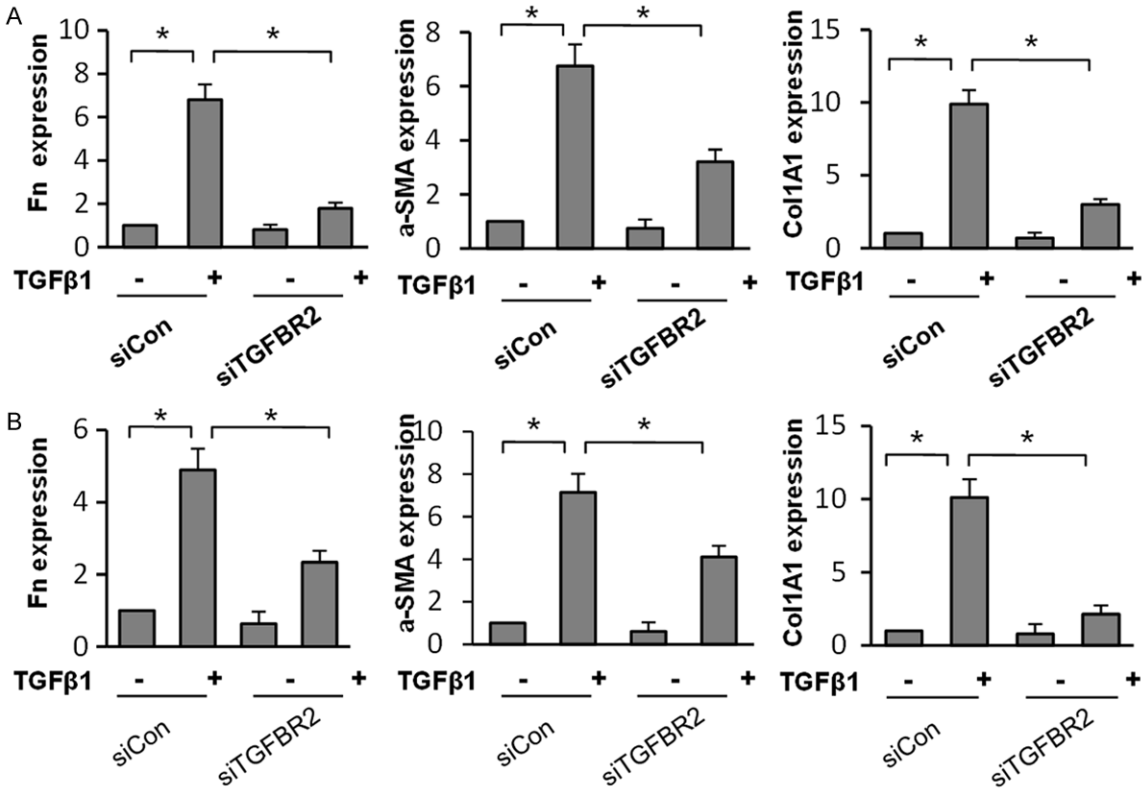


Figure 5. TGFBR2 downregulation inhibits the pro-fibrogenic activities of TGF-β1. (A, B) Fibroblasts HLF1 (A) and WI-38 (B) were transfected with negative control (NC) or TGFBR2 siRNA. Forty-eight hours after transfection, the fibroblasts were treated with TGF-β1 (200 pM) for 24 h. The levels of fibronectin, α-SMA, and Col1A1 mRNA were determined by real-time PCR. The data are expressed as mean ± SE of three independent experiments. *P<0.05.

co-transfected with miR-155 mimics and negative control target protector oligos (**Figure 3D**). These results indicate that TGFBR2 is a direct target of miR-155 in human lung fibroblasts.

miR-155 regulates TGFBR2 expression in human lung fibroblasts

Next, we transfected human lung fibroblasts with NC or miR-155 mimics. We found that miR-155 over-expression in human lung fibroblasts reduced endogenous TGFB2 mRNA and protein expression (**Figure 4A**). In contrast, down-regulating miR-155 expression with an anti-miR-155 inhibitor increased TGFBR2 expression (**Figure 4B**). These results suggest that the anti-fibrotic effects of miR-155 might be mediated through regulation of TGF-β1-related signaling events. Consistent with this hypothesis, increasing miR-155 levels attenuated Smad2 phosphorylation in response to TGF-β1 stimulation (**Figure 4B**).

TGFBR2 mediates the miR-155 suppression of pro-fibrogenic activities of TGF-β1

To investigate the potential role of TGFBR2 in miR-155-mediated suppression of fibrogenesis, we explored the biological functions of TGFBR2 by transfecting lung fibroblasts with a TGFBR2 siRNAs. Knockdown of endogenous TGFBR2 by siRNA resulted in decreased TGF-β1-induced transcription of fibronectin, Col1A1, and α-SMA in human pulmonary fibroblasts (**Figure 5A**, **5B**). This phenotype was similar to miR-155 overexpression. Next, we introduced a TGFBR2 construct lacking the 3'-UTR into lung fibroblasts. This resulted in the constitutive expression of TGFBR2, insensitive to miR-155. We found that co-transfection of TGFBR2 lacking the 3'-UTR in fibroblasts expressing miR-155 significantly blocked miR-155 supression of fibrogenesis (**Figure 6A**, **6B**). These results suggest that the suppression of the pro-fibrogenic activities of TGF-β1 by miR-155 is mediated by controlling TGFBR2 translation.

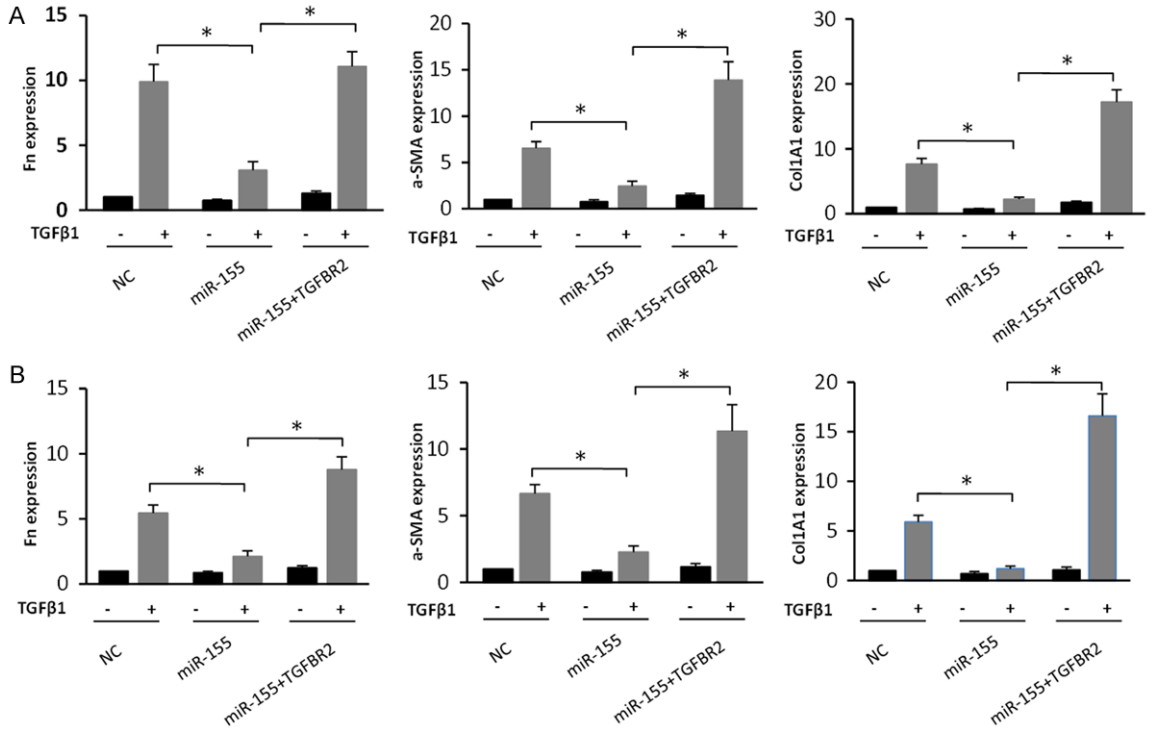


Figure 6. TGFBR2 mediates the miR-155 suppression of pro-fibrogenic activities of TGF-β1. (A, B) HLF1 (A) and WI-38 (B) fibroblasts were transfected with negative control (NC), miR-155, or TGFBR2 expression vectors. Forty-eight hours after transfection, the fibroblasts were treated with TGF-β1 (200 pM) for 24 h. Fibronectin, α-SMA, and Col1A1 mRNA levels were determined by real-time PCR. The data are expressed as mean ± SE of three independent experiments. *P<0.05.

Discussion

In this study, we have demonstrated that TGF-β1 downregulates miR-155 in human lung fibroblasts. miR-155 overexpression inhibited the pro-fibrogenic activities of TGF-β1. In addition, we identified TGFBR2 as a direct target of miR-155 that mediates the suppression of fibrogenesis induced by TGF-β1. Our study suggests an important role for miR-155 in fibrotic lung diseases, and indicates that miR-155 is a potent therapeutic miRNA for pulmonary fibrosis treatment.

Fibrosis is a chronic disease characterized by an excessive deposition of scar tissue in the affected organs. Fibroblast proliferation and the generation of provisional ECM are the primary tissue responses to injury [18]. Uncontrolled ECM production can lead to clinically important fibrotic diseases, including IPF and RIF [3, 19, 20]. Lung fibrosisis characterized by the histological appearance of usual interstitial pneumonia, including the presence of fibroblastic foci that are an aggregation of fibroblasts and myofibroblasts where fibroblasts proliferate, migrate, and contribute to excessive production of ECM [19]. Lung fibroblasts take a central role in the development of lung fibrosis. Fibroblasts produce very little matrix under normal conditions and maintain a fine network of β-actin stress fibers with few direct physical contacts to neighboring cells or ECM. In response to fibrogenic cytokines released by inflammatory cells and other cell types, fibroblasts differentiate into myofibroblasts [17]. Myofibroblasts produce collagens, fibronectins, and other matrixmolecules [21]. TGF-β1 is a prototypical factor for the induction of EMT and fibroblast differentiation [16]. In fibrotic lungs, a broad range of cells including fibroblasts, macrophages, and epithelial cells overexpresses TGF-β1. This expression leads to the dysregulation of normal lung homeostasis by increasing the synthesis and deposition of collagen and altering the balance of matrix metalloproteinases and their inhibitors [22]. Therefore, strategies to inhibit TGF-β signaling may lead to

developing novel therapeutics to treat pulmonary fibrosis.

miRNAs post-transcriptionally regulate the expression of target genes. By binding to partially complementary sequences in mRNAs, they degrade the targets and/or inhibit their translation, down-regulating protein expression [8]. A recent study suggests that a dysregulation of miRNAs is involved in many types of human diseases, including lung fibrosis [10]. A series of miRNAs that were differentially expressed in human IPF lungs have also been described as having pro-fibrotic or anti-fibrotic roles in animal models of lung fibrosis [23]. Potential mechanisms for this activity include, miRNAs that can directly regulate proinflammatory mediators, EMT, fibroblast proliferation and differentiation, TGF-β signaling, and ECM gene expression [23]. For example, let-7d, one of the first miRNAs discovered and extensively studied in human disease, had significantly decreased expression in IPF lungs. Let-7d inhibition caused a significant downregulation of epithelial markers such as E-cadherin and tight junction protein-1, and a significant upregulation in the expression of mesenchymal markers such as COL1 and α-SMA in a bleomycin-induced mouse model of lung injury [24, 25]. Many miRNAs have been found to have roles in the pathogenesis of pulmonary fibrosisin animal models. Recent studies have also identified the dysregulated expression of miR-155 in human pulmonary fibrosis. TGF-β1 treatment could lead to downregulation of miR-155 in human lung fibrosis [11]. However, the role of miR-155 in TGF-β-induced pulmonary fibrosis remains unclear. Therefore, in this study, we systemically studied the role of miR-155 in TGFβ-induced fibrogenesis. We confirmed that miR-155 inhibited the pro-fibrogenic activities of TGF-β1. Moreover, we identified TGFBR2 as a direct target of miR-155 that mediates the miR-155-suppression of fibrogenesis induced by TGF-β1. Therefore, our study suggests an important role for miR-155 in TGF-β1-induced lung fibrosis.

There are several limitations to our work. First, the evidence for a potential role of miR-155 is derived from experiments performed on human lung fibroblasts *in vitro*. Experiments that involve perturbations of miR-155 expression in animal models of fibrosis are required to prove that miR-155 plays an anti-fibrotic role *in vivo*. Second, although we provide compelling data about the effects of miR-155 in normal human lung fibroblasts, we did not study the effects of miR-155 in IPF fibroblasts. Therefore, a detailed analysis of a large number of primary lines from patients with IPF or RIF is required. Assessing the potential therapeutic value of administering miR-155 *in vivo* in animal models of fibrosis and detailed mechanistic analyses of its effects are the logical next steps.

In conclusion, our study provides evidence for the anti-fibrotic role of miR-155 in pulmonary fibrosis, which further highlights the potential role of miR-155 as a novel therapeutic target inpulmonary fibrosis.

Acknowledgements

This study was supported by the National Science Foundation of China (Grant number: 81572963); Natural Science Foundation of Shanghai (Grant number: 11ZR1407300); Shanghai Pujiang Program (Grant number: 15PJD008).

Disclosure of conflict of interest

None.

Address correspondence to: Zhengfei Zhu, Department of Radiation Oncology, Fudan University Shanghai Cancer Center, 270 Dong An Road, Shanghai 200032, China. Tel: 86-21-64175590; Fax: 86-21-64437657; E-mail: zfeizhu@aliyun.com

References

[1] Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol 2008; 214: 199-210.

[2] Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 2002; 3: 349-363.

[3] Yarnold J, Brotons MC. Pathogenetic mechanisms in radiation fibrosis. Radiother Oncol 2010; 97: 149-161.

[4] Todd NW, Luzina IG, Atamas SP. Molecular and cellular mechanisms of pulmonary fibrosis. Fibrogenesis Tissue Repair 2012; 5: 11.

[5] Emura M, Nagai S, Takeuchi M, Kitaichi M, Izumi T. In vitro production of B cell growth factor and B cell differentiation factor by peripheral blood mononuclear cells and bronchoalveolar lavage T lymphocytes from patients with idiopathic pulmonary fibrosis. Clin Exp Immunol 1990; 82: 133-139.

[6] Buttner C, Skupin A, Reimann T, Rieber EP, Unteregger G, Geyer P, Frank KH. Local production of interleukin-4 during radiation-induced pneumonitis and pulmonary fibrosis in rats: macrophages as a prominent source of interleukin-4. Am J Respir Cell Mol Biol 1997; 17: 315-325.

[7] Fernandez IE, Eickelberg O. The impact of TGF-beta on lung fibrosis: from targeting to biomarkers. Proc Am Thorac Soc 2012; 9: 111-116.

[8] Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 2009; 10: 126-139.

[9] Rajasekaran S, Rajaguru P, Sudhakar GP. MicroRNAs as potential targets for progressive pulmonary fibrosis. Front Pharmacol 2015; 6: 254.

[10] Pandit KV, Milosevic J, Kaminski N. MicroRNAs in idiopathic pulmonary fibrosis. Transl Res 2011; 157: 191-199.

[11] Pottier N, Maurin T, Chevalier B, Puissegur MP, Lebrigand K, Robbe-Sermesant K, Bertero T, Lino CC, Courcot E, Rios G, Fourre S, Lo-Guidice JM, Marcet B, Cardinaud B, Barbry P, Mari B. Identification of keratinocyte growth factor as a target of microRNA-155 in lung fibroblasts: implication in epithelial-mesenchymal interactions. PLoS One 2009; 4: e6718.

[12] Lekanne DR, Fijnvandraat AC, Ruijter JM, Moorman AF. Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. Anal Biochem 2002; 307: 63-69.

[13] Giovannetti E, Funel N, Peters GJ, Del CM, Erozenci LA, Vasile E, Leon LG, Pollina LE, Groen A, Falcone A, Danesi R, Campani D, Verheul HM, Boggi U. MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. Cancer Res 2010; 70: 4528-4538.

[14] Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 2005; 433: 769-773.

[15] Zhu Z, Xu Y, Zhao J, Liu Q, Feng W, Fan J, Wang P. miR-367 promotes epithelial-to-mesenchymal transition and invasion of pancreatic ductal adenocarcinoma cells by targeting the Smad7-TGF-beta signalling pathway. Br J Cancer 2015; 112: 1367-1375.

[16] Fernandez IE, Eickelberg O. The impact of TGF-beta on lung fibrosis: from targeting to biomarkers. Proc Am Thorac Soc 2012; 9: 111-116.

[17] Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The myofibroblast: one function, multiple origins. Am J Pathol 2007; 170: 1807-1816.

[18] Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 2002; 3: 349-363.

[19] Todd NW, Luzina IG, Atamas SP. Molecular and cellular mechanisms of pulmonary fibrosis. Fibrogenesis Tissue Repair 2012; 5: 11.

[20] Renzoni E, Srihari V, Sestini P. Pathogenesis of idiopathic pulmonary fibrosis: review of recent findings. F1000Prime Rep 2014; 6: 69.

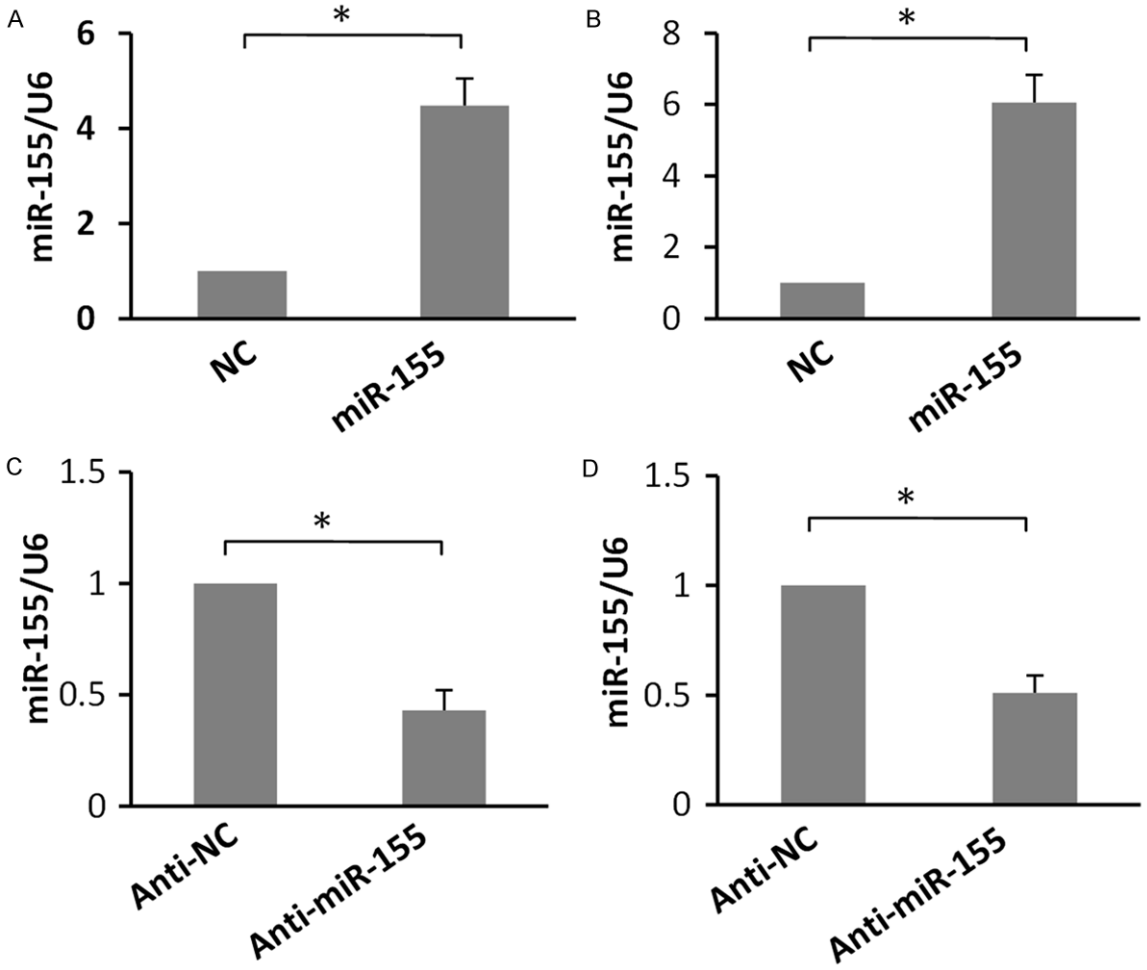
[21] Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol 2008; 214: 199-210.

[22] Verrecchia F, Mauviel A. Transforming growth factor-beta and fibrosis. World J Gastroenterol 2007; 13: 3056-3062.

[23] Rajasekaran S, Rajaguru P, Sudhakar GP. MicroRNAs as potential targets for progressive pulmonary fibrosis. Front Pharmacol 2015; 6: 254.

[24] Pandit KV, Corcoran D, Yousef H, Yarlagadda M, Tzouvelekis A, Gibson KF, Konishi K, Yousem SA, Singh M, Handley D, Richards T, Selman M, Watkins SC, Pardo A, Ben-Yehudah A, Bouros D, Eickelberg O, Ray P, Benos PV, Kaminski N. Inhibition and role of let-7d in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2010; 182: 220-229.

[25] Pandit KV, Milosevic J, Kaminski N. MicroRNAs in idiopathic pulmonary fibrosis. Transl Res 2011; 157: 191-199.



Supplementary Figure 1. Confirmation of the expression of mature miR-155 in human lung fibroblasts. (A, B) HLF1 (A) and WI-38 (B) fibroblasts were transiently transfected with 50 nM miR-155 mimics or control mimicsusing lipofectamine.miR-155 expression 48 h after transfection was confirmed by qRT-PCR. U6 was used as an internal control. (C, D) HLF1 (C) and WI-38 (D) fibroblasts were transiently transfected with anti-miR-155 inhibitors or anti-miR-C using lipofectamine. Inhibition of miR-155 expression 48 h after transfection was confirmed by qRT-PCR. U6 was used as an internal control.