

Original Article

MiR-338* suppresses fibrotic pathogenesis in pulmonary fibrosis through targeting LPA1

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Abstract: Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease involving pulmonary injury associated with tissue repair, dysfunction and fibrosis. MicroRNAs (miRNAs), as gene regulators, are assumed to regulate about one third of genes and thus play important roles in cellular functions including proliferation, growth, differentiation and apoptosis. Recent studies have indicated that some miRNAs may play critical roles in the pathogenesis of pulmonary fibrosis. In this study, we found that miR-338* (miR-338-5p), which has been found to be associated with tumor progression, was down-regulated in fibroblasts and TGF- β -induced lung fibrotic tissues. Over-expression of miR-338* can partly prevent the fibrotic process induced by TGF- β . Moreover, LPA1 was proven to be a downstream target of miR-338*. Lentivirus-mediated over-expression of miR-338* can alleviate lung fibrosis induced by bleomycin in mice. Taken together, our results suggest that miR-338* attenuates the pathogenesis of pulmonary fibrosis through targeting LPA1. Thus, miR-338* can be a potential therapeutic target for the treatment of IPF.

Keywords: Pulmonary fibrosis, miR-338*, bleomycin, TGF- β , LPA1

Introduction

Idiopathic pulmonary fibrosis (IPF) is a devastating, age-related disease characterized by the destruction of the gas-exchanging regions of the lung [1]. The annual incidence of IPF is rising and is estimated to be 4.6-16.3 per 100,000 people; the prevalence is 13-20 cases per 100,000 [2, 3]. Symptoms of IPF, including cough and dyspnea, often limit physical activity and lower the patients' quality of life. The course of this disease generally involves the progressive deterioration of lung functions, with a median survival time of 2.5 to 3.5 years after diagnosis [4]. The prognosis is very poor and treatment options are few. The pathological process of IPF generally involves pulmonary fibrosis and determination of the molecular mechanisms will facilitate the development of novel therapeutic modalities.

The pathological process of IPF remains not entirely clear; however, important evidence indicates that miRNAs play important roles in

this process [5, 6]. MiRNAs are a class of small RNA molecules that have been shown to be involved in various pathological processes such as cellular proliferation, tissue development and repair [7-9]. They are the key regulators of tissue phenotypes and potential targets for therapeutic interventions in various diseases. When miRNA array was performed to compare IPF and normal lung tissues, 10% of miRNAs were found to be significantly different and many of them were later verified and shown to have a potential role in this disease.

MiR-338* belongs to the miR-338 family and has been reported to be down-regulated in hepatocellular carcinoma and highly expressed in metastatic CRC tissues [10, 11]. Our previous reports also indicated that the main components in rhizomes of *I. tectorum*, tectorigenin, can enhance the expression of miR-338*, which targeted the downstream effector LPA1 and significantly inhibited the proliferation of pulmonary fibroblasts in bleomycin-treated rats [12]. These results suggested that miR-338*

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may play a role in the pathological process of pulmonary fibrosis, but little of the molecular mechanism has been investigated.

In our study, we also found that miR-338* was down-regulated in TGF- β - or bleomycin-induced pulmonary fibrosis, which were generally taken as the model of idiopathic pulmonary fibrosis. Moreover, LPA1 was shown to be the downstream target of miR-338* and silencing of LPA1 protected TGF- β induced pulmonary fibrosis. Additionally, over-expression of miR-338* overcomes TGF- β -induced pulmonary fibrosis *in vitro* and the inhibition of miR-338* increase LPA1 expression, thus preventing bleomycin-induced pulmonary fibrosis *in vivo*. Taken together, our study demonstrated that miR-338* may target LPA1 to regulate the pathological process of pulmonary fibrosis, meaning that miR-338* and LPA1 may serve as the therapeutic target for treating pulmonary fibrosis in the future.

Materials and methods

Reagents

Bleomycin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The alkaline hydrolysis kit was purchased from Jiancheng Bioengineering (Nanjing, China). RPMI 1640 medium, non-essential amino acids and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY, USA). Trizol was purchased from Invitrogen (Carlsbad, CA, USA). The high capacity cDNA synthesis kit and Fast SYBR Green Master Mix Kit were obtained from Applied Biosystems (Foster City, CA, USA). Rabbit anti-LPA1 polyclonal antibody (ab23698) were purchased from Abcam (Cambridge, United Kingdom). Mouse anti-GAPDH (sc-137179) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Poly-vinylidene fluoride (PVDF) membrane was purchased from Millipore (Bedford, MA, USA). ECLplus western blotting detection system was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

microRNA transfection

One day before transfection, cells were plated in the 6-well plate with the density of 40-50%. The cells were then treated or not treated with TGF-beta for 48 hours before transfection. Then the treated or not treated cells were trans-

ected with 2 ug of p-miR-338* or p-miR-control using lipofectamine 2000 (Invitrogen). 24 hours after transfection, the transfected cells were imaged under microscopy and collected for RNA isolation.

Bleomycin model

Balb/C mice (Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were maintained in a controlled environment and provided with water and standard rodent food. Forty mice were randomly divided into the following 2 groups (n=20/group): saline-water; BLM-water. Mice in the saline group were injected intratracheally with 2 ml/kg saline; the others were injected intratracheally with BLM (5 mg/kg, 2 ml/kg in saline). At day 14 following bleomycin or saline treatment, mice were sacrificed and lungs were collected for histology, immunohistochemistry, real-time PCR, and western blot analysis. All procedures involving animals were approved by the Ethics Committee for Animal Research of Medical School of Nanjing University.

RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). The qPCR reactions were performed using the SYBR Green ER™ qPCR SuperMix. The primers used for miR-338* were 5'-CTCAACTGGTGTCTGGAGTCG-GCAATTCAGTTGAGCAC TCAGC-3' and 5'-ACAC-TCCAGCTGGGAACAATATCCTGGTGC-3' and for HPRT1 were 5'-TGACACTGGCAAACAATGCA-3' and 5'-GGTCCTTTTCAC CAGCAAGCT-3'; HPRT1 was used as an endogenous control. Fold changes were calculated as the means of relative quantification ($2^{-\Delta\Delta Ct}$).

Immunohistochemistry

In vitro cultured pulmonary fibroblasts were pre-fixed with 4% paraformaldehyde for 30 min at room temperature. After washing with PBS, samples were incubated with PBS containing 0.1% Triton X-100 for 15 min. Following blocking with 3% bovine serum albumin (BSA) for 1 h, samples were stained with primary antibodies (rabbit anti-LPA1 polyclonal antibody) overnight at 4°C. Then, cells were incubated with corre-

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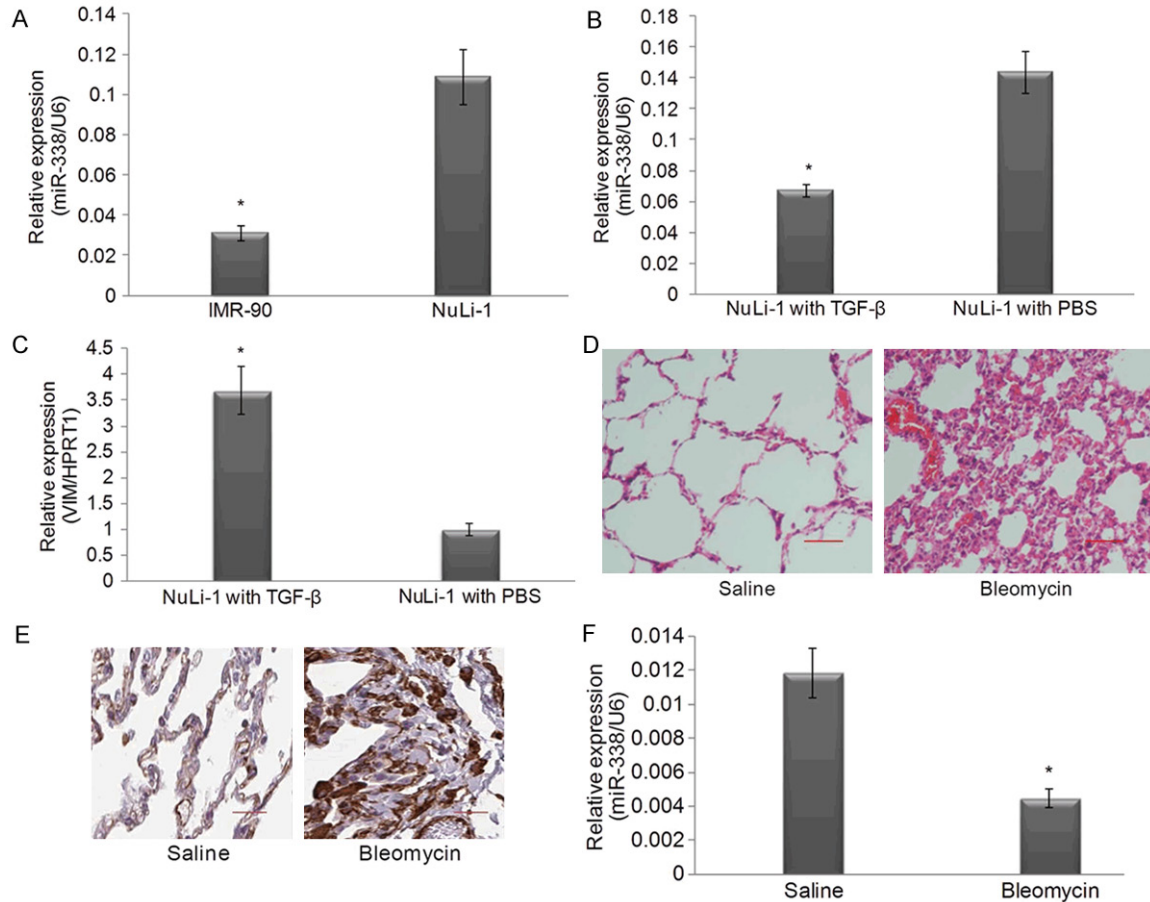


Figure 1. miR-338* was down-regulated in lung fibrosis cells or induced lung fibrosis cells and mice models. The pulmonary morphological examination of BLM-induced disease model was performed with hematoxylin-eosin (H&E) staining. A. miR-338* expression in fibrosis cells and in the control. B. miR-338* expression in TGF-β-induced fibrosis cells and in the control. C. VIM expression in TGF-beta induced fibrosis and in the control. D. H&E staining in BLM-induced and control groups. E. Immunofluorescence chemistry staining in BLM-induced and control groups. F. miR-338* expression in BLM-induced and control groups. The data indicate the mean ± SEM of three samples. *P < 0.05.

sponding secondary antibodies (goat anti-rabbit IgG-FITC; goat anti-mouse IgG-PE) for 1 h at 25°C. Eventually, samples were stained with DAPI for 5 min. Images were captured using an Olympus FluoView 1000 confocal microscope (Olympus America Inc., Melville, NY, USA).

Western-blot

Western blot was performed according to the protocol described previously [5]. Briefly, following separation on SDS-PAGE, proteins were transferred to a PVDF membrane. Protein bands were detected after incubation with rabbit anti-LPA1 poly-clonal antibody overnight at 4°C and HRP-goat anti-rabbit IgG for 2 h using the ECLplus western blotting detection system. GAPDH was used as an internal control. Blots were scanned using a Fluor Chem FC2 system

(Alpha Innotech, San Leandro, CA, USA). Western blots were quantified using the Image 1.42 software. Relative protein expression of LPA1 was calculated relative to GAPDH.

Dual-luciferase reporter assay

The 3'-UTR sequence of LPA1 predicted to interact with miR-338* or a mutated sequence with the predicted target sites were synthesized and inserted into pscheck2 vector (Promega, Madison, WI, USA). These constructs were named pGL3-LAP1-3'UTR-wt and pGL3-LAP1-3'UTR-mutant and used for transfecting 293T cells. The cells were cultured in 12-well plates and each transfected with 50 ng of pGL3-LAP1-3'UTR-wt or pGL3-LAP1-3'UTR-mutant together with 50 ng of pGL3 (Promega, Madison, WI, USA) containing firefly and Renilla

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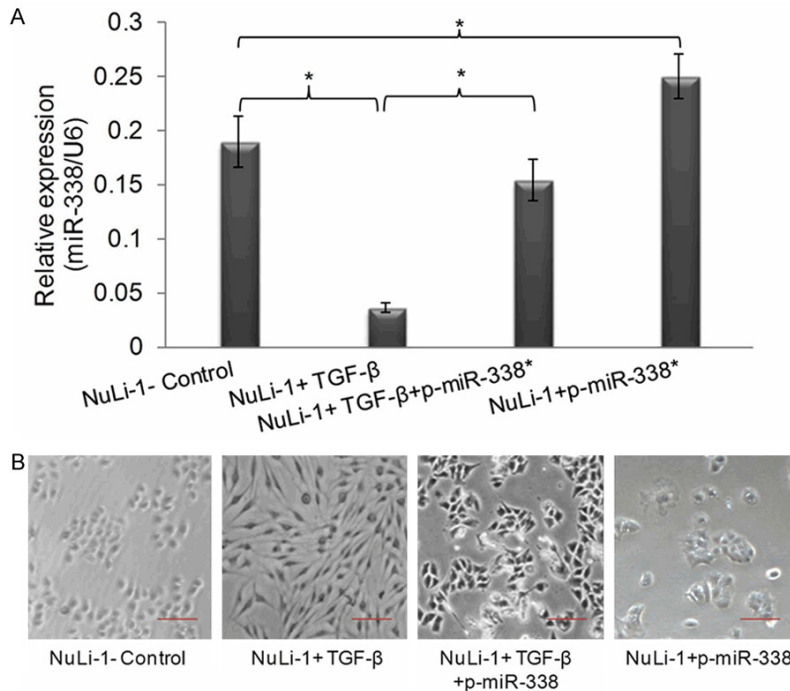


Figure 2. Over-expression of miR-338* overcomes TGF- β -induced pulmonary fibrosis *in vitro*. A. miR-338* was successfully transfected into TGF- β -induced pulmonary fibrosis cells. B. Fibrosis-like cells lost their phenotype and resembled the morphology of Nuli-1 cells when miR-338* was transfected. The data indicate the mean \pm SEM of three samples. * $P < 0.05$.

luciferase and 20 μ M of miR-338* or control using Lipofectamine 2000. Forty-eight hours after transfection, cells were harvested and assayed with the Dual-Luciferase Reporter Assay (Promega), according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean \pm standard deviation (SD). The data were analyzed using the SPSS Windows software, version 12.0. Statistical analyses were performed using analysis of variance (ANOVA) or Student's *t* test. *P* values < 0.05 were considered to be statistically significant.

Results

Evaluation of the pulmonary fibrosis model in mice

Histological analysis indicated that treatment with bleomycin resulted in enhanced pulmonary alveolus inflammation when compared to controls. Additionally, broadened alveolar septa

and increased fibroblasts were observed in multi-focus fibrosis area that differed in size (**Figure 1**).

miR-338* was down-regulated in lung fibroblast cells or induced lung fibrotic cells and mice models

Since miR-338* has been shown to be associated with pulmonary fibrosis models in rats, we first analyzed the expression of miR-338* in the human diploid fibroblast cell line-IMR-90 and human airway epithelial (HAE) cell line-NuLi-1 by quantitative RT-PCR. When compared with human lung airway epithelial cells, the expression of miR-338* was significantly lower by at least 2 to 4-fold in the examined fibroblast cell line (**Figure 1A**). Then, we investigated whether miR-338* was decreased in

induced fibrotic cells (TGF- β -treated NuLi-1 cells) or bleomycin-induced animal models. First, we used TGF- β -induced human lung airway epithelial cells and fibrosis cells as described. We detected the expression of miR-338* using a two-step quantitative RT-PCR. As compared with normal Nuli-1 cells, miR-338* was significantly decreased in TGF- β cultured Nuli-1 cells (**Figure 1B**). We have examined the additional marker of Vimentin by RT-qPCR and shown the effect of TGF- β induced fibrosis *in vitro* (**Figure 1C**). Together with **Figure 1A**, these results demonstrate that miR-338* was significantly down-regulated in fibroblasts or induced fibrosis cells, indicating that miR-338* may be associated with pathological processes of pulmonary fibrosis. Next, we investigated the expression of miR-338* in bleomycin-induced pulmonary fibrosis mice models. Consistent with the results from cell line experiments, miR-338* was significantly lower in the lung tissues of bleomycin-induced pulmonary fibrosis mice compared with saline-treated mice controls (**Figure 1D**). These results suggest that the

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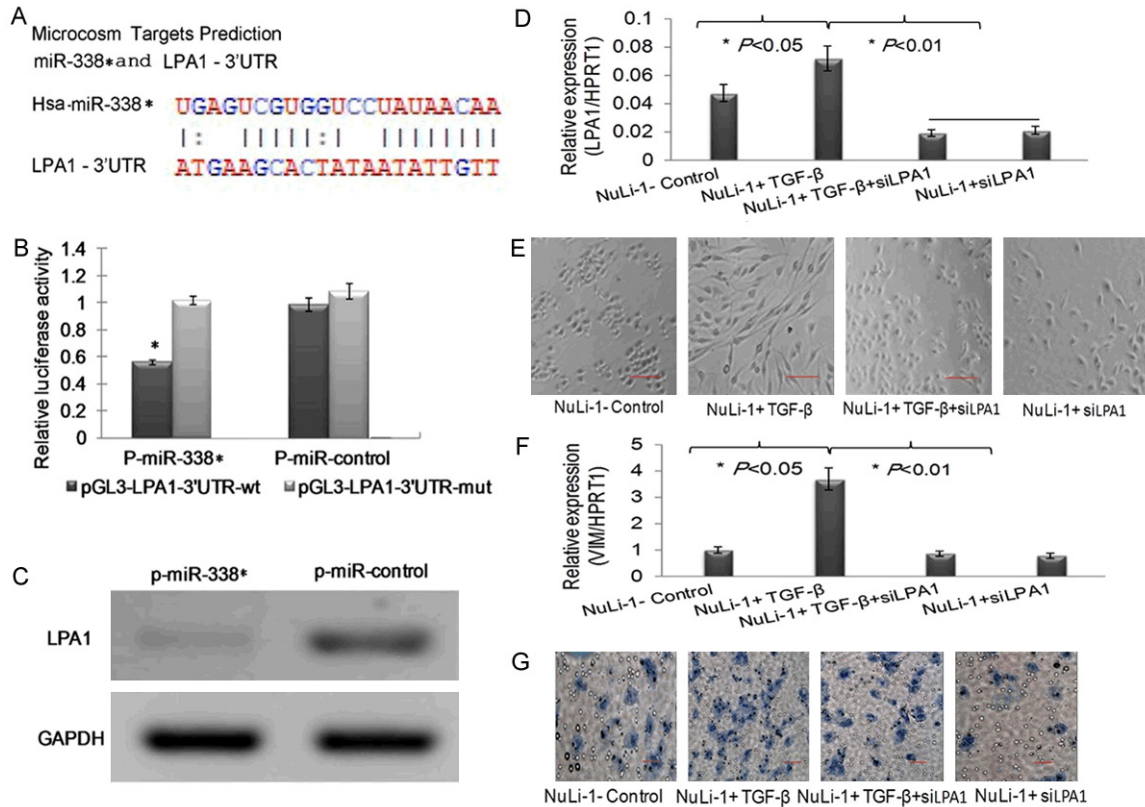


Figure 3. LPA1 was identified as the functional downstream target of miR-338* and silencing of LPA1 partially protected TGF- β -induced pulmonary fibrosis. **A.** LPA1 was predicted as the downstream target by Microcosm. **B.** LPA1 was demonstrated to be the downstream target of miR-338* by dual-luciferase reporting system. **C.** When miR-338* was up-regulated, LPA1 was down-regulated. **D.** LPA1 was inhibited in TGF- β -induced fibrosis cells. **E.** Fibrosis-like cells lost the phenotype and resembled the morphology of Nuli-1 cells when LPA1 was inhibited. **F.** Expression of VIM when LPA1 was knock-down. **G.** LPA1 also inhibits TGF-beta induced cell migration. The data indicate the mean \pm SEM of three samples. * $P < 0.05$.

expression of miR-338* may be involved in the initiation or development of pulmonary fibrosis.

Overexpression of miR-338* overcomes TGF- β -induced pulmonary fibrosis in vitro

To further investigate whether the overexpression of miR-338* can reverse the process of fibrosis in cell lines, we first stably transfected miR-338* into Nuli-1 TGF- β -induced fibrosis cells. As shown in **Figure 2A**, miR-338* was successfully transfected into TGF- β -induced fibrosis cells. Then, we used transmission electron microscopy to observe the morphology of these cells. We found that, compared with Nuli-1 cells, TGF- β -induced fibrosis cells showed a fibroblast-like cell phenotype, such as loss of cell-cell contact and the capacity to form viable spheroids. While stably transfected with miR-338*, fibroblast-like cells lose this phenotype,

reversing to the epithelial phenotype and resembling the morphology of Nuli-1 cells (**Figure 2B**). However, miR-338* transfection alone were not significantly different because the NuLi cells have already high expression of miR-338*. These results suggested that the forced expression of miR-338* may partially prevent the fibrosis induced by TGF- β .

LPA1 is the downstream target of miR-338*

To understand the mechanisms by which miR-338* prevents the fibrosis induced by TGF- β , we used several computational methods to identify the functional targets of miR-338* in humans. Among approximately hundreds of targets predicted by different miRNA target prediction programs, LPA1 was of particular interest. According to the microcosm online prediction, LPA1 is predicted to be a potential target of miR-338*.

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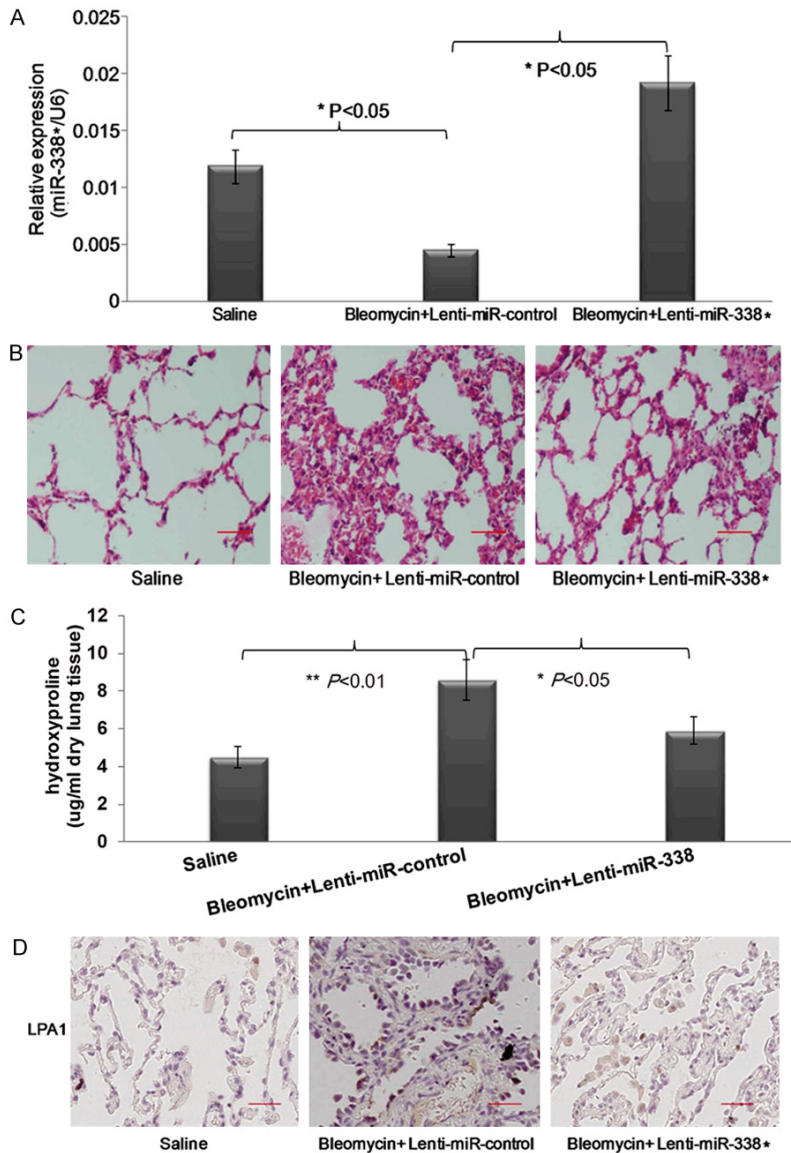


Figure 4. Lentivirus-mediated miR-338* inhibits LPA1 to prevent bleomycin-induced pulmonary fibrosis *in vivo*. **A.** miR-338* was successfully delivered into BLM-treated mice. **B.** H&E staining in lung tissues of lentivirus-mediated miR-338* or control-treated BLM-treated mice. **C.** When LPA1 was inhibited by miR-338*, bleomycin-induced pulmonary fibrosis was partially prevented. **D.** Immunohistochemistry staining demonstrated that LPA1 can be inhibited by miR-338* overexpression. These data indicate the mean \pm SEM of three samples. * $P < 0.05$.

To confirm that LPA1 is a direct functional target of miR-338*, we investigated whether miR-338* targets the 3'-UTR of LPA1 mRNA using the dual-luciferase reporter assay. According to the predicted target sites from Microcosm (Figure 3A), we cloned the wild-type 3'-UTR fragment containing these predicted sites into the pGL3 luciferase reporter vector (pGL3-LAP1-3'UTR). Another 3'-UTR fragment

with a mutation within each seed region was also cloned as a control (pGL3-LAP1-3'UTR-mut). We observed that only the co-transfection of P-miR-338* (not P-miR-control) and pGL3-LAP1-3'UTR (not the pGL3-LAP1-3'UTR-mut) significantly suppressed the luciferase activity by about 40% (Figure 3B). These data confirmed that LPA1 was a direct downstream target of miR-338*. We further measured the protein expression of LPA1 in Nuli-1 cells transfected with P-miR-338* or P-miR-control. The western blotting result also showed that overexpression of miR-338* down-regulated the endogenous protein expressions of LPA1 (Figure 3C).

To investigate whether miR-338* inhibits the fibrosis process via LPA1, we inhibited the LPA1 expression in TGF- β -induced fibrosis cells. As predicted, when LPA1 was knocked-down by siRNA, the morphology of fibroblast cells reversed to epithelial cells, resulting in their morphology resembling that of Nuli-1 cells (Figure 3D, 3E). LPA1 is known for its role in fibroblast migration. Knockdown of LPA1 also inhibits TGF-beta induced cell migration ability (Figure 3F).

Lentivirus-mediated miR-338 inhibits LPA1 to prevent bleomycin-induced pulmonary fibrosis in vivo*

We then examined whether miR-338* had the therapeutic potential for lung fibrosis in an established mouse model of bleomycin-induced pulmonary fibrosis. On day 14 after bleomycin treatment, Lentivirus-mediated miR-

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338* vectors or controls were constructed and delivered into the diseased lung using the SB-based gene transfer system, as described above. Quantitative RT-PCR showed that the lentivirus-based vector was capable of delivering miR-338* (**Figure 4A**). HE staining (**Figure 4B**) and hydroxyproline content assay (**Figure 4C**) showed that lentivirus-mediated miR-338* can prevent bleomycin-induced pulmonary fibrosis *in vivo*. Immunohistochemistry staining also clearly demonstrated that LPA1 can be inhibited by miR-338* overexpression (**Figure 4D**). Therefore, the Lentivirus-mediated miR-338* can prevent bleomycin-induced pulmonary fibrosis *in vivo* via its downstream target, LPA1.

Discussion

IPF is a chronic fibrotic interstitial lung disease of unknown causes that is untreatable [13]. Despite recent rapid progress in our understanding of lung fibrosis pathogenesis in mice, the initiation and development of IPF has not been fully elucidated in humans [14]. Alveolar epithelial cell injury and subsequent uncontrolled repair are the major pathological observations in patients with IPF [15]. MiRs are small RNA molecules that play critical roles in various physiological and pathological processes such as cellular proliferation, tissue differentiation and tissue repairs [16]. Thus, as gene regulators, miRs may play a role in the initiation or development of IPF [17]. Later, evidence has also demonstrated that, compared with control lung tissues, IPF tissues contained different miRNAs [18, 19].

TGF- β has a wide variety of biological functions, including the recruitment of inflammatory cells, regulation of the deposition of ECM components that contribute to fibrosis and induction of epithelial to mesenchymal transition in epithelial cells to fibroblasts and myofibroblasts [20]. Thus, TGF- β -induced lung epithelial cell lines were taken as fibroblast cell line models in this research. In our study, we used this approach to successfully establish a fibroblast cell line based on Nuli-1 cells. A recent study also showed that miR-338* was associated with the pathogenesis of IPF [12]. Of interest, we used RT-PCR to investigate the expression of miR-338* in Nuli-1 and TGF- β -induced fibrosis cell lines based on Nuli-1 cells. We found that miR-338* was significantly down-regulated in the

fibrosis cell line, which implied that it may play a role in the pathogenesis of pulmonary fibrosis.

Next, we overexpressed miR-338* in fibrosis cells and fibrotic lung tissues. We investigated the overexpression impact of miR-338* on pulmonary fibrosis. To our surprise, the forced expression of miR-338* can partly prevent fibrosis, regardless of whether induced by TGF- β cell lines or triggered by bleomycin in mice models. These results suggested that miR-338* partly plays a role in the pathogenesis of pulmonary fibrosis, promoting it as therapeutic target in the future. Further to this, the intriguing question is how miR-338* works in the pathogenesis of pulmonary fibrosis. To our knowledge, miRs generally target the 3'UTR of mRNA, thus downgrading or destroying mRNAs, negatively regulating genes in cells. We used computational software to search for the potential target of miR-338*, and lysophosphatidic acid receptor 1 (LPA1) was found to be the downstream target. Later, *in vivo* experiments demonstrated that LPA1 can be targeted by miR-338*.

LPA1, also known as EDG2, has been indicated to be involved in tumor cell invasion, differentiation and apoptosis [21]. Also, studies demonstrated that LPA1 may be associated with pulmonary fibrosis. For example, LPA1 levels are elevated in BAL after lung injury in a bleomycin-induced pulmonary fibrosis mice model and LPA1 knockout mice have reduced fibroblasts' recruitment. In addition, the inhibition of LPA1 can reduce the chemotaxis of fibroblasts in response to LPA1-enriched BAL from IPF patients [22]. AM152, an LPA1 antagonist, has been successfully investigated in a phase I clinical trial in IPF patients. In our study, as a key member of the LPA family, LPA1 was proven to be the downstream target of miR-338*. Thus, miR-338* may target LPA1 to attenuate the fibrosis induced by TGF- β or bleomycin.

Taken together, we found that miR-338* was down-regulated in fibrotic cells induced by TGF- β or tissues induced by bleomycin. LPA1 was a downstream target of miR-338*, through which miR-338* exerts its prevention function of lung fibrosis both *in vitro* and *in vivo*. Thus, miR-338* may be a potential therapeutic target to treat pulmonary fibrosis in the future.

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Disclosure of conflict of interest

None.

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