MicroRNA-101 attenuates pulmonary fibrosis by inhibiting fibroblast proliferation and activation

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Aberrant proliferation and activation of lung fibroblasts contribute to the initiation and progression of idiopathic pulmonary fibrosis (IPF). However, the mechanisms responsible for the proliferation and activation of fibroblasts are not fully understood. The objective of this study was to investigate the role of miR-101 in the proliferation and activation of lung fibroblasts. miR-101 expression was determined in lung tissues from patients with IPF and mice with bleomycin-induced pulmonary fibrosis. The regulation of miR-101 and cellular signaling was investigated in pulmonary fibroblasts in vitro. The role of miR-101 in pulmonary fibrosis in vivo was studied using adenovirus-mediated gene transfer in mice. The expression of miR-101 was down-regulated in fibrotic lungs from patients with IPF and bleomycin-treated mice. The down-regulation of miR-101 occurred via the E26 transformation-specific (ETS) transcription factor. miR-101 suppressed the WNT5a-induced proliferation of lung fibroblasts by inhibiting NFATc2 signaling via targeting Frizzled receptor 4/6 and the TGF-β-induced activation of lung fibroblasts by inhibition of SMAD2/3 signaling via targeting the TGF-β receptor 1. Adenovirus-mediated miR-101 gene transfer in the mouse lung attenuated bleomycin-induced lung fibrosis and improved lung function. Our data suggest that miR-101 is an anti-fibrotic microRNA and a potential therapeutic target for pulmonary fibrosis.

Idiopathic pulmonary fibrosis (IPF)2 is a lethal fibrotic lung disease characterized by enhanced fibroblast proliferation, collagen synthesis, extracellular matrix deposition, and alveolar epithelial type II cell hyperplasia. The IPF fibroblastic focus consists of activated fibroblasts in a collagen-rich matrix. Several cell types have been posited as a source for IPF fibroblasts, including (i) local resident pulmonary fibroblasts (1, 2), (ii) epithelial–to–mesenchymal transition (3–5), and (iii) circulating fibrocytes (6, 7). Recently, mesenchymal progenitor cells, isolated from human IPF lung tissue, have been shown to serve as a cell-of-origin for disease-mediating IPF fibroblasts (8). However, the relative contribution of each of these sources to the fibroproliferative process remains unclear. Nevertheless, the abnormal proliferation and activation of fibroblasts are thought to be the major factors driving fibrotic progression in IPF (9, 10).

MicroRNAs (miRNAs) have been implicated in a number of lung diseases, including lung cancer, asthma, lung inflammation, chronic obstructive pulmonary diseases, and IPF. Let-7 is expressed in alveolar epithelial cells and is down-regulated in IPF. The inhibition of let-7 results in epithelial–to–mesenchymal transition and collagen deposition (11). However, whether restoration of let-7 can prevent or reverse pulmonary fibrosis in animal models is unknown. Another down-regulated miRNA in IPF is miR-29, which is localized in interstitial cells and regulates fibrotic genes in fibroblasts in vitro (12). miR-29 is also a key player in the remodeling of the extracellular matrix in IPF (13) and has recently been shown to inhibit bleomycin-induced lung fibrosis (14). miR-21 is up-regulated in myofibroblasts in IPF, and antisense miR-21 attenuates bleomycin-mediated pulmonary fibrosis (15). Notably, let-7, miR-29, and miR-21 target TGF-β signaling and/or are regulated by TGF-β (11, 12, 15). Hypoxia-inducible miR-210 enhances fibroblast proliferation (16). miR-326 directly targets TGF-β, thereby reducing pulmonary fibrosis (17). Our recent studies have shown that miR-424 promotes myofibroblast differentiation during epithelial–to–mesenchymal transition (18). Because the expression of many miRNAs is altered in IPF, these studies underscore the need for further investigations to identify critical miRNAs that regulate key pathways for pulmonary fibrosis.

In this study, we identified miR-101 as one of the most downregulated miRNAs in IPF patients. miR-101 inhibited the proliferation and activation of lung fibroblasts by targeting the receptors of the non-canonical Wnt and TGF-β signaling pathways. Adenovirus-mediated gene transfer of miR-101 to the lung reduced pulmonary fibrosis in a pre-clinical mouse model.

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2 The abbreviations used are: IPF, idiopathic pulmonary fibrosis; RACE, rapid amplification of cDNA ends; m.o.i., multiplicity of infection; miRNA, microRNA; FVC, forced vital capacity; ANOVA, analysis of variance; NFAT, nuclear factor of activated T-cell; CsA, cyclosporin A; α-SMA, α-smooth muscle actin.

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Thus, our studies demonstrated that miR-101 is an anti-fibrotic miRNA.

**Results**

**miR-101 is down-regulated in IPF**

Using an miRNA microarray, we analyzed global miRNA expression profiles of the lung tissues from 28 IPF patients obtained from the Lung Tissue Research Consortium. The IPF patient specimens were classified into three groups according to the pre-bronchodilator forced vital capacity (FVC) % value: group 1, normal lung function (FVC% >80); group 2, mild lung restriction (FVC 50–80%); and group 3, severe lung restriction (FVC <50%). We found 29 miRNAs that were significantly altered between at least two groups (Table 1 and Fig. 1a). We verified the changes of 14 selected miRNAs using real-time PCR (Figs. 1b and 2).

We performed miRNA-target predictions and pathway analysis using TargetScan and KEGG. miR-101 had predicted targets in TGF-β and Wnt signaling that are involved in fibroblast proliferation and activation (Table 2). Furthermore, miR-101 had the greatest change in IPF lungs among the down-regulated miRNAs. Therefore, we chose miR-101 for further study.

There are two miR-101 isoforms, miR-101-1 and miR-101-2 in humans and miR-101a and miR-101b in mice. All of the miR-101 isoforms have the same mature sequence, with the exception of miR-101b, which has one base difference. Real-time PCR analyses in this study were based on the human miR-101 or mouse miR-101a mature sequences. Consistent with our findings using human lung tissue, we found that mouse miR-101a expression was reduced in the lung tissue from an experimental mouse model of bleomycin-induced pulmonary fibrosis (Fig. 1c). miR-101a down-regulation was also observed in fibroblasts isolated from bleomycin-treated mice (Fig. 1c).

It was reported that there is an miRNA processing defect in IPF, which may cause a more global decrease in multiple miRNAs (19). However, similar to mature miRNAs, the expression levels of pri-miR-101-a and pri-miR-101-b in the lung tissues of the bleomycin-treated mice were also lower than those of the control groups (Fig. 1d), suggesting that the miRNA processing defect is not the major mechanism for the down-regulation of miR-101 in IPF.

**miR-101 is regulated by the ETS transcription factor**

Human miR-101-2 is intragenic and located within the protein-coding RCL1 gene on chromosome 9, suggesting that it is co-regulated with the host RCL1 gene. In contrast, human miR-101-1 is intergenic and located in chromosome 1. Therefore, we examined the transcription start site of miR-101-1 using the RACE assay. We chose six primers at different positions upstream of precursor miR-101 for the 5’-RACE reaction (Table 5). Only one PCR product (500 bp) was obtained using the primer PROM-MIR-101-1-RE4 (GTGGCCGTCACAGGCATCCTTTCT) among all of the primers tested. Sequencing analysis revealed that the amplified RACE cDNA product was located upstream of miR-101-1. The sequence of the RACE product was used to search the NCBI and UCSC genome databases. We found several ESTs (including BU786820, BX476649, and AI275070) located downstream of the RACE primers.

**Table 1**

**Significantly dysregulated miRNAs in IPF samples**

Twenty eight IPF lung samples were classified as three groups according to the pre-bronchodilator forced vital capacity (FVC) % values: <50% predicted FVC group; 50–80% predicted FVC group, and >80% predicted FVC group. The numbers are ratios of miRNA expression of two groups. The statistical significance was determined by two-tailed Student’s t test considering unequal variance.

| miRNA       | 50–80% FVC to >80% FVC group | <50% FVC to >80% FVC group | <50% FVC to 50% >80% FVC group |
|-------------|-----------------------------|---------------------------|-------------------------------|
| hsa-miR-124 | 1.9                         | 5.0*                      | 2.6*                          |
| hsa-miR-215 | 1.2                         | 3.3*                      | 2.7*                          |
| hsa-miR-1300| 1.6                         | 2.7*                      | 1.7                           |
| hsa-miR-668 | 1.6                         | 2.6*                      | 1.6                           |
| hsa-miR-552 | 6.3*                        | 2.5*                      | 0.4*                          |
| hsa-miR-766 | 1.0                         | 2.0*                      | 1.9                           |
| hsa-miR-7   | 1.2                         | 2.0*                      | 1.7                           |
| hsa-miR-1,249| 2.9*                       | 1.8                       | 0.6                           |
| hsa-miR-493*| 0.4                         | 1.4                       | 3.7*                          |
| hsa-miR-181a-2*| 0.6                     | 1.3                       | 2.1*                          |
| hsa-miR-1297| 2.3*                        | 0.9                       | 0.4                           |
| hsa-miR-101 | 0.85                        | 0.21*                     | 0.24*                         |
| hsa-miR-977 | 0.68                        | 0.22*                     | 0.33                          |
| hsa-miR-887 | 0.52                        | 0.32*                     | 0.62                          |
| hsa-miR-27b | 0.55                        | 0.33*                     | 0.60                          |
| hsa-miR-878b| 0.54                        | 0.34*                     | 0.63                          |
| hsa-miR-5,688| 0.44                       | 0.35*                     | 0.8                           |
| hsa-miR-142-5p| 0.48*                    | 0.37*                     | 0.76                          |
| hsa-miR-1,297| 2.45*                     | 0.41*                     | 0.86                          |
| hsa-miR-1,249| 0.51                        | 0.41*                     | 0.81                          |
| hsa-miR-991-3p| 0.45                      | 0.42*                     | 0.94                          |
| hsa-miR-1,298| 0.73                        | 0.45*                     | 0.62                          |
| hsa-miR-888*| 0.52                        | 0.50*                     | 0.96                          |
| hsa-miR-5,194| 0.60                        | 0.52*                     | 0.85                          |
| hsa-miR-141 | 1.01                        | 0.53*                     | 0.53*                         |
| hsa-miR-877 | 1.32                        | 0.57                      | 0.43*                         |
| hsa-miR-589 | 1.35                        | 0.60                      | 0.44*                         |
| hsa-miR-299-3p| 2.12                      | 0.69                      | 0.32*                         |
| hsa-miR-195 | 1.55                        | 0.81                      | 0.52*                         |

*p < 0.05.

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product. These ESTs were assembled into a transcript and aligned with the human genome. The assembled transcript was primary miR-101-1, with a length of 1,183 bp. Primary miR-101-1 consists of three exons, with miR-101-1 located in exon 3 (Fig. 3a). The sizes of three exons are 129, 175, and 879 bp. Primary miR-101-1 has a typical poly(A) tail signal (AATAAA). The host miR-101-1 gene is 9,944 bp. The transcription start site is located at position chr1:65067704 (genome version hg38). The transcriptional start site distance to the pre-miR-
101-1 is 9,224 bp.

Promoter analysis using TRANSFAC 8.3 revealed that there were six and five predicted ETS transcription-binding sites in the 2.5-kb region upstream from the transcription start site of human miR-101-1 and miR-101-2, respectively (Fig. 3a). Therefore, we determined whether ETS1/2 affected miR-101 promoter activity. ETS1/2 increased the promoter activities of

Figure 1. miR-101 expression is down-regulated in fibrotic lungs. a, heat map of miRNA expression in IPF patients. Heat map of these dysregulated miRNAs was built by using GenePattern software (http://www.broadinstitute.org/cancer/software/genepattern). b, miR-101 expression in IPF lungs. miRNA microarray and real-time PCR were performed using total RNA isolated from the lung tissues of IPF patients. n = 10 for the <50% FVC group, n = 10 for the 50–80% FVC group, and n = 8 for the >80% predicted FVC group. Real-time PCR results were normalized to U6. Data were expressed as a ratio over the >80% FVC group. c and d, real-time PCR analysis showing the down-regulation of mature miR-101a, pri-miR-101a, and pri-miR-101b expression in the lungs (n = 6) and mature miR-101a expression in the fibroblasts (n = 3) isolated from saline (Sal)- and bleomycin (Bleo)-treated mice. The expression levels were normalized to U6 (for mature miR-101) or GAPDH (for pri-miR-101) and were expressed as a ratio of saline. The results are presented as the mean ± S.E. ANOVA followed by Tukey’s HSD test was performed for multiple comparisons. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
both miR-101-1 and miR-101-2 in HFL1 fibroblasts although their effects on miR-101-1 were bigger. (Fig. 3b). The overexpression of ETS1 and ETS2 was confirmed by Western blot analysis (Fig. 3c).

To determine which predicated ETS-binding sites are functional, we tested whether the mutation of selected ETS-binding sites abolished the ETS-induced miR-101 promoter activity. Considering that the conserved binding sites of a transcription factor are usually functional, we only mutated the conserved ETS-binding sites: binding sites 1 and 5 in the human miR-101-1 promoter and binding site 2 in the human miR-101-2 promoter. We found that ETS1- or ETS2-induced miR-101-1 promoter activities were significantly suppressed by the mutation of binding site 1, but not binding site 5, suggesting that ETS1/2 likely binds the binding site 1 in the miR-101-1 promoter. The mutation of binding site 2 in the miR-101-2 promoter had no effects on ETS1- or ETS2-induced miR-101-2 promoter activities (Fig. 3b). The ETS-binding sites that are responsible for the effects of ETS1/2 on miR-101-2 promoter activity remain to be determined.

To determine whether endogenous miR-101 expression was regulated by ETS1/2, we reduced the ETS1/2 protein level in CCD-8Lu fibroblasts using lentiviral shRNAs (Fig. 3d). Silencing of either ETS1 or ETS2 inhibited miR-101 expression by 50–60% (Fig. 3e). The ETS1 and ETS2 mRNA expression levels were significantly down-regulated in fibrotic lungs (Fig. 3f).

miR-101 inhibits fibroblast proliferation through WNT5a-NFATc2 signaling

The proliferation of fibroblasts is postulated to be one of the major contributors to the increase in the number of fibroblasts in the fibroblastic foci. A prior study has found that WNT5a is up-regulated in IPF and promotes fibroblast proliferation (20). We found that WNT5a and -5b were the major WNT ligands expressed in pulmonary fibroblasts from normal human lungs (HFL1) and IPF lungs (LL29) (Fig. 4a). Gain of function of WNT5a increased the proliferation of HFL1 and LL29 fibroblasts, whereas loss of function of WNT5a decreased their proliferation (Fig. 4, b–d); this result is consistent with a previous report (20).
miR-101 inhibits fibroblast activation via TGF-β/Smad2/3 signaling

Myofibroblasts are a cellular major component of fibroblastic foci. They are characterized by induced α-SMA expression and stress fiber formation, an enhanced contractile ability, and an increase in collagen synthesis and deposition (23, 24). Fibroblasts are stimulated to differentiate to myofibroblasts via TGF-β/Smad2/3 signaling. Overexpressing miR-101 in LL29 IPF fibroblasts inhibited TGF-β1-induced protein expression of α-SMA and the collagen COL1A1 and COL3A1 (7a) and mRNA expression of α-SMA and the collagens COL1A1, COL3A1, and COL4A1 (7b). In contrast, anti-miR-101 increased the protein and mRNA levels of α-SMA and the collagens in CCD-8 Lu normal lung fibroblasts (7c and d). Furthermore, miR-101 reduced TGF-β1-inducedcontractile activity as determined by the collagen gel assay and stress fiber formation (7e and f). ELISA showed that phosphorylated Smad2/3 were induced by TGF-β1 treatment and reached a peak at 30 min. miR-101 inhibited TGF-β1-induced Smad2/3 phosphorylation (8a). Real-time PCR showed that the mRNA expression of SMAD2 and SMAD3 was not significantly altered in the lung tissues of IPF patients (8b). These results suggest that miR-101 inhibits TGF-β-mediated fibroblast differentiation to myofibroblasts via SMAD2/3 signaling.

FZD4, FZD6, and TGFBR1 are the targets of miR-101

TargetScan predicts FZD4, FZD6, and TGFBR1 as potential targets of miR-101. There are two miR-101-binding sites in the 3′-UTRs of human FZD4 and TGFBR1 and one in FZD6. The binding sites of miR-101 in the 3′-UTRs of FZD4, FZD6, and TGFBR1 are conserved in humans, mice, cows, and dogs (http://www.targetscan.org) (3). We cloned each of the binding sites with flanking sequences downstream of the firefly luciferase gene under the control of the PGK promoter in the pmirGLO reporter. Transfection of miR-101 into HEK 293T cells inhibited the reporter activities of both FZD4-S1-UTR and FZD4-S2-UTR (Fig. 9a), indicating that miR-101 bound to both sites. However, miR-101 inhibited the TGFBR1-S2-UTR but not the TGFBR1-S1-UTR reporter activities, suggesting that miR-101 only bound to site 2, but not site 1, in the 3′-UTR of TGFBR1. miR-101 also inhibited the reporter activity of FZD6 3′-UTR (nucleotides 1–1,356). Infection of HLF1 fibroblasts with a lentivirus expressing miR-101 reduced the endogenous protein and mRNA levels of FZD4, FZD6, and TGFBR1 (9b and d), whereas anti-miR-101 increased the protein and mRNA expression of all three targets (9c and e). The mRNA levels of FZD4, FZD6, and TGFBR1 were up-regulated in the lungs of IPF patients (9f–h) and were inversely correlated with miR-101 expression (1b). Knockdown of TGFBR1 inhibited the

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#### Table 2

| KEGG pathway            | p value | No. of genes |
|-------------------------|---------|--------------|
| Fatty acid biosynthesis | 1.8E-20 | 2            |
| Proteoglycans in cancer | 8.8E-07 | 31           |
| Adherens junction       | 1.0E-05 | 19           |
| Hippo signaling pathway | 1.42E-15| 24           |
| Colorectal cancer       | 0.003232| 15           |
| Renal cell carcinoma    | 0.002687| 15           |
| Pathways in cancer      | 0.002989| 47           |
| Circadian entrainment   | 0.008563| 10           |
| TGF-β signaling pathway | 0.005452| 12           |
| Oocyte meiosis          | 0.010183| 23           |
| Viral carcinogenesis    | 0.018362| 28           |
| Axon guidance           | 0.019612| 15           |
| Protein processing in endoplasmic reticulum | 0.029436 | 13 |
| Ubiquitin-mediated proteolysis | 0.027623 | 23 |
| Glioma                  | 0.027623| 11           |
| MAPK-signaling pathway  | 0.029167| 35           |
| Cell cycle              | 0.030573| 23           |
| p53 signaling pathway   | 0.030573| 14           |
| Endocytosis             | 0.030573| 22           |
| Transcriptional misregulation in cancer | 0.030573 | 24 |
| Lysine degradation      | 0.030705| 6            |
| Pancreatic cancer       | 0.031673| 9            |
| Fatty acid metabolism   | 0.034377| 7            |
| Bladder cancer          | 0.043925| 9            |
| Amyotrophic lateral sclerosis (ALS) | 0.043925 | 8 |
| Pantothenate and CoA biosynthesis | 0.045609 | 3 |
| Dorso-ventral axis formation | 0.045609 | 9 |
| Progestosterone-mediated oocyte maturation | 0.047813 | 15 |
| Chronic myeloid leukemia | 0.047813 | 15 |
| Adrenergic signaling in cardiomyocytes | 0.047813 | 17 |
| Wnt signaling pathway   | 0.047813| 20           |
| Focal adhesion          | 0.047813| 28           |

WNT5a binds with the Frizzled receptor/Ror and activates the heterotrimeric G-protein and phospholipase C, leading to the generation of inositol 1,4,5-trisphosphate and an increase in Ca\(^{2+}\) (21). This activates calcineurin, which dephosphorylates NFAT and results in the translocation of NFAT into the nucleus (22). Treatment of LL29 cells with recombinant WNT5a led to NFATc2 nuclear translocation, as determined by immunofluorescence analysis (Fig. 5a). Cyclosporin A (CsA), an inhibitor of calcineurin, reduced WNT5a-induced fibroblast proliferation (Fig. 5b). Knockdown of Fzd4, Fzd6, and NFATc2 also inhibited WNT5a-induced fibroblast proliferation (Fig. 5c and d). Real-time PCR showed that WNT5a mRNA level was increased in IPF patients (Fig. 5e). This result is consistent with a previous report (20). NFATc2 mRNA level was also increased in the severe IPF groups, but it did not reach a significant level (Fig. 5f). These data indicate that WNT5a-induced fibroblast proliferation occurs through NFATc2 signaling in pulmonary fibroblasts.

To examine the effects of miR-101 on fibroblast proliferation, we overexpressed human miR-101 in LL29 fibroblasts with a miR-101-1 lentivirus and then stimulated them with WNT5a. miR-101 inhibited WNT5a-induced fibroblast proliferation by using the BrdU cell proliferation assay (Fig. 6a). This result was further confirmed by histological staining of BrdU, which showed the increased stained cell numbers and intensity in the WNT5a-treated cells (Fig. 6b). Moreover, miR-101 also suppressed NFATc2 signaling based on the observation that it decreased WNT5a-induced NFATc2 nuclear translocation and NFATc2 mRNA levels (Fig. 6c and d). Silencing Fzd4 and Fzd6 suppressed the WNT5a-induced NFATc2 nuclear translocation (Fig. 6e). These results suggest that miR-101 inhibits WNT5a-stimulated cell proliferation through WNT5a/NFATc2 signaling.
TGF-β1-induced mRNA expression of α-SMA, COL1A1, COL3A1, and COL4A1 (Fig. 10, a–d). Furthermore, overexpression of FZD4 or FZD6 rescued the inhibitions of miR-101 on fibroblast proliferation (Fig. 10, e and f). The overexpression of TGFBR1 also rescued the inhibition of miR-101 on the mRNA expression of α-SMA, COL1A1, and COL3A1 in

Figure 3. Regulation of miR-101 expression by ETS. a, genomic structure of human miR-101-1 and the schematic presentation of potential ETS-binding sites in the upstream regions of the human miR-101-1 and miR-101-2 promoters. TSS, transcription start site. b and c, effects of ETS1/2 on human miR-101-1 and miR-101-2 promoter activities without or with ETS-binding site mutations in fibroblasts. Conversed ETS-binding site 1 (CCGGAA) and site 5 (TTTCTA) in human miR-101-1 promoter were mutated to site 1 (CCActG) and site 5 (TTTagA). Conversed ETS-binding site 2 (ATTCCTC) in human miR-101-2 promoter was mutated to site 2 (ATagcaC). HFL1 cells were transfected with 50 ng of the promoter reporter construct of miR-101-1 wild type (WT), miR-101-1-mute1, miR-101-1-mute5, miR-101-2 WT, and miR-101-2-mute2, 100 ng of the pCMV-ETS1, pCMV-ETS2 or control (CON) vector, and the normalization TK vector for 48 h. Firefly and Renilla luciferase activities were measured. ETS1 and ETS2 protein levels were determined by Western blotting. Data were expressed as a fold change over the control vector. n = 3. d and e, miR-101 expression in ETS1/2 knockdown CCD-8Lu fibroblasts. miR-101 expression was determined by real-time PCR and normalized to U6 snRNA, n = 4. f, down-regulation of ETS1 and ETS2 mRNA expression in saline (Sal)- and bleomycin (Bleo)-treated mouse lung tissues. The expression of ETS1/2 was relative to GAPDH. n = 3. The results are presented as the mean ± S.E. ANOVA followed by Tukey's HSD test was performed for multiple comparisons. *p < 0.05; **p < 0.01; ***p < 0.001.
LL29 fibroblasts (Fig. 10, g–j). These results suggest that FZD4, FZD6, and TGFBR1 are miR-101 targets.

miR-101 attenuates bleomycin-induced pulmonary fibrosis in mice

We have found that miR-101 inhibits WNT5a-induced fibroblast proliferation and TGF-β1-induced fibroblast activation. Therefore, we hypothesized that inhibition of fibroblast proliferation and activation by miR-101 gain of function would reduce excessive extracellular matrix deposition and prevent the development of pulmonary fibrosis in response to bleomycin. To test this hypothesis, we examined the effect of overexpression of miR-101 in mouse lungs using adenovirus-mediated gene transfer on bleomycin-induced pulmonary fibrosis. The gene transfer resulted in a 2-fold increase in miR-101 expression in the lung (Fig. 11a). H&E analysis revealed reduced fibrosis in the miR-101-treated group (Fig. 11b), which was confirmed by quantitation of lung fibrosis using an Ashcroft score (Fig. 11c). miR-101 also inhibited lung collagen content as measured by hydroxyproline assay (Fig. 11d). Furthermore, miR-101 reduced bleomycin-induced COL1A1 and COL3A1 expression (Fig. 11, e and f). Importantly, lung function was improved in the miR-101-treated group, as evidenced by the decrease in elastance (Ers) and tissue elastance (H) (Fig. 11, g and h). The mRNA levels of NFATc2, FZD6, and TGFBR1 were increased in the lungs of mice with bleomycin-induced fibrosis, and miR-101 suppressed the increases (Fig. 11, i and j). The similar changes were observed for the FZD4 mRNA level but did not reach a significant level.

Discussion

In this study, we discovered that the miR-101 level was reduced in the lungs of patients with IPF. miR-101 expression was regulated by the ETS transcription factor. Notably, miR-101 reduced pulmonary fibrosis by inhibiting fibroblast proliferation and differentiation via the WNT5a/NFATc2 and TGF-β/Smad2/3 pathways (Fig. 12).

miR-101 was originally identified as a tumor suppressor, and its expression was reduced in many cancers due to genomic loss. miR-101 inhibited cell proliferation and invasion in cancer cells by targeting EZH2 (25) and inhibited autophagy and sensitized cancer cells to chemotherapeutic drug-induced cell death (26, 27). The effect of miR-101 on angiogenesis is contradictory, and both pro- and anti-angiogenic effects have been reported (28, 29). miR-101 enhanced LPS-induced pro-inflammatory cytokine production in macrophages via the activation of MAPK by targeting MAPK phosphatase-1 (30). Finally, miR-101 has been shown to inhibit liver and cardiac fibrosis (31, 32).

We identified the transcription start site of miR-101-1 using the RACE assay. Further analysis revealed that the primary miR-101-1 transcript was composed of three exons, and the mature miR-101 was located in exon 3. However, a recent
The report showed that the human primary miR-101-1 transcript consisted of two exons in hepatocyte-derived cells (33). The transcription start site in hepatocytes was located five nucleotides upstream of the transcription start site that we identified in lung cells. Alternative splicing and the transcription start site of the miR-101 gene in different cells may account for these differences. The presence of multiple ETS-binding sites in the miR-101-1 and miR-101-2 promoters and the decrease in ETS1/2 expression in bleomycin-treated lungs suggest that the ETS transcription factors may regulate miR-101 expression. ETS2 has been shown to be involved in the pathogenesis of pulmonary fibrosis (34).

Non-canonical Wnt signaling has been relatively less studied in IPF. We found that WNT5a and -5b served as the WNT ligands in pulmonary fibroblasts and that WNT5a induced fibroblast proliferation, which was consistent with a previous report (20). WNT5a induced fibroblast proliferation via the NFATc2 transcription factor, which was supported by the following observations: (i) WNT5a induced the nuclear translocation of NFATc2; (ii) inhibition of calcineurin decreased WNT5a-stimulated fibroblast proliferation; and (iii) reduction of FZD4/6 and NFATc2 protein levels by RNAi inhibited WNT5a-induced fibroblast proliferation.

Importantly, we discovered that miR-101 suppressed WNT5a-induced fibroblast proliferation. The following evidence supports that the miR-101 inhibition of fibroblast proliferation is the result of the repression of WNT5a/NFATc2 signaling: (i) FZD4/6 are the direct targets of miR-101; (ii) miR-101 inhibits NFATc2 nuclear translocation; (iii) miR-101 reduced WNT5a-induced NFATc2 mRNA levels; and (iv) miR-101 levels were inversely correlated with FZD4 and FZD6 expression in the lungs of IPF patients. Our studies revealed...
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**Graphs and Images:**
- **Graph a:** Bar graph showing cell proliferation with CON, WNT5a, miR-101, and miR-101+CON treatments.
- **Graph b:** Images of VC, miR-101, VC + WNT5a, and miR-101 + WNT5a treatments with Brdu and DAPI staining.
- **Graph c:** Western blot showing NFATc2 and H1 in nuclear extract with CON, miR-101, and miR-101+CON treatments.
- **Graph d:** Quantitative data of NFATc2 mRNA expression with CON, WNT5a, and miR-101 treatments.
- **Graph e:** Fluorescence images comparing shCON, shCON + WNT5a, shFDZ4/6, and shFDZ4/6 + WNT5a treatments with NFATc2 and DAPI staining.
that the molecular mechanism underlying the control of fibroblast expansion by miR-101 was the regulation of WNT5a-FZD4/6-NFATc2 signaling.

TGF-β stimulates collagen synthesis and extracellular matrix deposition (1, 2). TGF-β transduces its signals by binding to the transmembrane receptor TGFBR1, and it regulates the expression of numerous genes through their downstream effectors (the SMAD proteins). miR-101 inhibited TGF-β-induced fibroblast activation, as evidenced by the inhibition of collagen synthesis, reduced α-SMA mRNA and protein expression, decreased stress fiber formation, and enhanced contractile ability. The effect of miR-101 on fibroblast activation was due to its
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Figure 8. miR-101 inhibits fibroblast activation through TGF-β/Smad2/3 signaling. a, ELISA analysis showing increased Smad2/3 phosphorylation by TGF-β1 treatment in LL29 cells. miR-101 suppressed the TGFβ1-induced Smad2/3 phosphorylation. The results are presented as the mean ± S.E. n = 4–6, * p < 0.05. b, SMAD2/3 expression in IPF patients. Real-time PCR were performed using total RNA isolated from the lung tissues of IPF patients. n = 10 for the <50% FVC group, n = 10 for the 50–80% FVC group, and n = 8 for the >80% predicted. The expression level was relative to GAPDH.

inhibition of TGF-β/SMAD2/3 signaling, which was supported by the following observations. (i) TGFBR1 was identified as the target of miR-101 by the 3′-UTR reporter assay, and the endogenous TGFBR1 protein levels changed due to the manipulation of the miR-101 levels. This is consistent with a recent report in hepatic stellate cells (31). (ii) Silencing of TGFβ1R had similar effects to miR-101 on fibroblast activation. (iii) miR-101 inhibited the phosphorylation of SMAD2/3 induced by TGF-β1.

Emerging evidence suggests that suppressing up-regulated miRNAs or restoring the activities of the down-regulated miRNAs associated with disease could become a novel therapeutic strategy. Adeno-associated viruses have been used for the delivery of miRNAs in vivo to restore their activity; its therapeutic potential has been validated in a mouse model of lung carcinoma (35). Therapeutic delivery of miR-29 mimics by intravenous injections in the tail vein reversed pulmonary fibrosis in the bleomycin-induced mouse fibrosis model (14). In this study, adenovirus-mediated miR-101 lung delivery reduced the fibrotic changes in bleomycin-treated mouse lungs and also improved pulmonary function. Thus, miR-101 may be a potential therapeutic miRNA for the treatment of pulmonary fibrosis.

Experimental procedures

IPF lung tissues and RNA isolation

Twenty eight IPF patient lung tissue samples were obtained from the Lung Tissue Research Consortium. All of the samples were submerged in RNAlater solution and stored at −80 °C prior to use. Total RNA was isolated from these lung tissues using TRI Reagents (Molecular Research Center, Cincinnati, OH). The RNA concentration and quality were determined with the NanoDrop ND-1000 spectrophotometer (NanoDrop Tech., Rockland, DE) with an A260/A280 ratio of >1.8 and an A260/A230 ratio of >1.7.

miRNA microarray analysis

miRNA microarray slides were printed in-house using the miRCURY LNA™ ready-to-spot probe set 11.0 (Exiqon, Woburn, MA) composed of 1,282 human mature miRNAs. Total RNA was isolated from IPF lung tissues using TRI Reagents (Molecular Research Center, Cincinnati, OH). A common reference containing total RNAs pooled from 20 normal human tissues was purchased from Ambion (Grand Island, NY) (catalogue no. AM6000). Microarray hybridization and data analyses were performed as described previously (36). In brief, 400 ng of total RNAs from IPF lung tissues or the common reference were labeled with Hy3 or Hy5 using the miRCURY LNA™ microRNA power labeling kit (Exiqon, Woburn, MA). A dye swap was performed to eliminate dye bias. Each IPF sample was hybridized to an miRNA microarray slide with the common reference for 16–18 h at 56 °C. The hybridized slides were scanned with ScanArray Express (PerkinElmer Life Sciences). The images were analyzed using GenePix 5.0 pro (Axon Instruments, Inc., Union City, CA) for data extraction. The images and raw data were imported into the RealSpot software that was developed in our laboratory for quality control and Lowess normalization (37). miRNAs with an average quality index of less than 1 were filtered. A two-tailed Student’s t test assuming unequal variance was performed to identify differentially expressed miRNAs in the lung tissues of IPF patients. p < 0.05 was considered to be significant.

Real-time PCR

The miRNA expression levels were determined by SYBR Green I-based real-time PCR as described previously (38). One microgram of DNase-treated total RNA was poly(A)-tailed and purified by phenol/chloroform extraction. The poly(A)-tailed RNAs were reverse-transcribed into cDNA with an miRNA-specific primer (Table 3). The real-time PCR thermal conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Data were analyzed by the comparative ΔCt method using U6 small nucleolar RNA as an endogenous reference gene. The mRNA expression levels were also determined using real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The sequences of the primers used for real-time PCR are shown in Table 4.

RACE analysis

5′-RACE analysis was performed using the RExactSTART eukaryotic mRNA 5′- and 3′-RACE kit (EPICENTRE, Madison, WI) (catalogue no. E809010) according to the manufacturer’s instructions. PCR amplification of the 5′-end of human primary miR-101-1 was performed using the Racer 5′-primer and a gene-specific reverse primer (Table 5). The amplified RACE product was subjected to sequence analysis.
Construction of plasmids

3′-Untranslational region (3′-UTR) reporter vectors—The 3′-UTR of FZD4, FZD6, and TGFBR1 were amplified by PCR using specific primers (Table 6) from human genomic DNA (Promega, Madison, WI). The 3′-UTR fragments were inserted downstream of the firefly luciferase gene using the pmirGLO Dual-Luciferase miRNA target expression vector (Promega, Madison, WI) at the NheI and SalI sites. pmirGLO also contains the Renilla luciferase gene for normalization.

miRNA expression vectors—Mature miR-101-1 plus ∼200-bp flanking sequences at each end were amplified by PCR using specific primers (Table 6) from human genomic DNA. The

Figure 9. Identification of FZD4, FZD6, and TGFBR1 as miR-101 targets. a, 3′-UTR reporter assay: FZD4-UTR-S1 (position 1–1,432) containing miR-101-binding site 1 (position 537–543), FZD4-UTR-S2 (position 4,178–5,463) containing miR-101-binding site 2 (position 1,240–1,246), full length of FZD6-UTR, TGFBR1-UTR-S1 (position 66–1,524) containing miR-101-binding site 1 (position 460–466), or TGFBR1-UTR-S2 (position 3,210–4,749) containing miR-101-binding site 2 (position 3,993–3,999) were co-transfected into HEK 293T cells with miR-101-1. The relative luciferase activities were measured using the Dual-Luciferase® reporter assay system. n = 3. b and c, effect of overexpressing or knockdown miR-101 on the protein expression of FZD4, FZD6, and TGFBR1 in fibroblasts. LL29 cells were treated with a lentivirus expressing miR-101-1 or the virus control (VC) at an m.o.i. of 50 for 48 h (b) or CCD-8Lu fibroblasts were treated with a lentivirus expressing anti-miR-101 or the virus control (Anti-CON) at an m.o.i. of 50 for 72 h. c, Western blotting was performed to determine the protein expression of FZD4, FZD6, and TGFBR1. The protein samples used here were the same as mentioned in Fig. 7c. The GAPDH immunoblot shown in Fig. 7c was used again here. d, overexpression of miR-101 inhibits FZD4, FZD6, and TGFBR1 mRNA expression in fibroblasts. LL29 cells were treated with lentiviral miR-101 or the virus control at an m.o.i. of 50 for 48 h. The mRNA expression of FZD4, FZD6, and TGFBR1 was determined by real-time PCR and normalized to GAPDH. The results are presented as the mean ± S.E. n = 3. e, real-time PCR showing the increased mRNA levels of FZD4, FZD6, and TGFBR1 by anti-miR-101. CCD-8Lu fibroblasts were treated with lentiviral anti-miR-101 or the virus control at an m.o.i. of 50 for 72 h. The expression levels were relative to GAPDH from two cell preparations, each performed in duplicate. f–h, mRNA expression of FZD4, FZD6, and TGFBR1 in IPF patient lungs. The mRNA levels of FZD4, FZD6, and TGFBR1 were determined by real-time PCR and normalized to β-actin. n = 8–10. The results are presented as the mean ± S.E. Statistical analyses were performed by using Student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
fragments were inserted into adenoviral and lentiviral vectors at the XhoI and EcoRI sites as described previously (39, 40). The control vector was constructed with a similar size of genomic DNA that did not contain any miRNAs or stem-loop structures. shRNA vectors—All of the shRNAs were designed by the BLOCK-iTTM RNAi designer software from Invitrogen. shRNA vectors were inserted into the pSIH-H1 vector (System Biosciences, Mountain View, CA), which utilizes the H1 promoter to drive shRNA expression. The shRNA sequences are listed in Table 6. A control vector containing scrambled shRNA was purchased from System Biosciences.

FZD4, FZD6, and TGFBR1 expression vectors—The open reading frame of FZD4, FZD6, and TGFBR1 was amplified by PCR using specific primers (Table 6) from mouse cDNA clones purchased from OriGene (FZD4, catalogue no. MC203860; FZD6, catalogue no. MC204555; TGFBR1, catalogue no. MC203366).

Figure 10. Overexpression of FZD4, FZD6, or TGFBR1 rescues miR-101-mediated inhibition of fibroblast proliferation and activation. a–d, knockdown of TGFBR1 repressed the TGF-β1-induced mRNA expression of α-SMA, COL1A1, COL3A1, and COL4A1 in fibroblasts. LL29 cells were treated with lentivirus shRNA-TGFBR1 or the virus control at an m.o.i. of 50 for 48 h. Then, the cells were stimulated with 5 ng/ml TGF-β1 for 48 h. The α-SMA, COL1A1, COL3A1, and COL4A1 mRNA expression levels were determined by real-time PCR. The expression levels were relative to GAPDH from two cell preparations, each performed in duplicate. The results are presented as the mean ± S.D. **, p < 0.01. e and f, overexpression of FZD4 or FZD6 rescues the miR-101-mediated inhibition of fibroblast proliferation. LL29 cells were split into 96-well plates at 3,000 cells per well. After a 24-h culture, cells were infected with lentiviruses at a total of m.o.i. = 50 (miR-Con miR-101, GFP, FZD4, and FZD6 at m.o.i. = 25 each, virus control (VC) = miR-Con + GFP) for 48 h. Cells were starved for another 24 h and stimulated with WNT5a (1 μg/ml) for 12 h. FZD4 and FZD6 expression was determined by Western blotting by using anti-GFP antibody. Cell proliferation was determined by BrdU assay. Data were presented as mean ± S.E. Statistical analysis was performed by ANOVA and followed by Tukey’s HSD test. n = 4. **, p < 0.01. g–j, overexpression of TGFBR1 rescues miR-101-mediated inhibition of α-SMA, COL1A1, and COL3A1 mRNA expression. LL29 cells were infected with lentiviruses at a total of m.o.i. = 50 (miR-Con, miR-101, GFP, and TGFBR1 at m.o.i. = 25 each, virus control = miR-Con + GFP) for 48 h. Cells were then stimulated with TGFβ1 (5 ng/ml) for 48 h. Cells were collected for real-time PCR. The results are presented as the mean ± S.E., n = 3. ANOVA followed by Tukey’s HSD test was performed. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
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Supplementary Figures

**Figure S1.**

**a** miR-101a expression in different groups.

**b** Histological analysis of lung tissues from different groups.

**c** Asbestos Score in different groups.

**d** Hydroxyproline level in different groups.

**e** COL1A1 mRNA expression in different groups.

**f** COL3A1 mRNA expression in different groups.

**g** Ers (cm²/20 ml) in different groups.

**h** H (cm²/20 ml) in different groups.

**i** Nfatc2 mRNA expression in different groups.

**j** Fzd4, Fzd6, and Tgfbri mRNA expression levels in different groups.
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Figure 11. Adenovirus-mediated miR-101 gene transfer attenuates bleomycin-induced pulmonary fibrosis. On day 1, an adenovirus expressing miR-101-1 or the control adenovirus (VC) (5 × 10⁷ pfu per mouse) was delivered into the lungs of 6–8-week-old C57BL/6 mice by nasal instillation. On day 2, bleomycin (Bleo) (0.06 units per mouse) or the same volume of saline was intranasally instilled into the lungs. On day 15, the mice were killed, and the right lungs were fixed for histological analysis. a, real-time PCR analysis showing adenovirus-mediated overexpression of miR-101 in the mouse lung. The expression levels were relative to U6. b, H&E staining showing the fibrotic changes in the mouse lung induced by bleomycin. miR-101 attenuated the fibrotic changes in bleomycin-treated mouse lungs. Scale bar, 100 μm. c, Ashcroft score grade of pulmonary fibrosis. d, lung collagen content as measured using the QuickZyme hydroxyproline assay kit. e and f, real-time PCR analysis showing the increased COL1A1 and COL3A1 mRNA levels in bleomycin-treated mouse lungs and miR-101 suppression of the increase of COL1A1 and COL3A1 mRNA levels. The expression levels were relative to GAPDH. g and h, analysis of lung mechanics by Flexivent analysis. Ers and VC and bleomycin (80) and VC and bleomycin (109 pfu per mouse) was delivered into the lungs of 6–8-week-old C57BL/6 mice by nasal instillation. The expression levels were relative to U6. **, p < 0.01; ***, p < 0.001.

Production of lentiviruses and adenovirus

Lentiviruses were produced using the Lenti-X™ HTX packaging vectors (Clontech) in HEK 293T cells. For the production of the adenovirus, a pENTR vector was switched into the adenoviral vector pAd/PL-DEST using the gateway technique (Invitrogen). The obtained adenoviral vector was linearized by PciI and transfected into HEK 293A cells to produce virus. Adenovirus was further amplified by re-infecting HEK 293A cells. The adenoviruses were concentrated and purified with the Adenovirus Standard Purification ViralKit™ (VIRAPUR, San Diego). The virus titer was determined by infecting HEK 293T or HEK 293A cells with a series of dilutions of the viral stock and counting the virus-infected green fluorescent protein (GFP)-positive cells.

Cell culture

HEK 293T cells and lung fibroblasts (IMR-90, HFL1, CCD-8Lu, and LL29) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). HEK 293A cells were purchased from Invitrogen. IMR-90 and HFL1 cells were human diploid fibroblasts derived from fetal lungs. CCD-8Lu and LL29 cells are human fibroblasts isolated from the lungs of a normal adult and an IPF patient, respectively. The cells were grown and maintained with the following media supplemented with 10% fetal bovine serum (FBS): HFL1 and LL29 cells, F12K medium (Kaighn’s modification of Ham’s F-12 medium); and IMR-90 and CCD-8Lu, Eagle’s minimum essential medium.

Isolation of mouse lung fibroblasts

Primary lung fibroblasts were isolated from the lungs of saline or bleomycin-treated mice according to the previously described protocol (41). The cells were cultured in DMEM containing 10% FBS and used at passages 3–9.

Dual-Luciferase assay

For the 3′-UTR luciferase reporter assay, HFL1 cells were seeded onto a 96-well plate at a density of 2 × 10⁴ cells per well and transfected with 5 ng of a 3′-UTR luciferase reporter plasmid and 100 ng of an miRNA overexpression vector using Lipofectamine™ 2000 (Invitrogen). For the promoter luciferase reporter assay, HFL1 cells were seeded onto a
Table 3
Real-time PCR primers for miRNAs

| Primer name   | Primer sequence                  | Primer name   | Primer sequence                  |
|---------------|----------------------------------|---------------|----------------------------------|
| hsa-miR-101-RT| GCGACGACAGAATTTACGACTCATAGT      | hWNT7b-FW     | GCTTACGACAGACTCCAGCT          |
| hsa-miR-1-24-FW| GCGACGACAGAATTTACGACTCATAGT    | hWNT7b-FW     | GCTTACGACAGACTCCAGCT          |
| hsa-miR-1-249-FW| GCGACGACAGAATTTACGACTCATAGT    | hWNT9a-FW     | GCAAGCTCGCTAAGATGCT          |
| hsa-miR-1-289-FW| GCGACGACAGAATTTACGACTCATAGT    | hWNT11-FW     | GCTTACGACAGACTCCAGCT          |
| hsa-miR-1-297-FW| GCGACGACAGAATTTACGACTCATAGT    | hWNT11-FW     | GCTTACGACAGACTCCAGCT          |
| hsa-miR-1-297-FW| GCGACGACAGAATTTACGACTCATAGT    | hFZD1-FW      | GCGTTCGAGGGCGAGATTTA         |
| hsa-miR-1-297-FW| GCGACGACAGAATTTACGACTCATAGT    | mAET1-FW      | GACAGCTCGCTAAGATGCT          |
| hsa-miR-1-297-FW| GCGACGACAGAATTTACGACTCATAGT    | mAET1-FW      | GACAGCTCGCTAAGATGCT          |
| hsa-miR-1-297-FW| GCGACGACAGAATTTACGACTCATAGT    | mAET1-FW      | GACAGCTCGCTAAGATGCT          |
| hsa-miR-1-297-FW| GCGACGACAGAATTTACGACTCATAGT    | mAET1-FW      | GACAGCTCGCTAAGATGCT          |

Table 4
Real-time PCR primers for miRNAs

| Primer name   | Primer sequence                  | Primer name   | Primer sequence                  |
|---------------|----------------------------------|---------------|----------------------------------|
| hCOL1A1-FW    | GCGACGACAGAATTTACGACTCATAGT    | hWNT7b-FW     | GCTTACGACAGACTCCAGCT          |
| hCOL1A1-FW    | GCGACGACAGAATTTACGACTCATAGT    | hWNT7b-FW     | GCTTACGACAGACTCCAGCT          |
| hCOL1A1-FW    | GCGACGACAGAATTTACGACTCATAGT    | hWNT9a-FW     | GCAAGCTCGCTAAGATGCT          |
| hCOL1A1-FW    | GCGACGACAGAATTTACGACTCATAGT    | hWNT11-FW     | GCTTACGACAGACTCCAGCT          |
| hCOL1A1-FW    | GCGACGACAGAATTTACGACTCATAGT    | hFZD1-FW      | GCGTTCGAGGGCGAGATTTA         |
| hCOL1A1-FW    | GCGACGACAGAATTTACGACTCATAGT    | mAET1-FW      | GACAGCTCGCTAAGATGCT          |
| hCOL1A1-FW    | GCGACGACAGAATTTACGACTCATAGT    | mAET1-FW      | GACAGCTCGCTAAGATGCT          |
| hCOL1A1-FW    | GCGACGACAGAATTTACGACTCATAGT    | mAET1-FW      | GACAGCTCGCTAAGATGCT          |
| hCOL1A1-FW    | GCGACGACAGAATTTACGACTCATAGT    | mAET1-FW      | GACAGCTCGCTAAGATGCT          |

The forward primer (FW) orientation is A to G, and the reverse primer (RE) orientation is G to A.

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h is human; m is mouse. FW is forward, and RE is reverse.
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96-well plate at a density of $2 \times 10^4$ cells per well and transfected with 50 ng of the miR-101 promoter reporter vector and 100 ng of the pCMV-ETS1 vector, pCMV-ETS2 vector, or control vector (OriGene, Rockville, MD) using LipofectamineTM 2000. The TK plasmid, which expresses *Renilla* luciferase, was used as an internal control for normalization. The cells were collected 48 h after transfection, and firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase® reporter assay system (Promega, Madison, WI). The results were presented as the ratio of firefly to *Renilla* luciferase activities.

### Table 5

| Primer name       | Sequence                          | Position to pre-hsa-miR-101-1 |
|-------------------|-----------------------------------|-------------------------------|
| PROM-MIR-101-1-RE1 | CTTGGCTCTTCTGCTCTTGTG            | ~7094                         |
| PROM-MIR-101-1-RE2 | AAACGGCATGCACTGACCCCTCTCTT       | ~8067                         |
| PROM-MIR-101-1-RE3 | AACAGAACCACTGGCGCAACC             | ~8576                         |
| PROM-MIR-101-1-RE4 | GGTGCTCTGCACTGGCGCAACC            | ~14                           |
| PROM-MIR-101-1-RE5 | CGTACCTGACTGACGCGCGCA             | ~3472                         |
| PROM-MIR-101-1-RE6 | TGAAAGCCAGGCGCGCGCAAGA            | ~1596                         |

### Table 6

| Primer name                               | Sequence              |
|-------------------------------------------|-----------------------|
| pmiRGLO-FZD4-UTR-S1-FW                     | TATTGCTAGAAGTGGGACGAGT |
| pmiRGLO-FZD4-UTR-S1-RE                     | TTATGCTAGAAGTGGGACGAGT |
| pmiRGLO-FZD4-UTR-S2-FW                     | ATTAAGCTAGAAGTGGGACGAGT |
| pmiRGLO-FZD4-UTR-S2-RE                     | ATTAAGCTAGAAGTGGGACGAGT |
| pmiRGLO-FZD6-UTR-FW                       | TATTGCTAGAAGTGGGACGAGT |
| pmiRGLO-FZD6-UTR-RE                       | TATTGCTAGAAGTGGGACGAGT |
| pmiRGLO-TGFBR1-S1-UTR-FW                  | TATTGCTAGAAGTGGGACGAGT |
| pmiRGLO-TGFBR1-S1-UTR-RE                  | TATTGCTAGAAGTGGGACGAGT |
| pmiRGLO-TGFBR1-S2-UTR-FW                  | TATTGCTAGAAGTGGGACGAGT |
| pmiRGLO-TGFBR1-S2-UTR-RE                  | TATTGCTAGAAGTGGGACGAGT |

Cell proliferation

LL29 cells were split into a 96-well plate. On the 2nd day, the cells were infected with a lentivirus expressing miR-101, shRNA, or the control at a multiplicity of infection (m.o.i.) of 50. After 48 h of virus infection, the cells were serum-starved for 24 h and then stimulated for 12 h with 1 μg/ml of WNT5a. Cell proliferation was determined using the BrdU cell proliferation kit (Millipore, Billerica, MA). For BrdU staining, 10 μM BrdU (Abcam, Cambridge, MA) was added to the culture medium for 24 h. The cultures were fixed in 4% paraformaldehyde for 30 min, treated with 2 N HCl for 30 min, and then proceeded to...
were stimulated with TGF-β1 (5 ng/ml) for 0, 5, 15, 30, 60, and 120 min. The cells were lysed for an enzyme-linked immunoassay (ELISA) performed using the PathScan® Phospho-Smad2/3 Sandwich ELISA Kit (Cell Signaling) according to the manufacturer’s instructions.

Mouse model of bleomycin-induced pulmonary fibrosis

The animal procedures were approved by the Institutional Animal Care and Use Committee at Oklahoma State University. C57BL/6 male mice (6–8 weeks old) were divided into four groups: saline and control virus; saline and miR-101 virus; bleomycin and control virus; and bleomycin and miR-101 virus. On day 0, 60 μl of miR-101 adenovirus or control virus (5 × 10⁹ infectious units (IU) per mouse) were delivered into the lung intranasally. On day 1, 60 μl of bleomycin (0.06 units/mouse) or saline was delivered intranasally. On day 14, respiratory mechanics were determined using the FlexiVent (Scireq, Montreal, Canada). Then, the left lung was collected for the RNA, protein, and collagen content analyses. The right lung was fixed in 4% paraformaldehyde for histological analysis. The degree of fibrosis in the mouse lung was determined following the published method (42).

Hydroxyproline assay

The amount of collagen in the lung tissues was determined by the hydroxyproline assay according to the manufacturer’s protocol (QuickZyme Biosciences, Netherlands) and expressed as micrograms per mg of lung tissue.

Statistical analysis

The data presented in the figures are the means ± S.E. or standard deviation. Statistical analyses were performed using Student’s t test for two group comparisons, and analysis of variance (ANOVA), followed by Tukey’s HSD test for multiple comparisons. A p value <0.05 was considered to be significant.

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