MicroRNA-326 Regulates Profibrotic Functions of Transforming Growth Factor- β in Pulmonary Fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a fatal disorder resulting from the progressive remodeling of lungs, with no known effective treatment. Although transforming growth factor (TGF)- β has a well-established role in lung fibrosis, clinical experience with neutralizing antibodies to TGF- β has been disappointing, and strategies to directly suppress TGF- β 1 secretion are needed. In this study we used a combination of *in silico*, *in vitro*, and *in vivo* approaches to identify microRNAs involved in TGF- β 1 regulation and to validate the role of miR-326 in pulmonary fibrosis. We show that hsa-miR-326 regulates TGF- β 1 expression and that hsa-miR-326 levels are inversely correlated to TGF- β 1 protein levels in multiple human cell lines. The increase in TGF- β 1 expression during the progression of bleomycin-induced lung fibrosis in mice was associated with loss of mmu-miR-326. Restoration of mmu-miR-326 levels by intranasal delivery of miR-326 mimics was sufficient to inhibit TGF- β 1 expression and attenuate the fibrotic response. Moreover, human IPF lung specimens had markedly diminished miR-326 expression as compared with nonfibrotic lungs. Additional targets of miR-326 controlling TGF- β signaling and fibrosis-related pathways were

identified, and miR-326 was found to down-regulate profibrotic genes, such as Ets1, Smad3, and matrix metalloproteinase 9, whereas it up-regulates antifibrotic genes, such as Smad7. Our results suggest for the first time that miR-326 plays a key role in regulating TGF- β 1 expression and other profibrotic genes and could be useful in developing better therapeutic strategies for alleviating lung fibrosis.

Keywords: idiopathic pulmonary fibrosis; microRNAs; transforming growth factor- β signaling

Clinical Relevance

This study demonstrates that miR-326 is a central mediator in the pathogenesis of pulmonary fibrosis and acts by affecting multiple components of transforming growth factor- β signaling pathway. It could serve as a potential target for developing novel therapeutics in treating fibrotic diseases, including idiopathic pulmonary fibrosis.

Chronic fibrotic pulmonary disorders characterized by remodeling of the lungs, like in idiopathic pulmonary fibrosis (IPF), can be progressive and fatal due to

destruction of the lung architecture, impairment of gas exchange, and respiratory failure (1, 2). Despite progress in identifying key mechanisms, there are no effective

treatments for reversing the disease or for consistently attenuating its progression. A substantial body of scientific work has established the important role of profibrotic

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cytokines such as transforming growth factor (TGF)- β in the development of lung fibrosis (3, 4). In fibrotic lungs, TGF- β 1 is overexpressed by a broad range of cells, including fibroblasts, macrophages, and epithelial cells, and leads to the dysregulation of normal lung homeostasis by increasing synthesis and deposition of collagen and altering the balance of matrix metalloproteinases (MMPs) and their inhibitors (5). Preclinical models have found anti-TGF- β therapies to be efficacious, but the limited human trials with neutralizing antibodies or inhibitors like imatinib or pirfenidone have been somewhat disappointing (6–8). Therefore, in the absence of any efficacious treatment at the protein or receptor levels, clinical strategies to inhibit TGF- β may be improved through a better understanding of the mechanisms for fine-tuning of TGF- β 1 expression (9).

An important property of TGF- β 1 is its ability to activate its own mRNA expression and thereby increase its own secretion (10). This suggests a mode of local autocrine amplification of TGF- β 1 that makes systemic neutralization strategies difficult, consistent with the clinical experience. Specifically, circulating neutralizing antibodies are unlikely to sufficiently inhibit local concentrations of cytokines, especially when there are strong autocrine loops. Direct suppression of TGF- β synthesis or secretion should be a better strategy but has not been possible to date, although some inhibition was noted through pirfenidone via unclear mechanisms (11).

MicroRNAs (miRNAs) have emerged as a growing class of noncoding RNAs with a key role in networking and fine-tuning of gene expression at the post-transcriptional level (12, 13). Although TGF- β plays a central role in the pathogenesis of pulmonary fibrosis, the use of miRNA-based therapeutics targeting TGF- β 1 to prevent or reverse fibrosis remains unexplored. Such a strategy would have the advantages of using natural regulatory networks of TGF- β 1 suppression and the possibility of local targeting via aerosol delivery.

Previous evidence suggests that TGF- β 1 expression is regulated at the transcriptional level due to mRNA stability and posttranslational processing (14). In the present study, we report for the first time that miR-326 regulates TGF- β 1

expression post-transcriptionally. We further investigated the antifibrotic role of this miRNA and found that miR-326 is down-regulated in patients with IPF and during progression of bleomycin-induced pulmonary fibrosis in mice, whereas the overexpression of this miRNA attenuates disease pathogenesis and related phenotypes.

Materials and Methods

In Silico Identification of miRNA Binding Sites

All 470 human miRNAs (miRBase v9) were queried against human *TGF β 1* long 3' untranslated region (UTR) using RNAhybrid (using relaxed energy criteria) (see Table E1 in the online supplement). Prediction analysis against *TGF β 1* short 3'UTR (ENST00000221930) was also performed using RNAhybrid software (15).

Plasmids, Transfections, and Transcript Analyses

Plasmids were generated and transfected in cell lines as described in the online supplement. Luciferase assay was performed 24 hours after transfection as described previously (16). Development and validation of an epithelial–mesenchymal transition (EMT) model, RNA extraction, and transcript analyses were performed as detailed in the online supplement.

Human Lungs from Patients with and without IPF

After approval by the Mayo Clinic Institutional Review Board, lung samples were obtained from patients undergoing thoracic surgery at St. Mary's Hospital in Rochester, Minnesota. Details of individuals chosen for the study are provided in the online supplement.

Development of the Mouse Model of Pulmonary Fibrosis

Male C57BL/6 mice were obtained from the National Institute of Nutrition (Hyderabad, India) and maintained following guidelines approved by the Institutional Animal Ethics Committee. Mice were divided into six groups as indicated. Each group ($n = 6$) was named according to sensitization/treatment: Sham (normal controls treated with vehicle), Bleo (mice treated with 3.5 U/kg of bleomycin), Bleo/scrambled oligonucleotides (controls treated with

bleomycin and 120 μ g of scrambled oligonucleotide), Bleo/miR-326 (6 mg) (mice treated with bleomycin and 120 μ g of miR-326), Bleo/miR-326 (4 mg) (mice treated with 90 μ g miR-326), and Bleo/TGF- β 1 (mice treated with 120 μ g *TGF β 1* siRNA). A bleomycin-induced mouse model of pulmonary fibrosis was developed, and lung function parameters were calculated as described previously (17, 18) and detailed in the online supplement.

Measurement of Cytokines and Collagen Content

TGF- β 1, IL-13, IL-17, and collagen content were measured in lung homogenates as detailed in the online supplement. Biological activity of TGF- β 1 was measured by acidification of culture supernatant to release it from the latent complex.

Lung Histology and Western Blot Analysis

Paraffin-embedded lung sections were analyzed for histological changes as described previously (17). Immunofluorescence, *in situ* hybridization, immunohistochemistry, and Western blot analyses were performed following protocols described previously (17, 19) and are detailed in the online supplement.

Statistical Analysis

Data are expressed as mean $\pm$ SE. Significant differences between the two groups were estimated using unpaired Student's *t* test. Statistical significance was set at $P \leq 0.05$.

Results

miR-326 Targets TGF- β 1 3'UTR and Regulates Its Expression

To predict whether miRNA(s) could regulate TGF- β 1 expression, we used miRNA target prediction software as described in MATERIALS AND METHODS. Six miRNAs (miR-609, miR-18b, miR-133a, miR-133b, miR-199a-3p, and miR-326) were predicted to have potential binding sites in the *TGF β 1* 3'UTR (Table E1). To experimentally validate these predictions, *TGF β 1* 3'UTR was cloned into a luciferase reporter system and cotransfected with individual miRNA mimics (20 nM) in A549 cells as these cells constitutively expresses high level of TGF- β 1 and thus presumably may have lower levels of anti-TGF- β 1

miRNA(s). Cotransfection of *TGFβ1* 3'UTR with individual miRNAs, miR-326, and miR-609 significantly reduced luciferase expression (Figure 1A) by approximately 2-fold. However, Cel-67 (control miRNA) showed no significant effect on luciferase expression. Unpublished data from our lab and a recent report (20) suggest that two alternative 3'UTR variants of TGF-β1 exist across a range of human cells and that the predominant TGF-β1 isoform contains the shorter 3'UTR. We

observed that two binding sites for miR-326 were present in *TGFβ1* long 3'UTR, one of which was conserved in the shorter 3'UTR. However, no binding site for miR-609 was found in *TGFβ1* short 3'UTR. Because the shorter 3'UTR is the predominant form, we performed the subsequent experiments with miR-326. To further validate and confirm the specificity of miR-326, we cloned *TGFβ1* short 3'UTR into a luciferase reporter system and deleted the target site for miR-326 (see Figure E1A in

the online supplement). Although cotransfection of *TGFβ1* short 3'UTR (intact) with miR-326 significantly reduced luciferase expression, this down-regulation was not observed with *TGFβ1* short 3'UTR (mutant) (Figure 1B). Cel-67 showed no significant effect on luciferase expression.

To further investigate the biological effects of the miRNA, we transfected A549 cells with miR-326 mimics and stimulated them for 24 hours with a known inducer of TGF-β1 (epidermal growth factor [EGF]) (21)

Figure 1. Identification of post-transcriptional regulators of transforming growth factor (TGF)-β1 expression. (A) A549 cells were cotransfected individually with or without six candidate microRNA (miRNA) mimics (20 nM) or with Cel-67 together with *pMIR-TGFβ1* long 3' untranslated region (UTR) construct. Firefly Luciferase expression was measured and normalized with Renilla Luciferase values. (B) A549 cells were cotransfected with miR-326 mimics or Cel-67 together with *pMIR-TGFβ1* short 3'UTR (intact) or *pMIR-TGFβ1* short 3'UTR (mutant) luciferase construct as described in MATERIALS AND METHODS. Luciferase activity was measured and normalized to Renilla Luciferase expression. (C) A549 cells were transfected with miR-326 mimics or with Cel-67 at varying concentrations, and TGF-β1 protein levels were measured in cell-free culture supernatant after stimulation without or with epidermal growth factor (EGF) (100 ng/ml) for 24 hours. (D) Real-time PCR to detect relative *TGFβ1* transcript levels on ectopic expression of miR-326. (E) MCF7 cells were transfected with inhibitors of miR-326 and/or Cel-67 inhibitor control, and total TGF-β1 protein levels were measured by ELISA. TGF-β1 protein levels were normalized by total protein, transcript levels were normalized by β2-microglobulin, and miRNA levels were normalized by 18S RNA levels. Results were plotted as mean ± SE of three independent experiments. **P* < 0.05; #nonsignificant.

before measuring TGF- β 1 levels in the culture supernatants. The uptake of miR-326 during transfection was confirmed by real-time PCR, which indicated a significant increase in the expression of miR-326 in a dose-dependent manner (Figure E2A). We observed a significant down-regulation in TGF- β 1 levels, and this effect was found to be dose dependent as an increase in dose from 100 to 300 nM showed a corresponding decrease in TGF- β 1 levels (Figure 1C). A negative control oligonucleotide, Cel-67, showed no significant effect on TGF- β 1 expression. Also, we investigated the changes in the expression of miR-744, a recently reported miRNA involved in TGF- β regulation, during the transfection of miR-326 mimics (Figure E2A) and observed no significant increase in miR-744 levels. The mechanism of miRNA-mediated down-regulation was apparently due to degradation of *TGF β 1* transcripts (Figure 1D). In addition, transfection of MCF7 cells with miR-326 hairpin inhibitors resulted in a significant up-regulation in TGF- β 1 levels at 24 hours (Figure 1E). This effect was also dose-dependent; however, saturation was observed at higher doses, which may be attributed to system-level factors that determine the kinetics of regulation (22). We also measured the residual levels of miR-326 after treating the cells with various doses of miR-326 antagonists. The results showed that anti-miR-326 treatment could be achieved at a dose as low as 10 nM (Figures E2B–E2D).

miR-326 Shows Inverse Correlation with TGF- β 1 Levels in Various Cell Types

To understand the biological regulation of TGF- β 1 in different cell types, we cultured cell lines from diverse origins and measured the levels of total TGF- β 1 and miR-326 (Figure 2A). A correlation plot for TGF- β 1 versus miR-326 (Figure 2B) shows an inverse relationship, suggesting a possible correlation between the miRNA and TGF- β 1 expression. Because alveolar epithelial cells (A549) were observed to be the highest producers of TGF- β 1, we stimulated these cells with EGF and observed a rapid increase in TGF- β 1 protein levels 12 hours after stimulation. EGF stimulation potentiates an interesting transition in miR-326 levels, which peaks at 8 hours, followed by a progressive decline after 12 hours (Figure 2C). These reverse

kinetics further indicate that TGF- β 1 expression is inversely correlated with miR-326 level.

To demonstrate the functional importance of miR-326, we established an *in vitro* EMT model by stimulating human alveolar epithelial cells (A549) or normal human bronchial epithelial cells with IL-13 and TNF- α for 72 hours. There was a significant increase in TGF- β 1 production during EMT (Figure 2D), accompanied by a significant decrease in miR-326 levels (Figure 2E). Although ectopic expression of miR-326 mimics was able to rescue epithelial cells from EMT, treatment with anti-miR-326 promoted EMT and showed significant up-regulation of TGF- β 1 production (Figure 2F). Corresponding changes were observed by immunocytochemical staining for epithelial (cytokeratin 14) and mesenchymal (vimentin) markers during EMT (Figures 2G and 2H). We also confirmed these observations using real-time PCR (Figure 2I) and Western blot experiments (Figures 2J and 2K).

Lungs from Patients with IPF Have Lower Levels of miR-326

To determine if IPF is associated with loss of miR-326, we measured miR-326 and TGF- β 1 in lungs from patients with IPF ($n = 4$) and nonfibrotic control subjects ($n = 4$). TGF- β 1 levels and collagen content were found to be significantly higher in patients with IPF as compared with nonfibrotic control subjects (Figures 3A and 3B). IPF was associated with significantly lower levels of miR-326 (Figure 3C), suggesting that miR-326 may play a role in pulmonary fibrosis.

miR-326 Shows Inverse Kinetics with TGF- β 1 during Progression of a Bleomycin-Induced Mouse Model of Lung Fibrosis

To investigate the role of miRNAs in experimental lung fibrosis, we developed a mouse model of pulmonary fibrosis by intranasal administration of bleomycin as previously described (17, 23, 24) and as detailed in MATERIALS AND METHODS. We observed sequence homology for miR-326 in human and mouse (Figures E1B and E1C), and cotransfection of mouse *TGF β 1* 3'UTR construct with miR-326 mimics showed a significant down-regulation in luciferase levels, thus confirming a functional binding site for mmu-miR-326 in mouse *TGF β 1* 3'UTR (Figure E1D). We

also performed an *in vivo* time kinetics experiment and correlated changes in TGF- β 1 protein with endogenous mmu-miR-326 levels during the progression of bleomycin-induced lung fibrosis. We observed a significant increase in TGF- β 1 levels from 7 to 21 days (Figure 3D), which is in accordance with previously published reports (25). We also observed a progressive decline in miR-326 transcripts from 7 to 21 days (Figure 3D). A correlation plot further confirmed our *in vitro* observation that TGF- β 1 and miR-326 are inversely correlated (Figure 3E). Histological analyses also revealed significant pathological changes over the 21-day period. Masson's trichrome staining showed a marked increase in collagen deposition by Day 7, with peak pulmonary fibrosis developing between Days 14 and 21 (Figure 3F). Hematoxylin and eosin staining showed prominent architectural changes with a progressive increase in parenchymal distortion and fibrotic foci from Days 7 to 21 (Figures 3G and 3H). *In situ* hybridization of mouse lungs showed high cytoplasmic staining for miR-326 in control mice (Sham), which was found to be significantly decreased after treatment with 3.5 U/kg of bleomycin (Figures 3Ii, 3Iii, and 3J). The expression of miR-326 appeared specific to lung alveolar epithelial cells, which was confirmed by costaining with cytokeratin 14. In contrast, there was minimal background staining with scrambled control (Scr) probes, supporting the specificity of miR-326 staining (Figures 3Iiii and 3J).

mmu-miR-326 Affects Additional Genes Involved in TGF- β Signaling and Fibrosis-Related Pathways

Because miRNAs are known to functionally interact with multiple components within a cellular network, we explored the possibility that miR-326 targets additional fibrosis-related genes. Because uncontrolled proliferation of fibroblasts plays an important role in lung IPF (26), we cultured mouse fibroblasts (NIH/3T3) cells under fibroblast-proliferating conditions (in the presence of IL-13 and IL-1 β for 48 h) after transfection with mmu-miR-326 mimics and observed a significant down-regulation in TGF- β 1 production (Figure 4A). RNA from these cells was subjected to real-time PCR analysis for a panel of genes involved in mouse fibrosis. Cells transfected with *TGF β 1* siRNA and miR-326 subjected to the same treatment served to delineate

Figure 2. hsa-miR-326 regulates TGF-β1 levels in human epithelial cells. (A) TGF-β1 protein levels were measured by ELISA across cell lines of various origins (A549, MCF7, and MiaPaCa2 of epithelial origin; Jurkat of T-cell origin; and Raji of B-cell origin). miR-326 transcripts were measured as explained in MATERIALS AND METHODS, and the graph was plotted as mean $\pm$ SE for three independent experiments. (B) Correlation plot between TGF-β1 protein levels measured in cell lines of different origins with the levels of miR-326. Cell-free culture supernatants from unstimulated cells were subjected to TGF-β1 ELISA, and the levels of miR-326 were measured by real-time PCR. (C) A549 cells stimulated with or without EGF and TGF-β1 measured at different time points were correlated with endogenous miR-326 levels. (D) Alveolar (A549) cells or normal human bronchial epithelial (NHBE) cells were stimulated under epithelial-mesenchymal transition (EMT)-inducing conditions as described in MATERIALS AND METHODS, and TGF-β1 was measured in culture supernatants by ELISA (E) Real-time PCR to detect the levels of miR-326 in A549 and NHBE cells under EMT-inducing conditions. (F) A549 cells were transfected with miR-326, anti-miR-326, or scrambled controls before the induction of EMT, and TGF-β1 levels were measured in culture supernatants by ELISA after 72 hours. (G) Immunocytochemical staining for epithelial (Cytokeratin 14 [Cyto K]) and mesenchymal (Vimentin [Vim]) markers as described in MATERIALS AND METHODS. All micrographs were captured at 63 $\times$ magnification and are representative of two to three independent experiments. Scale bar: 20 μ m. DAPI, 4',6-diamidino-2-phenylindole. (H) Quantitative analysis for immunocytochemical staining was performed with ImageJ software. Real-time PCR (I), Western blot (J), and densitometry analysis (K) for EMT markers were performed with AlphaEaseFC 4.0 software.

genes affected by inhibition of TGF-β1 alone. Several genes were differentially regulated as compared with an unstimulated control group (Figure 4B). We observed a significant down-regulation in *Col1a2* and *Col3a1* genes, which are key

inducers of collagen production. We also observed a significant down-regulation in *Smad3* in miR-326-transfected cells, one of the positive modulators of TGF-β signaling pathway. We observed a significant up-regulation in *Smad7* and *Bmp7*, the well-

known negative regulators of TGF-β signaling. Furthermore, we constructed a molecular network by the software Ingenuity Pathway Analysis to identify additional genes affected by miR-326 (Figure E3). This molecular network

Figure 3. Involvement of miR-326 in human and mouse pulmonary fibrosis. (A and B) Total protein extracts were prepared using lungs from patients with idiopathic pulmonary fibrosis (IPF) ($n = 4$) and nonfibrotic control subjects ($n = 4$) and subjected to TGF- β 1 estimation by ELISA (A) or to quantitative assessment of total collagen levels by Sircol assay (B) as described in MATERIALS AND METHODS. (C) Total miRNA fraction was isolated from human lungs, and miR-326 transcripts were measured by real-time PCR. * $P < 0.05$. (D) After intranasal delivery of bleomycin (Bleo) on Day 0, TGF- β 1 protein levels were measured in total lung protein extracts at Days 3, 7, 14, and 21 by ELISA. Relative miR-326 transcript levels were measured using RNA from lung cells at the same time intervals. (E) Correlation plot between miR-326 and TGF- β 1 during the progression of bleomycin-induced pulmonary fibrosis. (F and G) Lung sections were stained using Masson's trichrome (MT) (F) and hematoxylin and eosin (H&E) (G) stains as described in MATERIALS AND METHODS. (H) Inflammation scoring was performed with ImageJ software. (I) *In situ* hybridization with labeled miR-326 or scrambled probes to determine the localization of miR-326. Bleo = bleomycin treated. (J) Quantitative analysis was performed with ImageJ software. All micrographs were captured at 10 $\times$ or 63 $\times$ magnification, and results were plotted as mean $\pm$ SE for two independent experiments. The data in A, B, and D were normalized by total protein. Scale bar (F, G, I): 20 μ m.

indicates that miR-326 interacts with several genes of TGF- β signaling and fibrosis-related pathways via direct or indirect interactions. Ets-1, which is a known target of miR-326 (27), also appeared as a potential target of miR-326 in this network. We further validated these interactions at the protein level. Western blot analyses confirmed a significant down-regulation of Smad3 and Ets-1 expression in miR-326-transfected cells, whereas an up-regulation was observed for Smad7

expression (Figure 4C). miR-326 up-regulates antifibrotic genes such as *Smad7*, *Bmp7*, and IL-10 to a greater extent as compared with TGF β 1 siRNA, whereas it down-regulates profibrotic genes such as *Col1a2*, *Col3a1*, and *Smad3*. To study the effect of miR-326 on the proliferation of fibroblasts, we performed a carboxyfluorescein succinimidyl ester proliferation assay and observed a significant decrease in the proliferation of NIH/3T3 cells when transfected with

miR-326 mimics as compared with Scr control (Figures 4D–4F).

Intranasal Delivery of mmu-miR-326 Reduces TGF- β 1 in the Bleomycin-Induced Model of Pulmonary Fibrosis

To determine the efficacy of miR-326 in reversing TGF- β 1-mediated fibrosis, we used the bleomycin-induced fibrosis model as detailed previously. miR-326 mimics were administered intranasally at two doses

Figure 4. Involvement of mmu-miR-326 in mouse fibroblast proliferation. (A) NIH/3T3 cells were transfected with miR-326 or *TGFβ1* siRNA, and TGF-β1 protein levels were measured after stimulation with or without IL-13/IL-1β combination. Results are presented as mean ± SE of three independent experiments. (B) RNA from transfected or control cells was subjected to real-time PCR array for mouse fibrosis genes, and a clustergram for selected genes was plotted as a representative of triplicate experiments using three independent samples for each lane. (C) Nuclear extracts (for Ets-1) and total cell extracts (for Smad3 and Smad7) were subjected to Western blot analyses as described in MATERIALS AND METHODS. Densitometry was performed for a representative of three independent experiments, and relative integrated density value [IDV] was plotted. (D–F) NIH/3T3 cells were seeded and stained with carboxyfluorescein succinimidyl ester (CFSE) the next day before transfection with miR-326 mimics. CFSE proliferation assay was performed on Days 1 and 2, and proliferation was measured using flow cytometry. The representative overlapped histogram is shown in (D). Region R2 (Day 0) in E has been gated to show the percentage of proliferated cells at Days 1 and 2 and is represented in the form of bar graph in F (neg con is Scr. Control).

(6 or 4 mg/kg) at the indicated time points (Figure 5A), and the efficacy of miRNA delivery was confirmed using previously established protocols (18). We measured the effects of intranasal delivery of miR-326 mimics on lung function parameters such as tissue elastance. Elastance was found to be higher in Bleo mice compared with saline control mice (Sham) and significantly decreased in mice treated with miR-326 and mice with *TGFβ1* siRNA compared

with Scr (Figure 5B). Corresponding changes in static compliance were noted (Figure E4A). Improvement in pulmonary function was accompanied by a significant down-regulation of TGF-β1 levels in mice treated with miR-326 or *TGFβ1* siRNA but not in Scr (Figure 5C). Immunofluorescence staining for TGF-β1 was confirmatory, showing significant dose-dependent down-regulation of TGF-β1 expression in miR-326-treated lung

sections as compared with a marked subepithelial expression in Bleo lungs (Figure 5D). Immunohistochemistry analyses also showed that although pSmad3 expression was high in Bleo mice as compared with control mice, it decreased in miR-326-treated lungs in a dose-dependent manner, suggesting that miR-326-mediated down-regulation of TGF-β1 resulted in decreased Smad3 phosphorylation (Figure 5E). Because MMPs have been

Figure 5. Exogenous administration of mmu-miR-326 reduces TGF- β 1 expression in fibrotic lungs. (A) Schematic representation of bleomycin-induced model for lung fibrosis, indicating time points for intranasal delivery of mmu-miR-326 mimics. (B) Lung function parameters were measured as described in MATERIALS AND METHODS, and elastance (E) was compared between different mice groups ($n = 6$). (C) Total protein extracts were prepared from mouse lungs, and TGF- β 1 levels were measured by ELISA. Results were normalized by total protein and are presented as mean $\pm$ SE of two independent experiments ($n = 6$). * $P < 0.05$. (D) Immunofluorescence staining was performed using primary antibody against TGF- β 1 and FITC-labeled secondary antibody as described in MATERIALS AND METHODS. (E and F) Immunohistochemical analyses were performed for pSmad3 (E) and MMP9 (F) in miR-326-treated lung sections as compared with scrambled (SCR) and sham controls. All micrographs were captured at 10 $\times$ magnification and are representative for two independent experiments. Nuclear localization of pSmad3 was captured at 100 $\times$ magnification. Scale bars: D, F = 20 μ m; E = 20 μ m, 15 μ m (100 $\times$). (G–I) Quantitative analysis was performed using ImageJ software. MMP9, matrix metalloproteinase 9.

implicated in pulmonary fibrosis (28), we investigated the levels of MMP9 in our model. We observed a significant increase in MMP9 expression in Bleo mice as compared with control mice, which was found to decrease in miR-326-treated lungs in a dose-dependent manner (Figure 5F). The results described in Figures 5D–5F were quantitatively analyzed by morphometric and statistical analysis using Image J software (Figures 5G–5I, respectively).

Intranasal administration of miR-326 mimics had no significant effect on the levels of IL-17 (Figure E4B), another cytokine implicated in this model. However, IL-13 showed a marginal decrease for miR-

326-treated and TGF β 1 siRNA-treated groups (Figure E4C). Most importantly, miR-326 administration in mice showed no effect on the general miRNA processing. For example, miR-21 levels were found to be elevated in Bleo mice as compared with control mice, as observed previously (29), but showed no significant change in miR-326-treated or scrambled oligonucleotide-treated groups (Figure E4D).

Intranasal Delivery of miR-326 Alleviates the Features of Bleomycin-Induced Fibrosis

After intranasal delivery of mmu-miR-326 mimics in Bleo mouse lungs, various

features of airway fibrosis were investigated as described in MATERIALS AND METHODS. Histological analyses revealed a marked increase in collagen deposition in the lung sections of Bleo mice as compared with control mice, with a visible decrease in lungs treated with miR-326 and TGF β 1 siRNA but not with scrambled oligonucleotide (Figure 6A). This was further confirmed by Sircol assay (Figure 6D). Prominent architectural changes, such as interstitial thickening, inflammation, and alveolar collapse were seen in Bleo lungs. These were markedly decreased in miR-326 and TGF β 1 siRNA-treated lungs but were unchanged

Figure 6. miR-326 delivery alters various histological features of bleomycin-induced fibrosis. Lung sections were examined using MT staining to assess the extent of collagen deposition (A) and H&E staining to assess inflammation as described in MATERIALS AND METHODS (B). (C) Inflammation scoring was performed with ImageJ software. (D) Total lung protein was subjected to Sircol assay for the quantitative assessment of collagen deposition. Results were normalized by total protein and are presented as means $\pm$ SE of two independent experiments ($n = 6$). $*P < 0.05$. (E–G) Immunohistochemical analyses for the expression of Smad3 (E), Smad7 (F), and Ets-1 (G) in miR-326-treated lung sections as compared with scrambled and sham controls. All micrographs were captured at 10 $\times$ magnification and are representative of two independent experiments. Scale bars: A, B, E–G = 20 μ m. (H–J) Quantitative analysis for E–G was performed by ImageJ software.

with Scr (Figure 6B). Because the *in vitro* results showed that miR-326 interacts with multiple genes involved in fibrosis, this was further explored in the bleomycin model. Immunohistochemistry studies showed that Smad3 and Ets-1 expression was increased in Bleo mice as compared with control mice and decreased in a dose-dependent manner in miR-326 treated lungs (Figures 6E and 6G, respectively). In contrast, Smad7,

a negative regulator of TGF- β signaling, was found to be significantly increased in miR-326-treated lungs as compared with Scr (Figure 6F). We also explored the therapeutic potential of miR-326 after the fibrotic phase of the bleomycin model was established (i.e., after 7 d) and found similar results (Figures E5A–E5E). Thus, our results showed that miR-326 regulates TGF- β 1 expression and that intranasal

administration of miR-326 alleviates the features of bleomycin-induced fibrosis.

Discussion

TGF- β controls a diverse set of cellular processes, including cell proliferation, recognition, differentiation, apoptosis, and several pathological conditions; therefore,

a deeper understanding of the regulation of TGF- β expression is of paramount clinical importance. In this study, we have explored the physiological posttranscriptional regulation of TGF- β 1 by miRNAs with a perspective toward identifying a novel mechanism for inhibiting TGF- β 1-induced lung fibrosis. Because previous preclinical trials with anti-TGF- β antibodies showed only moderate success, here we used a combination of *in vitro* and *in vivo* model systems to demonstrate the involvement of miR-326 in TGF- β 1 regulation. We report for the first time that miR-326 physiologically represses TGF- β 1 expression and that increased TGF- β 1 expression in a bleomycin-induced lung fibrosis model is associated with the loss of miR-326. Inhaled delivery of hairpin nucleotides mimicking miR-326 was sufficient to inhibit TGF- β 1 expression and attenuate the fibrotic process. Thus, administration of miR-326 mimics appears to be a promising mechanism to limit fibrotic processes.

Because the bleomycin-induced mouse model of fibrosis does not fully replicate all clinical features of human IPF, we extended our study using human IPF specimens and observed a similar inverse correlation of miR-326 expression with disease phenotype. Although we demonstrated the functional role of miR-326 using *in vitro* model of EMT, contradictory reports on EMT in IPF (30) indicate that these results may not be directly extrapolated to pulmonary fibrosis. However, many recent reports suggest the importance of miRNAs in pulmonary fibrosis (31–33), indicating the potential therapeutic importance of miRNAs in IPF. Because TGF- β 1-induced fibrosis is important in many diseases, it is

likely that our findings may have future clinical applications. Using online databases, we found that, besides human lungs, miR-326 is highly expressed in other cell types, such as blood cells, brain, etc. (Figure E6A). Furthermore, when we investigated the relative expression of miR-326 in naive mice organs, we observed that the levels of miR-326 were highest in mouse lungs, followed by kidney, spleen, and liver (Figure E6B). Another possible advantage of using physiological miRNA target-site-based nucleotide therapy, as compared with siRNAs (which target specific regions of the gene), is that related genes within a functional network share regulatory motifs. Molecular modeling with Ingenuity Pathway Analysis combined with leads from our fibrosis array data suggests a functional role of miR-326 in fibrosis by affecting multiple genes. We further demonstrate that key genes of TGF- β signaling pathway, including Smad3 and Smad7, are modulated by miR-326 at the protein level. Previously, miR-326 has been implicated as a positive regulator of Th17 differentiation and associated with the pathogenesis of multiple sclerosis by targeting Ets-1, a negative regulator of Th17 differentiation (27). Bleomycin-induced fibrosis is associated with a Th1/Th17 response at early time points (25); this finding supports our data, which show the presence of miR-326 during the onset of the disease. Our fibrosis array data also suggest a significant down-regulation in MMP-2 expression in miR-326 transfected cells, which has been previously implicated in the pathogenesis of pulmonary fibrosis (34). *TGF β 1* transcripts could be present with two distinct 3'UTR lengths (543 and 144 bases), although multiple human cell lines

predominantly express transcripts with the shorter 3'UTR (20). The importance of a longer *TGF β 1* 3'UTR transcript has been demonstrated previously (35) and has been shown to play an important role in TGF- β regulation by binding to HuR, a RNA stability factor. Thus, it is plausible that both forms of the *TGF β 1* transcript could play important biological functions in a context-dependent manner. However, in our bleomycin-induced lung fibrosis model, the shorter transcript seems to play a critical role. It has also been recently reported that hsa-miR-744 binds to the shorter *TGF β 1* transcript in human cells (20). However, no functional studies or importance of hsa-miR-744 in any disease pathology was reported by this group. Because miR-326 sites are present in shorter and longer transcripts, miR-326 could potentially be useful for inhibiting either form of the *TGF β 1* transcripts.

In conclusion, our results suggest for the first time that TGF- β 1 expression is regulated by miR-326 and that increased expression of this miRNA in mouse lungs results in the attenuation of bleomycin-induced lung fibrosis. Overall, our data suggest that miR-326 is a key mediator in the pathogenesis of lung fibrosis and a potential target for developing novel therapeutics in treating fibrotic diseases, including IPF. ■

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