Epigenetic Regulation of *miR-17~92* Contributes to the Pathogenesis of Pulmonary Fibrosis

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Rationale: Idiopathic pulmonary fibrosis (IPF) is a disease of progressive lung fibrosis with a high mortality rate. In organ repair and remodeling, epigenetic events are important. MicroRNAs (miRNAs) regulate gene expression post-transcriptionally and can target epigenetic molecules important in DNA methylation. The *miR-17~92* miRNA cluster is critical for lung development and lung epithelial cell homeostasis and is predicted to target fibrotic genes and DNA methyltransferase (DNMT)-1 expression.

Objectives: We investigated the *miR-17~92* cluster expression and its role in regulating DNA methylation events in IPF lung tissue.

Methods: Expression and DNA methylation patterns of *miR-17~92* were determined in human IPF lung tissue and fibroblasts and fibrotic mouse lung tissue. The relationship between the *miR-17~92* cluster and DNMT-1 expression was examined *in vitro*. Using a murine model of pulmonary fibrosis, we examined the therapeutic potential of the demethylating agent, 5'-aza-2'-deoxycytidine.

Measurements and Main Results: Compared with control samples, *miR-17~92* expression was reduced in lung biopsies and lung fibroblasts from patients with IPF, whereas DNMT-1 expression and methylation of the *miR-17~92* promoter was increased. Several miRNAs from the *miR-17~92* cluster targeted DNMT-1 expression resulting in a negative feedback loop. Similarly, *miR-17~92* expression was reduced in the lungs of bleomycin-treated mice. Treatment with 5'-aza-2'-deoxycytidine in a murine bleomycin-induced pulmonary fibrosis model reduced fibrotic gene and DNMT-1 expression, enhanced *miR-17~92* cluster expression, and attenuated pulmonary fibrosis.

Conclusions: This study provides insight into the pathobiology of IPF and identifies a novel epigenetic feedback loop between *miR-17~92* and DNMT-1 in lung fibrosis.

Keywords: microRNA; *miR-17~92*; pulmonary fibrosis; DNA methylation; DNMT-1

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Through the use of genome-wide studies, investigators have attempted to uncover the molecular changes responsible for the development of idiopathic pulmonary fibrosis (IPF). However, these studies have been met with little success. Recently, microRNAs (miRNA) have emerged as regulators of numerous genes and unique targets in IPF. The mechanisms by which miRNAs are altered in IPF remain unknown.

What This Study Adds to the Field

We identified an association between aberrant DNA methylation and miRNA expression in IPF. We report that miRNA expression in IPF is susceptible to epigenetic regulation. Based on this observation, our study suggests the potential for targeting DNA methylation events as a therapeutic approach for IPF.

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Idiopathic pulmonary fibrosis (IPF) represents the most aggressive form of interstitial lung disease with a median survival of 3–5 years (1). Failure to resolve epithelial cell injury in the lung is critical to the pathogenesis of IPF (2–4). In addition, epithelial-mesenchymal transition (EMT) (5), fibroblast proliferation and activation (6), and recruitment of inflammatory cells (7, 8) all contribute to extracellular matrix accumulation in the lung (7). The current study focused on identifying the molecular mechanisms underlying the pathogenesis of IPF.

Because changes in fibrotic gene expression (2, 9–11) and few genetic mutations have been identified in IPF (12, 13), we focused on microRNA (miRNA, miR) expression and epigenetic regulators in lung epithelial cells and fibroblasts. MiRNAs can either block translation or degrade target mRNAs (14, 15). Notably, a single miRNA can regulate upward of 30 genes. MiRNAs can be encoded in intronic or exonic DNA regions and encoded in their own open reading frame and controlled by DNA promoter elements, such as DNA methylation by DNA methyltransferases (DNMTs) of CpG islands (15, 16). Of the three DNMTs expressed in humans, DNMT-1 is associated with epigenetic repair of injured tissue, whereas DNMT-3a and -3b are critical for and *de novo* maintenance of DNA methylation (17, 18).

Based on previously observed changes in miRNA expression in IPF lung tissue (9), we focused on the *miR-17~92* cluster, which encodes six miRNAs within a single open reading frame (19, 20). In solid tumors, *miR-17~92* expression is elevated and targets antiangiogenic and fibrotic genes (19, 21–25), many of

which are altered in IPF (2, 9, 10). This cluster is also important in lung epithelial cell development (26) with high expression during embryonic development, and then declines into adulthood (15). Importantly, mice lacking the *miR-17~92* cluster, but not the duplicate paralog copies (*miR-106a~363* and *miR-106b~25*), have few lung epithelial cells and die from asphyxia at birth (26). Conversely, lung-specific *miR-17~92* overexpression in mice results in a highly proliferative undifferentiated lung epithelium (27). Thus, this miRNA cluster seems to maintain lung epithelial cell homeostasis, an important factor in effective lung repair.

Here, we demonstrate that epigenetic silencing of *miR-17~92* occurred in lung tissue and fibroblast cell lines from patients with IPF because of enhanced DNA methylation. Diminished *miR-17~92* expression inversely correlated to DNMT-1 expression. Introduction of the *miR-17~92* cluster in IPF lung fibroblasts reduced fibrotic gene and DNMT-1 expression, normalized cellular phenotype, and reduced DNA methylation of the cluster. We further investigated if this regulation was conserved in mice. In a murine model of pulmonary fibrosis, enhancing *miR-17~92* expression using a demethylating agent *in vivo* reduced fibrotic gene and DNMT-1 expression suggesting augmented *in vivo* lung repair. These data reveal a potential new therapeutic approach for IPF and intimate interplay among *miR-17~92*, DNMT-1 activity, and lung fibrosis.

METHODS

Primary Tissue Samples

Deidentified tissue samples from patients with IPF and chronic obstructive pulmonary disease (COPD) were acquired through the Lung Tissue Research Consortium. IPF and COPD samples were segregated into three FVC groups and two FVC groups, respectively. Patient clinical information and control samples were previously described (9).

Cell Culture and Treatments

Normal and IPF lung fibroblasts (American Type Culture Collection, Rockville, MD) were cultured per instructions. Cells ($2\text{--}3 \times 10^6$) were treated with 0.5–2 μM of 5'-aza-2'-deoxycytidine for 24–72 hours. Non-specific toxicity was measured. Fibroblasts (1×10^6) were transfected using siPORTNeoFX transfection reagent for 24 hours.

Bleomycin-induced Pulmonary Fibrosis and 5'-Aza-2'-Deoxycytidine Treatment in Mice

C57/BL6 mice (Jackson Laboratory, Bar Harbor, ME) were injected intraperitoneally with 0.035 U bleomycin/kg or vehicle (phosphate-buffered saline) (28). After 2 weeks, mice were injected intraperitoneally twice weekly with endotoxin-free 0.156 mg/kg 5'-aza-2'-deoxycytidine or dimethyl sulfoxide on alternating days in respect to bleomycin injections. Tissue and primary cells were isolated (28, 29).

Quantitative Real-Time Polymerase Chain Reaction Analyses

RNA was isolated and subjected to polymerase chain reaction analysis for miRNA and mRNA expression (9, 30). For mRNA analysis, the endogenous controls glyceraldehyde phosphate dehydrogenase and adenyl cyclase-associated protein-1 (CAP-1) were used for normalization. For miRNA analysis, several endogenous controls including *miR-191*, small nucleolar (sno) RNA, RNU38B, RNU48, snoRNA 202, and snoRNA 220 were evaluated for each experiment. The endogenous control with the most consistent Ct value and little variation was used for normalization as indicated.

Examination of DNA Methylation Patterns

DNA was isolated using Perfect Pure DNA Cultured Cell Kit (5 Prime Inc., Gaithersburg, MD) or QIAamp DNA Mini Kit (Qiagen, Valencia, CA) from cells or tissue, respectively. DNA methylation was analyzed

using the Methyl-Profiler DNA Methylation qPCR Primer Assays (SABiosciences/Qiagen, Frederick, MD) according to instructions.

miRNA Colocalization Studies

Antibodies recognizing AE 1/3, CD45, and CD31 were used to colocalize with *miR-19b* and *-20a* expression by *in situ* hybridization within fixed lung tissue (31, 32). *In situ* hybridization was performed as previously described (31) using 5'-digoxigenin-labeled LNA probes (1–2 pmol/ μL) for either *miR-19b* or *-20a*.

Luciferase Reporter Experiments

The 3' untranslated region (UTR) of DNMT-1 cloned into pGL-3 Luciferase reporter vector was provided by Dr. Muller Fabbri (The Ohio State University) (33) and the 3' UTR of Col13a in the pEXZ-MT05 reporter vector was purchased from GeneCopeia (Rockville, MD). The miRNA recognition sites were mutated as indicated in the online supplement. Firefly and Renilla luciferase activities were measured consecutively in HEK 293 cells transfected with reporter vectors (33).

Statistical Analysis

All data are expressed as the mean $\pm$ SD. One-way analysis of variance was performed with SPSS16 (SPSS Inc., Chicago, IL) and JMP/SAS v9.1 software (SAS Institute, Inc., Cary, NC). Holm method was used to adjust for multiplicity and control the overall type I error rate at $\alpha = 0.05$. To test for outliers, normality test was performed and none were identified. Statistical significance was defined as *P* less than 0.05.

RESULTS

miR-17~92 Expression Is Decreased in Lung Tissue from Patients with IPF

Given the predictive value of lung function in IPF patient outcomes (1), we stratified IPF lung tissue into severity groups based on FVC: group 1, FVC less than 50% (severe); group 2, FVC 50–80% (moderate); and group 3, FVC greater than 80% (mild). IPF lung tissue samples demonstrated reduced expression of pre-miRNAs (data not shown) and mature miRNAs encoded by the *miR-17~92* cluster compared with control lung tissue samples including pathologically normal lung tissue adjacent to lung cancer (Figure 1A). Based on miRNA target prediction software programs, the *miR-17~92* cluster was predicted to target several fibrotic genes including collagen, type I, $\alpha 1$ (Col1a1), transforming growth factor (TGF)- β , and metalloproteinases (Table 1). We validated that expression of the above mRNA targets of the *miR-17~92* cluster was increased in lung tissue from patients with IPF (see Figure E1A in the online supplement).

To determine if similar changes occurred in lung tissue from patients with other chronic lung diseases, we examined *miR-17~92* cluster expression in lung tissue from patients with COPD. In contrast to IPF lung tissue, we observed significant elevation of the miRNAs in lung tissue from patients with COPD compared with control tissue samples (see Figure E1B), suggesting that reduced lung *miR-17~92* cluster expression was not a nonspecific feature of chronic lung disease.

Detection of *miR-19b* and *-20a* by *In Situ* Hybridization

Members of the *miR-17~92* cluster, including *miR-19b* and *-20a*, were assessed in normal lung tissue by *in situ* hybridization (Figure 1B). Interestingly, *miR-19b* expression colocalized to lung epithelial cells expressing AE1/3 cytokeratin (see Figure E2). In IPF lung tissue, *miR-19b* and *-20a* were nearly undetectable in lung tissue from patients with moderate (50–80% FVC) and severe (<50% FVC) lung disease sections staining positive for α smooth muscle actin (α -SMA) and CD45 (see Figures E3 and E7). As expected, there was no nonspecific staining of miRNAs in tissue stained with scrambled probes. A probe recognizing

Figure 1. Decreased expression of the *miR-17~92* cluster in human idiopathic pulmonary fibrosis. (A) Expression of each microRNA (miRNA) from the *miR-17~92* cluster was determined by quantitative real-time polymerase chain reaction from control (n = 10), >80% FVC (n = 7), 50–80% FVC (n = 8), and <50% FVC (n = 9) lung tissue samples. Data were normalized to *miR-191*. Relative copy number (RCN, $2^{-\Delta\Delta C_T} \times 100$) was determined. Data are expressed as the average RCN $\pm$ SD, *P < 0.018 compared with control tissue. Comparison of the mild disease (80% FVC) with control tissue for *miR-19b* and *-92*, P = 0.1325 and 0.1320, respectively. (B) *In situ* hybridization was performed using LNA-modified DNA probes for *let-7c* (positive control) and *miR-19b* and *-20a* at a magnification of $\times 400$. Scrambled probes were used as a negative control. The arrows denote positively stained cells (dark blue/purple). Shown are representative images (n = 3 per group).

let-7c miRNA was used as a positive control and was similarly expressed among all tissue samples. Importantly, miRNAs from the *miR-17~92* cluster, including *miR-19b* and *-20a*, were expressed in freshly isolated lung epithelial and fibroblast cells from murine lung tissue (see Figure E4A).

Introduction of the *miR-17~92* Cluster in IPF-derived Lung Fibroblasts Results in Molecular and Phenotypic Changes

In IPF, fibroblast activation leads to extracellular matrix deposition (6, 34). Therefore, we analyzed *miR-17~92* expression in human normal and IPF lung fibroblast cell lines. Similar to IPF lung tissue, *miR-17~92* expression was decreased in IPF fibroblast cell lines compared with normal lung fibroblast cell lines (see Figure E4B). Gene profiles in IPF lung tissue are consistent with EMT and wound repair (2, 5, 9–11). Wound repair involves fibroblast migration to areas of damage (35) and cells undergoing EMT gain filopodia as they assume fibroblast phenotypes (5, 35). After transfecting *miR-17~92* in IPF and normal fibroblast cell lines, cell morphology, target gene, and *miR-17~92* cluster expression were assessed (Figure 2; see Figure E4B). Cytoskeleton morphology as

TABLE 1. miRNAs PREDICTED TO REGULATE GENE EXPRESSION INVOLVED IN IDIOPATHIC PULMONARY FIBROSIS

Genes	Predicted miRNAs					
COL13A1	miR-19a,b	miR-18a,b	miR-29a,b	miR-377	miR-544	
COL1A1	miR-92a,b	miR-19a,b	miR-29a,b,c	miR-224	miR-196a	
CTGF	miR-18a,b	miR-19a,b	miR-559	miR-600	miR-377	
VEGF	miR-17	miR-20a	miR-106a,b	miR-93	miR-363	
TGF-β	miR-17	miR-19a,b	miR-20a	miR-29a,b	miR-34b,c	
TSP-1	miR-17	miR-18a,b	miR-19a,b	miR-20a	miR-34a,c	
MMP-1	miR-20a	miR-19a,b	miR-29a,c	miR-190b	miR-22	
MMP-9	miR-19a,b	miR-92a,b	miR-581	miR-34a,c	miR-223	
MMP-7	miR-17	miR-18a,b	miR-34b,c	miR-106b	miR-122	

Definition of abbreviations: COL = collagen; CTGF = connective tissue growth factor; miRNA = microRNA; MMP = metalloproteinases; TGF-β = transforming growth factor-β; TSP-1 = thrombospondin-1; VEGF = vascular endothelial growth factor.

indicated by numerous filopodia and increased actin staining was different in untransfected and vector-transfected IPF lung fibroblasts compared with the normal lung fibroblast samples (Figures 2A and 2B). Introduction of the *miR-17~92* cluster into IPF cells reduced the actin staining to levels similar to the normal fibroblast (Figure 2B). Interestingly, overexpression of the *miR-17~92* cluster did not alter the normal lung fibroblasts (Figure 2B).

Transfection of the *miR-17~92* cluster in IPF fibroblasts reduced vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), *Col1a1*, and *Col13a1* expression compared with untransfected, mock-transfected, and vector-transfected cells (Figure 2C). In contrast, gene expression was relatively unchanged in normal fibroblast cell lines transfected with the *miR-17~92* cluster compared with control samples. VEGF and CTGF have been shown to be directly regulated by the *miR-17~92* cluster (19, 21, 22, 24, 25), whereas *Col1a* is indirectly targeted by *miR-17~92* regulation of TGF-β (23). Because *Col13a1* was a predicted target of the cluster, we confirmed that it is directly regulated by the miRNAs contained in the *miR-17~92* cluster (see Figure E4C). As a negative control, *HIF1α* mRNA expression, highly expressed in lung tissue (36, 37) and not a predicted target of the *miR-17~92* cluster, was unchanged in *miR-17~92*-transfected normal and IPF lung fibroblasts (see Figure E4D).

Altered DNA Methylation Patterns of the CpG Island in the *miR-17~92* Cluster Promoter in IPF

Because c-MYC transcriptionally regulates *miR-17~92* cluster expression (19, 20), we evaluated its expression in lung tissue from patients with IPF and found no differences in mRNA or protein expression in lung tissue or fibroblasts from patients with IPF (data not shown), suggesting alternative mechanisms accounted for decreased *miR-17~92* expression in IPF.

Interestingly, more than 80% of the *miR-17~92* promoter is heavily occupied with a large CpG island. We next hypothesized that this CpG island was aberrantly methylated in lung tissue from patients with IPF, reducing *miR-17~92* cluster expression. In lung tissue from patients with IPF, DNA methylation of the *miR-17~92* promoter was significantly increased compared with control lung tissue (Figure 3A). No unmethylated DNA was detected for the *miR-17~92* promoter in lung tissue from patients with moderate (50–80% FVC) and severe (<50% FVC) IPF, whereas unmethylated DNA in the *miR-17~92* promoter was present in mild disease (>80% FVC). Interestingly, a 20% increase in DNA methylation from baseline seen in mild disease samples seemed sufficient to reduce *miR-17~92* expression (Figure 1A). In contrast, control lung tissue had an equal distribution of unmethylated and methylated DNA in the *miR-17~92* cluster promoter. Similarly, the *miR-17~92* promoter

A.

B.

C.

Figure 2. Introduction of *miR-17~92* in idiopathic pulmonary fibrosis (IPF) lung fibroblasts induces phenotypic and molecular changes. Normal and IPF lung fibroblasts were transfected with an expression vector containing the *miR-17~92* cluster. Empty vector-transfected cells (vector) also served as a negative control. (A) After 24 hours, cells were stained with phalloidin and DAPI 0.5 μ g/ml then photographed using an Olympus inverted fluorescent at $\times 10$ magnification. Shown are representative images from three independent experiments. (B) To quantify actin fibers, the red fluorescence of phalloidin was quantitated and indicated as a percentage compared with cell number as enumerated by DAPI-positive nuclei. Shown is the average intensity $\pm$ SD. Significant decrease was apparent in *miR-17~92* transfected cells compared with vector-only transfected IPF cells, $*P = 0.0327$; differences in phalloidin staining for IPF vector-transfected IPF cells compared with untransfected ($P = 0.2549$) or vector-transfected ($P = 0.3534$) normal lung fibroblasts. (C) RNA was isolated and quantitative real-time polymerase chain reaction was performed for the indicated genes. Data were normalized using glyceraldehyde phosphate dehydrogenase as a housekeeping control. Data are expressed as the average relative copy number (RCN) $\pm$ SD from three experiments. Comparison was made between vector-only transfected IPF cells to the *miR-17~92* transfected IPF cells, $*P < 0.004$. COL = collagen; CTGF = connective tissue growth factor; VEGF = vascular endothelial growth factor.

was methylated in lung fibroblasts derived from patients with IPF (Figure 3B). Thus, our data suggested that silencing of the *miR-17~92* cluster occurred through DNA methylation of its promoter.

Inhibition of DNA Methylation in Lung IPF Fibroblasts Leads to Phenotypic and Genetic Changes

We next examined whether 5'-aza-2'-deoxycytidine, a compound that inhibits DNA methylation, restored *miR-17~92* cluster expression in IPF fibroblasts. Treating IPF fibroblasts with 5'-aza-2'-deoxycytidine for 24 hours increased *miR-17*, *-18a*, *-19b*, and *-20a* expression (Figure 4A). In contrast, there was no change in *miR-19a* and *-92a* expression. Interestingly, gene expression was different between

miR-17~92-transfected and 5'-aza-2'-deoxycytidine-treated IPF fibroblasts (Figure 4B). Notably, *Col13a1* expression was decreased in *miR-17~92*-transfected cells (Figure 2C) but not in 5'-aza-2'-deoxycytidine-treated cells (Figure 4B). Similar to cells transfected with the *miR-17~92* cluster (Figure 2A), IPF fibroblasts treated with 5'-aza-2'-deoxycytidine exhibited a more normal phenotype (see Figure E5A).

Increased Expression of DNMT-1 in IPF

To define the mechanisms regulating DNA methylation of the *miR-17~92* cluster in IPF lung tissue, we next examined DNMT-1 expression. DNMT-1 is the most abundant DNMT in mammalian cells (17, 18, 38) and is a predicted target of the *miR-17~92* cluster.

Figure 3. Increased DNA methylation of the *miR-17~92* promoter in idiopathic pulmonary fibrosis (IPF). (A) DNA was isolated from control lung tissue ($n = 3$) and individuals with IPF ($n = 3$ per severity group). Samples were selected from the cohort used in Figure 1. Data are presented as the average percent of unmethylated or methylated DNA of the *miR-17~92* promoter $\pm$ SD. Statistical difference was determined by comparing the % unmethylated with % methylated for each severity group, $*P < 0.003$. (B) DNA was isolated from normal or IPF fibroblasts. DNA methylation for the *miR-17~92* promoter was determined. Shown is the percent DNA methylation, $n = 6$ ($*P < 0.0001$ normal fibroblast compared with IPF fibroblast for unmethylated or methylated DNA).

Interestingly, DNMT-1 mRNA expression increased as FVC declined in IPF lung tissue and inversely correlated with *miR-17~92* cluster expression (Figure 5A). Similarly, DNMT-1 expression was elevated in IPF lung fibroblast cell lines compared with normal fibroblast cell lines (see Figure E5C). Introduction of the *miR-17~92* cluster in IPF fibroblast cell lines significantly decreased DNMT-1 expression compared with vector control-transfected samples (see Figure E5C). These observations suggest that DNMT-1 contributed to the hypermethylation of the *miR-17~92* promoter region in IPF lung tissue. Notably, treatment of the IPF lung fibroblast cell line with 5'-aza-2'-deoxycytidine also decreased DNMT-1 expression (Figure 5B).

To understand the relationship between DNMT-1 and *miR-17~92* expression, IPF lung fibroblast cell lines were treated with 5'-aza-2'-deoxycytidine in the presence of an antagomir to either *miR-19b* or *-20a* to reduce miRNA-specific expression. After treatment with the antagomir to *miR-19b*, treatment with 5'-aza-2'-deoxycytidine no longer decreased DNMT-1 expression (Figure 5B). In contrast, similar studies using the *miR-20a* antagomir did not alter DNMT-1 expression in the presence of 5'-aza-2'-deoxycytidine (Figure 5B), indicating that *miR-19b* primarily regulated DNMT-1 expression.

DNMT-1 Expression Is Directly Regulated by the *miR-17~92* Cluster

We identified seed sequences for *miR-17*, *-19b*, *-20a*, and *-92a* in the 3' UTR of DNMT-1. Using a luciferase system, transfection of *miR-17*, *-19b*, *-20a*, or *-92a* into HEK-293 cells expressing the 3'-UTR wild-type DNMT-1-luc construct reduced cellular luciferase activity, an effect abrogated by mutating the DNMT-1 seed sequence (Figure 5C). As expected, there was no change in DNMT-1 luciferase production in the presence of *miR-19a* and *-18a*, which do not have a predicted seed sequence in the 3' UTR for DNMT-1.

5'-Aza-2'-Deoxycytidine Alters Gene Expression in Murine Model of Pulmonary Fibrosis

Using a murine model of pulmonary fibrosis, we evaluated whether 5'-aza-2'-deoxycytidine treatment altered *miR-17~92* DNA methylation, fibrotic gene expression, and lung fibrosis. Similar to the pathologic fibrotic pattern observed in human IPF, mice given bleomycin twice weekly by intraperitoneal injection for 4 weeks develop subpleural fibrosis of the lung (28). In this model, fibrosis appears in the lungs within 2 weeks of treatment (28). To assess the use of demethylating agents in lung fibrosis, mice were treated for 2 weeks with intraperitoneal bleomycin injections before treatment with 5'-aza-2'-deoxycytidine or vehicle during the final 2 weeks of bleomycin treatment. As shown in Figure 6A, repeated systemic bleomycin treatment reduced *miR-17~92* cluster expression in murine lung tissue, which was abrogated by 5'-aza-2'-deoxycytidine treatment, even when started 2 weeks after bleomycin injections started. Although not significant ($P = 0.1348$), 5'-aza-2'-deoxycytidine treatment altered lung pathology after 2 weeks of bleomycin (Figures 6B and 6C) and significantly reduced collagen, VEGF, CTGF, and DNMT-1 gene expression (Figures 6D and 6E). Notably, 5'-aza-2'-deoxycytidine alone enhanced the endogenous expression of the *miR-17~92* cluster leading to a reduction in the indicated genes in the lung tissue. As predicted, 5'-aza-2'-deoxycytidine abolished bleomycin-induced DNA methylation of the *miR-17~92* cluster compared with mice treated with bleomycin alone (Figure 6F).

DISCUSSION

The molecular mechanisms underlying IPF are unknown. Lung injury followed by dysfunctional lung repair seems to be a mechanism for lung pathology in IPF (3, 4, 34). Insights from familial IPF also suggest a role for ineffective lung repair and remodeling

Figure 4. Treatment of idiopathic pulmonary fibrosis (IPF) lung fibroblasts with 5'-aza-2'-deoxycytidine liberates microRNA (miRNA) expression and down-regulates target mRNAs. IPF lung fibroblast cell line was either treated with the vehicle control dimethyl sulfoxide (DMSO) or treated with 0.5 μ M 5'-aza-2'-deoxycytidine (5'-Aza) for 24 hours. RNA was isolated and subjected to quantitative real-time polymerase chain reaction analysis for (A) miRNA or (B) fibrotic genes. Data were normalized to (A) RNU48 or (B) GAP-1 expression. Shown is the average relative copy number (RCN) $\pm$ SD from two independent experiments. COL = collagen; CTGF = connective tissue growth factor; VEGF = vascular endothelial growth factor.

(12) associated with enhanced DNA methylation (39). Moreover, lung epithelial homeostasis is critical in pulmonary fibrosis, because mutations in surfactant protein C, produced by lung epithelial cells, result in lung fibrosis (13). Given that ineffective lung epithelial cell repair and excessive collagen production are important in IPF (4, 34), we examined mechanisms connecting these processes. Thus, we focused on expression of the *miR-17~92* cluster, which is critically important in lung epithelial cell development (15, 26, 27) and targets critical fibrotic genes (19, 21–25). Here, we report that the *miR-17~92* cluster was decreased in lung tissue from patients with IPF accompanied by enhanced DNA methylation of its promoter. We also found decreased expression of several members from the paralog *miR-17~92* clusters (*miR-106a~363* and *miR-106b~25*) (data not shown) suggesting an unlikely compensatory role for these miRNAs. Moreover, a Food and Drug Administration–approved demethylating agent, 5'-aza-2'-deoxycytidine, increased the expression of the *miR-17~92* cluster. Notably, *miR-17~92* expression was reduced in lung tissue from patients with IPF but not in COPD lung tissue.

In IPF, changes in fibrotic gene expression in lung tissue are present, including metalloproteinases, collagen, and growth factors (2, 9–11). We therefore hypothesized that these changes were regulated by miRNAs and may contribute to disease pathogenesis. Notably, TGF- β –stimulated overexpression of *miR-21* is important in human and murine models of pulmonary fibrosis, and may amplify fibrogenesis by targeting negative regulators of TGF- β signaling in lung myofibroblasts (40). Targeting *miR-21*

in mice attenuates the effects of intratracheal bleomycin. Furthermore, several miRNAs are down-regulated in IPF including *let-7d* and *miR-17* and *-92a* (9, 41, 42).

Highly expressed genes in IPF were subjected to miRNA prediction analysis. Interestingly, miRNAs contained in the *miR-17~92* cluster were predicted not only to target numerous fibrotic genes, but also DNMTs that play important roles in epigenetic gene regulation suggesting that IPF may be a disease of aberrant DNA methylation (43, 44). Thus, we found that the *miR-17~92* cluster specifically targeted DNMT-1. Introducing the *miR-17~92* cluster into IPF lung fibroblasts reduced promoter methylation of the cluster and fibrotic gene expression.

An inverse relationship between the *miR-17~92* cluster and DNMT-1 expression was observed in IPF lung tissue. Importantly, DNMT-1 is recruited to sites of DNA damage where it regulates epigenetic events (18, 38). Notably, α -SMA expression is inversely regulated by DNMT expression in rat *ex vivo* fibroblasts and TGF- β –induced myofibroblast differentiation (43). Here, we report decreased expression of *miR-19b* and *-20a* from human lung tissue from patients with IPF accompanied by enhanced α -SMA expression (Figure 1; see Figure E3). Using either the *miR-17~92* cluster or 5'-aza-2'-deoxycytidine reduced DNMT-1 expression in human cells and reduced actin expression. Notably, we did not examine the relationship with DNMT expression and α -SMA expression in cells from mice with bleomycin-induced pulmonary fibrosis. In light of our findings and that of Hu and colleagues (43), it is possible that subtle differences for α -SMA regulation exist between species.

Figure 5. DNA methyltransferase (DNMT)-1 is altered in idiopathic pulmonary fibrosis (IPF) and a target of the *miR-17~92* cluster. (A) DNMT-1 expression was examined by quantitative real-time polymerase chain reaction from a select cohort of tissue samples shown in Figure 1 ($n = 3$, per each disease severity group and control tissue samples). Using glyceraldehyde phosphate dehydrogenase as an endogenous control, the average relative copy number (RCN) $\pm$ SD was calculated. Significance $*P < 0.05$ is shown. (B) IPF lung fibroblasts cells were transfected with or without *miR-19b* or *20a* antagomir to knock down (KD) their expression. After treatment with 5'-aza-2'-deoxycytidine (5'Aza) for 24 hours, RNA was extracted and DNMT-1 expression quantitated by quantitative real-time polymerase chain reaction. DNMT-1 expression was normalized to glyceraldehyde phosphate dehydrogenase in vehicle-treated untransfected cells. Shown is the average fold-change $\pm$ SD ($n = 3$). Significance for 5'Aza-treated cells compared with 5'Aza/*miR-19b* KD cells is $*P < 0.001$. No clinical difference was apparent between 5'Aza-treated cells and 5'Aza/*miR-20a* KD cells. (C) The wild-type (WT) or mutated (mut) 3' untranslated region for DNMT-1 was cloned into the pGL-3 Firefly luciferase vector and transfected in the presence of the indicated microRNAs (miRNAs) in HEK 293 cells. Irrelevant scrambled miRNA served as a control. Cells were also cotransfected with the Renilla luciferase construct, pRL-TK. Luciferase production was measured first for the Firefly luciferase followed by the Renilla luciferase from the culture supernatant after 24 hours. Firefly luciferase was normalized to the Renilla luciferase. Shown is the average luciferase production from eight independent experiments ($\pm$ SD). WT DNMT-1 was compared with the mutated DNMT-1 for each specified miRNA, $*P < 0.001$.

Proteins that bind methylated DNA can either repress or activate gene expression. These include methyl DNA binding proteins (MBD) 1 and MBD2 and methyl CpG binding protein (MeCP2) (44). Mice lacking MeCP2 have decreased collagen deposition and myofibroblast differentiation in response to intratracheal injection of bleomycin (44). Notably, the *miR-17~92* cluster is predicted to target MBD2 and MeCP2 but not MBD1. Thus, it is conceivable that epigenetic regulation by the *miR-17~92* cluster is critically regulated by targeting multiple proteins.

DNMTs are regulated by miRNAs in human cancer and some miRNAs are regulated by epigenetic events (45). In addition to the regulatory activity of *miR-19b* in regulating DNMT-1, *miR-29b* indirectly regulates DNMT-1 expression (45). Cushing and colleagues (46) found that increased TGF- β signaling suppresses *miR-29* family members in bleomycin-induced fibrosis. In human IPF lung tissue samples, we and others found changes in *miR-29* family members compared with control tissue samples (9, 42). More recently, Xiao and coworkers (47) demonstrated that enforced expression of *miR-29* in the lung of mice subjected to intratracheal bleomycin attenuates lung fibrosis. Although *miR-148a* and -152 also target DNMT-1 (48), we did not detect significant changes in their expression in fibrotic lung tissue (9).

Altering epigenetic changes with the chemotherapeutic drug 5'-aza-2'-deoxycytidine is clinically used in treating humans with

myelodysplastic syndrome and acute myelogenous leukemia (49). However, this drug is associated with myelosuppression caused by neutropenia, thrombocytopenia, and anemia. Interestingly, 5'-aza-2'-deoxycytidine treatment of IPF fibroblasts increased the expression of most miRNAs contained in the *miR-17~92* cluster and decreased the expression of fibrotic gene targets. Of note, the fibrotic genes studied here have single or multiple CpG islands. However, the promoters of most of these genes are unmethylated in control and IPF tissue (see Figure E6) suggesting a critical role for miRNAs in the regulation of their expression.

We investigated the preclinical use of 5'-aza-2'-deoxycytidine in murine pulmonary fibrosis induced by intraperitoneal bleomycin. Treatment of bleomycin-treated mice with 5'-aza-2'-deoxycytidine after the initiation of fibrosis reduced fibrotic and DNMT-1 gene expression while restoring *miR-17~92* cluster expression. Although lung pathology was not altered significantly in these mice, our data suggest that 5'-aza-2'-deoxycytidine treatment most likely does not mediate the breakdown of collagen already present but rather prevents the deposition of additional collagen. Bechtel and coworkers (50) found that 5'-aza-2'-deoxycytidine attenuated renal fibrosis by reducing fibrotic genes and decreasing DNMT-1-induced methylation of *RASAL1*, a regulator of Ras activation. Although TGF- β regulates *RASAL1* expression transcriptionally and through DNMT-1, it is important to note

Figure 6. *In vivo* treatment of mice with 5'-aza-2'-deoxycytidine attenuates bleomycin-induced fibrotic gene expression. Mice were treated with 0.035 U/kg twice weekly for 4 weeks with bleomycin (n = 4) or phosphate-buffered saline (PBS) alone (n = 4). A set of bleomycin-treated mice were injected with 0.156 mg/kg/wk of 5'-aza-2'-deoxycytidine intraperitoneally for the last 2 weeks of bleomycin treatment (n = 4) and designated as Bleomycin (4 wk) 5' Aza (2 wk). As a control, mice were treated with PBS alone for 4 weeks or PBS for 2 weeks and then 5'-aza-2'-deoxycytidine for 2 weeks without bleomycin (5' Aza [2 wk]). (A) RNA was extracted from the lung tissue and microRNA (miRNA) expression was examined by quantitative real-time polymerase chain reaction and normalized using snoRNA-202 expression. Shown is the average relative copy number (RCN) $\pm$ SD, *P value < 0.001 compared with bleomycin only treated mice. (B) Lung tissue was section and paraffin-embedded then subjected to trichrome staining to detect collagen deposition. The larger image is at $\times 10$ magnification, whereas the inset is a $\times 4$ magnification. Shown are representative images from a mouse from each group. (C) Trichrome sections were blindly assessed by a board-certified pathologist. The average arbitrary score $\pm$ SD is shown (n = 4 per group). Significance compared with bleomycin-only treated mice, *P < 0.0075 and **P = 0.1348. (D) RNA was subjected to quantitative real-time polymerase chain reaction for the indicated genes and (E) DNA methyltransferase (DNMT)-1 expression. CAP-1 served as an endogenous control for normalization for fibrotic genes and DNMT-1. Data are expressed as the relative copy number (RCN) $\pm$ SD, *P value < 0.001 compared with bleomycin-treated mice. (F) DNA were isolated and subjected to anal-

ysis for promoter DNA methylation of the *miR-17~92* cluster. Data presented is the average % unmethylated and % methylated, n = 3 mice per treatment group. *P value < 0.001 compared with bleomycin-only treated mice. COL = collagen; CTGF = connective tissue growth factor; VEGF = vascular endothelial growth factor.

that several miRNAs from the *miR-17~92* cluster are predicted to target *RASAL1* and TGF- β .

To our knowledge, this represents the first report showing that reexpression of the *miR-17~92* cluster using 5'-aza-2'-deoxycytidine leads to reduced fibrotic gene expression *in vitro* and *in vivo*. The current study suggests that epigenetic modifying drugs may have a therapeutic benefit for IPF.

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