MicroRNA-96 inhibits FoxO3a function in IPF fibroblasts on type I collagen matrix

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Idiopathic pulmonary fibrosis (IPF) is a lethal and progressive lung disease characterized by persistent (myo)fibroblasts and the relentless accumulation of collagen matrix. Unlike normal lung fibroblasts, IPF lung fibroblasts have suppressed forkhead box O3a (FoxO3a) activity, which allows them to expand in this diseased environment. microRNA-96 (miR-96) has recently been found to directly bind to the 3′-untranslated region of FoxO3a mRNA, which subsequently inhibits its function. We examined whether aberrantly low FoxO3a expression is in part due to increased miR-96 levels in IPF fibroblasts on polymerized collagen, thereby causing IPF fibroblasts to maintain their pathological properties. miR-96 expression was upregulated in IPF fibroblasts compared with control fibroblasts when cultured on collagen. In contrast, FoxO3a mRNA levels were reduced in most IPF fibroblasts. However, when miR-96 function was inhibited, FoxO3a mRNA and protein expression were increased, suppressing IPF fibroblast proliferation and promoting their cell death in a dose-dependent fashion. Likewise, FoxO3a and its target proteins p21, p27, and Bim expression was also increased in the presence of a miR-96 inhibitor in IPF fibroblasts. However, when control fibroblasts were treated with miR-96 mimic, FoxO3a, p27, p21, and Bim mRNA and protein levels were decreased. In situ hybridization analysis further revealed the presence of enhanced miR-96 expression in cells within the fibroblastic foci of IPF lung tissue. Our results suggest that when IPF fibroblasts interact with collagen-rich matrix, pathologically altered miR-96 expression inhibits FoxO3a function, causing IPF fibroblasts to maintain their pathological phenotype, which may contribute to the progression of IPF.

IPF; collagen matrix; miR-96; FoxO3a; proliferation; cell death

IDIOPATHIC PULMONARY FIBROSIS (IPF) is a lethal and progressive lung disease with unknown etiology. Currently there is no proven treatment available for IPF, and 40,000 people succumb from this disease every year (11, 15). IPF is characterized by the presence of persistent (myo)fibroblasts surrounded by a relentless accumulation of type I collagen-rich extracellular matrix, which eventually destroys lung function (2, 17, 18). Studies have shown that α-smooth muscle actin-expressing myofibroblasts are mainly found in the fibroblastic foci of IPF lung tissue (17, 28, 41). Similar to these findings, when IPF fibroblasts are cultured on type I collagen matrix, IPF fibroblasts maintain a proliferative and apoptosis-resistant fibroblast phenotype (28, 34, 41). In contrast, the proliferation of normal lung fibroblasts is suppressed and apoptosis increases in response to polymerized collagen (28, 41). Prior studies have shown that the aberrant PTEN/PI3K/Akt-dependent axis plays a major role in maintaining an IPF fibroblast phenotype on collagen (28, 29, 41, 42). One of the direct targets of Akt, FoxO3a, participates in regulating cell proliferation, apoptosis, differentiation, stress response, and glucose metabolism (12, 13, 32, 37). A myriad of evidence documents that FoxO3a alteration is closely linked to several types of cancer progression (9, 19, 26, 27, 36, 39, 43). Likewise, FoxO3a activity is also altered in IPF fibroblasts on collagen owing to inappropriate Akt activity, which subsequently confers a pathological IPF fibroblast phenotype (28). Prior studies revealed that Akt directly phosphorylates the crucial ser 253 residue, and this event drives the localization of FoxO3a protein to the cytoplasm (4, 5), thereby inhibiting FoxO3a-dependent functions on collagen (28, 30).

MicroRNAs (miRNAs) are a class of small noncoding RNA molecules known to contribute to the posttranscriptional regulation of gene expression (25). miRNAs bind to complementary sequences within mRNA molecules, thereby enhancing mRNA degradation or translational suppression (40). Several miRNAs are found to be associated with lung disease (7, 8, 44), and among them, miR21, miR155, miR-26a, and miR-17-92 have been linked to IPF pathogenesis (20, 22, 23, 10). Recently, microRNA-96 (miR-96) has been known to directly bind to 3′-untranslated region (UTR) of FoxO3a mRNA which subsequently inhibits its function (24). However, whether the deregulation of miR-96 in IPF fibroblasts on collagen is also responsible for FoxO3a suppression is currently unknown. Therefore, we hypothesized that miR-96 expression is increased when IPF fibroblasts interact with collagen matrix, suppressing FoxO3a function. To test this hypothesis, we first examined miR-96 levels in IPF and control fibroblasts cultured on collagen. We found that miR-96 expression is enhanced when IPF fibroblasts are cultured on collagen-rich matrix compared with that of control fibroblasts. In contrast, FoxO3a mRNA and protein expression was reduced in most IPF fibroblasts on collagen. However, the inhibition of miR-96 function increased FoxO3a mRNA and protein levels in IPF fibroblasts in a dose-dependent fashion. Furthermore, the proliferation of IPF fibroblasts was suppressed while viable cells were progressively reduced when miR-96 function is inhibited with various doses of miR-96 inhibitor. Also notable miR-96 positive cells were found in the fibroblastic foci of IPF patient lung tissues whereas the presence of cells expressing miR-96 was absent in normal lung alveoli. Our results suggest that highly expressed miR-96 also participates in the suppression of FoxO3a, con-
ferring IPF fibroblasts to maintain a pathological phenotype on collagen-rich matrix.

MATERIALS AND METHODS

Human subjects. This study involves the analysis of human IPF patient specimens. Primary fibroblast lines were obtained from unused, existing pathological human tissue samples. These cell lines were derived from lungs removed at the time of transplantation or death, and tissue samples were stripped of all identifiers and designated as waste (exemption 4). Exemption 4 includes research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects (http://grants.nih.gov/grants/policy/hsfaqs_aps_exempt.htm#291). All patients underwent procedures for diagnostic or therapeutic procedures. Written, informed consent was obtained on all patients prior to the procedure being performed. Use of human tissues was approved by the Institutional Review Board at the University of Minnesota. The diagnosis of IPF was supported by histology, physical examination, pulmonary function tests, and typical high-resolution chest computed tomographic findings of IPF. In all cases, the diagnosis of IPF was confirmed by microscopic analysis of lung tissue that demonstrated the characteristic morphological findings of usual interstitial pneumonia (41). All patients fulfilled the criteria for the diagnosis of IPF as established by the American Thoracic Society and the European Respiratory Society. To reduce technical variability, we routinely utilize cells between passages 5 and 7 because of concern that the phenotype of the cells is altered at higher passage. To address concerns of biological variability, we studied eight control cell lines and eight IPF cell lines.

Cell culture and three-dimensional type I polymerized collagen matrices. A total of 16 fibroblast lines (n = 8 each for control and IPF fibroblasts) were used for our study. Primary lung fibroblast lines were generated by explant culture in DMEM containing 20% FCS. Three-dimensional polymerized collagen matrices were prepared with 80% of type I collagen solution, 10% 10× DMEM, and 10% 1× DMEM, and pH was adjusted to 7.2 with 0.1 M NaOH. Collagen matrix was plated on cell culture dishes or plates and incubated for 3–4 h prior to seeding cells.

Reagents, antibodies, and chemicals. DMEM and FCS were purchased from Sigma-Aldrich (St. Louis, MO) and HyClone (Logan, UT), respectively. Bim, p27, and p21 antibodies were obtained from Cell Signaling (Beverly, MA), and FoxO3a and GAPDH antibodies were purchased from Millipore (Bedford, MA) and Santa Cruz (Dallas, TX), respectively. Type I collagen solution was obtained from Advanced BioMatrix (San Diego, CA). Synthetic human miR-96 inhibitor (hsa-miR-96-3p) and negative control inhibitor having no known homology to human gene sequences were purchased from Sigma-Aldrich. This miRNA inhibitor was designed with use of the mature miRNA sequence information from miRBase. To further elucidate the function of miR-96, human miR-96 mimic was obtained from Sigma-Aldrich. Protein expression was quantified by using LabWorks Image acquisition and analysis software version 4.6 (UVP BioImage Systems). For the inhibition of FoxO3a function in IPF fibroblasts, adenovirus expressing transactivation domain deleted FoxO3a (dominant negative FoxO3a) and GFP were purchased from Vector BioLabs (Eagleville, PA).

Real-time PCR, miR-96 inhibitor, and miR-96 mimic. We cultured 3 × 10^5 control and IPF fibroblasts on three-dimensional polymerized collagen matrices in serum-free DMEM medium for 24 h. Total RNA was then extracted using TRIzol (Invitrogen, Grand Island, NY) according to the manufacturer’s protocol. For the inhibition of miR-96, equal numbers of control or IPF fibroblasts cultured on polymerized collagen matrices were treated with 0, 0.5, 1, 2, and 5 nM miR-96 or control inhibitors for 24 h in serum-free medium. Then 1 μg of total RNA was used to generate complementary DNA (cDNA) using oligo(dT) (Roche Applied Science, Indianapolis, IN) and reverse transcriptase (Sigma-Aldrich). To measure miR-96 levels, a previously prepared 0.4 μg of total RNA from eight control and eight IPF fibroblasts was used for cDNA synthesis using miScript II RT kit (QIAGEN, Germantown, MD). Real-time RT-PCR was performed with miScript SYBR Green PCR kit (QIAGEN) by using Light Cycler 1.5 (Roche Applied Science) according to manufacturer’s instruction. The reaction conditions were as follows: denaturation at 95°C for 15 min and amplification by cycling 45 times at 95°C for 15 s, 55°C for 30 s, and 70°C for 30 s. RNase–2 was amplified together with miR-96 to normalize copy numbers of the miR-96 gene, and all primers were prevalidated primers from the kit (QIAGEN). Real-time RT-PCR for FoxO3a target genes was also performed with SYBR Green PCR kit (Roche Applied Science) with primers specific for human FoxO3a (forward: 5′-AAA TGT TCG TCG CGG CGG AAC-3′; reverse: 5′-GTC GCC CTTA TTC TCT TGG AAG TA-3′), p27 (forward: 5′-AGG AGA GCC AGG ATG AGC-3′; reverse: 5′-AAG AAT CGT CGG TCG CGT GC-3′), Bim (forward: 5′-GCC AGG CCT TCA ACC ACT AT-3′; reverse: 5′-ACC ATT CGT GGG TGG TCT TC-3′), or GAPDH (forward: 5′-TCC ATT GAC CTC AAC TAC ATG GT-3′; reverse: 5′-CCT TCT CCA TGG TGG TGA AGA-3′) by using Light Cycler 1.5 (Roche Applied Science) according to manufacturer’s instructions. The reaction conditions were as follows: denaturation at 95°C for 15 min and amplification by cycling 45 times at 95°C for 15 s, 60°C for 30 s, and 70°C for 30 s. For the miR-96 mimic treatment, 3 × 10^5 of control fibroblasts (n = 3) were transfected with 50 nM of human mir-96 mimic and cultured on three-dimensional polymerized collagen matrices in serum-free DMEM medium for 48 h. At the end of incubation, total RNA was extracted using TRIzol (Invitrogen) and cDNA was synthesized as previously described, and FoxO3a and its target p27, p21, and Bim mRNA expression were measured as previously described.

Western blot analysis. We treated 3 × 10^5 control or IPF fibroblasts cultured on polymerized collagen with 0, 0.5, 1, 2, and 5 nM of miR-96 inhibitor for 24 h. Cells were then lysed with 1 × cell lysis buffer (Cell Signaling) containing protease inhibitor (Roche Applied Science) and phosphatase inhibitor cocktail (Research Products International, Mount Prospect, IL). Cell lysates were collected after sonication for 15 s on ice, and SDS-PAGE was performed on 12–16% polyacrylamide gel. Separated protein was then electrotransferred to a PVDF membrane (Bio-Rad, Hercules, CA) by use of a Protein III tank transfer system (Bio-Rad). After blocking with 5% BSA in TTBS (0.1 M Tris, 0.9% NaCl, and 0.1% Tween 20) for 0.5 h, FoxO3a, p27, p21, and Bim expression was measured.

Cell proliferation and viability assay. The cell proliferation assay was conducted by using a Cell Proliferation ELISA BrdU kit (Roche Applied Science) according to manufacturer’s instructions. Briefly, control and IPF fibroblasts cells (n = 3; 1 × 10^4 cells/well on 96-well plate) were cultured on polymerized collagen in serum-free DMEM or DMEM containing 10% FBS for 24 h. Cells were treated with 0, 0.5, 1, 2, or 5 nM of miR-96 or control inhibitor for additional 24 h and were then labeled with 10 μM of bromodeoxyuridine (BrdU) (Promega) for an additional 6 h. After a fixation step, cells were incubated with anti-BrdU conjugated with peroxidase for 1.5 h and cell proliferation was then measured by using a 96-well plate reader at 370 nm (reference: 492 nm) followed by incubation with 3,3′,5′-tetramethylbenzidine (TMB) substrate for 10 min. The cell proliferation assay was also conducted with CellTiter 96 Aqueous One Solution cell proliferation assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, Madison, WI)]. Briefly, IPF cells (1 × 10^4 cells/100 μl/well) infected with adenovirus expressing dominant negative FoxO3a or empty GFP were cultured on a 96-well plate precoated with polymerized collagen and were then incubated in the presence of 5 nM of miR-96 or control
miR-96 expression is low in IPF fibroblasts cultured on collagen. A recent study showed that FoxO3a mRNA is suppressed by miR-96 (21). This finding suggests a possibility that FoxO3a mRNA is low in IPF fibroblasts on collagen matrix as a result of enhanced expression of miR-96. To test this possibility, control and IPF fibroblasts (n = 8, each) were attached to collagen matrix, and miR-96 levels were measured by real-time PCR (Fig. 1). Overall, miR-96 expression was twofold higher in IPF fibroblasts compared with that of control fibroblasts. This finding indicates that when IPF fibroblasts attach to polymerized collagen, miR-96 is abnormally high, which potentially inhibits FoxO3a mRNA.

FoxO3a mRNA and protein expression is reduced in IPF fibroblasts on collagen matrix owing to the alteration of miR-96. Since the miR-96 level is high in IPF fibroblasts when attached to polymerized collagen, we next sought to confirm whether FoxO3a mRNA levels were low in IPF fibroblasts as a result of enhanced miR-96 expression. To test this, we first examined FoxO3a mRNA levels in control and IPF fibroblasts cultured on collagen by real-time PCR. FoxO3a mRNA levels were approximately fivefold lower in IPF fibroblasts compared with that of control fibroblasts (Fig. 2A). To verify whether FoxO3a protein expression was also reduced, we next measured FoxO3a protein expression in control and IPF cells on collagen matrix. Like FoxO3a mRNA, FoxO3a protein levels were also approximately threefold lower in most IPF fibroblast lines compared with that of control fibroblasts (Fig. 2B).

**RESULTS**

miR-96 expression is low in IPF fibroblasts cultured on collagen. A recent study showed that FoxO3a mRNA is suppressed by miR-96 (21). This finding suggests a possibility that FoxO3a mRNA is low in IPF fibroblasts on collagen matrix as a result of enhanced expression of miR-96. To test this possibility, control and IPF fibroblasts (n = 8, each) were attached to collagen matrix, and miR-96 levels were measured by real-time PCR (Fig. 1). Overall, miR-96 expression was twofold higher in IPF fibroblasts compared with that of control fibroblasts. This finding indicates that when IPF fibroblasts attach to polymerized collagen, miR-96 is abnormally high, which potentially inhibits FoxO3a mRNA.

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**Fig. 1.** MicroRNA-96 (miR-96) expression is aberrantly high in idiopathic pulmonary fibrosis (IPF) fibroblasts on collagen matrix. Randomly selected 3 × 10^5 control and IPF fibroblasts (n = 8, each) were cultured on type I polymerized collagen in serum-free medium for 24 h, and cDNA was synthesized with use of miScript II RT. Real-time PCR was then carried out by using miR-96 primers as described in MATERIALS AND METHODS. RNU6−2 was used as an internal control. miR-96 expression was normalized to RNU6−2. Shown is the mean ± SD of miR-96 expression in 8 IPF and 8 control fibroblasts.

**Fig. 2.** FoxO3a mRNA and protein levels are low in IPF fibroblasts cultured on collagen matrix. Randomly selected 3 × 10^5 control and IPF fibroblasts (n = 8, each) were attached to collagen matrix, and FoxO3a mRNA and protein expression were measured by real-time PCR and Western analysis, respectively. A: FoxO3a mRNA expression in control and IPF fibroblasts cultured on collagen matrix. B: Representative FoxO3a protein expression determined by Western analysis.
2B, top and bottom). These data further support our hypothesis that inappropriately high miR-96 expression suppresses FoxO3a mRNA, which contributes in reducing FoxO3a protein levels in IPF fibroblasts cultured on collagen.

Since miR-96 expression is increased in response to IPF fibroblast attachment to collagen, we sought to use a synthetic miR-96 inhibitor to test the function of miR-96 on FoxO3a suppression. We first examined whether the miR-96 inhibitor can effectively suppress the miR-96 level when IPF fibroblasts are attached to collagen matrix by real-time PCR. miR-96 expression was 2.5-fold decreased compared with that of IPF cells treated with control inhibitor, showing that miR-96 level was successfully reduced when miR-96 inhibitor was used (Fig. 3A). To confirm whether aberrantly low FoxO3a mRNA expression is directly due to enhanced miR-96 expression in IPF fibroblasts, control and IPF cells were treated with various doses of the synthetic miR-96 inhibitor, and FoxO3a mRNA expression was then measured. FoxO3a mRNA levels in IPF fibroblasts were progressively increased in a dose-dependent fashion in the presence of the synthetic miR-96 inhibitor (Fig. 3B, $P < 0.02$, $P < 0.03$ based on two-sample $t$-test and a two-way ANOVA, respectively). These data showed that enhanced miR-96 expression directly targets FoxO3a mRNA in response to the IPF fibroblast’s attachment to collagen matrix. However, unlike IPF fibroblasts, FoxO3a mRNA expression remained high and was not significantly altered when control fibroblasts were attached to polymerized collagen in the presence of various doses of miR-96 inhibitor. This finding also suggests that since miR-96 expression is low when control fibroblasts are cultured to collagen matrix, the subsequent inhibition of preexisting low miR-96 levels has a relatively minor effect on FoxO3a mRNA expression. To examine the direct effect of miR-96 on FoxO3a protein expression, we also measured FoxO3a protein levels in control and IPF cells under the same conditions (Fig. 3C). FoxO3a protein expression in control fibroblasts was not significantly altered in the presence of 0.5 to 2 nM of inhibitor but increased when the highest dose of miR-96 inhibitor was used. In contrast, FoxO3a expression was low in IPF fibroblasts in the absence of miR-96 inhibitor but progressively increased in the presence of various doses of miR-96 inhibitor. Taken together, these data show that abnormally high miR-96 is responsible for the suppression of FoxO3a mRNA/protein expression in response to IPF fibroblast attachment to collagen-rich matrix.

**IPF fibroblast proliferation and viability are altered via FoxO3a when miR-96 is inhibited on collagen matrix.** FoxO3a inhibits cell proliferation and promotes cell death by increasing p27, p21, and Bim (3, 12, 33). Since miR-96 alteration clearly affects FoxO3a mRNA and protein expression, we next examined whether the inhibition of miR-96 increases FoxO3a target expression.
proteins, thereby regulating IPF fibroblast proliferation and their apoptosis. To test this, we first measured FoxO3a targets p27, p21, and Bim mRNA expression in the presence of miR-96 inhibitor in control and IPF cells on collagen matrix. The p27, p21, and Bim expression remained relatively unaltered when miR-96 inhibitor was used in control fibroblasts (Fig. 4A, top). In contrast, mRNA levels of these proteins were ~50% increased when IPF cells were treated with miR-96 inhibitor (Fig. 4A, bottom). These findings showed that miR-96 inhibition in IPF fibroblasts predominantly increases FoxO3a target mRNA levels. To confirm that the inhibition of FoxO3a mRNA by a miR-96 inhibitor also increases FoxO3a target protein expression, we next measured p27, p21, and Bim expression in control and IPF fibroblasts cultured on collagen in the presence of various doses of miR-96 inhibitor. The p27 and p21 protein levels in IPF fibroblasts were clearly lower (four- to fivefold) compared with levels of control fibroblasts in the absence of miR-96 inhibitor (Fig. 4B, top, lanes 1 and 6, and bottom, left and middle, respectively) but increased (3- to 3.5-fold, respectively) in the presence of 2 and 5 nM of miR-96 inhibitor. However, unlike IPF fibroblasts, p27 and p21 expression was high and remained relatively unaltered or had an approximately twofold increase in the presence of various doses of miR-96 inhibitor in control fibroblasts, respectively. In contrast, similar to the p21 level, Bim expression was low but increased ~2.6 fold when IPF fibroblasts were treated with 2 and 5 μM of miR-96 inhibitor (Fig. 4B, top and bottom right, P < 0.04 respectively). Unlike the case of the Bim level in IPF fibroblasts, Bim expression was highly expressed in the absence of miR-96 inhibitor and remained high in the presence of various doses of miR-96 inhibitor in control fibroblasts. These data suggest that when IPF fibroblasts are cultured on collagen, FoxO3a mRNA/protein expression is suppressed as a result of highly expressed miR-96, and the inhibition of enhanced miR-96 in IPF fibroblasts causes more robust effects on FoxO3a and its target proteins compared with the case of control fibroblasts.

To further test whether the induction of these proteins by miR-96 inhibition is correlated with the inhibition of proliferation and the increase in IPF fibroblast cell death, we next measured fibroblast proliferation and viability under the same conditions as described above. We previously found that IPF fibroblasts maintain a proliferative and a cell death-resistant phenotype on collage-rich matrix in serum-free condition (28, 29). To elucidate serum effects on fibroblast proliferation in the presence of miR-96 inhibitor, we next measured control and IPF fibroblast proliferation in the presence or absence of serum using a BrdU proliferation assay. When serum was present, there was no significant difference found in both cells in the presence or absence of miR-96 inhibitor (Fig. 5A, left). However, when IPF cells were attached to collagen in serum-free medium, they maintained enhanced proliferation in the absence of miR-96 inhibitor but their proliferation was progressively decreased in the presence of various doses of miR-96 inhibitor (Fig. 5A, right, P < 0.0001). In contrast, control fibroblast proliferation remained low and was relatively unaltered in the presence of miR-96 inhibitor at 24 h. These data suggest that serum had a minimal effect on proliferation in control fibroblasts, whereas in IPF fibroblasts, serum enhanced proliferation.

**Fig. 4.** miR-96 inhibition predominantly increases p27, p21, and Bim expression in IPF fibroblasts. We treated 3 × 10^5 control or IPF fibroblasts cultured on polymerized collagen with 5 nM of control or miR-96 inhibitor for 24 h in serum-free medium. A, top: fold change in p27, p21, and Bim mRNA expression after miR-96 or control inhibitor treatment in control fibroblasts (n = 3) by real-time PCR as described in MATERIALS AND METHODS. A, bottom: fold changes in p27, p21, and Bim mRNA expression in the presence of miR-96 or control inhibitor in IPF fibroblasts (n = 3). Values were determined with relative fold change in control or IPF fibroblasts after GAPDH normalization. B, top: representative Western analysis for p27, p21, Bim, and GAPDH expression in the presence or absence of various doses of miR-96 inhibitor and remained high in the presence of various doses of miR-96 inhibitor in control fibroblasts. These data suggest that when IPF fibroblasts are cultured on collagen, FoxO3a mRNA/protein expression is suppressed as a result of highly expressed miR-96, and the inhibition of enhanced miR-96 in IPF fibroblasts causes more robust effects on FoxO3a and its target proteins compared with the case of control fibroblasts.

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presence of miR-96 inhibitor. These results suggest that IPF cells have acquired pathological properties and maintain their abnormal phenotype in response to stress inducing environments such as serum-free conditions and polymerized collagen. Therefore, to find the function of miR-96 on cell death via FoxO3a, we further measured control and IPF fibroblast cell death at 48 h in the presence of various doses of miR-96 inhibitor in serum-free conditions. Viable IPF cells were also progressively decreased in a dose-dependent fashion (Fig. 5B). In contrast, similar to proliferation profiles, control cell viability was significantly low even in the absence of the miR-96 inhibitor and remained relatively unaltered in the presence of various doses of an inhibitor on collagen. These results also showed that miR-96 inhibition predominantly affects IPF fibroblast proliferation and viability presumably due to preexisting enhanced expression of miR-96. Taken together, our data further suggest that the aberrant miR-96 expression in IPF plays a role in acquiring pathological properties by inhibiting FoxO3a function that bestow a proliferative and cell death-resistant phenotype in response to collagen-rich matrix.

miR-96 regulates FoxO3a-dependent fibroblast proliferation and viability. Our data show that increased miR-96 suppresses FoxO3a mRNA expression, and this alteration promotes IPF fibroblast proliferation, enabling them to evade polymerized collagen-induced cell death. Since there is a possibility that miR-96 can also target proteins other than FoxO3a, thereby regulating IPF fibroblast proliferation and viability, we next sought to verify whether the acquisition of pathological IPF fibroblast properties on collagen matrix is mostly FoxO3a dependent. For this assay, IPF fibroblasts infected with adenovirus expressing dominant negative FoxO3a (DF) or empty vector (GFP) were treated with miR-96 inhibitor, and p27, p21, and Bim expression were also measured on collagen. If miR-96 mainly affects proteins other than FoxO3a, the inhibition of FoxO3a has little or no effect on the regulation of FoxO3a-target proteins. When FoxO3a function was inhibited by dominant negative FoxO3a in the presence of the miR-96 inhibitor, p27, p21, and Bim expression remained low compared with that expressing GFP alone (Fig. 6A, top, lanes 2 and 4). To confirm this result, we performed experiments using control (n = 3) and IPF fibroblasts (n = 3) under the identical conditions. The p21, p27, and Bim protein expression was two- to threefold reduced when FoxO3a function was repressed (Fig. 6B, black bars). These results demonstrated that enhanced miR-96 inhibits FoxO3a function in IPF fibroblasts, and the low expression of p21, p27, and Bim is FoxO3a dependent.

To confirm that FoxO3a reinition by dominant negative FoxO3a in the presence of a miR-96 inhibitor increases IPF fibroblast proliferation and protects cells from collagen matrix-induced cell death, we next measured IPF fibroblast proliferation and viability under the same conditions as described. IPF fibroblast proliferation increased (~15%) when IPF cells were treated with dominant negative FoxO3a in the presence of a miR-96 inhibitor (Fig. 6B, black bars). Likewise, viable cells also increased (~10%) when dominant negative FoxO3a was overexpressed in IPF cells in the presence of miR-96 inhibitor (Fig. 6C, black bars). Taken together, these results demonstrate that FoxO3a is a target of miR-96, and this FoxO3a inhibition by enhanced miR-96 expression plays a role in maintaining at

Fig. 5. Inhibition of miR-96 suppresses IPF fibroblast proliferation and promoted their cell death in serum deprived medium. A, left: % change of cell proliferation in 1 x 10^6 control (n = 3) and IPF fibroblasts (n = 3) on collagen matrix in medium containing 10% serum was measured at 24 h in the presence of various doses of miR-96 inhibitor by using bromodeoxyuridine (BrdU) ELISA as described in MATERIALS AND METHODS. No significance was found in serum containing medium. Right: % change of cell proliferation of control and IPF fibroblasts cultured in serum-free medium in the presence of various doses of miR-96 inhibitor. When IPF fibroblasts were cultured in serum-free medium, the percentage of proliferating cells decreased with increasing doses of miR-96 inhibitor in IPF fibroblasts, and the differences were statistically significant by a 2-way ANOVA (P value < 0.0001). In contrast, in control fibroblasts, the mean of cell proliferation percentages did not decrease with increasing doses of miR-96 inhibitor. The differences of the dose effect with use of the miR-96 inhibitor on cell proliferation percentages did not decrease with increasing doses of the miR-96 inhibitor. The differences of the dose effect with use of the miR-96 inhibitor on cell proliferation percentages between IPF and control fibroblasts were statistically significant (P value < 0.02 by a 2-way ANOVA). B: cell viability was determined at 48 h after various dosages of miR-96 inhibitor treatment in serum-free medium as described in MATERIALS AND METHODS. Shown is the % change of viable control and IPF fibroblasts on collagen matrix with the mean ± SD Control fibroblasts expressing an enhanced level of FoxO3a mRNA/protein and IPF cells expressing reduced FoxO3a mRNA/protein were used for fibroblast proliferation and a cell death assay. The assay was performed in triplicate. The percentage of viable cells decreased with increasing dosage of miR-96 inhibitor in IPF fibroblasts and the differences were statistically significant by a 2-way ANOVA (P value < 0.0001). In the control fibroblasts, the mean of cell viability percentages did not decrease with increasing doses of the miR-96 inhibitor. The differences of the dose effect using miR-96 inhibition on cell viability percentages between IPF and control fibroblasts were statistically significant (P value < 0.03 by a 2-way ANOVA). *Mean cell proliferation percentage in IPF fibroblasts was also statistically and significantly different from baseline group (0 nM miR-96 + 5 nM control inhibitor with IPF) by a 2-sample t-test (P value < 0.02). miR-96, miR-96 inhibitor; Control: control inhibitor.
least in part an IPF fibroblast phenotype on collagen-rich matrix.

**miR-96 mimic suppresses FoxO3a, p27, p21, and Bim expression in control fibroblasts.** Our results showed that miR-96 expression is abnormally high in IPF fibroblasts, and this alteration suppresses p27, p21, and Bim expression via reduced FoxO3a expression. In contrast, miR-96 is low in control fibroblasts on collagen, which maintain enhanced FoxO3a and its target proteins. To confirm the function of miR-96 in regulating the FoxO3a-dependent pathway, we next utilized the miR-96 mimic, and p27, p21, and Bim mRNA and protein expression were also measured in control fibroblasts cultured on collagen. Real-time PCR showed that FoxO3a, p27, p21, and Bim mRNA levels were clearly decreased when miR-96 mimic was used (Fig. 7A). Furthermore, when we measured FoxO3a and its target proteins, their expression was significantly decreased (Fig. 7B, top and bottom). Collectively, these data demonstrated that miR-96 alteration is linked to the regulation of FoxO3a, which confers the pathologically altered IPF fibroblast phenotype in response to collagen matrix via reducing FoxO3a and its target p27, p21, and Bim expression.

**Enhanced miR-96 expression in the fibroblastic foci of IPF patient lung tissues.** Fibroblastic foci are a hallmark of IPF (1, 11, 16) and we previously found that FoxO3a activity is suppressed in myofibroblasts in the fibroblastic foci of IPF lung tissue (30). Since our results showed that miR-96 expression is high in IPF fibroblasts on collagen, we further examined whether miR-96 is also expressed in the fibroblastic foci of IPF patient lung specimens using an in situ hybridization assay. miR-96-expressing cells were found in the fibroblastic foci of IPF lung tissue (Fig. 8, top and middle). In contrast, control lung tissue displayed a paucity of miR-96-positive cells (Fig. 8, bottom). These results suggest that miR-96 expression due to FoxO3a deregulation participates in conferring an IPF fibroblast phenotype in response to collagen-rich extracellular matrix.

**DISCUSSION**

IPF fibroblasts maintain highly proliferative and cell death-resistant properties in response to collagen-rich matrix by suppressing FoxO3a. We previously found that inappropriately high Akt activity phosphorylates FoxO3a, and this alteration participates in causing a pathological IPF fibroblast phenotype on collagen (28, 30). Although this finding showed that Akt is an important kinase in FoxO3a regulation in IPF fibroblasts, the question of whether the deregulation of microRNAs is also responsible for the suppression of FoxO3a was not ad-
dressed. Recent study showed that miR-96 regulates the FoxO3a transcripts (24), providing a possibility that alteration of microRNA is also linked to IPF. MicroRNA-96 is known to bind to the 3'-UTR of FoxO3a, thereby inhibiting its mRNA expression (24). Therefore, on the basis of this finding, it was a feasible concept that when IPF fibroblasts attach to polymerized collagen, miR-96 expression is aberrantly high, which subsequently inhibits FoxO3a mRNA. We first measured miR-96 expression in control and IPF fibroblasts cultured on collagen to test this concept. We found that miR-96 expression is enhanced in most IPF fibroblasts. However, the majority of IPF fibroblasts have low expression of FoxO3a mRNA. These results suggest that low FoxO3a mRNA expression is also due to highly expressed miR-96 in IPF fibroblasts. To test the direct link between miR-96 and FoxO3a mRNA, we inhibited miR-96 function using various doses of miR-96 inhibitor, and FoxO3a mRNA expression was measured. The FoxO3a mRNA level was progressively increased in a dose-dependent fashion when miR-96 inhibitor was used, showing that miR-96 alteration in IPF fibroblasts also participates in FoxO3a suppression.

IPF fibroblasts utilize the PTEN/Akt-dependent axis to inhibit FoxO3a function when interacting with collagen matrix (28, 30, 41). Unlike control fibroblasts, when IPF fibroblasts attach to collagen-rich matrix, Akt activity remains high and causes FoxO3a suppression by phosphorylating the crucial serine 253 residue. In this study, we further revealed that miR-96 expression is inappropriately high in IPF fibroblasts on collagen, and this alteration is also linked to the suppression of FoxO3a mRNA (Fig. 9). Thus our results showed that when IPF fibroblasts attach to polymerized collagen, the posttranscriptional and the posttranslational FoxO3a inhibitory mechanism by both miR-96 and Akt is engaged for the maximum suppression of FoxO3a function. These findings strongly suggest that IPF fibroblasts utilize multiple mechanisms that maintain their proliferation and cell death resistance in response to collagen-rich matrix, and a comprehensive approach to target the posttranscriptional/translational machinery may be effective for FoxO3a modulation. Since the IPF fibroblasts we analyzed in this study were from patients with various biological backgrounds, we examined miR-96 and mRNA FoxO3a expression in randomly selected multiple IPF and control fibroblasts to confirm whether miR-96 expression is strictly increased in the majority of IPF fibroblast lines. We found that although biological variability exists in cell to cell lines, overall miR-96 and FoxO3a mRNA are inversely correlated with each other. miR-96 inhibition using various doses of inhibitor further demonstrates that miR-96 participates in the regulation of FoxO3a function. Several pieces of evidence suggest a potential clinical application of FoxO3a, and the prospect of FoxO3a as a therapeutic target has recently been highlighted (1, 11, 21, 35, 38). In fact, the strong link between FoxO3a alteration and the progression of diseases has been previously described (19, 27, 36, 43). It is well known that the deregulation of FoxO3a is linked to various types of cancer including breast cancer (11, 14, 38), prostate cancer (35, 36), glioblastoma (37), and leukemia (6, 21). Low levels of FoxO3a may link to chemotherapy resistance in liver cancer and FoxO3a appears to possess antitumor properties in hepatocellular carcinoma (31). Likewise, we also found that FoxO3a mRNA/protein expression as well as FoxO3a target proteins are aberrantly low in response to IPF fibroblast attachment to collagen. Similar to the results obtained with the cell-collagen matrix interaction model,
miR-96 expression is also found in within the fibroblastic foci. Thus our study further provides a possibility that FoxO3a alteration by miR-96 is also linked to a myofibroblast phenotype in response to collagen-rich matrix and that the posttranscriptional and posttranslational modification of FoxO3a is a crucial underlying mechanism to explain how IPF fibroblasts acquire such pathological properties.

In summary, our results revealed the additional underlying mechanism of FoxO3a suppression when IPF cells are attached to collagen. On the basis of our new findings, the orchestration of synergistic FoxO3a suppression plays an important role in promoting IPF fibroblasts to acquire a pathological IPF fibroblast phenotype. Our findings suggest that the FoxO3a-specific targeting approach may be beneficial in managing IPF, and as our knowledge for FoxO3a continuously develops the clinical application of FoxO3a may be potentially promising to limit the progression of IPF.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

R.S.N. and J.I. conception and design of research; R.S.N., J.I., and P.H. performed experiments; R.S.N., J.I., Y.-Y.H., and P.H. analyzed data; R.S.N., J.I., Y.-Y.H., and P.H. interpreted results of experiments; R.S.N., J.I., Y.-Y.H., and P.H. prepared figures; R.S.N., J.I., Y.-Y.H., and P.H. drafted manuscript; R.S.N., J.I., Y.-Y.H., and P.H. edited and revised manuscript; R.S.N., J.I., and Y.-Y.H. approved final version of manuscript.

Fig. 8. Increased miR-96 expression in the fibroblastic foci of IPF patient lung tissue. Top and middle: paraffin-embedded normal and IPF lung tissues (n = 3 each) were obtained, and in situ hybridization analysis was performed as described in MATERIALS AND METHODS. Arrows represent miR-96-positive cells within the fibroblastic foci of IPF lung tissue in the magnified images. Scale bar represents 50 μm. Red represents nuclei. Insets: 200 μm images of the fibroblastic foci in IPF lung tissues. Arrows in insets represent areas of the fibroblastic foci magnified. Bottom: miR-96 expression in normal lung alveoli. Scale bar represents 50 μm.

Fig. 9. Proposed model for the role of miR-96 in IPF fibroblasts on collagen-rich matrix. When IPF fibroblasts are cultured on polymerized collagen, miR-96 expression is enhanced, which facilitates the inhibition of FoxO3a mRNA and protein expression. This alteration subsequently suppresses FoxO3a target proteins, which bestows IPF fibroblasts to maintain a pathological IPF fibroblast phenotype.
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