miR-34a Inhibits Lung Fibrosis by Inducing Lung Fibroblast Senescence

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Abstract

Cellular senescence has been implicated in diverse pathologies. However, there is conflicting evidence regarding the role of this process in tissue fibrosis. Although dysregulation of microRNAs is a key mechanism in the pathogenesis of lung fibrosis, it is unclear whether microRNAs function by regulating cellular senescence in the disease. In this study, we found that miR-34a demonstrated greater expression in the lungs of patients with idiopathic pulmonary fibrosis and in mice with experimental pulmonary fibrosis, with its primary localization in lung fibroblasts. More importantly, miR-34a was up-regulated significantly in both human and mouse lung myofibroblasts. We found that mice with miR-34a ablation developed more severe pulmonary fibrosis than did wild-type animals after fibrotic lung injury. Mechanistically, we found that miR-34a induced a

senescent phenotype in lung fibroblasts because this microRNA increased senescence-associated β -galactosidase activity, enhanced expression of senescence markers, and decreased cell proliferative capacities. Consistently, we found that primary lung fibroblasts from fibrotic lungs of miR-34a-deficient mice had a diminished senescent phenotype and enhanced resistance to apoptosis as compared with those from wild-type animals. We also identified multiple miR-34a targets that likely mediated its activities in inducing senescence in lung fibroblasts. In conclusion, our data suggest that miR-34a functions through a negative feedback mechanism to restrain fibrotic response in the lungs by promoting senescence of pulmonary fibroblasts.

Keywords: lung fibrosis; microRNA; pulmonary fibroblast; cellular senescence

Interstitial lung diseases comprise a group of parenchymal lung disorders characterized by uncontrolled production of extracellular matrix (ECM) that results in thickening and scarring of the pulmonary interstitium (1–3). Idiopathic pulmonary fibrosis (IPF) is the most prevalent form of interstitial lung diseases, which is relentlessly progressive and usually leads to death within 2–3 years of diagnosis (1–4). Despite extensive investigation,

effective treatments for IPF remain disappointingly limited.

Cellular senescence is a process in which cells undergo stable cell cycle arrest and develop distinctive phenotypic features (5, 6). It was described originally as a state caused by finite proliferative capacity of normal human diploid fibroblasts in culture (7). Later studies demonstrated that this process could also be triggered by diverse stresses including

DNA damage, reactive oxygen species, oncogene activation, and excessive mitogenic stimulation (5, 6). Cellular senescence has been widely associated with organism aging and has been shown to play critical roles in many chronic diseases (5, 6, 8). However, there is also overwhelming evidence that this process is one of the most important antitumorigenic mechanisms and that it is essential to normal embryonic development (5, 6).

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Clinical Relevance

The role of cellular senescence in tissue fibrosis has been controversial, with both profibrotic and antifibrotic effects reported in the literature. We demonstrated that miR-34a functioned as a negative feedback checkpoint to restrain lung fibrosis by promoting lung fibroblast senescence. Our study provided important mechanistic input for clarifying the role of this cellular event in tissue fibrotic disorders. Our study is the first to demonstrate that specific microRNAs participate in the pathogenesis of pulmonary fibrosis by regulating the senescence of lung fibroblasts. Our study also establishes miR-34a as a novel therapeutic target for treating tissue fibrosis.

The role of cellular senescence in tissue fibrosis has been controversial. Although this process has been shown to be beneficial in tissue repair in models of liver and skin fibrosis, senescent cells are evidently present in the fibrotic lesions of many organs (9–14). We also found previously that prolonged senescence played a role in the failure of fibrosis resolution in aged mouse lungs (11). These seemingly incompatible discoveries reflect the functional complexity of cellular senescence in fibrosis regarding different cells, tissues, and ages under investigations.

microRNAs (miRNAs) are small noncoding RNAs, 20–25 nucleotides in length that play important roles in a variety of pathologies (15). Dysregulation of miRNAs has been shown to make major contributions to lung fibrosis by causing characteristic phenotypic alterations in discrete pulmonary cells, such as profibrotic polarization of alveolar macrophages, mesenchymal transition of lung epithelial cells, and myofibroblastic differentiation of lung fibroblasts (16–22). However, whether miRNAs regulate the senescence of pulmonary cells or how this potentially miRNA-modulated process participates in the pathogenesis of lung fibrosis has not been studied.

In this study, we found that miR-34a was up-regulated in fibrotic lungs, with a majority of its localization in lung fibroblasts. We found that miR-34a ablation aggravated bleomycin-induced lung

fibrosis in mice. We determined that, mechanistically, miR-34a promoted senescence of lung fibroblasts, which was diminished in the fibroblasts of the fibrotic lungs of miR-34a-deficient mice. We also identified multiple miR-34a targets that likely mediated its pro-senescence activities in lung fibroblasts. Our data suggest that miR-34a functions in a negative feedback loop to restrain lung fibrosis by promoting lung fibroblast senescence. Some of the results of this study have been reported previously in the form of an abstract (23).

Materials and Methods

Experimental Pulmonary Fibrosis Model

C57BL/6 mice (10–12 wk old) were purchased from the Jackson Laboratory (Bar Harbor, ME) and Charles River Laboratories (Wilmington, MA). miR-34a^{-/-} mice were from the Jackson Laboratory. The bleomycin-induced lung fibrosis model was described previously (16). The animal protocol was approved by the University of Alabama Institutional Animal Care and Use Committee.

Cell Lines

Human lung fibroblast line MRC-5 was purchased from American Type Culture Collection (Manassas, VA).

Human Lung Tissues

IPF lungs, histologically normal lungs of human patients with lung cancer, and failed donor lungs were obtained from the University of Alabama Tissue Procurement and Cell Culture Core. The protocol was approved by the institutional review board at the University of Alabama at Birmingham.

miRNA Transfection

Lung fibroblasts were transfected with miRNA mimics or inhibitors using HiPerFect Transfection Reagent (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Detailed information is provided (*see online supplement*).

Isolation of Primary Lung Fibroblasts and Epithelial Cells

Primary human or mouse lung fibroblasts and lung epithelial cells were harvested as described previously (*see online supplement*) (24, 25). This method has been performed routinely in our

laboratory and consistently yields lung epithelial cells with ~95% purity and lung fibroblasts with 100% purity.

Hydroxyproline Content Determination

Mouse right lungs were homogenized in 2 ml H₂O. One hundred milliliters of homogenates was mixed with 100 μ l 12N HCl, and the samples were incubated at 120°C for 3 hours. Hydroxyproline contents were then determined according to the manufacturer's instructions.

Masson's Trichrome Assay

The assay was performed using the Trichrome Stain (Masson) kit from Sigma-Aldrich (St. Louis, MO).

Immunohistochemistry

Immunohistochemistry was performed as described previously (16).

Quantitative Real-Time Polymerase Chain Reaction

miR-34a levels were determined by TaqMan MicroRNA Assay, and small nucleolar RNAs sno135 (mouse) and RNU48 (human) were used as internal references (Applied Biosystems, Foster City, CA). RNA levels of protein coding genes were determined by real-time polymerase chain reaction using the SYBR Green Master Mix Kit (Roche, Mannheim, Germany). Primer sequences are provided (*see online supplement*).

Western Blotting

Western blotting was performed as described previously (26). Mouse anti- α -tubulin, anti- β -actin, and anti- α -smooth muscle actin (α -SMA) antibodies were from Sigma. Rabbit anti-p21 was from Proteintech (Rosemont, IL). Rabbit anti-plasminogen activator inhibitor-1 (PAI-1) antibody was from Molecular Innovations (Novi, MI). Rabbit anti-cleaved caspase-3 antibody was from Cell Signaling (Danvers, MA). Mouse anti-fibronectin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-collagen 1 antibody was from SouthernBiotech (Birmingham, AL).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Staining

Apoptotic cells were detected by the ApopTagPlus Fluorescein In Situ Apoptosis Detection Kit according to the manufacturer's instructions.

Apoptosis Assay

Cells were collected and stained with fluorescein isothiocyanate-annexin V and propidium iodide according to the manufacturer's instructions. Positive cells were determined by flow cytometry.

Cellular Senescence Assay

Cellular senescence was evaluated by determining cellular senescence-associated β -galactosidase (SA- β -gal) activity with either the Cellular Senescence Assay Kit (Millipore, Temecula, CA) or the MarkerGene Cellular Senescence Microtiterplate Assay Kit (Marker Gene Technologies, Eugene, OR) according to the manufacturer's instructions.

Cell Proliferation Assay

Cell proliferative activity was determined with the BrdU Cell Proliferation Assay Kit

(Cell Signaling, Danvers, MA) according to the manufacturer's instructions.

Statistical Analysis

One-way analysis of variance, followed by the Bonferroni test, was used for multiple group comparisons. The Student's *t* test was used for comparison between two groups. $P < 0.05$ was considered statistically significant. (For detailed information on the methods and reagents used in this study, see online supplement.)

Results

miR-34a Is Up-Regulated in Fibrotic Lungs and Lung Myofibroblasts

We previously performed miRNA profiling on RNAs isolated from the lungs of mice

that were treated intratracheally with or without bleomycin, a widely used model for pulmonary fibrosis (16). In our continuing effort to delineate the role of up-regulated miRNAs in the pathogenesis of this disease, we became intrigued by miR-34a because this miRNA was demonstrated to be a tumor-suppressive miRNA that was capable of inducing cancer cell senescence (27–29), which plays profound roles in many pathophysiological processes (30). We initially performed real-time polymerase chain reaction assays and confirmed that miR-34a was significantly up-regulated in the lungs of mice that were treated with bleomycin (Figure 1A and Figure E1A in the online supplement). miR-34a also demonstrated greater expression in IPF lungs than in normal human lungs (Figure 1B),

Figure 1. miR-34a is up-regulated in fibrotic lungs and lung myofibroblasts. (A) C57BL/6 mice (10–12 wk old) were intratracheally instilled with bleomycin (1.5 U/kg body weight in 50 μ l saline). At Day 0, Day 14, and Day 21 after bleomycin treatment, mice were killed and lungs were collected. Levels of miR-34a were determined by real-time polymerase chain reaction (PCR) ($n = 3$; mean $\pm$ SEM). ** $P < 0.01$ compared with saline group. (B) miR-34a levels in normal lungs and idiopathic pulmonary fibrosis (IPF) lungs were determined by real-time PCR ($n = 10, 14$). (C) Primary lung epithelial cells and fibroblasts were isolated from lungs of mice. RNA was purified and the same amount of RNA was used for reverse-transcription reaction. Levels of miR-34a were then determined by real-time PCR, using mouse small nucleolar RNA Sno135 as the internal reference gene for the normalization of miR-34a levels in different pulmonary cells ($n = 3, 3$; mean $\pm$ SEM). * $P < 0.05$ compared with lung epithelial group. (D) Mice were treated intratracheally with saline or bleomycin. At Day 21 after bleomycin treatment, mice were killed and lungs were collected. Primary lung fibroblasts were isolated, and levels of miR-34a were determined by real-time PCR ($n = 4, 12$; mean $\pm$ SEM). * $P < 0.05$ compared with saline group. (E) miR-34a levels in IPF lung fibroblasts (average age, 63.1 ± 7.0 yr old) and those from age-matched normal subjects (average age, 63.8 ± 8.3 yr old) were determined by real-time PCR ($n = 12, 14$; mean $\pm$ SEM). ** $P < 0.01$ compared with normal control group. BLM, bleomycin; con, control; Fbs, fibroblasts; myo-Fbs, myofibroblasts.

suggesting that miR-34a up-regulation is a common phenomenon in lung fibrosis in both humans and mice.

To further characterize miR-34a expression in fibrotic lungs, we examined miR-34a levels in the respective pulmonary cells that are affected directly in this disease. Levels of miR-34a were markedly higher in lung fibroblasts than in lung epithelial cells of mouse lungs (Figure 1C). Consistent with the findings in total lungs, expression of miR-34a was also increased in myofibroblasts from fibrotic lungs compared with in lung fibroblasts from normal animals (Figure 1D and Figure E1B in the online

supplement). Of note, lung epithelial, but not alveolar macrophage, miR-34a was up-regulated at an early time point after bleomycin treatment (Figure E1B). Furthermore, we assessed miR-34a levels in IPF myofibroblasts and lung fibroblasts from age-matched normal subjects. We found that miR-34a was significantly up-regulated in these IPF cells (Figure 1E). As expected, fibroblasts from IPF lungs displayed a myofibroblastic and senescent phenotype reflected by greater expression of myofibroblasts marker α -SMA and the senescence marker p21 in these cells (Figures E2A and E2B). Taken together, these data suggest that miR-34a

participates in the regulation of phenotypic alterations of lung fibroblasts during the process of pulmonary fibrosis.

miR-34a Protects Mice from Experimental Lung Fibrosis

The finding of elevated miR-34a expression in fibroblasts of fibrotic lungs prompted us to question if miR-34a participated in the pathogenesis of pulmonary fibrosis. To test this hypothesis, we compared the severity of lung fibrosis in wild-type mice and in mice with miR-34 ablation (miR-34a^{-/-}) that were treated intratracheally with bleomycin. As shown in Figure 2A, there was significantly more

Figure 2. miR-34a protects mice from experimental lung fibrosis. Wild-type (WT) or miR-34a^{-/-} mice (10–12 wk old) were instilled intratracheally with saline or bleomycin (1.5 U/kg body weight in 50 μ l saline). Three weeks after bleomycin instillation, the mice were killed, and lungs were collected. (A) Hydroxyproline levels in the right lungs were determined ($n = 4, 11, 3$, and 16; mean $\pm$ SEM). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B) mRNA levels of collagen type I α 1 (Col1A1) and fibronectin (Fn) in bleomycin-treated lungs were determined by real-time PCR ($n = 5, 4$; mean $\pm$ SEM). *** $P < 0.001$ compared with bleomycin WT group. (C) Lung sections were prepared from above experiments. Hematoxylin and eosin (H&E) staining, Masson's trichrome staining, and immunohistochemistry assay for α -smooth muscle actin (α -SMA) were performed (original magnification $\times 20$). (D) Levels of miR-34a in the lungs of WT and miR-34a^{-/-} mice were determined by real-time PCR ($n = 5, 5$; mean $\pm$ SEM).

collagen deposition in the lungs of bleomycin-treated miR-34a^{-/-} mice than in the lungs of bleomycin-treated wild-type animals, as indicated by hydroxyproline levels in the respective lungs. Notably, the hydroxyproline levels in saline-treated wild-type and miR-34a^{-/-} mice were virtually the same (Figure 2A). The increased collagen content in the lungs of bleomycin-treated miR-34a^{-/-} mice was in accord with a greater expression of ECM genes, collagen I, and fibronectin in the lungs of these animals (Figure 2B). Furthermore, histological examination of lung sections, trichrome staining of collagen deposition, and *in situ* immunostaining of myofibroblast marker

α -SMA all confirmed markedly increased pulmonary fibrosis in the lungs of bleomycin-treated miR-34a^{-/-} mice (Figure 2C). As expected, there was no miR-34a expression in the lungs of miR-34a^{-/-} mice (Figure 2D). Given that miR-34a was up-regulated in fibroblasts from both IPF and mouse fibrotic lungs, these data suggest that miR-34a functions in a feedback mechanism to dampen pulmonary fibrosis.

miR-34a Does Not Affect Transforming Growth Factor- β 1-Induced Myofibroblastic Differentiation

To delineate the mechanism by which miR-34a protects mice from pulmonary

fibrosis, we investigated the interregulations between miR-34a and transforming growth factor (TGF)- β 1 in lung fibroblasts, primarily because TGF- β 1 is arguably the most important profibrotic molecule in tissue fibrosis (31). First, we found that although miR-34a was up-regulated in lung myofibroblasts, miR-34a expression remained unaltered during TGF- β 1-induced myofibroblastic differentiation of normal lung fibroblasts (Figure E3A). We then performed miR-34a overexpression or knockdown experiments by transfecting human lung fibroblasts with miR-34a mimics or inhibitors, followed by TGF- β 1 treatment. As shown in Figures E3B–E3D, miR-34a did not affect TGF- β 1-induced

Figure 3. miR-34a induces lung fibroblast senescence. (A–D) Human lung fibroblasts MRC-5 were transfected with 50 nM control mimics or mimics for miR-34a. Three days after transfection, senescence-associated β -galactosidase (SA- β -gal) activities in the cells were determined (A and B). Levels of p21, plasminogen activator inhibitor-1 (PAI-1), nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4), and hyaluronan synthase (HAS2) were determined by real-time PCR (C and D) ($n = 3$; mean $\pm$ SD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control mimics group. (E) MRC-5 cells were transfected with 50 nM control mimics or miR-34a mimics. Three days after transfection, cells were incubated with medium containing 5-bromo-2'-deoxyuridine (BrdU) for 6 hours. BrdU incorporation was determined ($n = 4$; mean $\pm$ SD). ** $P < 0.01$ compared with control miR group. (F) MRC-5 cells in 24-well plates were transfected with control mimics or miR-34a mimics. Cells were trypsinized and counted at the indicated time points ($n = 3$; mean $\pm$ SD). * $P < 0.05$, ** $P < 0.01$ compared with control miR group. con miR, control microRNA; OD, optical density.

myofibroblastic differentiation, as demonstrated by there being no obvious difference in TGF- β 1-activated expression of α -SMA and ECM molecules, fibronectin, and type 1 collagen between control and miR-34a-modified lung fibroblasts. These data suggest that the antifibrotic activity of miR-34a in mice is independent of TGF- β 1-induced lung myofibroblastic differentiation.

miR-34a Induces Lung Fibroblast Senescence

miR-34a was shown previously to regulate cancer cell senescence (27–29). Furthermore, cellular senescence has been implicated in the pathogenesis of tissue fibrosis (9–12). Given that miR-34a was up-regulated in the fibroblasts of bleomycin-treated mouse lungs and IPF lungs, we decided to examine whether miR-34a induced lung fibroblast senescence, and we tested whether this could be the mechanism behind the protection of mice from lung fibrosis afforded by miR-34a. To answer these questions, we transfected lung fibroblasts with control or miR-34a mimics and evaluated the cellular senescence status in these cells. We found that miR-34a overexpression induced cellular senescence, as shown by elevated SA- β -gal activity and increased expression of the senescence markers p21 and PAI-1 in miR-34a-transfected cells (Figures 3A–3C). In addition, we found that the expression of nicotinamide adenine dinucleotide phosphate oxidase 4, the up-regulation of which was shown previously in senescent IPF lung fibroblasts (11), was increased in lung fibroblasts transfected with miR-34a (Figure 3D). In contrast, hyaluronan synthase 2, the ablation of which was shown recently to induce lung fibroblast senescence (32), was down-regulated by miR-34a (Figure 3D). In further support of the pro-senescence activity of miR-34a, we found that miR-34a inhibited fibroblast proliferation, as indicated by decreased 5-bromo-2'-deoxyuridine incorporation, and that it slowed growth in miR-34a-transfected cells (Figures 3E–3F).

miR-34a Knockdown Inhibits Senescence of Lung Fibroblasts

Next, we investigated whether miR-34a knockdown could produce an effect opposite to that produced by miR-34a

overexpression on lung fibroblast senescence. We first confirmed previous studies by finding that low-dose bleomycin (0.01 U/ml) could induce senescence in lung fibroblasts (Figure 4A) (33). We found that miR-34a was markedly up-regulated by bleomycin in these cells (Figure 4B), suggesting an association of miR-34a induction with lung fibroblast senescence. More importantly, knockdown of miR-34a significantly diminished the bleomycin-induced senescent phenotype, indicated by lessened SA- β -gal activity and decreased expression of the senescence markers p21 and PAI-1

in miR-34a knockdown cells (Figures 4C–4F).

Fibroblast Senescence Is Attenuated in the Fibrotic Lungs of miR-34a^{-/-} Mice

Because we had found that miR-34a was able to induce fibroblast senescence *in vitro*, we next determined whether miR-34a had similar activity in the fibroblasts of mouse lungs. Lung fibroblasts were isolated from wild-type mice that were given saline or bleomycin, and expression of the senescence markers p21 and PAI-1 was evaluated. As shown in Figures 5A

Figure 4. miR-34a knockdown inhibits senescence of lung fibroblasts. (A and B) Human lung fibroblasts MRC-5 were treated with low-dose bleomycin (0.01 U/ml) for 2 days. (A) SA- β -gal activities in the cells were measured, and (B) levels of miR-34a were determined by real-time PCR ($n = 3$; mean $\pm$ SD). ** $P < 0.01$ compared with control group. (C–F) MRC-5 cells were transfected with 50 nM control inhibitors or inhibitors against miR-34a. Two days after transfection, cells were treated with PBS or bleomycin (0.01 U/ml) for 24 hours. Levels of (C) miR-34a, (E) p21, and (F) PAI-1 were determined by real-time PCR. (D) SA- β -gal activities in the cells were measured ($n = 3$; mean $\pm$ SD). * $P < 0.05$, ** $P < 0.01$ compared with PBS anti-con group; # $P < 0.05$, ## $P < 0.01$ compared with bleomycin anti-con group.

and 5B), lung fibroblasts from bleomycin-treated mice demonstrated increased senescent phenotype, as indicated by greater expression of p21 and PAI-1 in these cells compared with their expression in lung fibroblasts from saline-treated animals. Given that lung fibroblasts are the major source of fibrotic matrix, and given the fact that the senescent phenotype is also characterized by arrested cell proliferation, the elevated senescent phenotype demonstrated by pulmonary fibroblasts from fibrotic lungs suggests that fibroblast senescence is a beneficial mechanism that protects animals from developing fibrosis in response to profibrotic injury. In support of this hypothesis, we found that the senescent phenotype was diminished significantly in lung fibroblasts from bleomycin-treated miR-34a^{-/-} mice, as compared with that in lung fibroblasts from bleomycin-treated wild-type animals (Figure 5C). In addition, we found that lung fibroblasts from bleomycin-treated mice demonstrated increased p53 expression (Figure E4),

concordant with the up-regulation of its target p21 in these cells. Furthermore, p53 elevation was attenuated in lung fibroblasts from bleomycin-treated miR-34a^{-/-} mice (Figure E4), which was also consistent with the diminished senescent phenotype observed in these cells (Figure 5C). More significantly, these data were in accord with the more severe lung fibrosis found in bleomycin-treated miR-34a^{-/-} mice, compared with that found in the wild-type animals (Figures 2A–2C).

miR-34a Promotes Lung Fibroblast Apoptosis

We continued to characterize the functional significance of the miR-34a-induced lung fibroblast senescence. We found that miR-34a sensitized lung fibroblasts to staurosporine-induced apoptosis, as shown by greater pro-caspase-3 cleavage and increased annexin V/propidium iodide-positive staining in staurosporine-treated miR-34a-transfected cells as compared with those in staurosporine-treated control

fibroblasts (Figures 6A and 6B). In contrast, staurosporine-induced fibroblast apoptosis was diminished by miR-34a knockdown (Figure 6C). Furthermore, primary lung fibroblasts from miR-34a^{-/-} mice were more resistant to staurosporine-induced apoptosis than were primary lung fibroblasts from wild-type animals (Figure 6D). Given that fibroblast apoptosis is believed to be beneficial in alleviating lung fibrosis (34), these data suggest that miR-34a protects mice from lung fibrosis by promoting lung fibroblast apoptosis and thus eliminating the source of fibrotic matrix-producing myofibroblasts. We found that, in accord with this hypothesis, cell apoptosis was diminished in the lungs of bleomycin-treated miR-34a^{-/-} mice compared with that in the lungs of bleomycin-treated wild-type animals (Figures 6E and 6F).

miR-34a Targets Multiple Genes that Regulate Cellular Senescence

We found that miR-34a promoted cellular senescence in lung fibroblasts. We next examined the expression of miR-34a targets that potentially mediated these effects. As shown in Figure 7A, there was decreased expression of multiple genes that are involved in senescent phenotypic alterations, such as Sirt1, cyclin E2, cyclin D1, and E2F3, in lung fibroblasts that were transfected with miR-34a. In contrast, miR-34a knockdown significantly enhanced the endogenous expression of these targets in lung fibroblasts (Figure 7B). More importantly, there was a general trend toward enhanced expression of these genes in fibroblasts of fibrotic lungs of miR-34a^{-/-} mice as compared with that of their respective wild-type counterparts (Figure 7C).

Discussion

Since it was first described in the late 1950s, cellular senescence has been associated with organism aging (8). However, it was not until recently that the causal link between these two events was firmly established (30, 35, 36). Cellular senescence has been recognized generally as a harmful process because senescent cells are identified readily in the lesions of many chronic diseases, and elimination of these cells apparently improves fitness in animals. However, there is also compelling

Figure 5. Fibroblast senescence is attenuated in the fibrotic lungs of miR-34a^{-/-} mice. (A and B) WT mice (10–12 wk old) were instilled intratracheally with bleomycin (1.5 U/kg body weight in 50 μ l saline). Three weeks after bleomycin treatment, the lungs were collected, and primary lung fibroblasts were isolated. (A) mRNA levels of p21 and PAI-1 were determined by real-time PCR and (B) protein levels by Western blotting, respectively ($n = 5, 5$; mean $\pm$ SEM). ** $P < 0.01$, *** $P < 0.001$ compared with saline group. (C) WT and miR-34a^{-/-} mice were treated intratracheally with bleomycin as in A and B. Three weeks after bleomycin treatment, the lungs were collected, and primary lung fibroblasts were isolated. Protein levels of p21 and PAI-1 were determined by Western blotting, and densitometric analyses were performed using ImageJ. * $P < 0.05$ compared with WT bleomycin group.

Figure 6. miR-34a promotes lung fibroblast apoptosis. (A and B) Human lung fibroblasts MRC-5 were transfected with 50 nM control mimics or mimics for miR-34a. Two days after transfection, the cells were treated with vehicle DMSO or staurosporine (Stau) (200 nM). Nine hours after treatment, the cells were collected, and (A) cleaved caspase-3 was determined by Western blotting and (B) annexin V/propidium iodide staining was analyzed by flow cytometry ($n=3$; mean $\pm$ SD). $^{**}P < 0.01$ compared with DMSO control miR group; $^{##}P < 0.01$ compared with Stau control miR group. (C) MRC-5 cells were transfected with 50 nM control inhibitors or inhibitors against miR-34a. Two days after transfection, the cells were treated with DMSO or Stau (200 nM) as in B, and annexin V/propidium iodide assay was performed ($n=3$; mean $\pm$ SD). $^{***}P < 0.001$ compared with DMSO anti-con group; $^{##}P < 0.01$ compared with Stau anti-con group. (D) Primary lung fibroblasts were isolated from WT and miR-34a^{-/-} mice (10–12 wk old). Cells were treated with or without Stau (300 nM) for 6 hours. Annexin V staining, followed by flow cytometry, was performed ($n=5$; mean $\pm$ SEM). $^{*}P < 0.05$ compared with the WT group; $^{#}P < 0.05$ compared with the WT+ group. (E and F) WT and miR-34a^{-/-} mice were treated intratracheally with bleomycin (1.5 U/kg body weight in 50 μ l saline). Three weeks after instillation, the mice were killed, and lung sections were prepared. (E) Cell apoptosis in the lungs was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (green fluorescence). (F) The number of TUNEL+ cells was counted (original magnification $\times 20$). $^{**}P < 0.01$ compared with bleomycin WT group.

Figure 7. miR-34a targets multiple genes that regulate cellular senescence. (A) MRC-5 cells were transfected with control mimics or miR-34a mimics. Two days after transfection, the levels of Sirt1, cyclin E2, cyclin D1, and E2F3 were determined by real-time PCR ($n = 4$; mean $\pm$ SD). * $P < 0.05$, ** $P < 0.01$ compared with control miR group. (B) MRC-5 cells were transfected with control inhibitors or inhibitors against miR-34a. Three days after transfection, levels of Sirt1, cyclin E2, cyclin D1, and E2F3 were determined by real-time PCR ($n = 3$; mean $\pm$ SD). * $P < 0.05$ compared with anti-con group. (C) WT and miR-34a^{-/-} mice (10–12 wk old) were instilled intratracheally with bleomycin. Three weeks after treatment, the lung fibroblasts were harvested. Levels of Sirt1, cyclin E2, and cyclin D1 were determined by real-time PCR ($n = 3$, 3 for Sirt1 and cyclin E2; $n = 7$, 6 for cyclin D1). * $P < 0.05$ compared with bleomycin WT control. E2F3, E2F transcription factor 3; sirt1, sirtuin1.

evidence demonstrating that senescence and apoptosis constitute the two most important tumor-suppressive mechanisms (5, 6, 30, 37, 38). These studies suggest that the functional consequence of cellular senescence is clearly context contingent.

A similar complexity has also been reflected in the role of this cellular process in tissue fibrosis (9–12, 39). For example, although it was shown convincingly that fibroblast senescence functioned to restrain fibrosis during cutaneous wounding healing and liver fibrosis, our previous studies also demonstrated that senescent pulmonary fibroblasts were more resistant to apoptosis, a phenotype that was likely associated with the failed resolution of lung fibrosis in aged animals (9–11, 40). These seemingly paradoxical findings will

create serious hurdles in the further delineation of the precise role of cellular senescence in tissue fibrotic disorders.

It has been recognized that there exist three types of senescence: acute, embryonic, and chronic (8, 30). Although they all exhibit common phenotypic features, they may have subtle differences that dictate their roles in a unique context. miR-34a-induced senescence of lung fibroblasts in pulmonary fibrosis, which is also known as stress-induced senescence, appears to be an acute response because the comparable average age between subjects with IPF and normal subjects negates an age-associated miR-34a up-regulation in these diseased lung fibroblasts. Stress-induced senescence has been found to be beneficial in general in many pathologies

(8, 30). Chronic cellular senescence associated with aging is otherwise detrimental and has been implicated in the development of age-related diseases (8, 30). This difference may account for the conflicting role of cellular senescence in different models of tissue fibrosis. On another note, it is possible that senescence of pulmonary cells other than lung fibroblasts, such as alveolar epithelial cells and endothelial cells, does play a detrimental role in the initiation and progression of lung fibrosis. Therefore, the role of miR-34a in the senescence of those pulmonary cells and how the potentially miR-34a-modulated process regulates response to fibrotic injury are interesting open questions that are worth further investigations.

As a further on this ambiguity, although the findings regarding the role of fibroblast senescence in the pathogenesis of organ fibrosis were at times conflicting, we would like to stress that most of the evidence presented thus far was not established causally. These shortcomings call for studies to put it under direct test by using approaches to manipulate the senescence phenotype itself, for example, to eliminate senescent fibroblasts or inactivate master senescence regulators such as p16, p21, and p53, during the pathogenesis of this disease. Only then will the role of this fundamental cellular process in fibrosis be much better understood.

In this study, we showed that miR-34a induced lung fibroblast senescence. We also found that miR-34a was capable of promoting lung fibroblast apoptosis. However, it should be noted that these findings do not necessarily suggest a correlation between miR-34a-induced senescence and enhanced cell death. In addition, they contrast with the common assumption that senescent cells are refractory to apoptosis. Despite these arguments, the possibility still exists that miR-34a generates some noncanonical signaling that facilitates mutual propagation of cell senescence and death.

We found that miR-34a was neither regulated by TGF- β 1 nor did it modulate TGF- β 1 profibrotic activity in lung fibroblasts, which contrasts with the features of most miRNAs that have been identified thus far to be involved in the pathogenesis of lung fibrosis (18, 21, 41). This study and previous investigations suggest that the activities of this special

class of noncoding RNAs are astonishingly complex. However, the functional diversity of miRNAs also presents additional challenges for targeting these molecules to achieve maximal therapeutic efficacy without causing excessive unfavorable side effects.

Although we showed that senescence of lung fibroblasts protects mice from pulmonary fibrosis by limiting proliferation and promoting apoptosis of these cells, thereby reducing the primary source of fibrotic matrix-making myofibroblasts, there are likely other mechanisms by which this cellular process could exert beneficial effects in the pathology. One hypothesis is that soluble factors produced by senescent lung fibroblasts, often recognized as senescence-associated secretory phenotype (SASP) (42), which include a number of proinflammatory cytokines, could skew the profibrotic M2 phenotype of lung macrophages to an antifibrotic M1 state (43). Indeed, it has been shown previously that reprogramming lung macrophages to the M1 phenotype diminishes experimental lung fibrosis in mice (44, 45). In addition, proteinases such as matrix metalloproteinases are also major components of SASP (40, 42). These

enzymatic activities could also help break down the collagenous matrix in fibrotic lungs. Our group is currently investigating these hypotheses by examining the SASP of lung fibroblasts from bleomycin-treated wild-type and miR-34a^{-/-} mice and testing their biological activities. We also want to point out that SASP may also contain a profibrotic mediator such as TGF- β 1, which could add complexity to the effect of senescent cells in context-specific settings.

We acknowledge that the increased miR-34a expression in IPF fibroblasts seems not readily compatible with a beneficial role in lung fibrosis. Therefore, although the number of IPF fibroblast lines was inadequate statistically to be stratified for further analysis, it remains relevant to determine whether miR-34a levels are associated with some other clinical parameters in IPF-like progression and exacerbation.

We identified multiple miR-34a targets that might mediate its activity in inducing lung fibroblast senescence. However, we must acknowledge that most of these regulations were modest. Although this is true for most other miRNAs, particularly regarding their activities *in vivo*, we must

caution against a conclusion that the effect of miR-34a on lung fibrosis is mediated exclusively by one or more of these targets. It is likely that additional mediators are involved and may be just the collective moderate effects from multiple targets that ultimately produce the senescence-inducing activity of miR-34a in lung fibroblasts.

Conclusions

In conclusion, we found that miR-34a was up-regulated in the lung fibroblasts of fibrotic lungs in both humans and mice. We found that miR-34a-deficient mice developed more severe lung fibrosis than did their wild-type counterparts. Mechanistically, miR-34a functioned as a negative checkpoint to dampen the fibrotic response by promoting senescence of lung fibroblasts. To our knowledge, our study provides the first evidence that miRNA serves as a functional link between cellular senescence and lung fibrosis. More importantly, our study establishes miR-34a as a novel therapeutic target for the treatment of fibrotic diseases, including pulmonary fibrosis. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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