Yin Yang 1 Is a Novel Regulator of Pulmonary Fibrosis

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Rationale: The differentiation of fibroblasts into myofibroblasts is a cardinal feature of idiopathic pulmonary fibrosis (IPF). The transcription factor Yin Yang 1 (YY1) plays a role in the proliferation and differentiation of diverse cell types, but its role in fibrotic lung diseases is not known.

Objectives: To elucidate the mechanism by which YY1 regulates fibroblast differentiation and lung fibrosis.

Methods: Lung fibroblasts were cultured with transforming growth factor (TGF)- β or tumor necrosis factor- α . Nuclear factor (NF)- κ B, YY1, and α -smooth muscle actin (SMA) were determined in protein, mRNA, and promoter reporter level. Lung fibroblasts and lung fibrosis were assessed in a partial YY1-deficient mouse and a YY1^{+/f} conditional knockout mouse after being exposed to silica or bleomycin.

Measurements and Main Results: TGF- β and tumor necrosis factor- α up-regulated YY1 expression in lung fibroblasts. TGF- β -induced YY1 expression was dramatically decreased by an inhibitor of NF- κ B, which blocked I- κ B degradation. YY1 is significantly overexpressed in both human IPF and murine models of lung fibrosis, including in the aggregated pulmonary fibroblasts of fibrotic foci. Furthermore, the mechanism of fibrogenesis is that YY1 can up-regulate α -SMA expression in pulmonary fibroblasts. YY1-deficient (YY1^{-/-}) mice were significantly protected from lung fibrosis, which was associated with attenuated α -SMA and collagen expression. Finally, decreasing YY1 expression through instilled adenovirus-cre in floxed-YY1^{+/f} mice reduced lung fibrosis.

Conclusions: YY1 is overexpressed in fibroblasts in both human IPF and murine models in a NF- κ B-dependent manner, and YY1 regulates fibrogenesis at least in part by increasing α -SMA and collagen expression. Decreasing YY1 expression may provide a new therapeutic strategy for pulmonary fibrosis.

Keywords: nuclear factor- κ B; α -smooth muscle actin; idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a progressive, chronic interstitial lung disease associated with high mortality (median survival of newly diagnosed patients is $\sim$ 3 yr) and a uniformly poor prognosis (1). IPF is the pathologic hallmark of interstitial lung diseases and is characterized by increased deposition of

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Lung fibrosis including human idiopathic pulmonary fibrosis (IPF) is caused by accumulation of myofibroblasts in injured lung. Myofibroblasts are derived from three sources: (1) proliferation and differentiation of fibroblasts, (2) epithelial mesenchymal transition, and (3) fibrocytes from circulation. However, the mechanism of accumulation of myofibroblasts in injured lung is unknown.

What This Study Adds to the Field

We found that a transcription factor, Yin Yang 1, plays an important role in fibroblast differentiation in lung fibrosis. Decreasing YY1 expression can inhibit differentiation of fibroblasts mediated by nuclear factor- κ B and can decrease lung fibrosis in silica- and bleomycin-treated mice.

extracellular matrix proteins including collagen. This lethal lung disorder presents major clinical challenges, because effective therapeutic agents for reversing lung fibrosis have not yet been discovered (2). One current hypothesis is that IPF represents a chronic injury and repair response to specific environmental insults, such as silica or asbestos. However, the precise molecular mechanisms underlying persistent fibroblast activation remain poorly understood.

After inhalation of profibrotic stimuli (e.g., asbestos, silica, or bleomycin), alveolar macrophages and neutrophils produce cytokines including transforming growth factor (TGF)- β 1 and tumor necrosis factor (TNF)- α that contribute to lung inflammation and fibrosis by different mechanisms (3, 4). TGF- β plays an essential role in wound healing and matrix molecule deposition. It induces myofibroblast differentiation and alveolar remodeling *in vivo* (5), and overexpression of this potent profibrotic mediator leads to progressive fibrosis in mice, with minimal inflammation (6). TNF- α also contributes to lung fibrosis (7), and its effects may be mediated through activation of other growth factors. For example, TNF- α -deficient mice are protected against bleomycin-induced lung inflammation via reduced apoptosis of inflammatory cells (8). Inhibition of TNF- α with infliximab may stabilize the progression of pulmonary fibrosis associated with collagen vascular disease (9).

Myofibroblasts are derived from epithelial to mesenchymal transition (EMT) (10), circulating fibrocytes (11), or resident fibroblasts, in response to lung injury or chronic inflammation induced by stimuli, such as silica and bleomycin, and are now recognized as major effector cells in pulmonary fibrosis. They are characterized by the expression of α -smooth muscle actin (α -SMA), enhanced proliferation, and synthesis of extracellular matrix proteins (12), and are thought to be derived from fibroblasts via the activity of TGF- β and other stimuli (13). In pulmonary fibrosis, myofibroblasts acquire resistance to apoptosis, which may account for the increased number of

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these cells present in fibroblastic foci (14). These myofibroblasts accumulate in the injured lung and block alveolar gas exchange. Preventing the formation of myofibroblasts would provide protection against lung fibrosis, and this could be achieved by inhibition of α -SMA expression in lung fibroblasts (15).

Yin Yang 1 (YY1) is a ubiquitously expressed zinc finger transcription factor that can either activate or repress gene transcription, and plays an important role in cellular proliferation, differentiation, and apoptosis. Growing evidence indicates that YY1 contributes to the pathogenesis of cancer and inflammation (16). TNF- α -induced YY1 represses Fas expression, providing a mechanism whereby YY1 contributes to TNF- α -induced cell survival (17). In fibroblasts, TNF- α induces YY1 in a nuclear factor (NF)- κ B-dependent manner (18), supporting a link between the NF- κ B pathway and YY1 expression (17). YY1 can bind to and activate the type I and type II collagen and α -SMA gene promoters in fibroblasts (19–21). Although these reports suggest that YY1 may play a role in fibrotic responses, very little is known about the expression or function of YY1 in fibrotic conditions *in vivo*.

Embryonic fibroblasts from YY1-deficient mice demonstrated reduced proliferation *in vitro* in proportion to the levels of YY1 protein expression. This finding indicates that YY1 controls fibroblast proliferation in a gene dosage-dependent manner (22). When designing the present study, we considered that YY1 overexpression is associated with hyperproliferation and increased differentiation of cells, and that YY1 can regulate collagen gene expression (23). Therefore, we investigated the role of YY1 in fibrotic lung diseases using tissues and cells from human subjects. Here we report that YY1 expression is markedly increased in lung tissue obtained from human subjects with IPF and in two mouse models of lung fibrosis. Using loss-of-function strategies, we show that YY1 plays an important and previously underappreciated role in lung fibrosis by regulating fibroblast activation and differentiation. Some of the results of these studies have been previously reported in the form of an abstract (24).

METHODS

Cell line, Animals, and Silica or Bleomycin Administration

The WI-38 and LL97A human fibroblast lines were purchased from ATCC (Manassas, VA). 293FT cells were obtained from Invitrogen (Carlsbad, CA). YY1 heterozygotes (YY1^{+/-}) and conditional knockout YY1 (YY1^{ff}) mice were obtained from Y. Shi (Harvard University, Cambridge, MA). All animals were grouped according to age (6–12 wk) or weight (20–35 g). YY1^{+/-} mice were on a C57BL/6 background (≥ 6 generations). YY1^{ff} mice were on a C57BL/6 background (four generations). These mice received 200–300 μ g of silica or three units of bleomycin per kg of body weight by intratracheal injection. All animals were housed under specific pathogen-free conditions at the National Institutes of Health (NIH) in an American Association for the Accreditation of Laboratory Animal Care-approved facility. The University of Rochester Medical Center animal care and use committee approved all experimental procedures.

Histopathologic and Immunohistochemical Examination

Lungs were fixed with 10% buffered formalin and embedded in paraffin. Hematoxylin and eosin and Masson's trichrome stains were used for analysis of pathologic changes. Immunohistochemical staining for α -SMA and YY1 tissues was performed according to a previous paper (25). The severity of fibrosis was evaluated in stained sections by an individual who was blinded to the genotypes of the mice. For area analysis of fibrotic changes, a quantitative fibrotic scale (Ashcroft scale) was used (26) based on hematoxylin and eosin

staining. A numerical fibrotic score (Ashcroft scale) was obtained as follows: the severity of the fibrotic changes in each lung section was given as the mean score from the observed microscopic fields. Twenty fields within each lung section were observed at a magnification of $\times 10$, and each field was assessed individually for fibrotic severity and allotted a score from 0 (normal) to 8 (total fibrosis). The severity score for each field was averaged and presented as the average for each lung section. To avoid bias, all histologic specimens were evaluated in a blinded fashion. Each specimen was scored independently by three observers and the mean of their individual scores was taken as the fibrotic score.

Western Blot Analysis and Quantitative Polymerase Chain Reaction

Cells or lung tissues were solubilized in lysis buffer. Western blot, mRNA extract, and quantitative polymerase chain reaction (PCR) were performed according to previous protocols (27). Data were analyzed using NIH Image J software or by the $2^{-\Delta(\Delta C_T)}$ method and normalized to the expression of the GAPDH housekeeping gene. The sequences of the primers are listed in Table E1 (see online supplement).

Luciferase Assay and Construction of YY1 and α -SMA Luciferase Reporter

Plasmids containing the wild-type YY1 promoter (or a YY1 promoter with a mutant NF- κ B binding site) driving a luciferase reporter gene were kindly presented by Dr. Denis C. Guttridge (Ohio State University). Transiently transfection protocol was the same as a previous paper (28). We transiently transfected the reporter constructs into WI38 cells, which were then stimulated with TGF- β for 24 hours or with TNF- α for 6 hours. Using the same procedure, we cotransfected a p65 (NF- κ B) plasmid with wild-type or mutated YY1 promoter constructs. Construction of α -SMA luciferase reporter is shown in the online supplement.

Immunofluorescent Staining and Lentiviral shRNA, Lentiviral-cre, and Adenoviral-cre

LL97A cells, derived from human fibroblasts obtained from patients with IPF, were transduced with YY1 shRNA, and were plated in four-well chamber slides. Cells were supplemented with Dulbecco's modified Eagle medium and 10% fetal bovine serum at 37°C for 4 days. Anti- α -SMA antibody (1A4; DAKO Cytomation, Carpinteria, CA), anti-collagen (LF-67; Larry W. Fisher, Ph.D., Matrix Biochemistry Section, Craniofacial and Skeletal Diseases Branch of NIH), and anti-YY1 antibody (H-414; Santa Cruz, Santa Cruz, CA) were stained according to a previous paper (25). The cells were examined with a Zeiss fluorescence microscope. Package and transduction of lentiviral shRNA and adenoviral-cre are listed in the online supplement.

Statistical Analysis

Quantitative PCR, YY1 promoter reporter assay, and histologic score of lung fibrosis were analyzed for statistical significance by using the paired Student *t* test using Microsoft Excel software. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

YY1 Is Up-Regulated by TGF- β and TNF- α in Lung Fibroblasts

Lung fibrosis results from proliferation and differentiation of fibroblasts in response to profibrotic cytokines, such as TGF- β and TNF- α . To test the possibility that these cytokines induce YY1 expression, we grew a human lung fibroblast line (WI38) *in vitro* and exposed cells to TGF- β for different times. We found that YY1 expression was up-regulated by TGF- β , as demonstrated by Western blot (Figure 1A) and quantitative PCR (Figure 1B). Using the same protocol as TGF- β , we determined that YY1 protein and mRNA expression are also increased by

TNF- α stimulation in WI38 cells (Figures 1C and 1D). Taken together, these data indicate that YY1 is up-regulated by the profibrotic cytokines TGF- β and TNF- α in human lung fibroblasts. Next we investigated the mechanisms by which YY1 is up-regulated by these cytokines.

NF- κ B Mediates YY1 Expression in Lung Fibroblasts after TGF- β Stimulation

Recent studies have found that TNF- α can up-regulate YY1 expression through NF- κ B activation in tumor cells (17). To determine whether TGF- β induces YY1 expression through NF- κ B in lung fibroblasts, first we evaluated if NF- κ B was activated by TGF- β . Fibroblasts (WI38) were stimulated with TGF- β after being serum starved for 24 hours. NF- κ B and phospho I- κ B expression were determined by immunoblot. We found that NF- κ B and phospho I- κ B were up-regulated by TGF- β in fibroblasts, beginning at 4 hours (Figure 2A). NF- κ B can up-regulate YY1 expression by directly binding to the YY1 promoter in cancer cells (18). Second, we used an I- κ B inhibitor (Bay11-7085), which blocks I- κ B degradation and quenches NF- κ B activity. We found that incubation with Bay11-7085 abolished TGF- β -induced YY1 expression (Figure 2B). The quantitative data of Figure 2B is shown in Figure E5A. Next, we studied TGF- β -induced YY1 promoter activity with a wild-type YY1 promoter reporter construct and a construct in which a putative NF- κ B binding site was mutated (18). Transfected cells were stimulated with TGF- β , and reporter gene activity was analyzed by luminometry. Interestingly, YY1 promoter activity was significantly increased in lung fibroblasts in response to both TGF- β stimulation and cotransfection with the NF- κ B subunit p65 (RelA; Figure 2C). We found that disrup-

Figure 1. Transforming growth factor (TGF)- β or tumor necrosis factor (TNF)- α induces Yin Yang 1 (YY1) protein expression and mRNA transcription in lung fibroblasts. After serum starvation for 24 hours, WI38 cells were treated with 10 ng/ml of TGF- β (A) or TNF- α (C) for the indicated times in serum-free Dulbecco's modified Eagle medium. Extracts from whole cells were subjected to immunoblot analysis with anti-YY1 or anti- β -actin antibody. The mRNA expression of YY1 was detected by quantitative polymerase chain reaction with TGF- β (B) or TNF- α (D) stimulation. The data are presented with standard errors derived from at least three independent experiments performed in triplicate. * P < 0.05 and ** P < 0.01 by the Student *t* test.

tion of a potential NF- κ B binding site on the YY1 promoter inhibited the enhancing effect of NF- κ B, suggesting that this involves direct binding to the YY1 promoter (Figure 2D). Together, these findings suggest that the pathway leading to YY1 up-regulation by TGF- β is mediated through NF- κ B in lung fibroblasts.

YY1 Expression Is Increased in Human IPF and in Fibrotic Mouse Models

We next wanted to determine whether YY1 is induced in human IPF and in mouse models of lung fibrosis. To address these possibilities, we used immunohistochemistry (IHC) and immunoblot assays separately performed with anti-YY1 and anti-fibroblast-specific protein (FSP) 1 antibodies. In lung sections from humans with IPF, we conducted IHC staining with anti-YY1 or anti-FSP1 antibodies, and found that YY1 was overexpressed together with the fibroblast marker FSP1 (Figure 3A). Double staining of the IPF lung in Figure 3A with anti-YY1 and anti-FSP1 antibodies is shown in Figure E3C and confirms

Figure 2. Yin Yang 1 (YY1) overexpressed in lung fibroblasts by transforming growth factor (TGF)- β is mediated by nuclear factor (NF)- κ B. The fibroblasts (WI38) were treated with 10 ng/ml of TGF- β for the indicated time in serum-free Dulbecco's modified Eagle medium. Phosphorylated I- κ B and NF- κ B were determined by immunoblot (A). WI38 cells were pretreated with 5 μ M of NF- κ B inhibitor (Bay 11-7085) for 1 hour. They were then treated with 10 ng/ml of TGF- β for 8 hours in serum-free Dulbecco's modified Eagle medium. Extracts from whole cells were immunoblotted with anti-YY1 or anti- β -actin antibody (B). WI38 were transiently cotransfected with a vector control (pSG5) or 2.5 μ g of NF- κ B (p65) plasmid, 6 μ g of wild-type YY1 promoter, and mutant YY1 promoter constructs by electroporation and were then treated with phosphate-buffered saline or 10 ng/ml of TGF- β for 24 hours. Wild-type YY1 promoter reporter activity was induced by cotransfection with NF- κ B (p65) (C). Activity of a YY1 promoter construct with a disrupted NF- κ B binding site (Mutant-YY1-LUC) was decreased in WI38 cells (D). Data are presented with standard errors derived from at least three independent experiments performed in triplicate. ** P < 0.01 or *** P < 0.001 (untreated pSG5 vs. untreated p65; TGF- β -treated pSG5 vs. TGF- β -treated p65, and compared as untreated or treated cells transfected with YY1 or p65 alone or combination). All reporter data were normalized by empty vector control. Relative light units (RLU) data are shown from at least three independent experiments performed in triplicate.

Figure 3. Yin Yang 1 (YY1) protein expression is increased in the lungs of patients with idiopathic pulmonary fibrosis (IPF) and silica- and bleomycin-challenged mice. Hematoxylin and eosin staining shows that highly inflammatory lung tissue was found in IPF and silica- and bleomycin-induced pulmonary fibrosis. Immunohistochemistry (IHC) staining was used to identify the expression of YY1 and fibroblast-specific protein 1 (FSP1) in lungs from human subjects with IPF ($n = 5$) separately (A). YY1 expression was near and in FSP1-positive cells. IHC was used to identify the expression of YY1 in lung tissues from silica-treated C57Bl6 mice ($n = 8$ mice per group) (D), and from bleomycin-treated C57Bl6 mice ($n = 5$ mice per group) (G). α -Smooth muscle actin (SMA), YY1 and FSP1 expression were determined. These are compared with normal subjects or saline-treated C57Bl6 mice, respectively. Representative samples obtained from patients, silica-treated mice, and bleomycin-treated mice are shown. Pictures are obtained at the original magnification of $\times 63$. Extracts from whole lung tissues were subjected to immunoblotting with anti-YY1 and anti- β -actin antibodies. YY1 expression is up-regulated in whole lung tissue lysis of patients with IPF (B), and silica- (E) and bleomycin-induced fibrosis in mice (H). YY1 transcription level is up-regulated in whole lung tissues of IPF, and silica- and bleomycin-induced fibrosis. The mRNA expression of YY1 in lung tissue derived from IPF subjects ($n = 5$) (C), silica-treated mice (F, samples are same as D), and bleomycin-treated mice (I, samples are same as G) were assessed by quantitative polymerase chain reaction. * P < 0.05 or ** P < 0.01 by the Student t test. Exposures were repeated three times.

YY1 overexpression in FSP1-positive fibroblasts. Furthermore, lung tissues from patients with IPF were homogenized and YY1 protein and mRNA were determined by Western blot and quantitative PCR, respectively. Both YY1 protein (Figure 3B) and mRNA (Figure 3C) from lungs of patients with IPF were increased. These data show that YY1 is increased in lung tissue and lung fibroblasts from human subjects with IPF.

Silica and bleomycin are widely used to induce lung fibrosis in mice, and act via different mechanisms. We next investigated whether YY1 expression was also up-regulated in these fibrotic models. Wild-type mice were exposed to silica, bleomycin, or saline control using standard approaches (see METHODS), and lungs were excised and analyzed 21 days later using IHC. Interestingly, YY1 overexpression was localized to FSP1- and α -SMA-positive cells in both silica- (Figure 3D) and bleomycin-induced lung fibrosis (Figure 3G). Magnification $\times 10$ of the lung in Figures 3D and 3G is shown as supplementary data (Figures E2A and E2B). Immunoblot analysis confirmed that total YY1 expression was up-regulated in both silica (Figure 3E) and bleomycin mouse models (Figure 3H). Using quantitative PCR, we also found that YY1 transcript levels were increased both in silica- (Figure 3F) and bleomycin-exposed

mice (Figure 3I). Taken together, these findings indicate that lung fibrotic responses are associated with enhanced expression of YY1 mRNA and protein in both human subjects and two widely used mouse models.

YY1 Can Directly Up-Regulate α -SMA in Fibroblasts

We further explored the mechanism by which YY1 may regulate fibroblast differentiation and activation. α -SMA is a hallmark of myofibroblasts and is well-accepted as a marker of pulmonary fibrosis. Although YY1 can bind to the α -SMA promoter, it is unknown whether it up-regulates or down-regulates α -SMA promoter activity (21, 29). To investigate the ability of YY1 to regulate α -SMA promoter activity, we created a murine 740-bp construct driving the firefly luciferase gene (α -SMA-Luc). This construct was transfected with or without a YY1 expression vector into WI38 cells. Using gel shift assays or CHIP assays, we confirmed that nuclear YY1 can directly bind to the α -SMA promoter *in vitro* and *in vivo* (Figures E1A–E1C). Overexpressed YY1 alone significantly increased α -SMA reporter activity, and this effect was further enhanced by TGF- β stimulation (Figure 4A). The enhancing effects of YY1 on α -SMA-Luc activity were further augmented by cotransfection with NF- κ B. This finding suggests that TGF- β

magnification of $\times 40$. (B) Representative examples of three independent experiments are shown.

Figure 4. A total of 6 μ g of murine α -smooth muscle actin luciferase reporter (α -SMA-Luc), control vector or 2.5 μ g of Yin Yang 1 (YY1) and p65 (alone or in combination) were transiently transfected into WI38 cells by electroporation, respectively, followed by treatment with phosphate-buffered saline or 10 ng/ml of transforming growth factor (TGF)- β for 24 hours. * $P < 0.05$ or ** $P < 0.01$ (compare as untreated or treated groups in transfected YY1 or p65 alone or recombination, respectively) (A). All reporter data were normalized by empty vector control. Relative light units (RLU) data are shown from at least five independent experiments performed in duplicate. LL97A cells were transduced with control lentivirus vector shRNAs (control-lenti-shRNAs) or lentivirus vector YY1 shRNAs (YY1-lenti-shRNAs) for 3 days. Puromycin was then used to select transduced cells. The selected LL97A cells were immunostained with anti- α -SMA (green) and anti-YY1 (red) antibodies. Alternatively, they were immunostained with anti-collagen I (green) and anti-YY1 (red) antibodies. Pictures are presented at the original

induces α -SMA promoter activity mediated by NF- κ B and YY1. Therefore, in addition to type I collagen (19), the α -SMA gene represents another potential YY1 target in lung fibroblasts.

We then tested whether reducing the expression of YY1 would inhibit α -SMA or collagen expression in human lung fibroblasts. A fibroblast cell line (LL97A) was derived from a human subject with IPF and infected using different YY1 lentiviral shRNA constructs. After transduction with two controls and two YY1 lentiviral shRNA constructs, LL97A cells were allowed to recover for 3 days and then selected in puromycin to enrich for transduced cells. Figure 4B shows that decreased YY1 expression inhibited both α -SMA and collagen expression in human lung fibroblasts, as determined by immunofluorescence staining. These data suggest that knockdown of YY1 expression in lung fibroblasts inhibits fibroblast activation. Taken together, these data provide support for the idea that α -SMA is a previously unrecognized and important target of YY1 in lung fibroblasts and suggest that YY1 may be a potential therapeutic target for fibrotic lung disease through decreasing α -SMA and collagen.

Partial YY1-deficient Mice Are Protected from Lung Fibrosis after Exposure to Silica

We next investigated whether decreasing YY1 expression is able to protect against lung fibrosis in mice. To test this hypothesis, we exposed partially YY1-deficient mice (YY1^{+/-}), or littermate controls on the C57BL/6 background, to silica particles by intratracheal injection. Three weeks later, mice were killed and the lungs stained using IHC, and connective tissue was analyzed using Masson trichrome staining. We found that lung fibrosis was markedly decreased in YY1^{+/-} compared with littermate mice. Expression of the myofibroblast marker α -SMA, collagen, FSP1-positive cells, and inflammation were markedly greater in wild-type mice compared with YY1^{+/-} littermates (Figure 5A). Magnification $\times 10$ of the lung in Figure 5A is shown in Figure E2C. Lung fibrosis scores from heterozygote YY1^{+/-} and wild-type mice were evaluated by the Ashcroft scale. Whole lung was scored using a double-blind method. Using this approach, lung fibrosis was significantly diminished in YY1^{+/-} mice compared with wild-type littermates (Figure 5B). In addition, collagen I protein expression was greatly reduced in YY1^{+/-} mice (Figure E5C). This suggests that lung fibrosis was significantly inhibited in YY1^{+/-} mice, and that decreased YY1 expression protects against lung fibrosis.

Treatment Using Conditional Deletion with Adeno-cre in YY1^{f/f} Mice

To determine if inducible deletion of YY1 could be used in a therapeutic regimen, we used floxed-YY1 mice (YY1^{f/f}) in which lox P sites were stably integrated into the introns between YY1 exon 1. Both wild-type and YY1^{f/f} mice were exposed to silica particles using our standard protocol, except that both groups then received an intratracheal adenoviral vector expressing cre recombinase on Day 12, and lungs were harvested 21 days later. This strategy likely targets multiple lung cell types including lung epithelial cells, which also express YY1 (Figure E3A), and could potentially differentiate into fibroblasts in mice exposed to silica (30). Figure 6 shows that when compared with wild-type mice that received silica followed by adenoviral-cre, the induction of lung fibrosis, and α -SMA, collagen, FSP1, and YY1 expression, were all markedly attenuated in YY1^{f/f} mice (Figure 6A). Arrows indicate that YY1 was decreased in epithelial cells of YY1^{f/f} mice. Magnification $\times 10$ of Figure 6A is shown in Figure E2D. Lung fibrosis was attenuated in YY1^{f/f} mice as determined by Ashcroft scored using a double-blind method (Figure 6B). YY1 mRNA and protein expression were similarly reduced in YY1^{f/f} compared with wild-type mice exposed to adenovirus-cre, as expected (Figures 6C and 6D). These suggest that partially decreasing YY1 expression by adeno-cre in type 1 epithelial cells (Figure E3A) and potentially other cell types can protect against lung fibrosis by reducing accumulation of fibroblasts.

DISCUSSION

YY1 is a versatile transcription factor with pivotal roles in normal biologic processes, such as development, differentiation, replication, and cell proliferation, and is increasingly linked with pathologic conditions. For example, YY1 overexpression is strongly linked with cancer development and progression (31). In this study, we demonstrate that YY1 is overexpressed by fibrotic cytokines TGF- β and TNF- α in lung fibroblasts, and that this involved activation of NF- κ B. In addition, we found that YY1 expression is up-regulated in or near FSP1- and α -SMA-positive cells in human IPF and two murine models

Figure 5. The expression of α -smooth muscle actin (SMA) and collagen is decreased in Yin Yang 1 (YY1)^{+/-} compared with littermate mice. Lung sections from silica-treated YY1^{+/-} or littermate mice were evaluated 21 days after silica treatment (n = 6 mice per group). Hematoxylin and eosin staining and immunohistochemistry (IHC) were used to compare histologic features and the expression of α -SMA, FSP1, and collagen in YY1^{+/-} compared with wild-type mice. Pictures are presented at their original magnification of $\times 63$ (A). The $\times 10$ magnification of A is shown in Figure E2C. (B) The fibrosis grade-score for lung tissue sections was evaluated with a double-blind method using the Ashcroft scale (26). n = 10 $^{**}P < 0.01$ by the Student *t* test.

of lung fibrosis. In addition YY1 expression in lung fibroblasts isolated from mice instilled with bleomycin was dramatically increased, compared with fibroblasts from mice instilled with phosphate-buffered saline (Figure E5B). Furthermore, we found that YY1 can directly up-regulate α -SMA expression in pulmonary fibroblasts leading to fibroblast differentiation. Using gene-

targeted mice and a conditional deletion strategy, we found that reducing YY1 expression protects against lung fibrosis *in vivo*. These novel data will be considered in turn.

Pulmonary fibrosis results from activation and differentiation of fibroblasts, which overexpress extracellular matrix proteins and α -SMA in the injured lung. In this study, we found

Figure 6. Lung fibrosis was decreased by conditional deletion of Yin Yang 1 (YY1) expression in YY1^{f/f} mice. Hematoxylin and eosin staining and immunohistochemistry (IHC) were used to compare with histologic features and the expression of α -smooth muscle actin (SMA), FSP1, YY1, and collagen in YY1^{f/f} compared with WT mice that received silica followed by adenoviral-cre recombinase. C57BL6 wild-type mice and YY1^{f/f} littermates (n = 5 per group) were instilled with silica (200 μ g/mouse), followed 11 days later by adeno-cre (4×10^8 pfu). After 21 days, lung sections were harvested for analysis of IHC, protein, and mRNA. The expression of α -SMA and collagen is decreased in YY1^{f/f} mice compared with WT mice. Pictures are presented at their original magnification

of $\times 63$ (A). The fibrosis grade-score for the lung tissue sections was evaluated by double-blind scoring using the Ashcroft scale (n = 4 mice per group) (B). $^{*}P < 0.05$ by the Student *t* test. Lung sections obtained from adenoviral cre and silica-treated YY1^{f/f} mice and WT mice were evaluated by quantitative polymerase chain reaction (C) and Western blot (D). Lung sections were ground in liquid nitrogen and divided for extraction of RNA or lysis for Western blot. Representative Western blots are shown.

that fibrotic cytokines induced YY1 expression, and that YY1 mRNA and protein were up-regulated in mouse model of lung fibrosis, and in lungs from human subjects with IPF (Figure 3A). We focused on lung fibroblasts because lung fibroblasts ultimately control deposition of extracellular matrix and their differentiation into myofibroblasts characterizes the injury and repair response. Furthermore, we found that both α -SMA and collagen expression were reduced in YY1-deficient mice. We did not find that YY1 was overexpressed in type II alveolar epithelial cells (Figure E3B), and macrophages (Figure E3C, *middle panel*) in the human IPF and in the lung of bleomycin-instilled mouse (Figure E3C, *top panel*). We concluded that YY1 expression by fibrotic cytokines seems to contribute to differentiation of fibroblasts into α -SMA expressing myofibroblasts in lung fibrosis. The precise molecular mechanisms for this effect requires further study, including the potential for YY1 directly to regulate genes involved in fibroblast activation.

In addition to TNF- α and TGF- β , other cytokines and stimuli may induce YY1 expression in lung fibroblasts. Silica and asbestos particles were recently shown to activate the Nalp3 containing inflammasome and induce caspase-1-driven processing of mature IL-1 β (32), which we also found up-regulated fibroblast YY1 expression *in vitro* (Figure E4). Additionally, we found that cytokine-dependent induction of YY1 required the transcription factor NF- κ B, in keeping with prior studies in other cell types (17, 33). This implied that blockade of the NF- κ B pathway might protect against lung fibrosis by decreasing YY1 expression. Interestingly, a recent report demonstrated that Bay11-7085 decreased silica-induced inflammation and collagen deposition (34), although potential effects of NF- κ B inhibition on YY1 expression were not investigated in that report. Thus, in addition to promoting the expression of cytokines, chemokines, and other inflammatory mediators, these data suggest that by inducing YY1 expression, NF- κ B promotes the expression of genes involved in tissue repair responses. In other models, YY1 expression mediated by NF- κ B can down-regulate Fas and death receptor 5 (17, 35) expression, suggesting a mechanism by which lung myofibroblasts may be protected from apoptosis. Smads are also activated by TGF- β during lung fibrosis, and interestingly Smad3 can bind to different domains of YY1 (36). It will be interesting in future studies to investigate interactions between YY1 and other transcription factors in lung fibrosis and fibrosis in other organs. Because TGF- β and TNF- α are universally implicated in injury and repair and remodeling, we predict that YY1 will play a role in fibroblast differentiation and activation in diverse tissues, and that the paradigms we established in this report should be generalizable.

In this study, we demonstrate that YY1 directly binds to the proximal α -SMA promoter (in keeping with a recent report [37]), and that overexpressed YY1 enhances α -SMA gene expression. In contrast, using shRNA-mediated knock-down of YY1 expression in human lung fibroblasts (Figure 4C), we found that loss of YY1 markedly reduces α -SMA expression. Because YY1 can directly bind to the promoter regions of both the collagen (19) and α -SMA genes and is required for their expression, we concluded that YY1 directly regulates two crucial genes (α -SMA and collagen) involved in fibroblast activation and myofibroblast differentiation.

Lung myofibroblasts derive from multiple sources including resident cells, circulating fibrocytes (11), or via EMT (10, 38). Our observation that intratracheally administered adeno-cre attenuates fibrotic reactions supports a potential role for YY1 in EMT, because our strategy likely targeted epithelial cells. This approach can be potentially used for a therapeutic strategy. Future studies

are needed in this area. In addition to regulating myofibroblast gene expression, YY1 may also confer resistance to apoptosis (39), which is known to be induced during lung fibrotic responses after exposure to silica (40), TGF- β (41), and TNF- α (17, 42). Thus, decreasing YY1 expression may attenuate fibrotic responses by multiple mechanisms, in keeping with a growing role for YY1 in cell survival in response to injury (43, 44).

In addition to direct effects in fibroblasts, decreasing YY1 expression may inhibit early inflammation during injury and repair responses, and may help to protect against lung fibrosis by this mechanism. In support of this idea, we detected lower levels of inflammatory cytokines in bronchoalveolar lavage fluids from silica-challenged YY1^{+/-} compared with wild-type controls (Figure E6). Because complete YY1 deficiency in mice results in early embryonic lethality, heterozygous mice (YY1^{+/-}) were used in this report. YY1 heterozygous mice express about 50% of normal YY1 levels (22, 45). Thus, even partial reduction of YY1 levels can protect against lung fibrosis in mice. Future studies should expose the possibility that YY1 may be a therapeutic target in lung fibrosis, where new therapies are urgently needed.

YY1 is generally considered a constitutively expressed nuclear phosphoprotein, but there is growing evidence that YY1 expression can be dynamically regulated, including by compounds in therapeutic use. For example, YY1 and NF- κ B expression are diminished in B cells by rituximab, which is an anti-CD20 antibody used for treating B-cell non-Hodgkin lymphoma (39). Nitric oxide can also inhibit YY1 expression in human tumor cells (46) and in lung fibroblasts, which may explain some of the therapeutic benefits of inhaled nitric oxide in lung fibrosis (47). In addition to regulating gene expression directly at the transcriptional level, YY1 is intimately involved in chromatin remodeling and epigenetic imprinting. For example, YY1 associates with histone H3-specific methyltransferases to its target promoters (48), and together with Polycomb group family members generates high levels of H3-K27 trimethylation that are needed to maintain a repressed chromatin state (49–51). It will be interesting in future studies to determine whether YY1 regulates fibroblast activation or differentiation *in vivo* via epigenetic mechanisms.

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References

- Khalil N, O'Connor R. Idiopathic pulmonary fibrosis: current understanding of the pathogenesis and the status of treatment. *CMAJ* 2004; 171:153–160.
- Gharaee-Kermani M, Hu B, Thannickal VJ, Phan SH, Gyetko MR. Current and emerging drugs for idiopathic pulmonary fibrosis. *Expert Opin Emerg Drugs* 2007;12:627–646.
- Hardie WD, Le Cras TD, Jiang K, Tichelaar JW, Azhar M, Korfhagen TR. Conditional expression of transforming growth factor- α in adult mouse lung causes pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L741–L749.
- Liu JY, Sime PJ, Wu T, Warshamana GS, Pociask D, Tsai SY, Brody AR. Transforming growth factor-beta(1) overexpression in tumor

- necrosis factor- α receptor knockout mice induces fibroproliferative lung disease. *Am J Respir Cell Mol Biol* 2001;25:3–7.
5. Leask A, Abraham DJ. TGF- β signaling and the fibrotic response. *FASEB J* 2004;18:816–827.
 6. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor- β 1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997;100:768–776.
 7. Ortiz LA, Lasky J, Hamilton RF Jr, Holian A, Hoyle GW, Banks W, Peschon JJ, Brody AR, Lungarella G, Friedman M. Expression of TNF and the necessity of TNF receptors in bleomycin-induced lung injury in mice. *Exp Lung Res* 1998;24:721–743.
 8. Kuroki M, Noguchi Y, Shimono M, Tomono K, Tashiro T, Obata Y, Nakayama E, Kohno S. Repression of bleomycin-induced pneumopathy by TNF. *J Immunol* 2003;170:567–574.
 9. Antoniou KM, Mamoulaki M, Malagari K, Kritikos HD, Bouros D, Siafakas NM, Boumpas DT. Infliximab therapy in pulmonary fibrosis associated with collagen vascular disease. *Clin Exp Rheumatol* 2007;25:23–28.
 10. Kuroishi S, Suda T, Fujisawa T, Ide K, Inui N, Nakamura Y, Nakamura H, Chida K. Epithelial-mesenchymal transition induced by transforming growth factor- β 1 in mouse tracheal epithelial cells. *Respirology* 2009;14:828–837.
 11. Andersson-Sjoland A, de Alba CG, Nihlberg K, Becerril C, Ramirez R, Pardo A, Westergren-Thorsson G, Selman M. Fibrocytes are a potential source of lung fibroblasts in idiopathic pulmonary fibrosis. *Int J Biochem Cell Biol* 2008;40:2129–2140.
 12. Sheppard KA, Rose DW, Haque ZK, Kurokawa R, McInerney E, Westin S, Thanos D, Rosenfeld MG, Glass CK, Collins T. Transcriptional activation by NF- κ B requires multiple coactivators. *Mol Cell Biol* 1999;19:6367–6378.
 13. Roy SG, Nozaki Y, Phan SH. Regulation of α -smooth muscle actin gene expression in myofibroblast differentiation from rat lung fibroblasts. *Int J Biochem Cell Biol* 2001;33:723–734.
 14. Horowitz JC, Rogers DS, Sharma V, Vittal R, White ES, Cui Z, Thannickal VJ. Combinatorial activation of FAK and AKT by transforming growth factor- β 1 confers an anoikis-resistant phenotype to myofibroblasts. *Cell Signal* 2007;19:761–771.
 15. Meyer-Ter-Vehn T, Katzenberger B, Han H, Grehn F, Schlunck G. Lovastatin inhibits TGF- β -induced myofibroblast transdifferentiation in human tenon fibroblasts. *Invest Ophthalmol Vis Sci* 2008;49:3955–3960.
 16. Gordon S, Akopyan G, Garban H, Bonavida B. Transcription factor YY1: structure, function, and therapeutic implications in cancer biology. *Oncogene* 2006;25:1125–1142.
 17. Huerta-Yepez S, Vega M, Garban H, Bonavida B. Involvement of the TNF- α autocrine-paracrine loop, via NF- κ B and YY1, in the regulation of tumor cell resistance to Fas-induced apoptosis. *Clin Immunol* 2006;120:297–309.
 18. Wang H, Hertlein E, Bakkar N, Sun H, Acharyya S, Wang J, Carathers M, Davuluri R, Guttridge DC. NF- κ B regulation of YY1 inhibits skeletal myogenesis through transcriptional silencing of myofibrillar genes. *Mol Cell Biol* 2007;27:4374–4387.
 19. Riquet FB, Tan L, Choy BK, Osaki M, Karsenty G, Osborne TF, Auron PE, Goldring MB. YY1 is a positive regulator of transcription of the Col1a1 gene. *J Biol Chem* 2001;276:38665–38672.
 20. Miao K, Potter JJ, Anania FA, Rennie-Tankersley L, Mezey E. Identification of two repressor elements in the mouse α 2(I) collagen promoter. *Arch Biochem Biophys* 1999;361:7–16.
 21. Favot L, Hall SM, Haworth SG, Kemp PR. Cytoplasmic YY1 is associated with increased smooth muscle-specific gene expression: implications for neonatal pulmonary hypertension. *Am J Pathol* 2005;167:1497–1509.
 22. Affar el B, Gay F, Shi Y, Liu H, Huarte M, Wu S, Collins T, Li E, Shi Y. Essential dosage-dependent functions of the transcription factor yin yang 1 in late embryonic development and cell cycle progression. *Mol Cell Biol* 2006;26:3565–3581.
 23. Chinnappan D, Xiao D, Ratnasari A, Andry C, King TC, Weber HC. Transcription factor YY1 expression in human gastrointestinal cancer cells. *Int J Oncol* 2009;34:1417–1423.
 24. Lin X, Georas S, Guo J. Yin Yang 1 a critical regulator of pulmonary fibrosis [abstract]. *Am J Respir Crit Care Med* 2008;177:A743.
 25. Hindman HB, Swanton JN, Phipps RP, Sime PJ, Huxlin KR. Differences in the TGF- β 1-induced profibrotic response of anterior and posterior corneal keratocytes in vitro. *Invest Ophthalmol Vis Sci* 2010;51:1935–1942.
 26. Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol* 1988;41:467–470.
 27. Guo J, Lin X, Williams MA, Hamid Q, Georas SN. Yin-Yang 1 regulates effector cytokine gene expression and T(H)2 immune responses. *J Allergy Clin Immunol* 2008;122:195–201.
 28. Guo J, Casolaro V, Seto E, Yang WM, Chang C, Seminario MC, Keen JC, Georas SN. Yin Yang-1 activates IL-4 gene expression in T cells. *J Biol Chem* 2001;276:48871–48878.
 29. Bushel P, Kim JH, Chang W, Catino JJ, Ruley HE, Kumar CC. Two serum response elements mediate transcriptional repression of human smooth muscle α -actin promoter in ras-transformed cells. *Oncogene* 1995;10:1361–1370.
 30. Guarino M, Tosoni A, Nebuloni M. Direct contribution of epithelium to organ fibrosis: epithelial-mesenchymal transition. *Hum Pathol* 2009;40:1365–1376.
 31. Castellano G, Torrisi E, Ligresti G, Malaponte G, Militello L, Russo AE, McCubrey JA, Canevari S, Libra M. The involvement of the transcription factor Yin Yang 1 in cancer development and progression. *Cell Cycle* 2009;8:1367–1372.
 32. Dostert C, Petrilli V. Asbestos triggers inflammation by activating the Nalp3 inflammasome. *Med Sci (Paris)* 2008;24:916–918.
 33. Baritaki S, Suzuki E, Umezawa K, Spandidos DA, Berenson J, Daniels TR, Penichet ML, Jazirehi AR, Palladino M, Bonavida B. Inhibition of Yin Yang 1-dependent repressor activity of DR5 transcription and expression by the novel proteasome inhibitor NPI-0052 contributes to its TRAIL-enhanced apoptosis in cancer cells. *J Immunol* 2008;180:6199–6210.
 34. Di Giuseppe M, Gambelli F, Hoyle GW, Lungarella G, Studer SM, Richards T, Yousem S, McCurry K, Dauber J, Kaminski N, et al. Systemic inhibition of NF- κ B activation protects from silicosis. *PLoS ONE* 2009;4:e5689.
 35. Huerta-Yepez S, Vega M, Escoto-Chavez SE, Murdock B, Sakai T, Baritaki S, Bonavida B. Nitric oxide sensitizes tumor cells to TRAIL-induced apoptosis via inhibition of the DR5 transcription repressor Yin Yang 1. *Nitric Oxide* 2009;20:39–52.
 36. Kurisaki K, Kurisaki A, Valcourt U, Terentiev AA, Pardali K, Ten Dijke P, Heldin CH, Ericsson J, Moustakas A. Nuclear factor YY1 inhibits transforming growth factor β - and bone morphogenetic protein-induced cell differentiation. *Mol Cell Biol* 2003;23:4494–4510.
 37. Ellis PD, Martin KM, Rickman C, Metcalfe JC, Kemp PR. Increased actin polymerization reduces the inhibition of serum response factor activity by Yin Yang 1. *Biochem J* 2002;364:547–554.
 38. Radisky DC, Kenny PA, Bissell MJ. Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT? *J Cell Biochem* 2007;101:830–839.
 39. Bonavida B. Rituximab-induced inhibition of antiapoptotic cell survival pathways: implications in chemo/immunoresistance, rituximab unresponsiveness, prognostic and novel therapeutic interventions. *Oncogene* 2007;26:3629–3636.
 40. Gambelunghe A, Antognelli C, Del Buono C, Piccinini R, Ugolini B, dell'Omo M, Murgia N, Talesa VN, Muzi G. Crystalline silica can induce oxidative stress by inhibiting glyoxalase system in bronchial epithelial cells. *G Ital Med Lav Ergon* 2007;29:397–399.
 41. Honda K, Kimura A, Dong RP, Tamai H, Nagato H, Nishimura Y, Sasazuki T. Immunogenetic analysis of silicosis in Japan. *Am J Respir Cell Mol Biol* 1993;8:106–111.
 42. Zhao HW, Hu SY, Barger MW, Ma JK, Castranova V, Ma JY. Time-dependent apoptosis of alveolar macrophages from rats exposed to bleomycin: involvement of tnfr receptor 2. *J Toxicol Environ Health A* 2004;67:13911406.
 43. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1 α transcriptional complex. *Nature* 2007;450:736–740.
 44. Grubich L, Juhl-Christensen C, Rethmeier A, Olesen LH, Aggerholm A, Hokland P, Ostergaard M. Gene expression profiling of Polycomb, Hox and Meis genes in patients with acute myeloid leukaemia. *Eur J Haematol* 2008;81:112–122.
 45. Donohoe ME, Zhang X, McGinnis L, Biggers J, Li E, Shi Y. Targeted disruption of mouse Yin Yang 1 transcription factor results in perimplantation lethality. *Mol Cell Biol* 1999;19:7237–7244.
 46. Hongo F, Garban H, Huerta-Yepez S, Vega M, Jazirehi AR, Mizutani Y, Miki T, Bonavida B. Inhibition of the transcription factor Yin Yang 1 activity by S-nitrosation. *Biochem Biophys Res Commun* 2005;336:692–701.
 47. Yung GL, Kriett JM, Jamieson SW, Johnson FW, Newhart J, Kinninger K, Channick RN. Outpatient inhaled nitric oxide in a patient with

- idiopathic pulmonary fibrosis: a bridge to lung transplantation. *J Heart Lung Transplant* 2001;20:1224–1227.
48. Rezai-Zadeh N, Zhang X, Namour F, Fejer G, Wen YD, Yao YL, Gyory I, Wright K, Seto E. Targeted recruitment of a histone H4-specific methyltransferase by the transcription factor YY1. *Genes Dev* 2003;17:1019–1029.
49. Caretti G, Di Padova M, Micales B, Lyons GE, Sartorelli V. The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. *Genes Dev* 2004;18:2627–2638.
50. van Leenders GJ, Dukers D, Hessels D, van den Kieboom SW, Hulsbergen CA, Witjes JA, Otte AP, Meijer CJ, Raaphorst FM. Polycomb-group oncogenes EZH2, BMI1, and RING1 are overexpressed in prostate cancer with adverse pathologic and clinical features. *Eur Urol* 2007;52:455–463.
51. Nekrasov M, Klymenko T, Fraterman S, Papp B, Oktaba K, Kocher T, Cohen A, Stunnenberg HG, Wilm M, Muller J. Pcl-PRC2 is needed to generate high levels of H3–K27 trimethylation at Polycomb target genes. *EMBO J* 2007;26:4078–4088.