

Pulmonary Silicosis Alters MicroRNA Expression in Rat Lung and miR-411-3p Exerts Anti-fibrotic Effects by Inhibiting MRTF-A/SRF Signaling

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To identify potential therapeutic targets for pulmonary fibrosis induced by silica, we studied the effects of this disease on the expression of microRNAs (miRNAs) in the lung. *Rattus norvegicus* pulmonary silicosis models were used in conjunction with high-throughput screening of lung specimens to compare the expression of miRNAs in control and pulmonary silicosis tissues. A total of 70 miRNAs were found to be differentially expressed between control and pulmonary silicosis tissues. This included 41 miRNAs that were upregulated and 29 that were downregulated relative to controls. Among them, miR-292-5p, miR-155-3p, miR-1193-3p, miR-411-3p, miR-370-3p, and miR-409a-5p were found to be similarly altered in rat lung and transforming growth factor (TGF)- β 1-induced cultured fibroblasts. Using miRNA mimics and inhibitors, we found that miR-1193-3p, miR-411-3p, and miR-370-3p exhibited potent anti-fibrotic effects, while miR-292-5p demonstrated pro-fibrotic effects in TGF- β 1-stimulated lung fibroblasts. Moreover, we also found that miR-411-3p effectively reduced pulmonary silicosis in the mouse lung by regulating *Mrtfa* expression, as demonstrated using biochemical and histological assays. In conclusion, our findings indicate that miRNA expression is perturbed in pulmonary silicosis and suggest that therapeutic interventions targeting specific miRNAs might be effective in the treatment of this occupational disease.

INTRODUCTION

Occupational exposure to silica dust particles remains a major cause of pulmonary fibrosis.¹ Although this form of pulmonary fibrosis can largely be prevented by implementing strict occupational regulations, it remains an important cause of respiratory morbidity and mortality in many countries throughout the world, such as China and other developing countries.^{2,3}

MicroRNAs (miRNAs) are non-coding single-stranded RNA molecules with a length of 20–24 nt. They play a key role in the post-transcriptional regulation of a large number of genes.⁴ Indeed, it is estimated that 60% of all transcripts are regulated by miRNAs,

illustrating the essential role that miRNAs play in almost all physiological and pathological processes.⁵ Because individual miRNAs tend to influence the expression of many functionally related genes, it has long been proposed that the targeting of specific miRNA species may be a useful strategy to ameliorate disease.⁶ For example, specific miRNAs have been shown to regulate entire sets of fibrosis-related genes in pulmonary fibrosis, and targeting these molecules has been shown to reduce the severity of this disease in experimental models.⁷

To date, very little is known about the role of miRNAs in the pathogenesis of pulmonary silicosis. However, studies have shown that certain miRNAs are altered in lung tissues in some silicosis model systems.^{8,9} For example, Yuan et al.¹⁰ have shown that miR-542-5p attenuates pulmonary silicosis by targeting integrin α 6. And Yan et al.¹¹ have shown that miR-503 has similar effects by reducing the epithelial-mesenchymal transition through a phosphatidylinositol 3-hydroxy kinase p85-dependent mechanism. Likewise, miR-19a-19b-20a has been shown to suppress transforming growth factor (TGF)- β 1-induced activation of fibroblasts in both bleomycin- and silica-induced pulmonary fibrosis models.¹² In this study, we sought to identify the full spectrum of miRNAs that are altered as a consequence of pulmonary silicosis and to explore the consequences of these changes on signaling pathways associated with this disease, with the aim of identifying new putative therapeutic targets.¹³ Using the inhalational model, we found that a large number of miRNAs were differentially expressed after chronic exposure to silica dust, including 41 miRNAs that were upregulated and 29 that were downregulated. Moreover, we found that 10 of the miRNAs demonstrating the most significantly altered expression in lung tissues following

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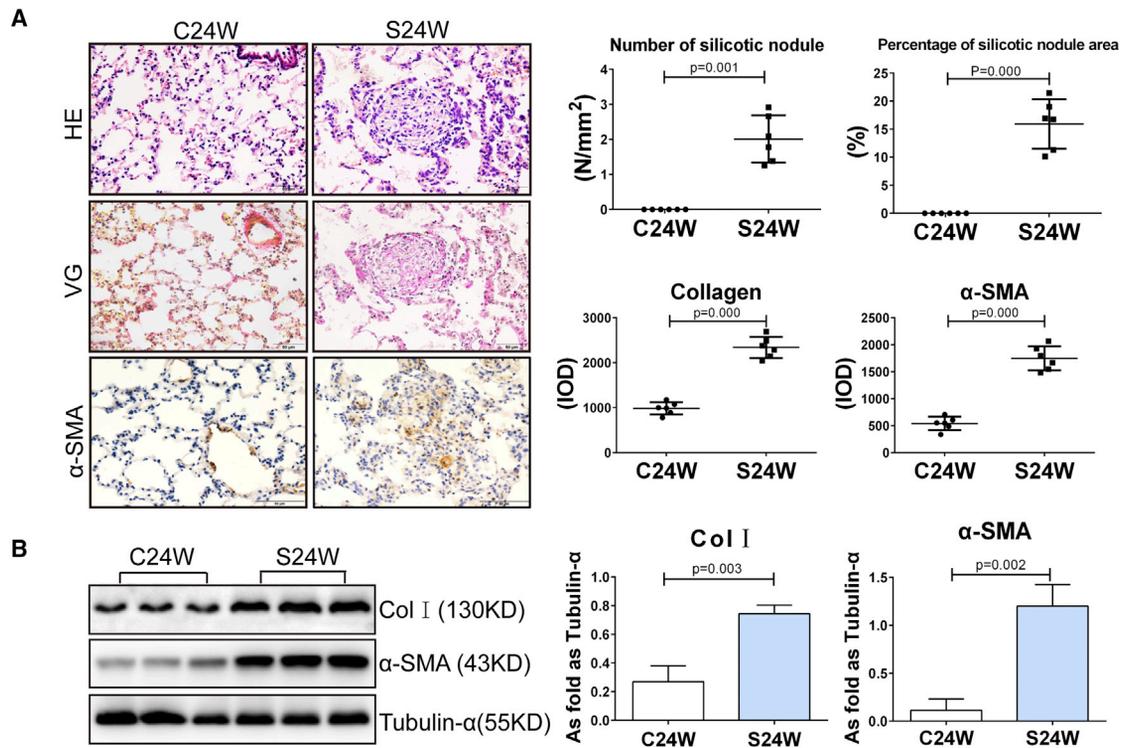


Figure 1. Silicosis in Rats Induced by Inhalation of SiO₂

(A) H&E staining, VG staining, and α -SMA immunohistochemical (IHC) staining in rat lung (scale bars, 50 μ m). (B) The increasing levels of Col I and α -SMA in silicotic rats measured by western blot. (Data indicate mean \pm SD; n = 6 independent experiments.)

silica exposure also showed similar changes in primary lung fibroblasts induced with TGF- β 1.

Activated lung myofibroblasts are a major contributor to the pathogenesis of pulmonary fibrosis and are characterized by positive expression of α -smooth muscle actin (α -SMA). The differentiation of fibroblasts to myofibroblasts can be induced by TGF- β 1 and is associated with excessive extracellular matrix deposition.^{14,15} It has been well documented that serum response factor (SRF) and its transcription cofactor, myocardin-related transcription factor A (MRTF-A), can regulate the expression of α -SMA at the transcriptional level,¹⁶ and they have also been reported to promote the synthesis of collagen type I (Col I) and play a key role in pulmonary fibrosis.¹⁷ In the present study, we found that many of the miRNAs differentially expressed in pulmonary fibrosis had effects on myofibroblast differentiation and the production of extracellular matrix *in vitro*. Additionally, using bioinformatics prediction analysis, we identified the *Mrtfa* gene as a target of miR-411-3p. In further studies designed to examine the connection between miR-411-3p and *Mrtfa*, we showed that *in vivo* administration of miR-411-3p could significantly attenuate pulmonary silicosis in mice. Taken together, our findings indicate that pulmonary silicosis has marked effects on the expression of miRNAs in the lung and suggest that targeting specific miRNAs could be effective in reducing morbidity and mortality in this occupational disease.

RESULTS

Silicosis Alters miRNA Expression in the Rat Lung

To determine the effects of pulmonary silicosis on the expression of miRNAs in the lung, we exposed rats to aerosolized silica dust particles daily for 24 weeks. This model system readily induced fibrotic remodeling in the lung typical of pulmonary silicosis, as has been reported previously.¹⁸ This included the accumulation of large numbers of silicotic lung nodules, extensive deposition of interstitial collagen, and increased numbers of α -SMA-positive cells (Figure 1A). We also found that Col I and α -SMA protein levels were significantly increased in the lungs of these animals relative to those in controls ($p < 0.05$; Figure 1B).

Having validated our model of pulmonary silicosis, we next examined the effects of silicosis on the expression of miRNAs in the lung. Selecting only those miRNAs whose expression significantly differed from that of control lung tissues (cutoff threshold of $|\log_2(\text{fold change})| \geq 1$ and $p < 0.05$), we identified 70 miRNAs that were differentially expressed in the silicotic lung. This included 41 miRNAs whose expression was increased and 29 whose expression was decreased. Clustering analysis and miRNA profiles are shown in Figure 2A and Table S1, respectively, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis is shown in Figure 2B. Gene Ontology (GO) and KEGG pathway analyses for the top five upregulated and downregulated miRNAs are shown in Figures 2C and 2D.

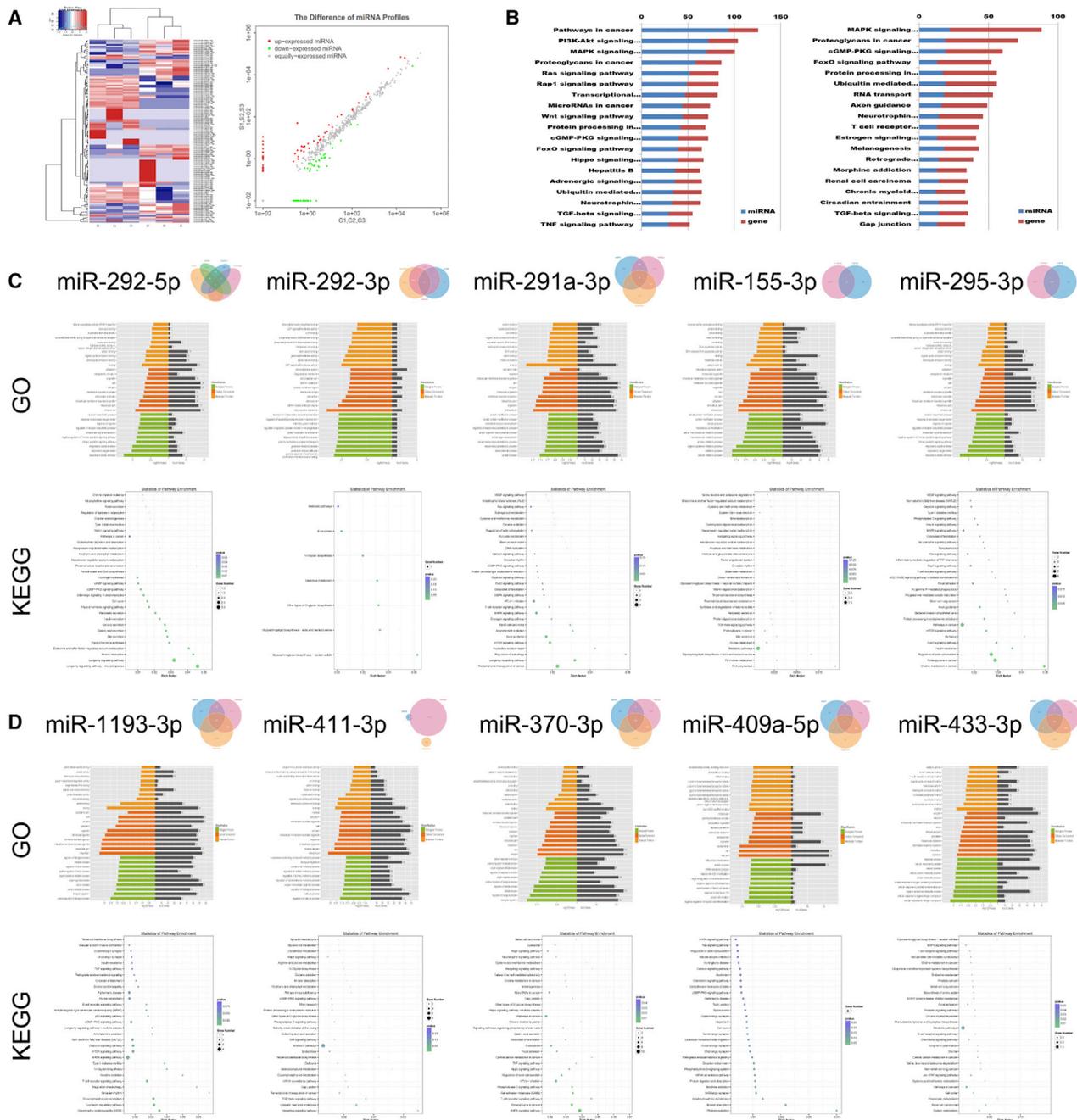


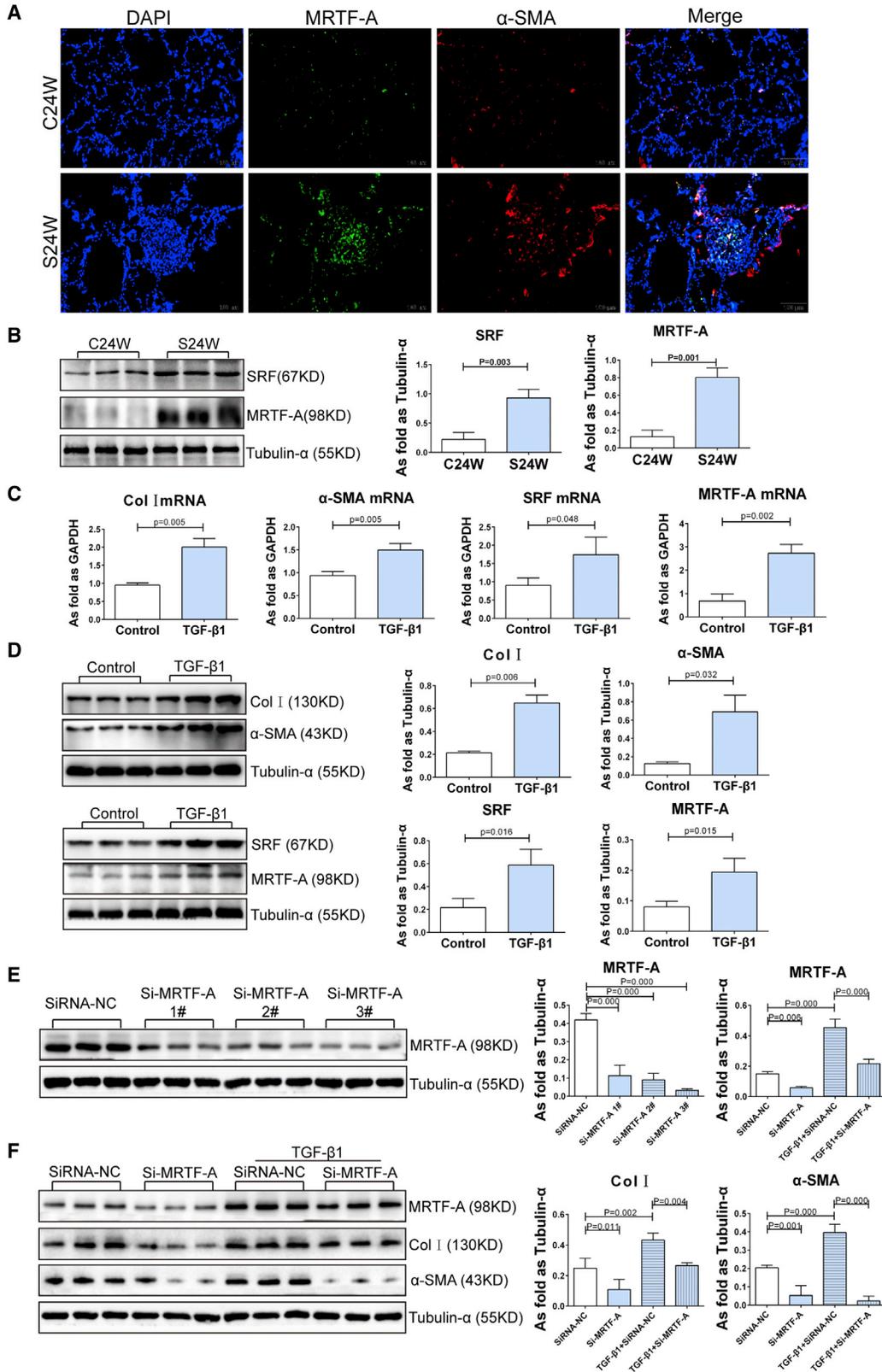
Figure 2. The Bioinformatics Analysis of Dyregulated miRNAs in Silicotic Rats

(A) The cluster analysis of miRNA profiles. (B) The KEGG pathway of upregulated miRNAs (left) and downregulated miRNAs (right). (C and D) The GO (C) and KEGG (D) pathway analyses of regulated mRNAs by 10 top changes of miRNAs.

MRTF-A Participates in Myofibroblast Differentiation in Silicosis

Transcription of the contractile protein α -SMA has been reported to be mediated by the transcription factor SRF along with its co-activator, MRTF-A.¹⁹ In our previous study, we found that SRF levels were elevated in silicotic rats and also in TGF- β 1-treated lung fibroblasts.¹⁴ As shown in Figure 3, in this study, we also observed co-

expression of MRTF-A and α -SMA in silicotic lesions of rat lung tissue, and this was associated with increased MRTF-A and SRF protein levels in silicotic lungs. In addition, the expression of MRTF-A and SRF, as well as of Col I and α -SMA, were upregulated in lung fibroblasts induced by TGF- β 1. Furthermore, knockdown of MRTF-A by small interfering RNA (siRNA) suppressed Col I and α -SMA levels



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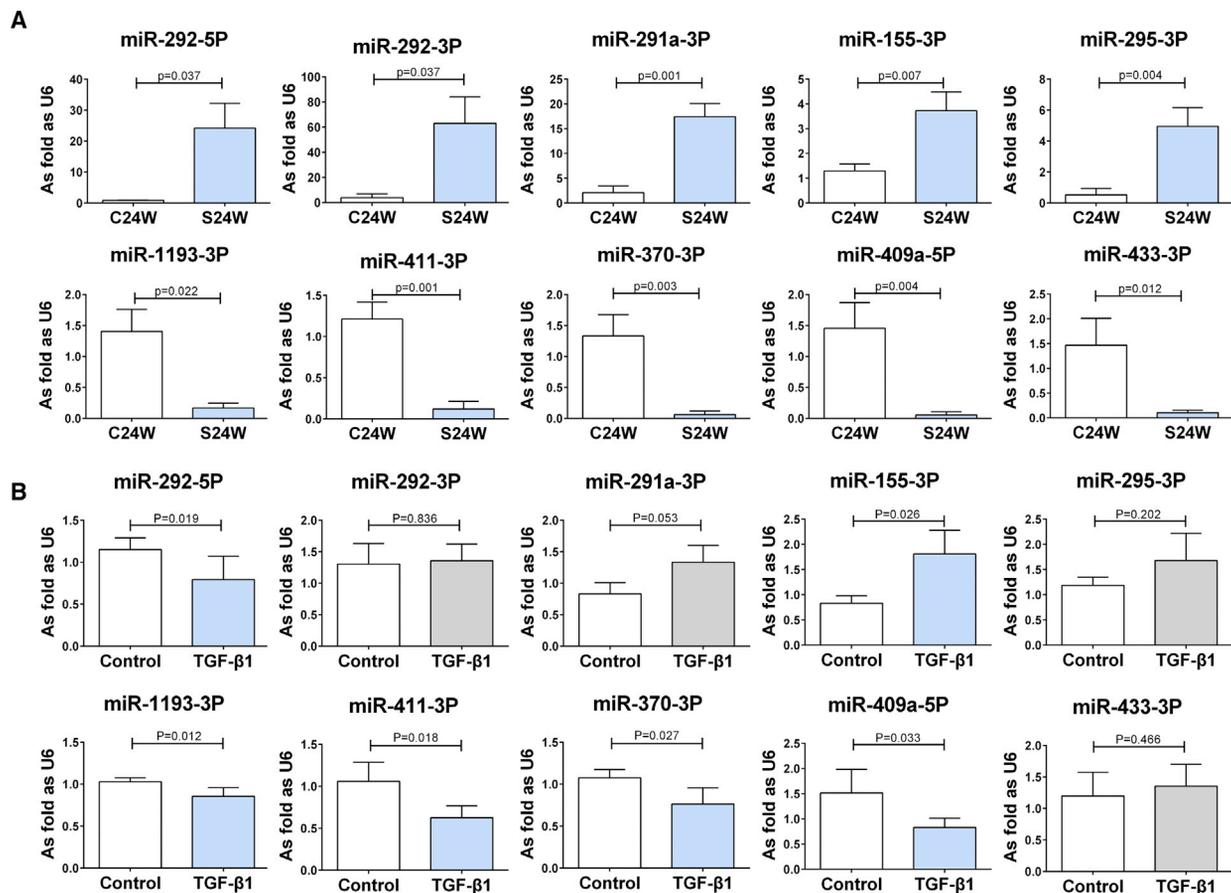


Figure 4. The Levels of miRNAs Identified in Rats or in Fibroblasts Measured by Fluorescent Quantitative Real-Time PCR

(A) The levels of miR-292-5p, miR-292-3p, miR-291a-3p, miR-155-3p, and miR-295-3p increased in silicotic rats accompanied with downregulation of miR-1193-3p, miR-411-3p, miR-370-3p, miR-409a-5p, and miR-433-3p. (B) The level of miR-155-3p was increased accompanied with reduced levels of miR-292-5p, miR-1193-3p, miR-411-3p, miR-370-3p, and miR-409a-5p measured by quantitative real-time PCR. (Data indicate mean \pm SD; n = 3 independent experiments.)

in lung fibroblasts induced by TGF- β 1. These results indicate that MRTF-A plays a role in pulmonary silicosis and that it contributes to myofibroblast differentiation induced by TGF- β 1 by regulating the key effector proteins Col I and α -SMA.

miRNA Expression Is Altered in the Silicotic Rat Lung and TGF- β 1-Induced Fibroblasts

To validate the findings from the high-throughput miRNA screen, we used quantitative real-time PCR to examine the expression of the top five upregulated and downregulated miRNAs in silicotic rat lungs and TGF- β 1-treated lung fibroblasts. As shown in Figure 4A, the quantitative real-time PCR analysis confirmed the changes in expression of these miRNAs in the lungs of silicotic rats, when compared with the control group. Similar directional changes in the expression of

various miRNAs were identified in cultured lung fibroblasts induced by TGF- β 1 (Figure 4B). This included a significant upregulation in miR-155-3p and a significant downregulation in miR-292-5p, miR-1193-3p, miR-411-3p, miR-370-3p, and miR-409a-5p ($p < 0.05$). Taken together, these results suggest that changes in the miRNA expression profile in pulmonary silicosis are, at least in part, downstream of TGF- β 1 receptor activation.

miRNAs Regulate Extracellular Matrix Production and Myofibroblast Formation

To assess whether the miRNAs altered in pulmonary silicosis impact on lung fibroblast behavior, we transfected cultured lung fibroblasts with different miRNA mimics and inhibitors. As showed in Figure 5 and Figure 6, mimics of miR-292-5p, miR-155-3p, and miR-295-3p markedly

Figure 3. MRTF-A Participates in Myofibroblast Differentiation in Silicosis

(A) The co-expression of MRTF-A and α -SMA in lung of rats (scale bars, 100 μ m). (B) Protein levels of SRF and MRTF-A were increased in silicotic rats. (C and D) The (C) mRNA levels and (D) protein levels of Col, α -SMA, SRF, and MRTF-A were increased in lung fibroblasts induced by TGF- β 1. (E) The effective siRNA screened by western blotting. (F) siRNA-MRTF-A reduced the levels of MRTF-A, COL I, and α -SMA. (Data indicate mean \pm SD; n = 3 independent experiments.)

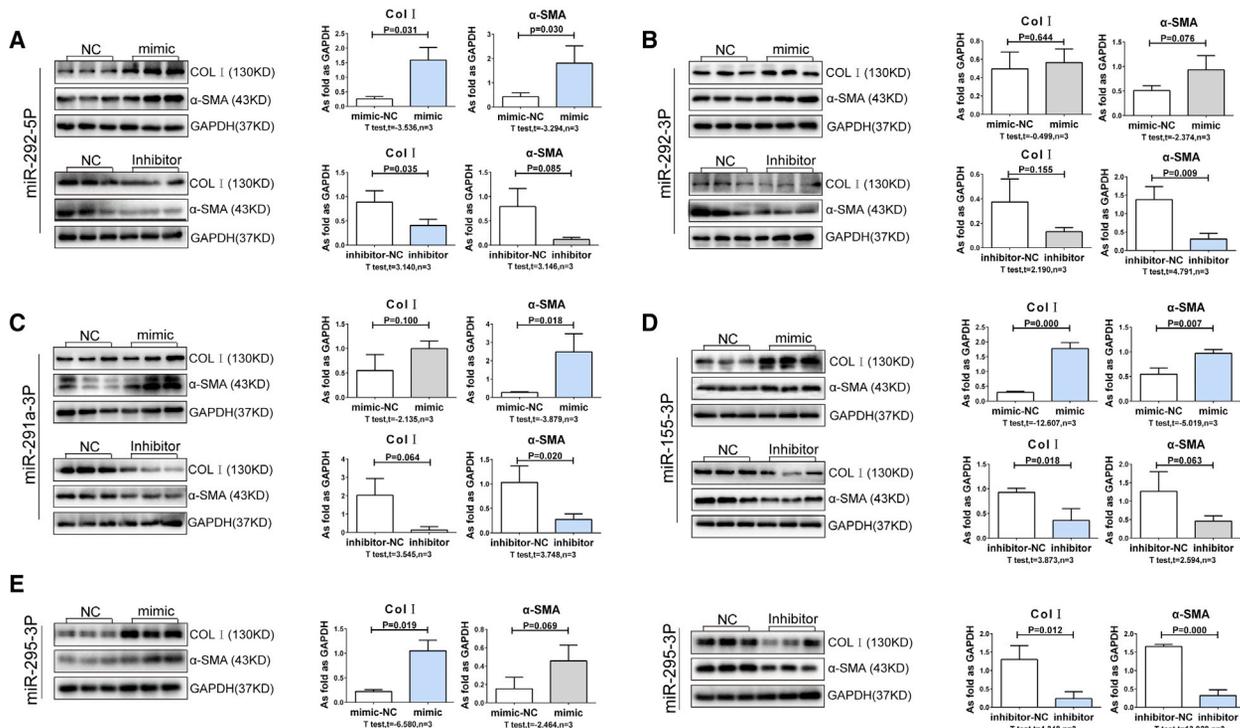


Figure 5. The Mimics and Inhibitors of Upregulated miRNAs Regulate Col I and α -SMA in Lung Fibroblasts in FBS-free Medium

(A–E) The expressions of Col I and α -SMA in lung fibroblasts in FBS-free medium and treated with mimics and inhibitors of (A) miR-292-5p, (B) miR-292-3p, (C) miR-291a-3p, (D) miR-155-3p, and (E) miR-295-3p. (Data indicate mean \pm SD; n = 3 independent experiments.)

increased Col I expression, signifying an increase in extracellular matrix production. Moreover, we found that mimics of miR-292-5p, miR-291a-3p, and miR-155-3p promoted myofibroblast differentiation, as demonstrated by the increase in α -SMA expression. In contrast to these findings, we found that mimics of miR-411-3p and miR-370-3p attenuated Col I levels, while mimics of miR-1193-3p, miR-411-3p, miR-409a-5p, and miR-433-3p inhibited α -SMA expression ($p < 0.05$), indicating the antagonistic roles that miRNAs play, and the intricate balance in target gene expression they must maintain, in lung fibroblasts.

Next, we studied the effects of each miRNA on lung fibroblasts cultured in the presence of TGF- β 1. Consistent with findings in unstimulated cells, we found that mimics of miR-292-5p, miR-292-3p, miR-291a-3p, and miR-155-3p increased Col I expression, whereas mimics of miR-292-5p, miR-292-3p, miR-291a-3p, and miR-295-3p promoted α -SMA expression in TGF- β 1-treated lung fibroblasts (Figure 7). Likewise, mimics of miR-1193-3p, miR-411-3p, miR-370-3p, and miR-409a-5p attenuated Col I expression, while mimics of miR-1193-3p, miR-411-3p, miR-370-3p, and miR-433-3p inhibited TGF- β 1-induced α -SMA expression ($p < 0.05$; Figure 8).

miR-411-3p Inhibits TGF- β 1-Induced Myofibroblast Differentiation by Regulating *Mrtfa*

Given the aforementioned findings, we next searched for putative targets of miR-411-3p using the TargetScan online prediction tool

(http://www.targetscan.org/vert_72/). TargetScan analysis identified *Mrtfa* as a potential target gene of miR-411-3p (Figure 9A). To confirm that *Mrtfa* was, indeed, a target of miR-411-3p, we performed luciferase reporter assays using the 3' UTR of *Mrtfa*, which harbors the putative miR-411-3p target site. As shown in Figure 9B, cells co-transfected with the *Mrtfa*-3' UTR-WT (wild-type) reporter plasmid and miR-411-3p mimic demonstrated significantly reduced luciferase expression when compared with cells transfected with this reporter and the miR-411-3p negative control ($p < 0.05$). These findings indicate that MATF-A is a direct target of miR-411-3p.

In addition to confirming *Mrtfa* as a target of miR-411-3p in reporter assays, we also found that treatment with miR-411-3p mimic suppressed Col I, α -SMA, and MRTF-A mRNA levels in fibroblasts cultured with or without TGF- β 1 (Figures 9C–9E). Furthermore, MRTF-A protein expression was also reduced in these cells (Figures 10A and 10B). mRNA and protein levels for SRF were also reduced in unstimulated fibroblasts following treatment with miR-411-3p mimic (Figures 9D and 10A). Conversely, treatment with an miR-411-3p inhibitor resulted in an increase in mRNA levels for Col I, α -SMA, and MRTF-A in fibroblasts induced by TGF- β 1 (Figure 9E) and an increase in the protein expression of MRTF-A and SRF in these cells, regardless of TGF- β 1 treatment (Figures 10A and 10B).

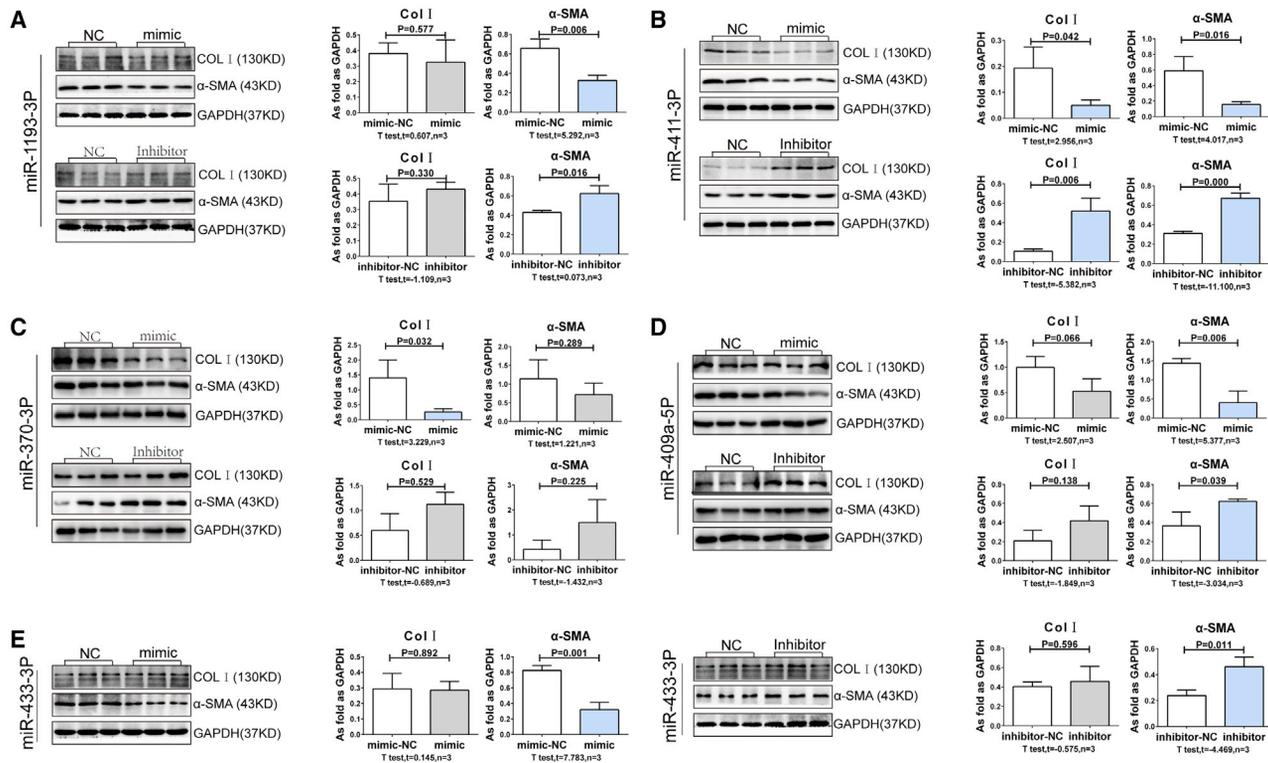


Figure 6. The Mimics and Inhibitors of Downregulated miRNAs Regulate Col I and α -SMA in Lung Fibroblasts in FBS-free Medium

(A–E) The expressions of Col I and α -SMA in lung fibroblasts in FBS-free medium and treated with mimics and inhibitors of (A) miR-1193-3p, (B) miR-411-3p, (C) miR-370-3p, (D) miR-409a-5p, and (E) miR-433-3p. (Data are mean \pm SD; n = 3 independent experiments.)

miR-411-3p Attenuates Silica-Induced Pulmonary Fibrosis *In Vivo*

Because miR-411-3p inhibited the expression of pro-fibrotic factors *in vitro*, we next examined whether administering this miRNA could ameliorate pulmonary silicosis in an experimental model of this disease. First, we confirmed by *in situ* hybridization that miR-411-3p levels were decreased in silicotic lesions of rat lung tissues and also verified similar reductions in mouse and human silicotic tissues using normal lung tissues as a control (Figure 11).

Next, to assess the therapeutic potential of miR-411-3p in pulmonary silicosis, we administered a miR-411-3p agomir to mice 1 week after oropharyngeal instillation of silica dust. As showed in Figure 12A, histological analysis revealed that lung fibrosis was attenuated by miR-411-3p agomir treatment at day 14 after silica administration. *In situ* hybridization also revealed a concomitant increase in miR-411-3p levels in the lung tissue of mice that were treated with agomir (Figure 12B). Moreover, we found that administration of miR-411-3p agomir to mice markedly reduced mRNA and protein levels for MRTF-A as well as α -SMA and Col I (Figures 12C and 12D). In addition, miR-411-3p agomir treatment improved the lung function of mice exposed to silica (Figure 13). These results indicate that miR-411-3p can also regulate MRTF-A *in vivo* and can exert potent anti-fibrotic effects in the mouse lung.

DISCUSSION

In this study, we report on the miRNA expression profile associated with pulmonary silicosis in the rat lung. Although several studies have described changes in miRNAs in mouse models,^{8,9} our study performed a detailed assessment of miRNA expression in a chronic dust inhalation model that mimics the exposure and histological findings in humans.¹³ From this work, we identified 70 miRNAs that are differentially expressed in the silicotic rat lung when compared with controls. Furthermore, we verified these findings through a variety of approaches and also demonstrated similar changes for some of these miRNAs in lung fibroblasts exposed to TGF- β 1. Mechanistic studies confirmed that many of these miRNAs are involved in regulating extracellular matrix production and/or myofibroblast differentiation, and we showed that miR-411-3p has potent anti-fibrotic effects *in vivo* and *in vitro*.

Among the miRNAs dysregulated in our study are several that have been shown to be involved in tissue fibrosis in other organs. For example, miR-21-5p has been implicated as a serum biomarker in patients with idiopathic pulmonary fibrosis.²⁰ Moreover, miR-146b-5p and miR-146b-3p have been shown to be upregulated in various models of kidney injury,²¹ while miR-183-5p has been shown to be a significant predictor of experimentally induced liver fibrosis.²²

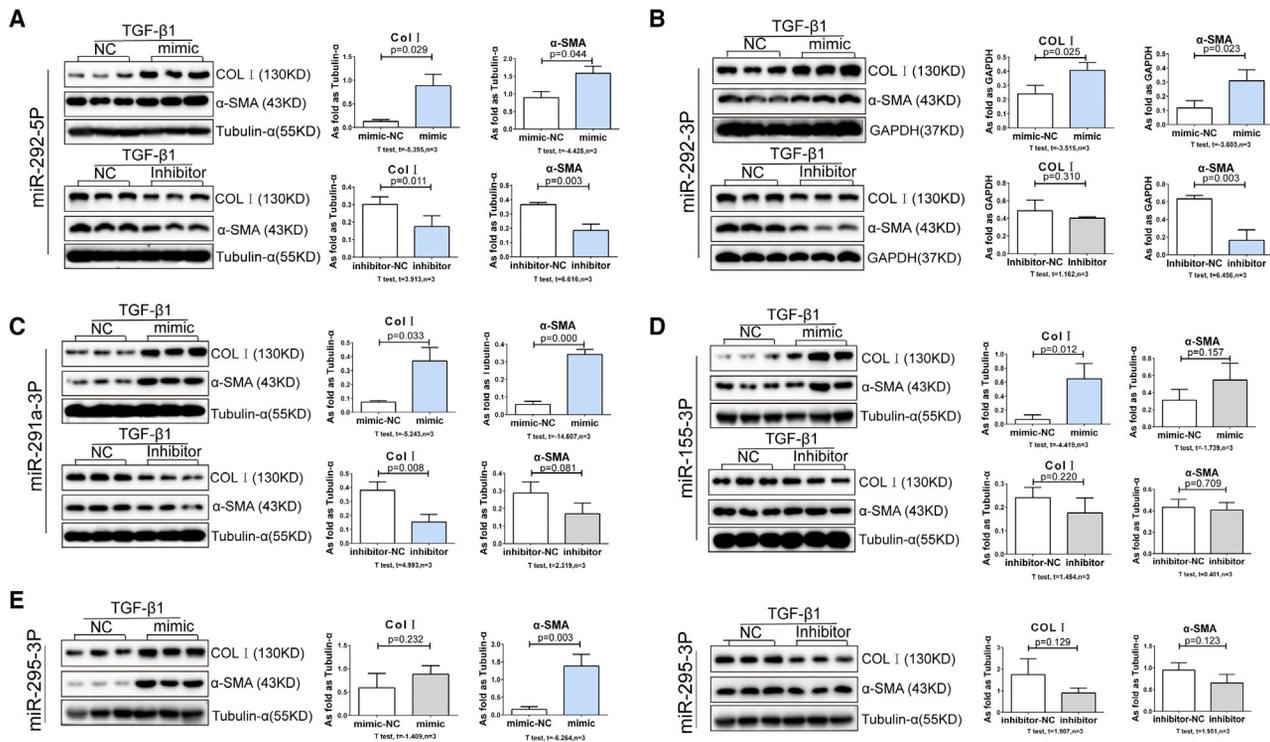


Figure 7. The Mimics and Inhibitors of Upregulated miRNA Regulates Col I and α -SMA in TGF- β 1-Induced Lung Fibroblasts

(A–E) The expressions of Col I and α -SMA in lung TGF- β 1-treated fibroblasts by transfecting with mimics and inhibitors of (A) miR-292-5p, (B) miR-292-3p, (C) miR-291a-3p, (D) miR-155-3p, and (E) miR-295-3p. (Data indicate mean \pm SD; n = 3 independent experiments.)

Although the focus of our study was pulmonary silicosis, we believe that our findings may have broader implications for the pulmonary fibrosis field. This is because several of the miRNAs that were altered in the silicotic lung were also altered in cultured fibroblasts exposed to TGF- β 1. Moreover, we have shown that mimics and inhibitors of these miRNAs have profound effects on either extracellular matrix production or myofibroblast formation.

Recently, a miR-292-5p inhibitor was shown to protect against myocardial ischemia reperfusion injury through activation of the peroxisome-proliferator-activated receptor signaling pathway.²³ Also, miR-292-3p was noted to be one of the most abundant miRNAs in syncytial-exosome-enriched extracellular vesicles.²⁴ Embryonic stem cells have been reported to package miR-291-3p into exosomes that demonstrate anti-senescence activity upon transfer to human dermal fibroblasts, thereby accelerating the excisional skin wound-healing process in aged mice.²⁵ In monocytes and macrophages induced by lipopolysaccharide (LPS), a miR-155-3p mimic promoted increased levels of interleukin (IL)-6.²⁶ miR-411-3p has been reported to suppress the hypoxia-inducible factor 1 α /vascular endothelial growth factor/p38 pathway and to exert an anti-cancer role by promoting apoptosis and inhibiting cell growth, invasion, and migration.²⁷ A potential association between miR-370-3p and pneumonia has been identified from studies in which the normal human fibroblast WI-38 cell line was induced with LPS.²⁸ In the present study, we provide preliminary experimental evidence for

roles for 10 miRNAs in silicosis and fibroblast activation, following their identification as the top differentially expressed genes in a rat model of pulmonary silicosis. We have also identified target genes regulated by these miRNAs that are associated with fibrosis-related signaling pathways, highlighting the potential utility of miRNA-based therapies in the treatment of silicosis.

Myofibroblast differentiation, which is characterized by increased cellular expression of α -SMA, is one of the causes of SRF-dependent collagen deposition in silicosis.¹⁴ MRTF-A, functioning as a co-activator for the SRF-mediated transcription of CARG-box-containing genes, plays a central role in fibroblast activation, myofibroblast differentiation, and collagen deposition.²⁹ MRTF-A also physically interacts with the promoter of the *COL1A1* gene to facilitate histone acetylation and RNA polymerase II recruitment.³⁰ In the present study, we identified the 3' UTR of *Mrtfa* mRNA as a potential target binding site for miR-411-3p, using the TargetScan online tool. Our subsequent *in vitro* assays demonstrated that miR-411-3p is a negative regulator of TGF- β 1-dependent lung fibroblast induction. Given these findings, we examined the therapeutic effects of miR-411-3p *in vivo* and found that it had potent anti-fibrotic effects, not only reducing the histological and biochemical indicators of lung fibrosis but also restoring physiological lung function in animals. We found that miR-411-3p attenuated silicosis by regulating *Mrtfa* expression, resulting in the downregulation of α -SMA and Col I protein levels.

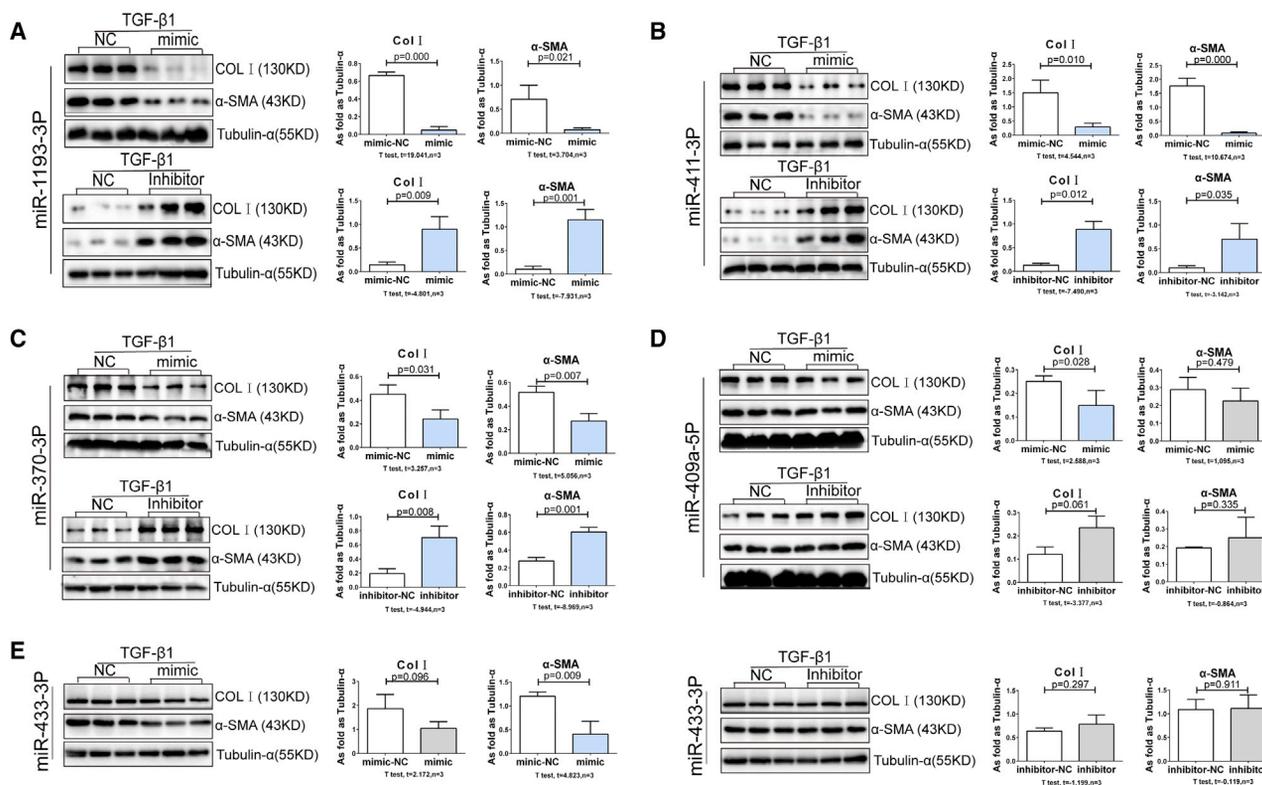


Figure 8. The Mimics and Inhibitors of Downregulated miRNA Regulates Col I and α -SMA in TGF- β 1-Induced Lung Fibroblasts

(A–E) The expressions of Col I and α -SMA in lung TGF- β 1-treated fibroblasts by transfecting with mimics and inhibitors of (A) miR-1193-3p, (B) miR-411-3p, (C) miR-370-3p, (D) miR-409a-5p, and (E) miR-433-3p. (Data indicate mean \pm SD; n = 3 independent experiments.)

In a previous study, we have demonstrated that miR-411-3p inhibits lung fibroblast viability and migration induced by Smurf2/TGF- β 1 signaling.³¹ Taken together, these findings provide strong support for the notion of miRNA targeting as a therapeutic strategy in the treatment of pulmonary silicosis.

Our study demonstrates that miRNA expression is significantly altered in pulmonary silicosis and suggests that therapeutic approaches aimed at restoring miR-411-3p levels may be effective in the treatment of this occupational lung disease. Whether targeting other miRNAs identified in our study would have similar therapeutic effects on pulmonary silicosis is a focus of future investigation.

MATERIALS AND METHODS

Animal Experiments

Three-week-old male Wistar rats, weighing approximately 80 g, were purchased from Vital River Laboratory Animal Technology (Beijing, China). Pulmonary silicosis was induced using previously described methods.¹³ In brief, rats were placed in a HOPE MED 8050 exposure apparatus (HOPE Industry and Trade, Tianjin, China) for 3 h/day for 24 consecutive weeks. Cabinet temperature, humidity and pressure were set at 20°C–25°C, 70%–75%, and –50 to +50 Pa, respectively. The oxygen concentration was maintained at 20%–23%, and the air flow rate was set at 3.0–3.5 mL/min. SiO₂ dust particle concentration

in the cabinet was 50 \pm 10 μ g/m³, with 80% of particles less than 5 μ m in size (S5631, Sigma-Aldrich). Rat lung tissues were harvested at week 24 from the start of the protocol. All animal experiments were approved by the Ethics Committee for Animal Experimentation of North China University of Science and Technology (2013-038) and were in accordance with the guidelines set by the National Institutes of Health.

In vivo therapeutic studies using miR-411-3p were performed in mice. Mice were divided into four groups: (1) control, (2) silica,^{32,33} (3) silica plus agomir-Negative control (NC), and (4) silica plus agomir miR411-3P (n = 6 animals per group).^{31,34} In these studies, pulmonary silicosis was induced by administering a one-time dose of SiO₂ dust into the posterior oropharynx.³⁵ Silica dust (5- μ m silica particles, S5631, Sigma-Aldrich) was administered at a concentration of 100 mg/kg. One week after silica dust administration, 0.05 mL control (agomir-NC) or agomir miR411-3P (5 nmol/mL, RiboBio, Guangzhou, China) was administered by tail vein injection.³¹ Animals were sacrificed at day 14 after silica administration, and harvested lungs were then stored at –80°C for subsequent analysis.

Cell Culture Studies

Rat lung fibroblasts were cultured in 25 cm² dishes in Dulbecco's modified Eagle's medium (BI-SH0019, Biological Industries,

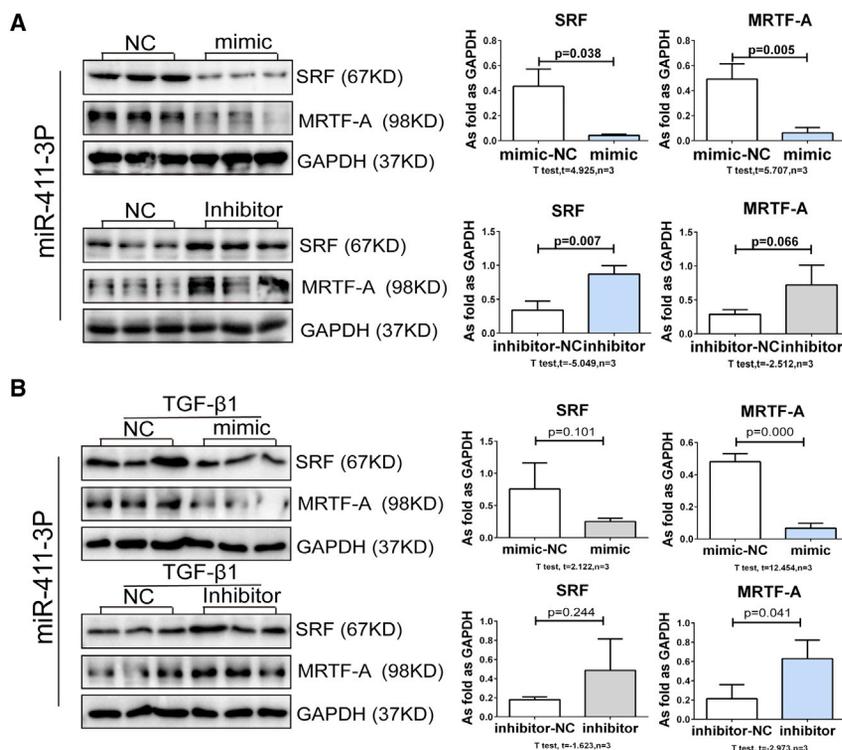


Figure 10. miRNA-411-3p Inhibited MRTF-A in Lung Fibroblasts Activated by TGF- β 1

(A and B) The protein levels of SRF and MRTF-A in (A) lung fibroblasts and (B) TGF- β 1-induced lung fibroblasts transfected with miR-411-3p mimic/inhibitor. (Data indicate mean \pm SD; n = 3 independent experiments.)

In Situ Hybridization of miR-411-3p

Paraffin-embedded lung sections were dewaxed and re-hydrated. Pepsin was used to expose nucleic acid fragments. Hybridization reactions were performed overnight at 38°C–42°C using the miR-411-3p probe sequence 5'-GGTTA GTGGACCGTGTACATA-3'. Sections were washed several times and then incubated with blocking solution. Digoxin-labeled biotinylated antibody was applied for 2 h at 37°C, followed by several washing steps. Cy3-labeled streptavidin-biotin complex was then applied for 1 h at 37°C. Visualization of miR-411-3p-stained cells was performed using standard fluorescence microscopy.

Luciferase Reporter Assay

MRTF-A (MKL1), WT, and mutant (Mut) 3' UTRs were synthesized and cloned into the XhoI/SalI restriction sites of the pmirGLO vector, downstream of the Firefly luciferase reporter gene. The vector also contains a control Renilla luciferase gene for the normalization of the Firefly luciferase signal. pmirGLO-MKL1-3' UTR-WT or pmirGLO-MKL1-3' UTR-Mut constructs were transfected into 293T cells using Lipofectamine 2000 Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Cells were lysed at 48 h post-transfection, and luciferase activity was then measured using the Dual-Luciferase Reporter Assay System (Promega, E1910, Madison, WI, USA). Firefly luciferase activity was normalized to that of Renilla luciferase. All assays were performed in triplicate. Ratios of Firefly luciferase and Renilla luciferase relative light unit (RLU) values were used for comparisons between treatment groups.

Histology and Immunostaining

Lung tissues were stained with hematoxylin and eosin (H&E), Van Gieson's solution (VG), or Sirius red for the semiquantitative analysis of collagen levels. Immunostaining was performed according to published methods.^{14,18} In brief, dewaxed tissue sections were treated with 0.3% H₂O₂ to quench endogenous peroxidases and then subjected to antigen retrieval using a high-pressure method. Samples were incubated overnight at 4°C with a primary antibody against α -SMA (ab32575, Abcam), followed by incubation with a secondary antibody (PV-6000, Beijing Zhongshan Jinqiao Biotechnology, Beijing, China) at 37°C for 30 min before the application of 3,3'-diaminobenzidine tetrahydrochloride substrate (ZLI-9018, ZSGB-BIO, Beijing, China). Brown staining, signifying α -SMA-positive cells, was visualized by light microscopy. Co-staining of

in miRNA expression levels between groups was calculated by the formula: fold change = $|\log_2(\text{test RPM}/\text{control RPM})|$, where RPM refers to reads per million mapped reads. Only those miRNAs demonstrating a $|\log_2(\text{fold change})| \geq 1$ ($p < 0.05$) difference in expression between samples were considered as differentially expressed.

Quantitative Real-Time PCR Analysis

Reverse transcription (TakaRa, Clontech) was performed according to the manufacturer's instructions. Target amplification by quantitative real-time PCR was carried out using the TB Green Premix Ex Taq II (TakaRa, Clontech) system. Each 20- μ L reaction contained cDNA (2 μ L), TB Green Premix Ex TaqII Mix (10 μ L), and specific forward and reverse miRNA primers (5 μ M, 0.8 μ L). Primer sequence details are shown in Tables S3 and S4. Thermocycling was conducted as follows: initial denaturation at 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s. U6 snRNA was used as the internal reference for miRNA, and GAPDH was used as the internal reference for Col I, α -SMA, SRF, and MATF-A. The results were calculated using the $2^{-\Delta\Delta C_t}$ method.

Analysis of Differentially Expressed miRNAs

Differentially expressed miRNAs were determined using the TargetScan program (<http://www.targetscan.org/>). Functional categories were ascertained using GO analysis (<http://www.geneontology.org>) and the KEGG pathway analysis (<https://www.genome.jp/kegg/pathway.html>).

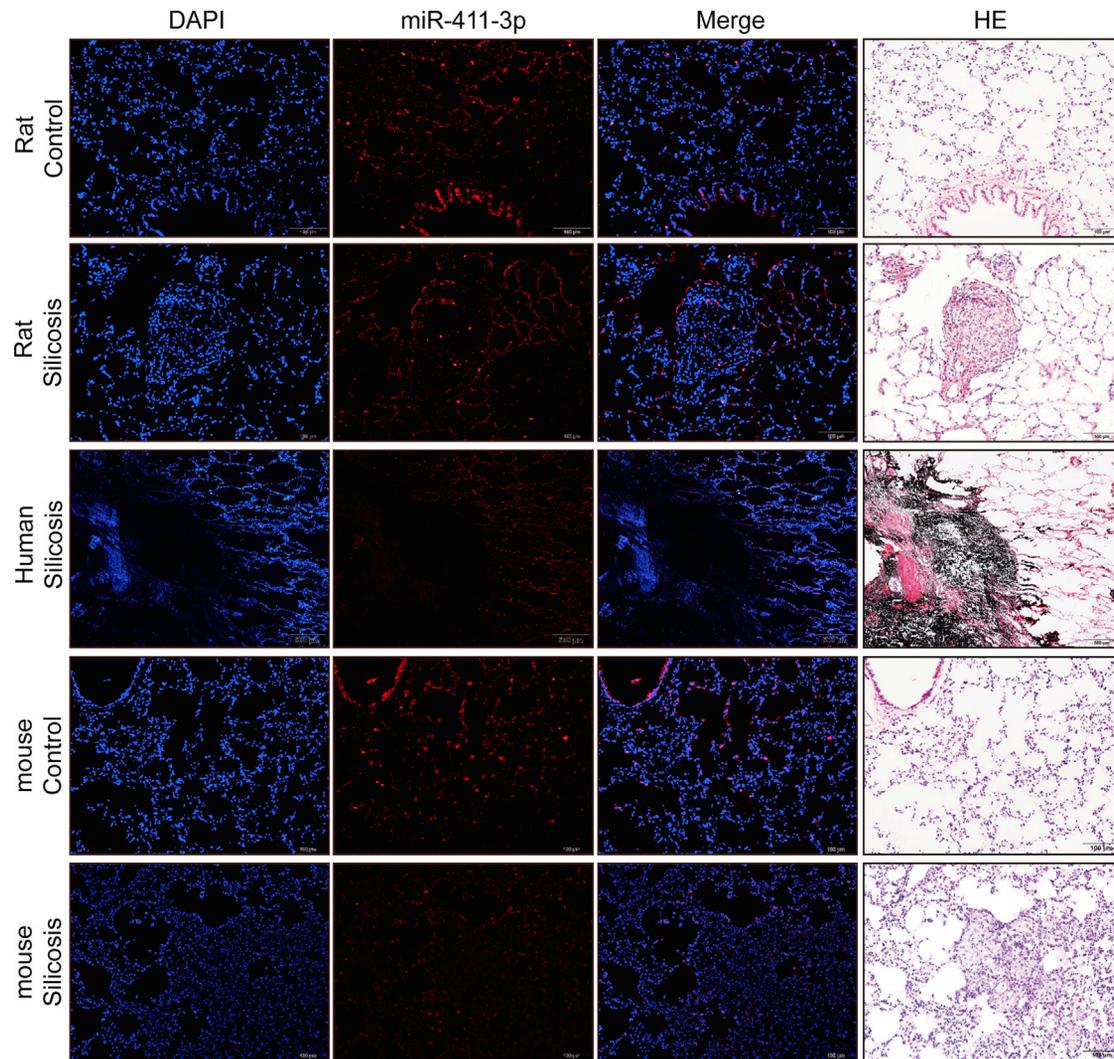


Figure 11. The Location of miR-411-3p in Lung Tissue Observed with *In Situ* Hybridization

Nuclear was stained by DAPI (blue), and miR-411-3p was marked by Cy3 (red) in immunofluorescence staining. H&E staining indicated the morphological change at the same location of immunofluorescence staining (scale bars: rat control, 100 μ m; rat silicosis, 100 μ m; human silicosis, 500 μ m; mouse control, 100 μ m; mouse silicosis, 100 μ m).

α -SMA and MATF-A was performed according to our previously published method¹⁸ using primary antibodies against α -SMA (ab7817, Abcam) and MATF-A (A12598, ABclonal).

Western Blotting

Western blotting was performed according to standard protocols using a Bicinchoninic acid (BCA) protein concentration determination kit (70-PQ0012, MultiSciences) and primary antibodies against Col I (ab34710, Abcam), α -SMA (ab32575, Abcam), SRF (AF6160, Affinity), MATF-A (A12598, ABclonal), and α -tubulin (sc-53029, Santa Cruz Biotechnology).^{13,18} Membranes were probed with a peroxidase-conjugated, affinity-purified secondary antibody to rabbit/mouse IgG (H+L, 074-1506/074-1806, Kirkegaard and Perry Laboratories, Gaithersburg, MD). Target bands were visualized by the addition of ECL Prime Western Blotting Detection Reagent

(RPN2232, GE Healthcare, Hong Kong, China). The expression of proteins of interest was normalized to that of the α -tubulin control.

Lung Function

Respiratory parameters were assessed in whole-body plethysmograph chambers (Buxco FinePointe Whole Body Plethysmography, Buxco Research Systems, St. Paul, MN, USA) according to published methods.³⁶ In brief, rats were placed in the chambers and allowed to acclimatize for 10 min before measurements were performed (respiratory parameters are shown in Table S5). Lung function in mice was normalized to body weight.³⁷

Statistical Analyses

The SPSS v.22.0 software package (SPSS, Chicago, IL, USA) was used to perform statistical analyses. All results are presented as

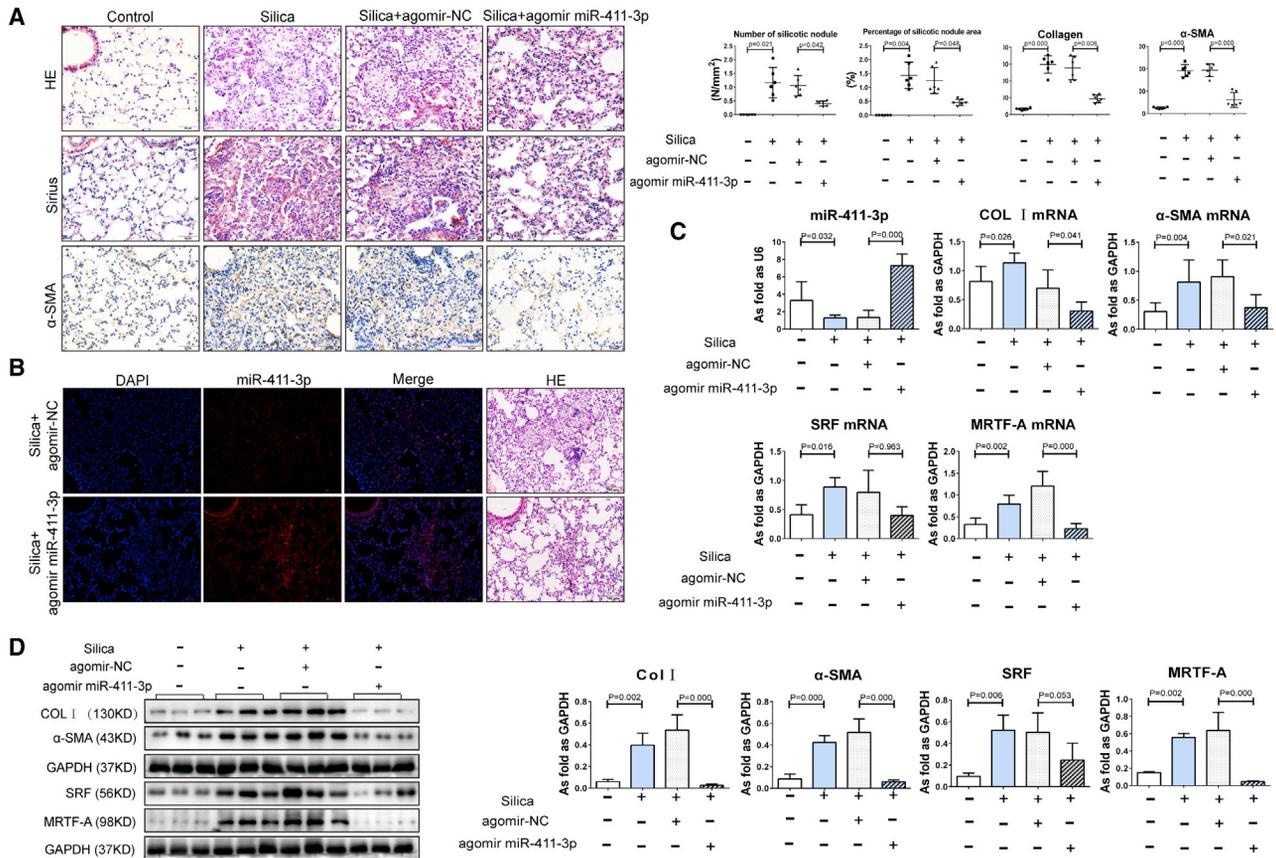


Figure 12. Agomir of miR-411-3p Inhibited Silicosis in Mice Exposed to Silica

(A) H&E, Sirius red staining, and positive expression of α -SMA showed silicotic lesions induced by SiO₂ in the silica and the silica+agomir-NC groups. Treatment with agomir miR-411-3p could reduce silicotic lesions compared to the silica+agomir-NC group (scale bars, 50 μ m). (B) The miR-411-3p expression was shown by *in situ* hybridization (scale bars, 100 μ m). (C) The level of miR-411-3p was reduced in the silica and the silica+agomir-NC groups and was increased in the agomir miR-411-3p group. The mRNA levels of Col I, α -SMA, SRF, and MRTF-A were increased in the silica and the silica+agomir-NC groups and were decreased in the agomir miR-411-3p group. (D) The protein levels of Col I, α -SMA, SRF, and MRTF-A were increased in the silica and the silica+agomir-NC groups and were decreased in the agomir miR-411-3p group. (Data indicate mean \pm SD; n = 6 independent experiments.)

mean \pm standard deviation (SD). Analysis of variance (ANOVA) was performed when comparing more than two groups, using the least significant difference test where variance was constant and Tamhane's test where variance was unequal. The independent samples t test was used to compare the means of two groups. Statistical significance was set at $p < 0.05$, with a 95% confidence interval.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2020.05.005>.

AUTHOR CONTRIBUTIONS

H.X.: data collection, analysis, drafting the manuscript, and final approval of the manuscript; X.G., D.X., Z.W., Shumin Li, W.C., Shifeng Li, F.J., Y.L., and X.Y.: data collection, analysis, and interpretation; D.X., Z.W., Shifeng Li, and H.L.: collection of experimental samples; X.G. and H.X.: writing the manuscript; H.X.

and F.Y.: study concept and design. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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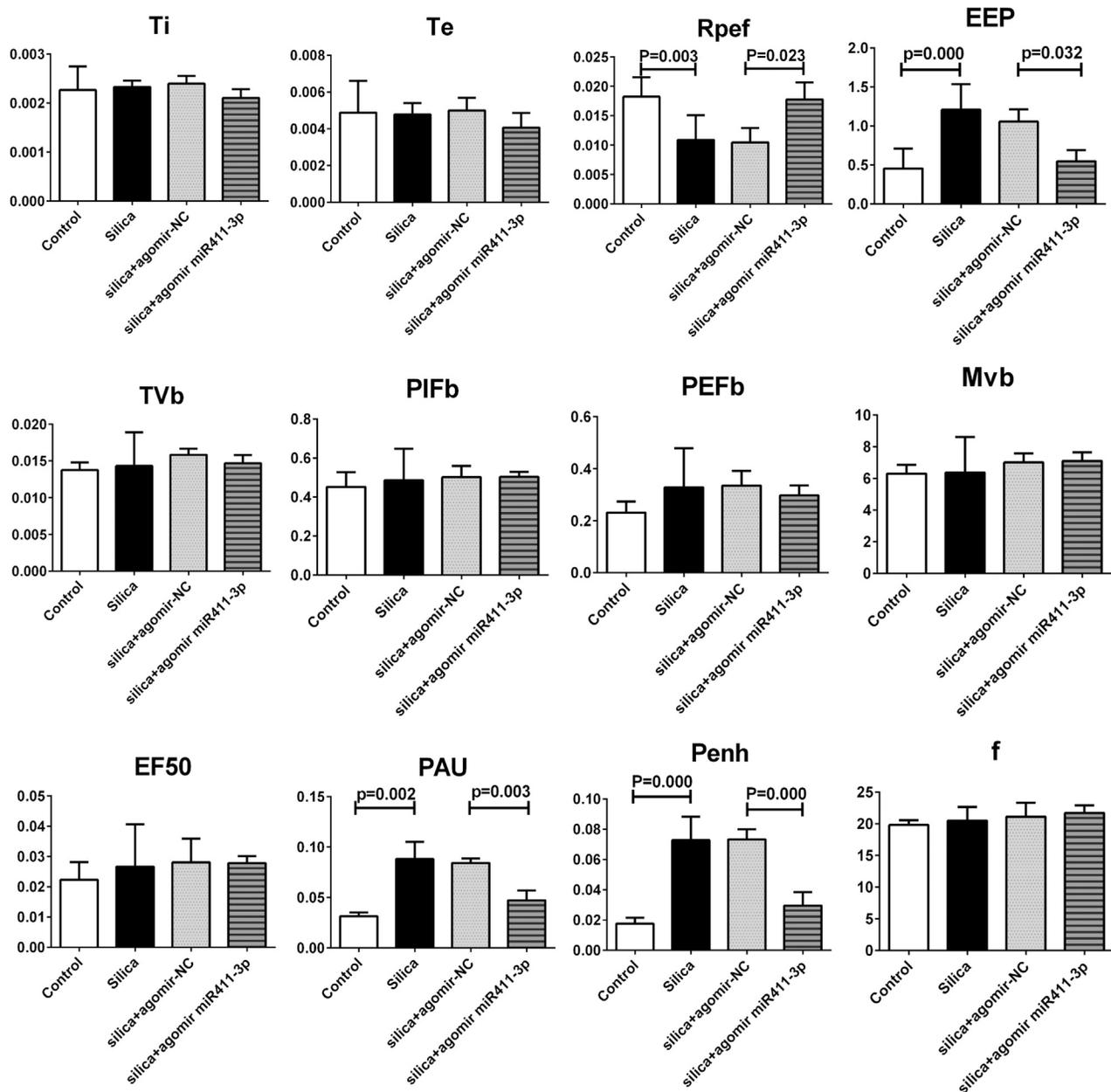


Figure 13. Agomir of miR-411-3p Regulated Lung Function in Mice Exposed to Silica

Agomir of miR-411-3p upregulated the level of Rpef and downregulated the level of EEP, PAU, and Penh. (Data indicate mean \pm SD; n = 6 independent experiments.)

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