

Inhibition of miR-155-5p Exerts Anti-Fibrotic Effects in Silicotic Mice by Regulating Meprin α

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Silicosis is a fatal profession-related disease linked to long-term inhalation of silica. The present study aimed to determine whether meprin α , a master regulator of anti-fibrotic peptide *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), is diminished by miR-155-5p in silicotic and control lung macrophages and fibroblasts upon activation. NR8383 macrophages, primary lung fibroblasts, and mouse embryonic fibroblasts were used to evaluate the expression and function of meprin α and miR-155-5p. *In vitro* meprin α manipulation was performed by recombinant mouse meprin α protein, actinonin (its inhibitor), and small interfering RNA knock-down. Macrophage and fibroblast activation was assessed by western blotting, real-time PCR, matrix deposition, and immunohistochemical staining. The roles of meprin α and miR-155-5p were also investigated in mice exposed to silica. We found that the meprin α level was stably repressed in silicotic rats. *In vitro*, silica decreased meprin α , and exogenous meprin α reduced activation of macrophages and fibroblasts induced by profibrotic factors. miR-155-5p negatively regulated *Mep1a* by binding to the 3' untranslated region. Treatment with anti-miR-155-5p elevated meprin α , ameliorated macrophage and fibroblast activation, and attenuated lung fibrosis in mice induced by silica. The sustained repression of meprin α and beneficial effects of its rescue by inhibition of miR-155-5p during silicosis indicate that miR-155-5p/meprin α are two of the major regulators of silicosis.

INTRODUCTION

As a natural tetra peptide with anti-fibrotic and anti-inflammatory properties, *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) prevents collagen deposition and myofibroblast differentiation in rats exposed to silica.¹ Recently, Ac-SDKP was shown to be released by the nephron from hydrolyzed thymosin β 4 (T β 4) by meprin α .^{2,3} Meprin α belongs to the astacin family and metzincin superfamily of metalloproteases, which targets a wide variety of substrates including basement membrane proteins, cytokines, adherens junction proteins, growth factors, protein kinases, bioactive peptides, and cell surface proteins.^{4,5}

Soluble meprin α and membrane-bound meprin A (heterodimer of meprin α and β subunits) have been identified based on their different cellular localizations and oligomeric forms.⁶ In peripheral blood mononuclear cells, meprin α promotes the production of tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) by increasing nuclear factor κ B (NF- κ B) transcriptional activity induced by lipopolysaccharide (LPS).⁷ In addition, meprin α cleaves the N-terminal domain of monocyte chemoattractant protein 1 (MCP-1) and promotes the loss of biological activity of MCP-1.⁸ Meprin α also removes both C- and N-terminals of type I procollagen, thereby releasing mature collagen I and contributing to the integrity of connective tissue in skin.⁹ Many studies have indicated that meprin α and β play important roles in fibrosis by regulating inflammation and the extracellular matrix (ECM).⁴ However, unlike in meprin β knockout (KO) mice, meprin α KO does not decrease collagen deposition or tissue density.¹⁰ In the present study, macrophages and fibroblasts were treated with recombinant meprin α , actinonin (an inhibitor of meprin α), and small interfering RNA (siRNA)-meprin α to explore its roles in collagen deposition. Our study provides another explanation linking deregulation of meprin α expression to the development of pulmonary fibrosis. We found that recombinant meprin α attenuated collagen synthesis in macrophages and fibroblasts.

Further analyses showed that *Mep1a* was a target gene regulated by miR-155-5p. Global microRNA (miRNA) expression profiling showed that miR-155-5p was increased in silicotic rats. A recent study indicated that the level of miR-155-5p is significantly increased in plasma from arterial and coronary sinuses of patients with advanced heart failure.¹¹ In addition, miR-155-5p promotes fibrosis of proximal tubule cells and epithelial-mesenchymal transition (EMT) by modulating transforming growth factor β (TGF- β)1 under hypoxic

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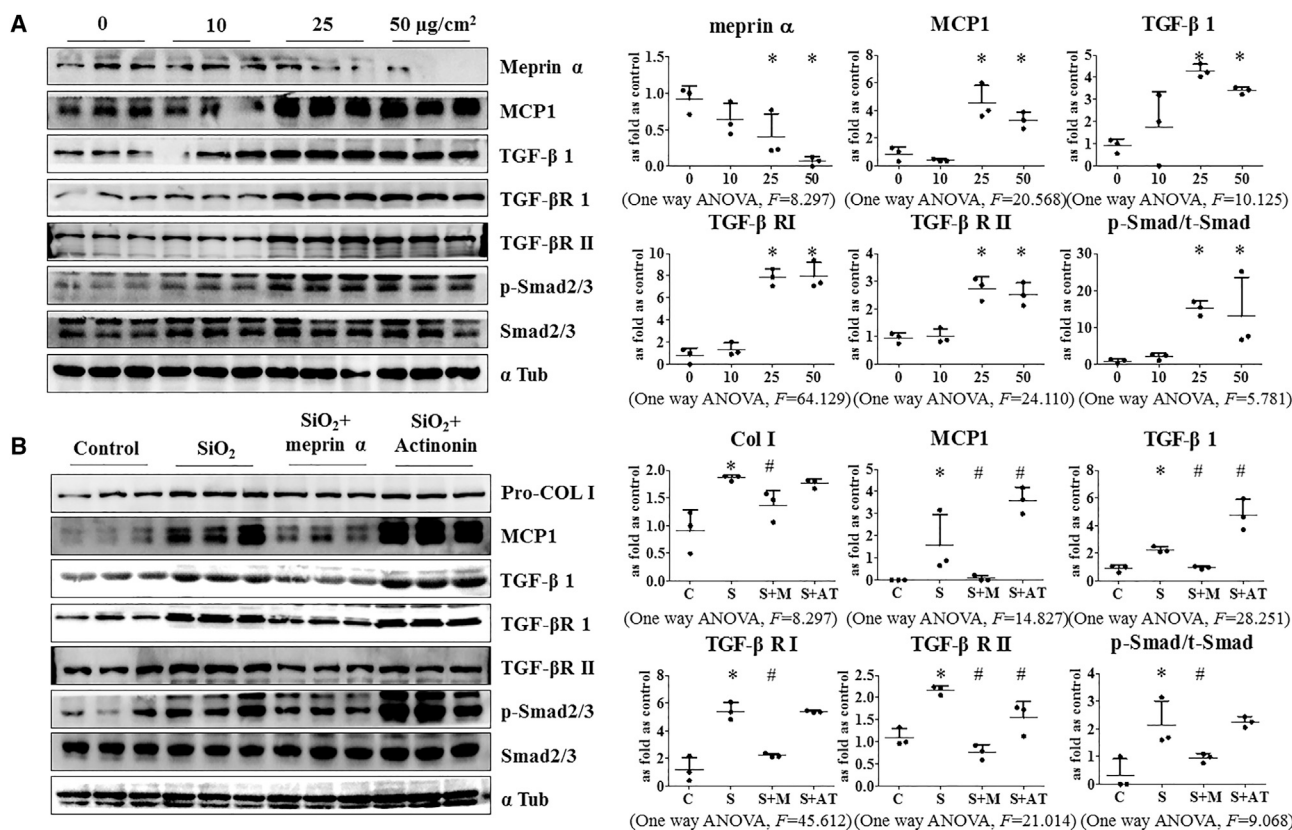


Figure 1. Recombinant Meprin α Inhibits Activation of NR8383 Macrophages Induced by SiO₂

(A) Protein expression of meprin α , MCP-1, TGF- β 1, TGF- β R I, TGF- β R II, and p-Smad2/3 was measured in NR8383 cells treated with 10, 25, and 50 $\mu\text{g}/\text{cm}^2$ silica. * Compared with control group, $p < 0.05$. (B) Expression of pro-COL I, MCP-1, TGF- β 1, TGF- β R I, TGF- β R II, and p-Smad2/3 was measured in NR8383 cells treated with SiO₂, SiO₂ plus meprin α , and SiO₂ plus actinonin. * Compared with control group, $p < 0.05$; # Compared with SiO₂ group, $p < 0.05$. Data are presented as the mean \pm SD. $n = 3$ per group.

conditions.¹² Studies have also shown that miR-155 is a crucial miRNA for the development of fibrosis, and depletion of miR-155 abrogates collagen synthesis in both animal models of fibrosis and human fibroblast cell lines derived from fibrotic lesions.¹³

We predicted the existence of binding sites in the *Mep1a* 3' untranslated region (UTR) for miR-155-5p by online bioinformatics analysis (TargetScan).¹⁴ Therefore, we hypothesized that miR-155-5p/meprin α may be involved in regulation of the activation process of macrophages and fibroblasts during silicosis. In this study, we verified that miR-155-5p inhibited the mRNA and protein levels of meprin α by binding to the *Mep1a* 3' UTR. Moreover, treatment with a miR-155-5p inhibitor attenuated collagen deposition, myofibroblast differentiation, and macrophage activation *in vitro* and *in vivo*.

RESULTS

Silica Induces a Reduction of Meprin α in NR8383 Cells, and Meprin α Inhibits Macrophage Activation

Because silica is a classical activator of macrophages,¹⁵ we examined the level of meprin α in NR8383 cells treated with silica. As shown in

Figure 1A, silica increased the levels of MCP-1, TGF- β 1, TGF- β receptor (TGF- β R) I, TGF- β R II, and phosphorylated Smad2/3 (p-Smad2/3) in NR8383 cells in a dose-dependent manner. Silica also reduced the protein level of meprin α . To assess whether induction of meprin α exerted anti-fibrotic or pro-fibrotic effects, we treated NR8383 cells with SiO₂ and mouse recombinant meprin α or actinonin (an inhibitor of meprins). Of note, treatment with mouse recombinant meprin α reduced MCP1 and TGF- β 1 signaling in NR8383 cells and inhibited the enhancing effect of silica on pro-collagen I (pro-COL I), MCP-1, TGF- β 1, TGF- β R I, TGF- β R II, and p-Smad2/3 expression (Figure 1B). In contrast, treatment with actinonin increased the levels of MCP-1, TGF- β 1, TGF- β R II, and p-Smad2/3 in NR8383 cells treated with silica.

Meprin α Inhibits TGF- β 1 Signaling Activation in Fibroblasts Induced by Silica-Treated Macrophages

Several studies suggest that macrophage activation induced by silica initiates lung fibrosis characterized by fibroblast activation and collagen synthesis.^{16,17} Fibroblasts activated by TGF- β 1 or activated macrophages *in vitro* are classical models in lung fibrosis studies.¹ Therefore, we subsequently explored the effects of meprin α on fibroblasts treated

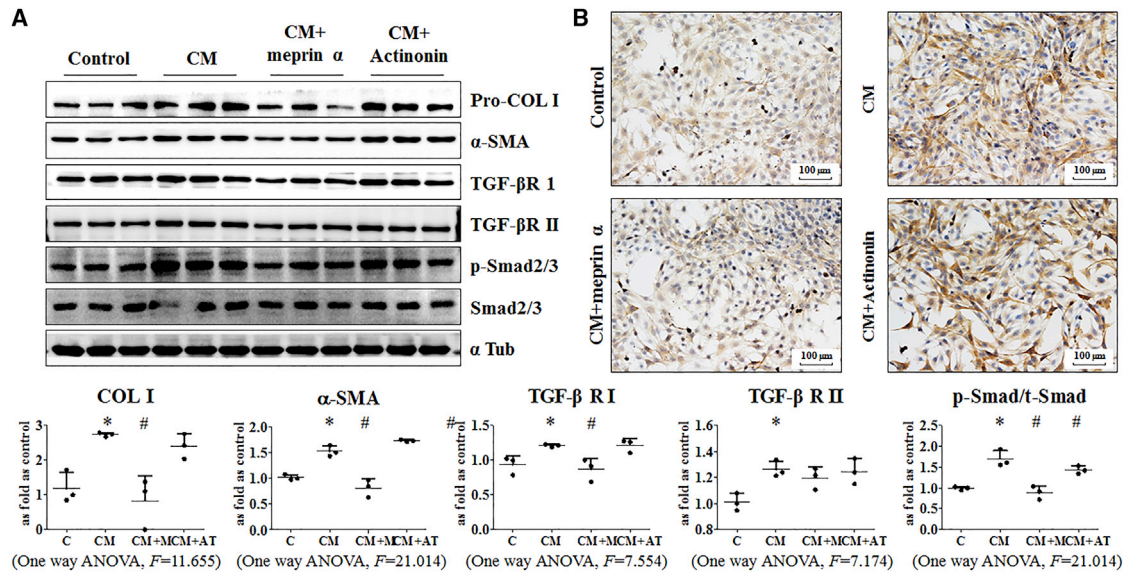


Figure 2. Recombinant Meprin α Inhibits the Fibrotic Response in Fibroblasts Treated with CM of Silica-Treated Macrophages

(A) Protein expression of pro-COL I, α -SMA, TGF- β R I, TGF- β R II, and p-Smad2/3 was measured in lung fibroblasts treated with CM plus meprin α or actinonin. Data are presented as the mean \pm SD. $n = 3$ per group. * Compared with control group, $p < 0.05$; # Compared with CM group, $p < 0.05$. (B) Expression of α -SMA in lung fibroblasts observed by IHC staining (Bar = 100 μ m).

with conditioned medium (CM) of NR8383 macrophages exposed to silica. As expected, CM readily induced fibroblast activation as indicated by enhanced expression of pro-COL I, α -smooth muscle actin (α -SMA), TGF- β R I, TGF- β R II, and p-Smad2/3 (Figure 2; Figure S2A). Treatment with meprin α reversed the profibrotic effects on fibroblast activation induced by CM of silica-treated NR8383 cells. Furthermore, TGF- β 1 treatment reduced the level of meprin α in murine embryonic fibroblasts (MEFs) (Figure 3A). Similar to NR8383 cells, mouse recombinant meprin α decreased expression of pro-COL I and α -SMA in fibroblasts treated with TGF- β 1, and treatment with actinonin or *Mep1a* siRNA promoted pro-COL I and α -SMA expression enhanced by TGF- β 1 (Figures 3B–3D). Overall, these findings indicate that exogenous meprin α reduces activation of macrophages and fibroblasts induced by profibrotic factors.

Silica Induces miR-155-5p and Negatively Regulates *Mep1a*

Next, we determined the effect of silica inhalation on lung meprin α expression levels. As shown in Figures 4A and S2B, silicotic rats exposed to silica for 4, 12, and 24 weeks had a significant decrease in their lung level of meprin α . Western blot analysis also demonstrated increases in the levels of pro-COL I, α -SMA, MCP-1, and TGF- β 1 in rats exposed to silica for 24 weeks.

Using a miRNA microarray, dozens of various miRNAs were found in silicotic rats, and miR-155-5p was increased in rats exposed to silica (data not shown). A recent study indicated that miR-155 is a crucial miRNA for the development of fibrosis.¹³ According to the predictive search by the analysis software, we found a target binding site of miR-155-5p in the *Mep1a* 3' UTR.

To assess the relationship of miR-155-5p with *Mep1a*, we first confirmed that upregulation of miR-155-5p was accompanied by a low level of *Mep1a* in the lung after silica exposure. As shown in Figure 4B, we detected a decrease in the mRNA level for meprin α in lungs of rats exposed to silica. Moreover, this downregulation of *Mep1a* was associated with a significant increase of miR-155-5p in rat lungs. Furthermore, we observed that treatment with the miR-155-5p agomir reduced the mRNA and protein levels of meprin α , and significantly increased levels of meprin α were found after treatment with the miR-155-5p antagonist in RAW 264.7 cells (Figure 4C). Luciferase reporter assays demonstrated that *Mep1a* was a target of miR-155-5p (Figure 4E). Taken together, these data support the concept that miR-155-5p negatively regulates *Mep1a* by binding to its 3' UTR.

miR-155-5p Promotes Macrophage and Fibroblast Activation by Inhibiting Meprin α

To determine whether miR-155-5p is important for macrophage and fibroblast activation *in vitro*, we applied the miR-155-5p agomir and antagonist to NR8383 cells for 24 h. As expected, activation of miR-155-5p by the agomir enhanced the levels of MCP-1, TGF- β 1, TGF- β R I, TGF- β R II, and p-Smad2/3 in NR8383 cells treated with SiO₂ (Figure 5). To explore the role of miR-155-5p in macrophage-fibroblast crosstalk, we used CM of NR8383 cells treated with SiO₂ plus agonist miR-155-5p (agomiR-155-5p) or antagonist miR-155-5p (antamiR-155-5p) to treat lung fibroblasts. As shown in Figures 6 and S2C, the CM of NR8383 cells treated with SiO₂ plus agomiR-155-5p increased the levels of pro-COL I, MCP-1, TGF- β 1, TGF- β R I, TGF- β R II, and p-Smad2/3 in fibroblasts, and treatment with antamiR-155-5p resulted in the opposite effect. Similarly, we found

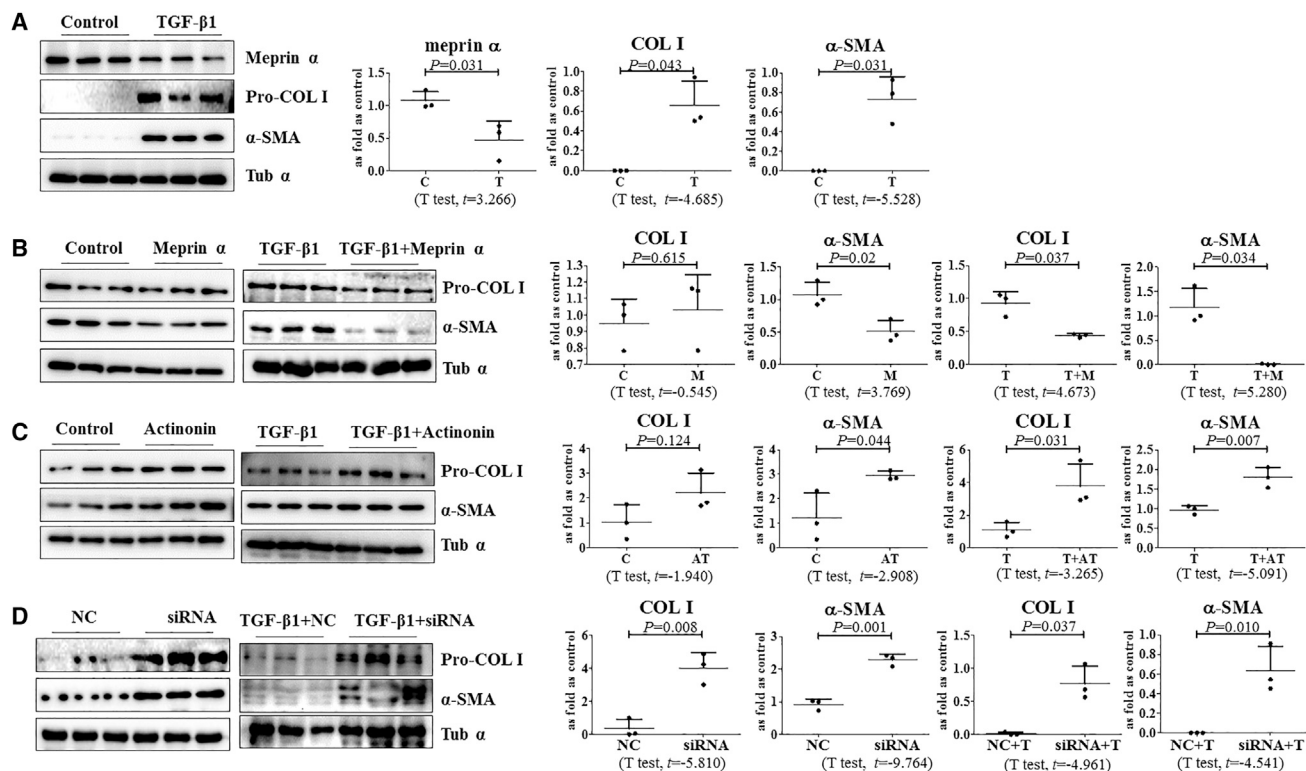


Figure 3. Recombinant Meprin α Inhibits Collagen Deposition in Fibroblasts Treated with TGF- β 1

(A) Protein expression of meprin α , pro-COL I, and α -SMA was measured in lung fibroblasts treated with or without TGF- β 1. (B–D) Expression of pro-COL I and α -SMA was measured in lung fibroblasts treated with (B) meprin α or TGF- β 1 plus meprin α and (C) actinonin or TGF- β 1 plus actinonin, and in (D) MEFs transfected with *Mep1a* siRNA or TGF- β 1 plus *Mep1a* siRNA. Data are presented as the mean \pm SD. $n = 3$ per group.

that the miR-155-5p agomir also promoted pro-COL I and α -SMA expression in fibroblasts treated with TGF- β 1 (Figure 7). In contrast, the miR-155-5p antagonir largely reduced the effects of profibrotic factors in macrophages and fibroblasts treated with SiO₂, CM, or TGF- β 1, indicating that inhibition of miR-155-5p may exert an anti-fibrotic effect.

miR-155-5p Antagonir Abrogates Lung Fibrosis in Mice Exposed to Silica

Using an acute silicotic model, we also found a significant decrease in the lung level of meprin α and higher levels of pro-COL I, α -SMA, MCP-1, and TGF- β 1 in mice exposed to silica for 3, 7, and 14 days compared with the control (Figure 8A). Because inhibition of miR-155-5p reduced macrophage and fibroblast activation, we considered that inhibiting miR-155-5p might reduce fibrotic responses to silica *in vivo*. To test this hypothesis, we administered the miR-155-5p antagonir to mice exposed to silica.

We observed that treatment with the miR-155-5p antagonir overcame the suppression of lung functions in response to silica exposure (Figures 8B and 8C; Tables S1–S16) and significantly attenuated fibrotic remodeling in silica-exposed lungs, as assessed by histology (H&E and Van Gieson's [VG] staining). Consistent with our *in vitro* observations,

treatment with the miR-155-5p antagonir augmented the expression of meprin α and dramatically decreased pro-COL I, α -SMA, MCP-1, and TGF- β 1 in the lungs (Figure 9; Figure S2D). In addition, treatment with the antagonir resulted in an increase of meprin α . Taken together, these data support the concept that augmenting meprin α by inhibiting miR-155-5p suppresses macrophage and fibroblast activation and prevents fibrotic remodeling in the lungs.

DISCUSSION

This study demonstrated that increasing miR-155-5p is an essential response to silica, which promotes pulmonary fibrosis by inhibiting meprin α . Specifically, we observed that the response to silica in lungs is to upregulate miR-155-5p and decrease the level of meprin α . Enhanced production of meprin α by blocking miR-155-5p was required to maintain ECM homeostasis by inhibiting pro-COL I, α -SMA, MCP-1, and TGF- β 1/Smad2/3 signaling *in vitro*. Importantly, we showed that augmenting meprin α expression by a miR-155-5p antagonir attenuated collagen deposition and reduced silica-induced pulmonary fibrosis, suggesting that upregulating meprin α or inhibition of miR-155-5p might be an effective therapy for fibrotic lung diseases.

Our study provides another explanation linking enhancement of meprin α expression to the development of pulmonary fibrosis. It is

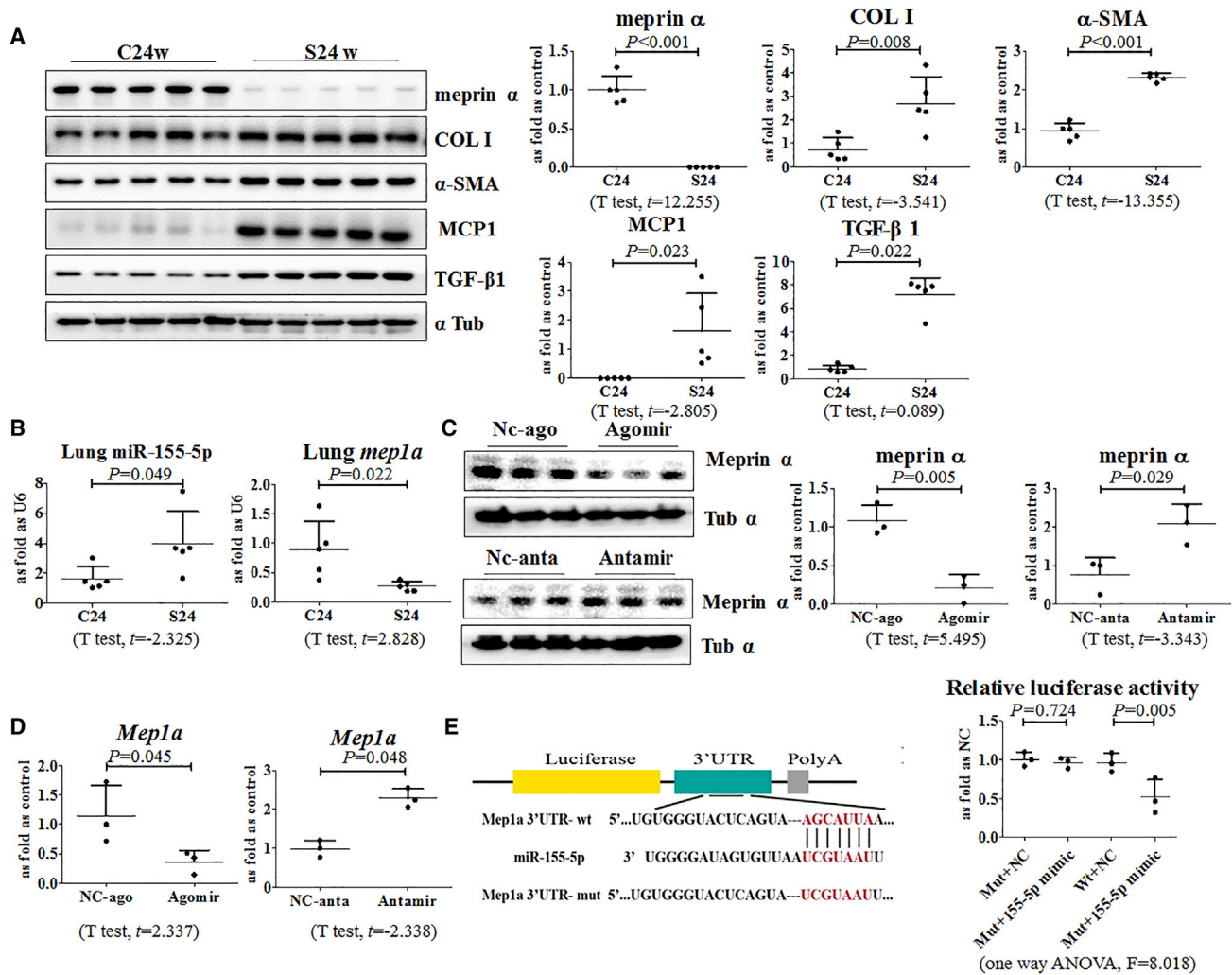


Figure 4. miR-155-5p Negatively Regulates *Mep1a*

(A) Protein expression of meprin α , pro-COL I, TGF- β 1, α -SMA, and MCP-1 was detected by western blotting and quantified. (B) Levels of miR-155-5p and *Mep1a* in rat lungs. Data are presented as the mean \pm SD. $n = 5$ per group. (C and D) Protein (C) and (D) mRNA (D) levels of meprin α in RAW264.7 cells treated with agomiR-155-5p or anti-miR-155-5p. (E) Luciferase reporter assay demonstrating *Mep1a* was a target of miR-155-5p. Data are presented as the mean \pm SD. $n = 3$ per group.

recognized that meprin α releases C- and N-propeptides from type I procollagen⁹ and type III procollagen,¹⁸ which contributes to the generation of mature collagen molecules that spontaneously assemble into collagen fibrils. Furthermore, meprin α hydrolyses basement membrane components such as collagen IV, nidogen-1, and fibronectin.¹⁹ It is well known that meprin α targets cytokines and chemokines to regulate inflammatory processes and control immune cell recruitment by its proinflammatory or anti-inflammatory roles.⁴ It is difficult to determine the precise roles of meprin α in fibrosis because of the wide range of its substrates and various proteases with similar effects. Data from meprin α KO mice also showed that reducing meprin α does not inhibit bleomycin-induced lung fibrosis, which can be explained by other proteases being activated as a compensatory mechanism.¹⁰ In the present study, we speculated that enhancing meprin α expression in macrophages induced by

silica and subsequently inhibiting macrophage and fibroblast activation to maintain ECM homeostasis would suppress fibrotic remodeling.

Based on the global miRNA expression profiling of silicotic rats, miR-155 was increased in rats exposed to silica. Several studies have documented that miR-155 is consistently upregulated in fibrotic disorders, and its ablation downregulates collagen synthesis.¹³ First, we found that miR-155-5p expression was increased and *Mep1a* expression was decreased in silicotic rats. It has been reported that activated macrophages secrete miR-155-containing exosomes that are taken up by cardiac fibroblasts and promote the inflammatory response.²⁰ Treatment with agomiR-155-5p activated macrophages and fibroblasts, and miR-155-5p-treated macrophage-derived medium promoted collagen synthesis in fibroblasts. Collectively, we found that

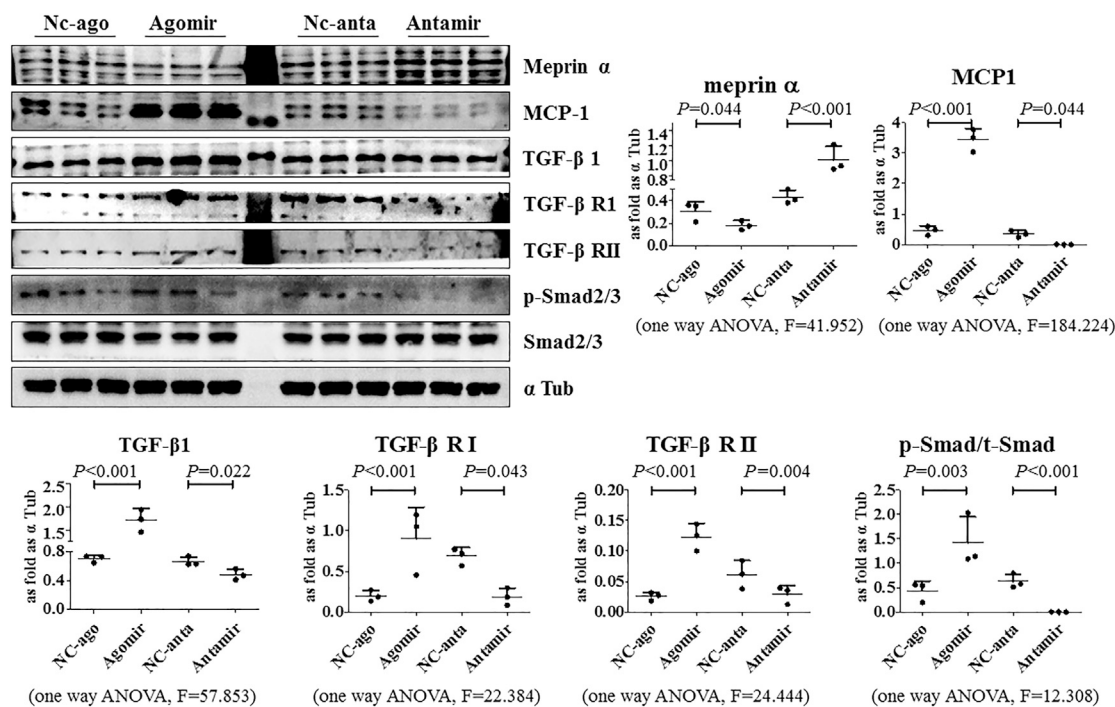


Figure 5. miR-155-5p Decreases the Levels of Meprin α and Promotes Macrophage Activation Induced by SiO₂

Levels of meprin α , MCP-1, TGF- β 1, TGF- β R I, TGF- β R II, and p-Smad2/3 were measured in NR8383 cells treated with SiO₂, and treated with agomiR-155-5p or antamiR-155-5p. Data are presented as the mean \pm SD. n = 3 per group.

meprin α is involved in miR-155-5p-mediated profibrotic effects in silicosis, and that miR-155-5p deletion or exogenous meprin α may be a new therapeutic strategy for silicosis.

Biological activity of meprin α is mainly characterized by the substrate specificity, tissue distribution, and cellular localization.²¹ When considering *in vitro* studies, it will be important to determine whether meprin α negatively regulated by miR-155-5p leads to inhibition of EMT in alveolar epithelial cells of the lung. Furthermore, a single miRNA has various and overlapping target genes, and a single gene can be regulated by several miRNAs. In future studies, it will be important to clarify the precise mechanisms of crosstalk between miRNAs and meprin α to inhibit fibrosis.

Based on the above observations, we conclude that miR-155-5p, which is upregulated in silicotic rats, suppresses *Mepr1a*, thereby inducing macrophage and fibroblast activation as well as collagen deposition to promote lung fibrosis. Enhancing meprin α expression, particularly by reducing miR-155-5p, protects against pulmonary fibrosis.

MATERIALS AND METHODS

Animals and Surgical Procedures

All experimental and surgical procedures were approved (2013-038 and 2017-025) by the Ethics Committee for Animal Experimentation of North China University of Science and Technology, which com-

plies with the US National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Male Wistar rats (80 \pm 10 g, 3 weeks old) were purchased from Vital River Laboratory Animal Technology (SCXY 2009-0004, Beijing, China). The rats were provided with free access to water and food and housed in a temperature-controlled chamber at 22°C–24°C with a 12-h light/12-h dark cycle. We established the silicotic model using a HOPE MED 8050 exposure control apparatus (HOPE Industry and Trade, Tianjin, China) as reported previously.²² The rats were placed in the apparatus suffused with SiO₂ (s5631, Sigma-Aldrich, St. Louis, MO, USA; ground and then heated at 180°C for 6 h). The settings of the exposure control apparatus were as follows: exposure chamber volume, 0.3 m³; pressure, –50 to +50 Pa; oxygen concentration, 21%–23%; cabinet temperature, 20°C–25°C; humidity, 70%–75%; flow rate of SiO₂, 3.0–3.5 mL/min; mass concentration in the cabinet, 40 μ g/m³. Each animal was exposed for 3 h per day.

To observe the anti-fibrotic effect of miR-155-5p, specific pathogen-free male C57BL/6 mice weighting 15 \pm 3 g at 8 weeks of age were purchased from Vital River Laboratory Animal Technology. Animals were randomly divided into three groups as follows (n = 6): agonist miRNA negative control (agomiR-NC), 0.05 mL of 0.9% saline containing 5 nmol ago-NC; silica plus agomiR-NC, 0.05 mL of 0.9% saline containing 5 nmol ago-NC plus

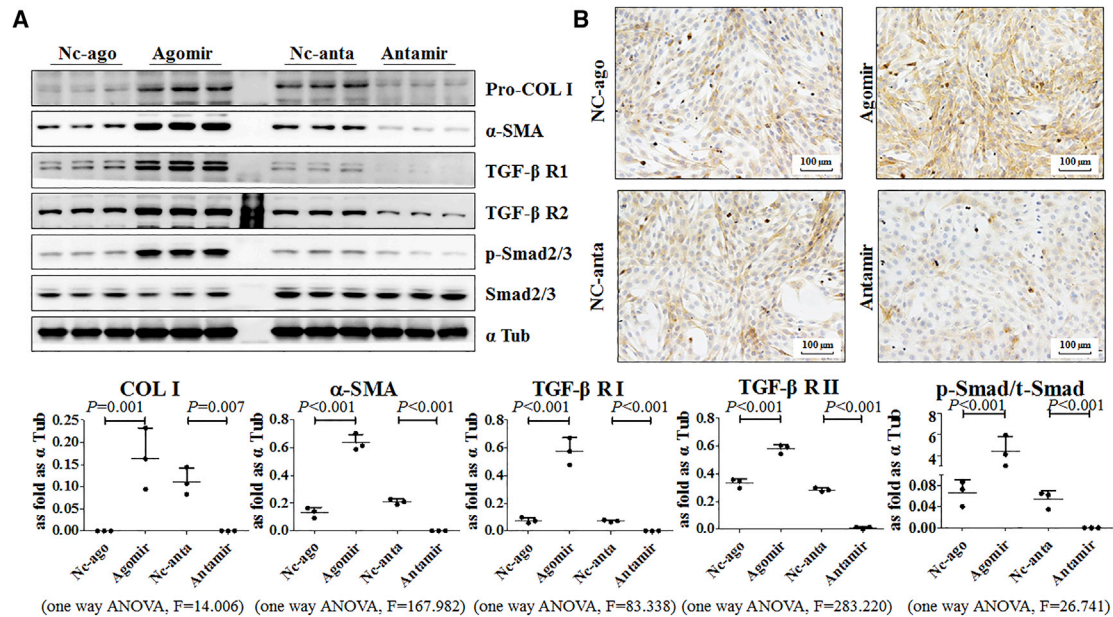


Figure 6. miR-155-5p Decreases the Levels of Meprin α and Promotes Fibroblast Activation Induced by CM of Macrophages

(A) Levels of meprin α , MCP-1, TGF- β 1, TGF- β R I, TGF- β R II, and p-Smad2/3 were measured in fibroblasts treated with CM derived from silica-treated NR8383 cells, and treated with agomiR-155-5p or antamiR-155-5p. Data are presented as the mean \pm SD. $n = 3$ per group. (B) Expression of α -SMA in lung fibroblasts observed by IHC staining (scale bars, 100 μ m).

2.5 mg SiO₂; silica plus agomiR-155-5p group, 0.05 mL of 0.9% saline containing 5 nmol agomiR-155-5p plus 2.5 mg SiO₂ administered via trachea instillation.^{23,24} Subsequently, 2.5 nmol of ago-NC or agomiR-155-5P was injected via the tail vein at 1 week after silica treatment. The mice were sacrificed on day 14 after silica administration, and their lungs were isolated and stored at -80°C until analysis.

In Vitro Experiments

The NR8383 murine monocyte/macrophage cell line and MEFs were obtained from the Cell Bank of the Chinese Academy

of Sciences (Shanghai, China). Rat primary lung fibroblasts were isolated from rat lung tissue and cultured as described previously.¹

Cells were plated in 25-cm² flasks and cultured with or without one or more of the following: silica (50 $\mu\text{g}/\text{cm}^2$);²⁵ recombinant human TGF- β 1 (5 ng/mL, 240-B, R&D Systems, USA);¹ recombinant mouse meprin α protein (100 nmol/L, 4007-ZN, R&D Systems);⁵ actinonin (20 $\mu\text{mol}/\text{L}$, c3331, APExBIO, USA);³ and LY364947 (59 nmol/L, 13341, Cayman Chemical, MI, USA). miR-155-5p (RiboBio, China) and *Mepla*-siRNA (RiboBio, China) transfections were carried out

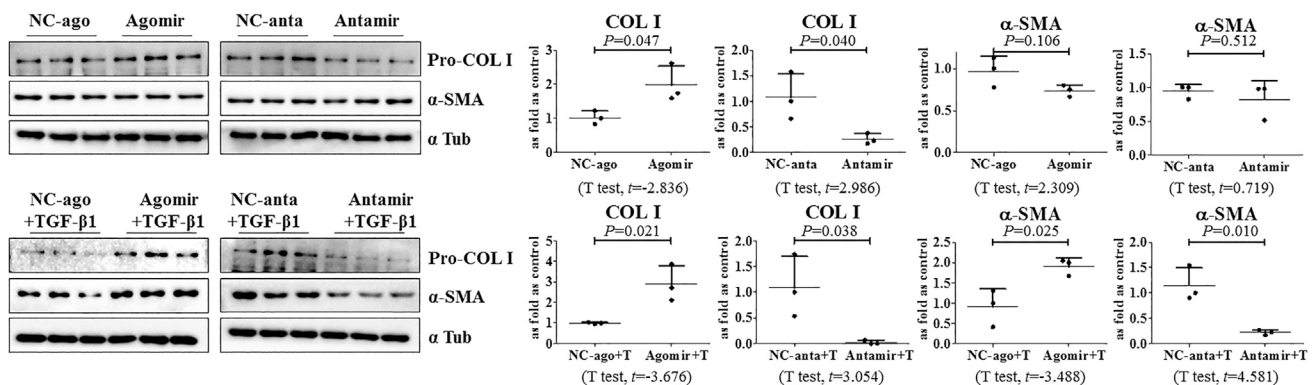


Figure 7. miR-155-5p Increases the Levels of Pro-COL I and α -SMA in Fibroblasts

Levels of pro-COL I and α -SMA were measured by western blotting in MEFs treated with or without TGF- β 1, and treated with agomiR-155-5p or antamiR-155-5p. Data are presented as the mean \pm SD. $n = 3$ per group.

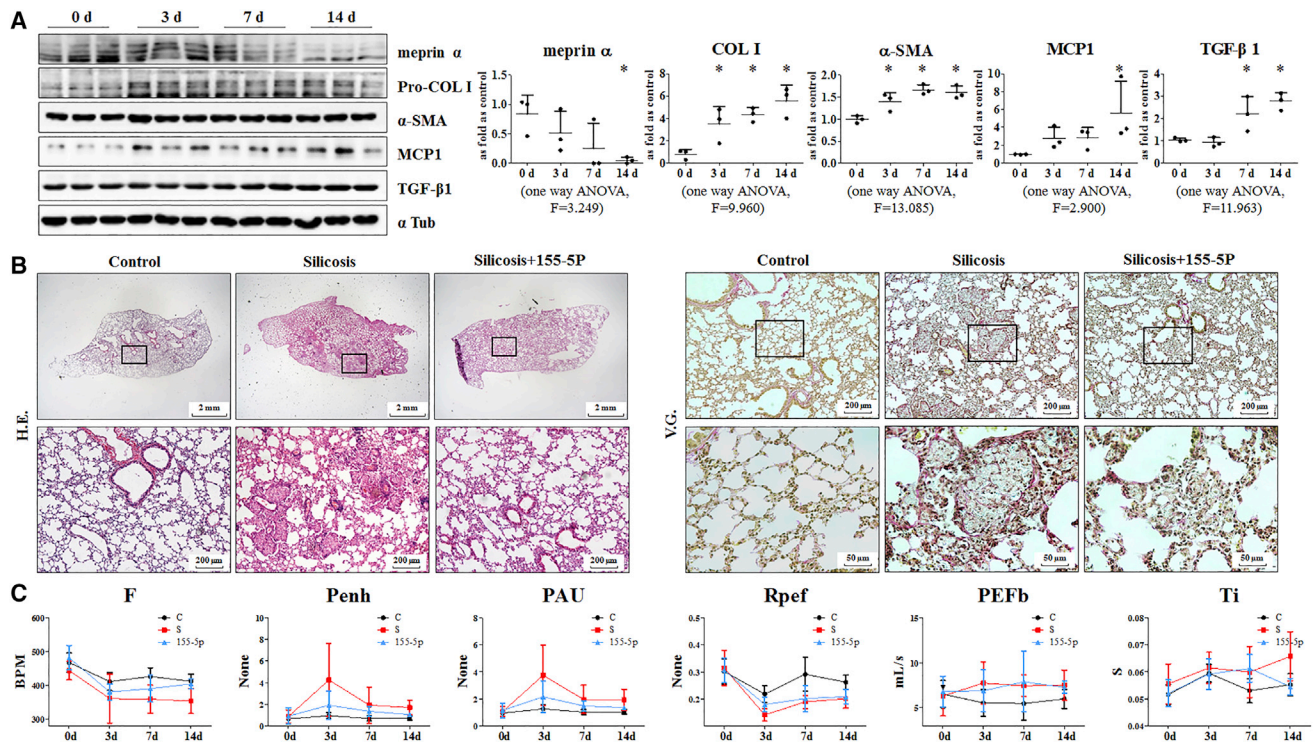


Figure 8. Inhibition of miR-155-5p Improves the Lung Functions of Mice Exposed to Silica
 (A) Protein expression of meprin α , α -COL I, TGF- β 1, α -SMA, and MCP-1 was detected by western blotting in mice exposed to silica for 3, 7, and 14 days. * Compared with 0 d group, $p < 0.05$; Data are presented as the mean \pm SD. $n = 3$ per group. (B) HE staining and VG staining of lung tissue in mice exposed to silica (Bars = 2 mm or 200 μ m). (C) Lung functions of mice exposed to silica and treated with anti-miR-155-5p. Data are presented as the mean \pm SD. $n = 6$ per group.

using Lipofectamine 2000 (203749, Invitrogen, USA), according to the manufacturers' recommendations.

Cells were transfected with NC-agomiR (sense, 5'-UUUGUACUA CACAAAAGUACUG-3'; antisense, 5'-CAGUACUUUUGUGUAG UACAAA-3'), agomiR-155-5P (sense, 5'-UUA AUGCUAAUUGUG AUAGGGGU-3'; antisense, 5'-ACCCCUAUCACAAUAGCAU UAA-3'), and NC-antagomiR (sense, 5'-CAGUACUUUUGUGUA GUACAAA-3') or antagomiR miR-155-5p (sense, 5'-ACCCCUA UACAAUUGCAUUA-3').

Cells were also transfected with NC-siRNA (sense, 5'-UUCUCCG AACGUGACACGU(dTdT)-3'; antisense, 5'-ACGUGACACGUU CGGAGAA(dTdT)-3'), *Mep1a*-siRNA-001 (sense, 5'-CGAUACAG AUGUUGGUGAA(dTdT)-3'; antisense, 5'-UUCACCAACAUCUG UAUCG(dTdT)-3'), *Mep1a*-siRNA-002 (sense, 5'-GACUGAAUC GAAUGUACAA(dTdT)-3'; antisense, 5'-UUGUACAUUCGAUUC AGUC(dTdT)-3'), or *Mep1a*-siRNA-003 (sense, 5'-GUCUAUGA UUGGUGAUCAA(dTdT)-3'; antisense, 5'-UUGAUCACCAAUCA UAGAC(dTdT)-3'). Based on the preliminary experiment shown in Figure S1A, *Mep1a*-siRNA-002 was used in the following experiments.

After 24 h, the supernatant was collected, centrifuged to remove cell debris, and immediately frozen at -80°C until analysis. Whole-cell

lysates were used for western blotting or measurement of mRNA expression by real-time PCR.

Immunohistochemistry and Immunofluorescence Staining

Immunostaining was performed on lung sections and cells after antigen retrieval and quenching endogenous peroxidases with 3% H_2O_2 . The sections were subsequently incubated with primary antibodies against α -SMA (1:200 dilution, ab32575, Abcam, Cambridge, UK) and meprin α (1:200 dilution, ab232892, Abcam) overnight at 4°C . After washing, the secondary antibody (PV-6000, Beijing Zhongshan Jinqiao Biotechnology, China) was applied, and then the sections were developed with 3,3',9-diaminobenzidine (ZLI-9018, ZSGB-BIO, Beijing, China).

Western Blot Analysis

Lung tissues and cells were homogenized in ice-cold hypotonic buffer (10 \times hypotonic buffer, 10% protease and 1% phosphatase inhibitors [400-10, Active Motif, USA], 0.1% detergent, and 0.1% of 1 M DTT). After centrifugation (14,000 \times g, 15 min, 4°C), the supernatant was collected according to the manufacturer's instructions (40010, Active Motif, Carlsbad, CA, USA). The protein concentration was determined by a BCA assay kit (PQ0012, Multi Sciences, China). Protein samples (10 μ g) were solubilized in 5 \times sample buffer (AS0001-5, Seracare, USA), heated at 95°C for 10 min, centrifuged at 3,000 \times g for

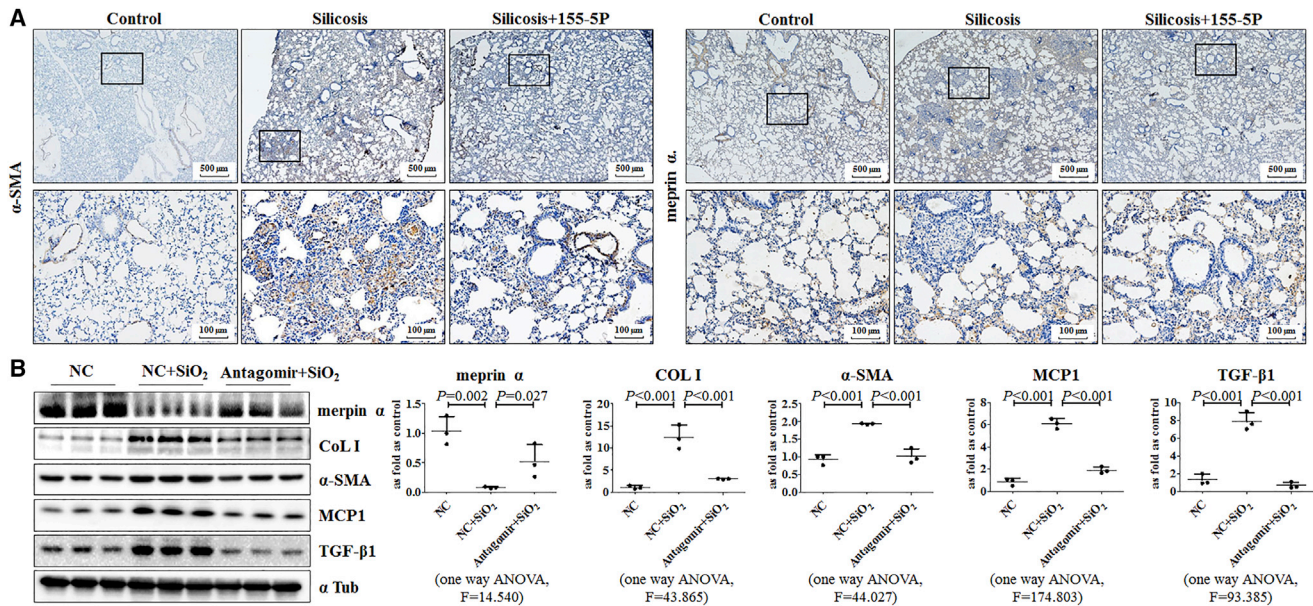


Figure 9. Inhibition of miR-155-5p Increases the Levels of Meprin α and Attenuates Lung Fibrosis in Mice Exposed to Silica

(A) Expression of α -SMA and meprin α measured by IHC staining (scale bars, 500 μ m and 100 μ m). (B) Levels of meprin α , pro-COL I, α -SMA, MCP-1, and TGF- β 1 in mouse lungs measured by western blotting. Data are presented as the mean \pm SD. n = 3 per group.

1 min, loaded on a 12% Tris-HCl-SDS-polyacrylamide gel, and separated for 1 h at 120 V. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (31337600, Roche Diagnostics, Germany) and then blocked with 5% BSA for 1 h at room temperature, followed by incubation overnight at 4°C with a specific primary antibody against meprin α , COL I (ab34710, Abcam), α -SMA, MCP-1 (DF7577, Affinity, USA), TGF- β 1 (ARG56894, Arigo Biolaboratories, Taiwan, China), TGF- β RI (A16983, ABclonal, Wuhan, China), TGF- β RII (ARG59501, Arigo), p-Smad2/3 (8828s, Cell Signaling Technology, MA, USA), or Smad2/3 (5678, Cell Signaling Technology), followed by incubation with goat anti-rabbit or anti-mouse secondary antibodies (074-1506/074-1806, Kirkegaard & Perry Laboratories, USA) at a dilution of 1:5,000 in blocking buffer. After three washes with Tris-buffered saline with Tween 20 (TBST), all immunoblots were visualized using ECL prime western blotting detection reagent (RPN2232, GE Healthcare). The results were normalized against the Tub α expression level and corresponding control. Information about antibodies (e.g., molecular weight) is shown in Figure S1B.

RNA Isolation and Analysis

Total RNA was isolated using an RNeasy Mini-Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions, and quantified with a NanoDrop spectrophotometer. Bulge-Loop miR-155-5p reverse transcriptase (RT) primers (ssD0904071006, RiboBio, China), Bulge-Loop U6 RT primer (ssD115584401), and a RevertAid first strand cDNA synthesis kit (K1622, Thermo Scientific) were used for reverse transcription of miRNAs. Each 10 μ L of reaction volume in a 200- μ L tube consisted of 5 \times buffer (2 μ L), RiboLock RNase inhibitor (20 U/ μ L, 0.5 μ L), 10 mM 2'-deoxynucleoside 5'-triphosphate

(dNTP) mix (1 μ L), RevertAid Moloney murine leukemia virus (M-MuLV) RT (200 U/ μ L, 0.5 μ L), 1–5 ng of total RNA, and RNase-free water to the final volume of 10 μ L. Reverse transcription was conducted using a PCR system (Bio-Rad, USA) under the following conditions: 42°C for 60 min and then 70°C for 5 min. The reverse transcription reaction product was directly used in PCR.

An RT kit (ZR102, Zomanbio) was used for reverse transcription of meprin- α mRNA. Each 20 μ L of reaction volume in a 200- μ L tube consisted of total RNA (5–50 ng), RT enzyme mix (3 μ L), RT reaction mix (7 μ L), and double-distilled H₂O (ddH₂O) to the final volume of 20 μ L. Reverse transcription was conducted using the PCR system under the following conditions: 45°C for 15 min and 85°C for 5 min. The reverse transcription reaction product was directly used in PCR.

Amplification by real-time PCR was carried out using 2 \times SYBR qPCR mix (ZF102-1, Zomanbio). The primer sequences were as follows: (1) Bulge-Loop miR-155-3P forward primer (ssD115584402) and Bulge-Loop miR reverse primer (ssD089261711); Bulge-Loop U6 forward primer (ssD0904071006) and Bulge-Loop U6 reverse primer (ssD0904071107); (2) mouse *Mep1a*: forward primer, 5'-CTGATGACTACGGGCCATT-3'; reverse primer, 5'-GAGTATGTGTTGCGGTGCAG-3'; mouse *Actb*: forward primer, 5'-AGATGTGATCAGCA AGCAG-3'; reverse primer, 5'-AGATGTGGATCAGCAAGCAG-3'; and (3) rat *Mep1a*: forward primer, 5'-AGCAGCTGTACCATTAAAGTATCT-3'; reverse primer, 5'-TGAAAGAGGTC CAAGC CTGC-3'; and rat *Gapdh*: forward primer, 5'-GGTGAAGTCCGGTGTGAACG-3'; reverse primer, 5'-CTCGC TCCTGGAAGATGGTG-3'. Each 20 μ L of reaction volume contained a

cDNA sample (1–10 ng, 2 μ L), 10 μ L of 2 \times SYBR qPCR mix, 0.4 μ L of ROX Reference Dye, 0.5 μ L of forward primer, 0.5 μ L of reverse primer, and 6.6 μ L of RNase-free H₂O. Thermocycling was conducted in a real-time PCR system (Applied Biosystems, USA) as follows: 94°C for 2–3 min, followed by 35–45 cycles of denaturation at 94°C for 15 s, 55–65°C for 30 s, 72°C for 30 s, and then 72°C for 5–10 min. Subsequently, a melting curve procedure was carried out. U6, β -actin, or GAPDH was used as the internal reference to measure miR-155-5p or meprin α expression. The results were calculated by the $2^{-\Delta\Delta C_T}$ method.

Luciferase Reporter Assays

H293K cells were seeded in six-well plates 24 h prior to transfection. According to the manufacturer's protocol, the cells were transiently cotransfected with 5 ng of wild-type/mutant reporter plasmid and agomiR-NC/agomiR-155-5p or antamiR-NC/antamiR-155-5p using Lipofectamine 2000. Firefly and Renilla luciferase activities were measured by a Dual-Luciferase assay (E1910, Promega, Madison, WI, USA) after transfection for 48 h. Firefly luciferase activity was normalized to Renilla luciferase activity, and the ratio of firefly luciferase activity to Renilla luciferase activity was obtained.

Lung Function Assessment

Respiratory parameters were assessed in whole-body plethysmograph (WBP) chambers (FinePointe WBP, Buxco Research Systems, USA), following the manufacturer's protocol. Mice were placed into the whole-body plethysmographic chambers. After a few minutes for stabilization, lung function detection included an adaptation period (10 min), atomization period (1 s), reaction period (5 min), and recovery period (1 min).

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 software. Two group comparisons were analyzed by the unpaired Student's *t* test, whereas multiple group comparisons were performed by one-way analysis of variance followed by Tukey's *post hoc* analysis. Statistical significance was considered as $p < 0.05$ with a 95% confidence interval.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2019.11.018>.

AUTHOR CONTRIBUTIONS

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