

ACE2 Attenuates Epithelial-Mesenchymal Transition in MLE-12 Cells Induced by Silica

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Purpose: The role of angiotensin-converting enzyme 2 (ACE2) in silicosis remains unknown, although previous studies have suggested that ACE2 may be beneficial. We, therefore, investigated the effect of ACE2 on silicosis, particularly with regard to its role in regulating the epithelial-mesenchymal transition (EMT) induced by silica, with the aim to uncover a new potential target for the treatment of pulmonary fibrosis.

Materials and Methods: We employed wild-type mice treated with diminazene aceturate (DIZE, an ACE2 activator, 15 mg/kg/day for 4 weeks), *hACE2*-transgenic mice (overexpress the *ACE2* gene), and the mouse lung type II epithelial cell line treated with DIZE (10^{-7} M for 48 h) or angiotensin-(1-7) [Ang-(1-7)] (10^{-4} M for 48 h), following induced fibrotic responses to determine the protective potential of ACE2. Silicosis models were established by orotracheal instillation of SiO₂ (2.5 mg/mouse). Immunostaining was used to determine α -smooth muscle actin (α -SMA) expression. The activities of angiotensin-converting enzyme (ACE) and ACE2 and the levels of angiotensin II (Ang II) and Ang-(1-7) were detected by enzyme-linked immunosorbent assay. The mRNA expression of *ACE* and *ACE2*, and protein expression of the renin-angiotensin system (RAS) components and EMT indicators were studied by qRT-PCR and Western blot, respectively.

Results: DIZE treatment and overexpression of ACE2 markedly inhibited the formation of silica-induced lung fibrosis and increased the level of E-cadherin, with concomitant down-regulation of pro-collagen, vimentin, and α -SMA via RAS signaling. Furthermore, DIZE and Ang-(1-7) attenuated the EMT and collagen deposition induced by silica in MLE-12 cells. Moreover, these effects were abrogated by MLN-4760 (a specific ACE2 inhibitor) and A779 (a specific Mas receptor blocker).

Conclusion: The overexpression of *ACE2* and treatment with DIZE can ameliorate EMT in silicotic mice via activation of the ACE2-Ang-(1-7)-Mas receptor axis, and these changes are accompanied by suppression of the ACE-Ang II-AT1 receptor axis.

Keywords: angiotensin-converting enzyme 2, angiotensin-converting enzyme, silicosis fibrosis, diminazene aceturate, renin-angiotensin system, transgenic mice

Introduction

Silicosis is a major occupational health hazard throughout the world.¹ Previous studies by our group found that macrophage activation, myofibroblast differentiation, and epithelial-mesenchymal transition (EMT) are important factors in the pathogenesis of silicosis,^{2,3} while several other studies have confirmed that activation of the local renin-angiotensin system (RAS) in lung tissue is involved in the development of pulmonary fibrosis.⁴⁻⁶ However, the pathomechanisms of silicosis remain to be fully elucidated.

Inhibition of the upstream angiotensin-converting enzyme (ACE)/Ang II/angiotensin receptor type 1 (AT1R) pathway and activation of the downstream angiotensin-converting enzyme 2 (ACE2)/Ang-(1-7)/Mas receptor pathway are two feasible treatment strategies with demonstrated efficacy in various pulmonary disease models.⁷ Our recent studies also showed that Ac-SDKP (N-acetyl-seryl-aspartyl-lysyl-proline) exerts an anti-silicotic effect through activation of the ACE2-Ang-(1-7)-Mas axis.^{8,9}

Our previous study found that the expression of ACE, Ang II and AT1 increased while that of ACE2 decreased in local lung tissue in mice with acute lung injury induced by limb ischemia-reperfusion. After preventive intervention with DIZE for 4 weeks, the lesions of the lung injury were attenuated, and the production of Ang-(1-7) and Mas receptor protein were up-regulated in the lung, whereas the opposite trend was observed for Ang II and the AT1 receptor protein. Compared with wild-type mice, the application of DIZE in *hACE2* (human ACE2) transgenic mice resulted in similar expression changes but to a greater magnitude.¹⁰ Moreover, we observed high ACE2 expression in the epithelial cells of the lung, and ACE2 inhibited myofibroblast differentiation induced by Ang II in fibroblasts.⁸ We, therefore, hypothesized that increased ACE2 expression may have a beneficial effect on alveolar epithelial cells. Several studies have shown that diminazene aceturate (DIZE) is an activator of ACE2 and that treatment with DIZE is beneficial for myocardial, liver, and biliary fibrosis in mice.¹¹⁻¹³ In addition, DIZE combined with physical exercise potentiates the reduction of bleomycin-induced pulmonary fibrosis.¹⁴

Therefore, in the present study, we focused on the effect of ACE2 on silicosis, particularly with regards to its role in regulating EMT induced by silica. For this purpose, we used silica-treated *hACE2* (human ACE2)-transgenic mice and MLE-12 cells to explore the respective effects of endogenous overexpression of ACE2 and exogenous activation (DIZE intervention) on silicosis, with the aim to uncover a new potential target for the treatment of pulmonary fibrosis.

Materials and Methods

Animals and Experimental Procedures

Ninety-eight- to twelve-week-old male ICR mice, weighing 30 ± 3 g, were used in the experiments. The wild-type and F1 generation of the human *ACE2* (*hACE2*)-transgenic ICR mice were kindly supplied by the Institute of

Laboratory Animal Sciences, CAMS & PUMC (Beijing, China), and fed in the Laboratory Animal Center of North China University of Science and Technology. All mice were housed in a specific pathogen-free facility and were maintained under conditions of constant temperature (23–25°C), humidity (40–50%), and a 12-h light/dark cycle. Animals were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978), and the experimental procedures were subject to the approval of the Ethics Committee of the North China University of Science and Technology (approval number 2013–038 and 2017–025).

Wild-type mice were randomly divided into five groups ($n = 10$ each) as follows: 1) wild-type control group, 2) wild-type silicosis model group, 3) DIZE (15 mg/kg/day, i. p., D7770; Sigma-Aldrich, St. Louis, MO, USA)¹⁵ treatment group, 4) DIZE + A779 (5 mg/kg/day, s.c., 4030357; Bachem, Bubendorf, Switzerland)¹⁶ treatment group, and 5) DIZE + MLN-4760 (1 mg/kg/day, s.c. 530,616; Millipore, Darmstadt, Germany)¹⁷ treatment group. The *hACE2*-transgenic ICR mice were randomly divided into four groups ($n = 10$ each) as follows: 1) *hACE2*-transgenic control group; 2) *hACE2*-transgenic silicosis model group, 3) A779 treatment group, and 4) MLN-4760 treatment group.

Mice were anesthetized by 1% pentobarbital sodium (50 mg/kg, i.p.),¹⁸ except for the wild-type and *hACE2*-transgenic control groups (intratracheal administration of 50 μ L saline), all mice were subjected to once-off intratracheal administration of 50 μ L SiO₂ suspension (50 mg/mL, equal to 2.5 mg/mouse, Cat. No. 274739, mean particle size $<5 \mu$ m; Sigma-Aldrich)¹⁸ at the same time as receiving the treatment specific to each experimental group. The mice were sacrificed after 4 wk of treatment, and their lungs were isolated. Left lung tissues were fixed with 4% paraformaldehyde solution, and the rest of the lungs were stored at -80°C until analysis.

Cell Culture and Experimental Procedures

MLE-12 cells (mouse lung type II epithelial cell line) were obtained from the American Type Culture Collection (CRL-2110; ATCC, Manassas, VA, USA). MLE-12 cells were grown as monolayer cultures routinely in DMEM/F-12 50/50 medium (10-092-CVR; Mediatech, VA, USA) supplemented with 10% fetal bovine serum (10099141;

Gibco, Thermo Fisher Scientific, Madison, WI, USA) and 1% penicillin and streptomycin (P06-07001; Pan Biotech, Aidenbach, Germany) and maintained at 5% CO₂ and 37°C.

After serum starvation for 24 h, the cells were cultured with various combinations of the following: SiO₂ (50 µg/cm³), 10⁻⁷ mol/L Ang-(1-7) (4084113; Bachem),¹⁹ 10⁻⁵ mol/L A779, 10⁻⁴ mol/L DIZE,²⁰ and 10⁻⁵ mol/L MLN-4760.²¹ Cells were harvested for total RNA and protein extraction after treatment for 48 h.

Immunohistochemistry (IHC)

Immunostaining for α-smooth muscle actin (α-SMA, EPR5368; Epitomics, Burlingame, CA, USA) was performed on lung sections as described previously.^{8,9} Brown staining indicated samples positive for α-SMA expression.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA method was used to determine ACE/ACE2 activity and Ang II/Ang-(1-7) levels in the lung tissue and serum samples. For this purpose, 100 mg of lung tissue was rinsed with phosphate buffer saline (PBS), homogenized in 1 mL of PBS, and stored overnight at -20°C. Two freeze-thaw cycles were then performed to disrupt the cell membranes, and the homogenates were centrifuged at 5,000 ×g, 4°C for 5 min. The resulting supernatants were removed and stored at -80°C. The samples were then centrifuged again after thawing before the assay. Serum samples were allowed to clot overnight at 4°C before centrifugation at 1,000 ×g, 4°C for 20 min. The supernatant was harvested immediately and stored at -80°C for later analysis. Then, the ACE (MB-0005A; Jiangsu Meibiao Biological Technology, Yancheng, China)/ACE2 (MB-3569A; Jiangsu Meibiao Biological Technology) activity and Ang II (CSB-E04495m; Cusabio, Baltimore, MD, USA)/Ang-(1-7) (CSB-E13763m; Cusabio) concentration in the lung tissue and serum were detected with the ELISA kits according to the manufacturer's instructions. Enzyme activity was expressed in U/L and peptide concentration in pg/mL.

Total RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR (qRT-PCR)

Lung tissue was ground in liquid nitrogen and total RNA was extracted with TRIzol reagent (15,596-018; Invitrogen,

Carlsbad, CA, USA) according to a standard method. RNA samples (2 µg each) were reverse-transcribed to synthesize cDNA (ZR102-2; Beijing Zoman Biotechnology, Beijing, China). Lung mRNA expression of ACE and ACE2 was evaluated by qRT-PCR (2× SYBR qPCR Mix, ZF102-2; Beijing Zoman Biotechnology) according to the manufacturer's instructions on an ABI 7500 Real-Time PCR System (Thermo Fisher Scientific).

The primer sequences (synthesized by Thermo Fisher Scientific) were: ACE sense, 5'-CAGTGTCTACCCCCAAGCAT-3'; ACE antisense, 5'-CTTCCATCAAAGACCC TCCA-3';²² ACE2 sense, 5'-CCTTCTCAGCCTTGTTCG TGTAC-3'; ACE2 antisense, 5'-TGCCCAGAGCCTAGA GTTGTAGTC-3';²³ β-actin sense, 5'-GTCGTACCACAGG CATTGTGATGG-3'; and β-actin antisense, 5'-GCAATGC CTGGGTACATGGTGG-3'.²² Expression levels were normalized with the 2^{-ΔΔCt} formula based on the Ct values of the respective RNA samples relative to the housekeeping gene, β-actin.

Protein Isolation and Western Blot

Lung tissues and cells were homogenized in ice-cold RIPA lysis buffer containing 1% protease inhibitor for 30 min. The lysates were centrifuged at 13,800 ×g, 4°C for 20 min and the supernatants were then recovered. Protein concentrations were measured with a bicinchoninic acid protein assay kit [PQ0012; Multisciences (Lianke) Biotech, Hangzhou, China]. The supernatants were boiled in 5× bromophenol blue sample buffer for 5 min, and 10 µL (20 µg) of each sample was applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to a polyvinylidene fluoride membrane. The membranes were then blocked with 5% non-fat milk and hybridized with a primary antibody against each of α-SMA (EPR5368; Epitomics), collagen I (pro-Col, AF7001; Affinity Biosciences, OH, USA), collagen III (pro-Col III, AF0136; Affinity Biosciences), vimentin (Vim, ARG57462; Arigo Biolaboratories, Hsinchu, Taiwan), E-cadherin (E-cad, A11453; ABclonal Technology, Boston, MA, USA), ACE (EPR2757; Epitomics), ACE2 [EPR4435(2); Epitomics], AT1 (EPR3873; Epitomics), Mas (sc-390453; Santa Cruz Biotechnology, Dallas, TX, USA), Ang II (GTX37789; GeneTex Inc., San Antonio, TX, USA) and Ang-(1-7) (P1787; EIAab Science Co., Wuhan, China) at 4°C overnight. ECL and horseradish peroxidase-conjugated secondary antibodies toward rabbit (5220-0336; SeraCare, Samuel, CA, USA) and mouse (5220-0341; SeraCare) IgG were used for detection. Protein expression was normalized to that of

GAPDH (AC036; ABclonal) or tubulin (AF7010; Affinity Biosciences).

Data Presentation and Statistical Analysis

SPSS 17.0 statistical software (SPSS Inc, Chicago, IL, USA) was used to analyze the data. All data were expressed as the mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) and the least significant difference (LSD) test were used to determine the significance of differences between groups. Statistical significance was defined as $p < 0.05$.

Results

DIZE Inhibits SiO₂-Induced EMT and Attenuates Silicotic Fibrosis

Based on histology using immunohistochemistry staining for α -SMA, we observed nodule formation with extensive α -SMA expression in the nodular lesions and pulmonary interstitial region (Figure 1A), and large regions of silicosis were observed, indicating fibrotic response in the lung tissue of silicotic mice (Figure 1B). Treatment with DIZE overcame the fibrotic remodeling and reduced the silicotic area in silica-exposed lungs. Western blotting (Figure 1C) indicated that treatment with DIZE dramatically decreased the protein expression of α -SMA, Vim, pro-Col I, and pro-Col III in the lungs (Figure 1D–G) while augmenting the expression of E-cad (Figure 1H). Furthermore, we observed that treatment with MLN-4760 (an ACE2 inhibitor) or A779 (a Mas receptor blocker) reversed the suppression of silicosis by DIZE, with significantly increased expression of α -SMA, Vim, pro-Col I, and pro-Col III expression in the lungs along with markedly reduced expression of E-cad (Figure 1D–H).

We also measured RAS activity in the lung tissue and serum samples to analyze the role of circulating and local RAS in silicosis. Serum ACE2 activity was decreased in silicotic mice and this effect was reversed by DIZE treatment, while the level of ACE activity and the concentration of Ang II, and Ang-(1–7) in the serum were unchanged (Figure 2A–D). In the lung tissue, treatment with DIZE decreased the activity of ACE but increased the activity of ACE2, while the concentration of Ang II decreased and that of Ang-(1–7) increased (Figure 2E–H). Furthermore, the beneficial effects of DIZE on the lung tissue were blocked by MLN-4760 and A779. These results implied that circulatory and local RAS may perform distinct physiological and pathological roles in mice exposed to silica.

Additionally, Western blotting results (Figure 3A) indicated that treatment with DIZE resulted in increased protein and mRNA levels of ACE2 (Figure 3B and C) and elevated protein levels of Mas (Figure 3D), accompanied by reduced protein and mRNA expression of ACE (Figure 3E and F) and reduced protein expression of AT1R (Figure 3G). All of these changes were reversed by A779 and MLN-4760 treatment. These results indicated that the activation of ACE2 by DIZE suppressed the lung fibrotic response toward exposure to silica.

Overexpression of hACE2 Exerts an Anti-Fibrotic Effect in Mice Exposed to Silica

To assess whether augmenting endogenous ACE2 has anti-fibrotic effects in vivo, murine overexpression of *hACE2* was employed. We detected fewer nodules, lower α -SMA positive expression, and a lower proportion of silicotic area in the silicotic *hACE2*-transgenic mice compared with silicotic wild-type mice (Figure 4A and B), as well as sustained decreases in α -SMA, Vim, pro-Col I, and pro-Col III (Figure 4C–G), and a slight increase of E-cad (Figure 4H) in *hACE2* mice exposed to silica in comparison with wild-type silicotic mice.

The ACE2 activity in the lung tissue and serum (Figure 5A and B), as well as the protein and mRNA expression levels of ACE2 in the lung tissue (Figure 6A–C), were higher in the silicotic *hACE2*-transgenic mice compared with the silicotic wild-type mice. Similarly, the concentration of Ang-(1–7) in the lung tissue and serum was higher in the *hACE2*-transgenic group (Figure 5C and D). This was accompanied by decreased ACE activity in the lung tissue (unchanged in the serum) (Figure 5E and F) and decreased ACE protein and mRNA expression levels in the lungs of the *hACE2*-transgenic group (Figure 6D and E). Ang II concentration was also lower in the lung tissue (unchanged in the serum) in the *hACE2*-transgenic group compared with the wild-type group (Figure 5G and H), with increased Mas and lower AT1R protein levels present in the lung tissue from the transgenic group (Figure 6F and G). Thus, the overexpression of *ACE2* was shown in vivo to have a beneficial effect on silicosis by upregulating the ACE2-Ang-(1–7)-Mas receptor axis and suppressing the ACE-Ang II-AT1 receptor axis.

Ang-(1–7) Inhibits SiO₂-Induced EMT in MLE-12 Cells

We treated MLE-12 alveolar type II epithelial cells with SiO₂. Immunohistochemistry staining (Figure 7A) and Western

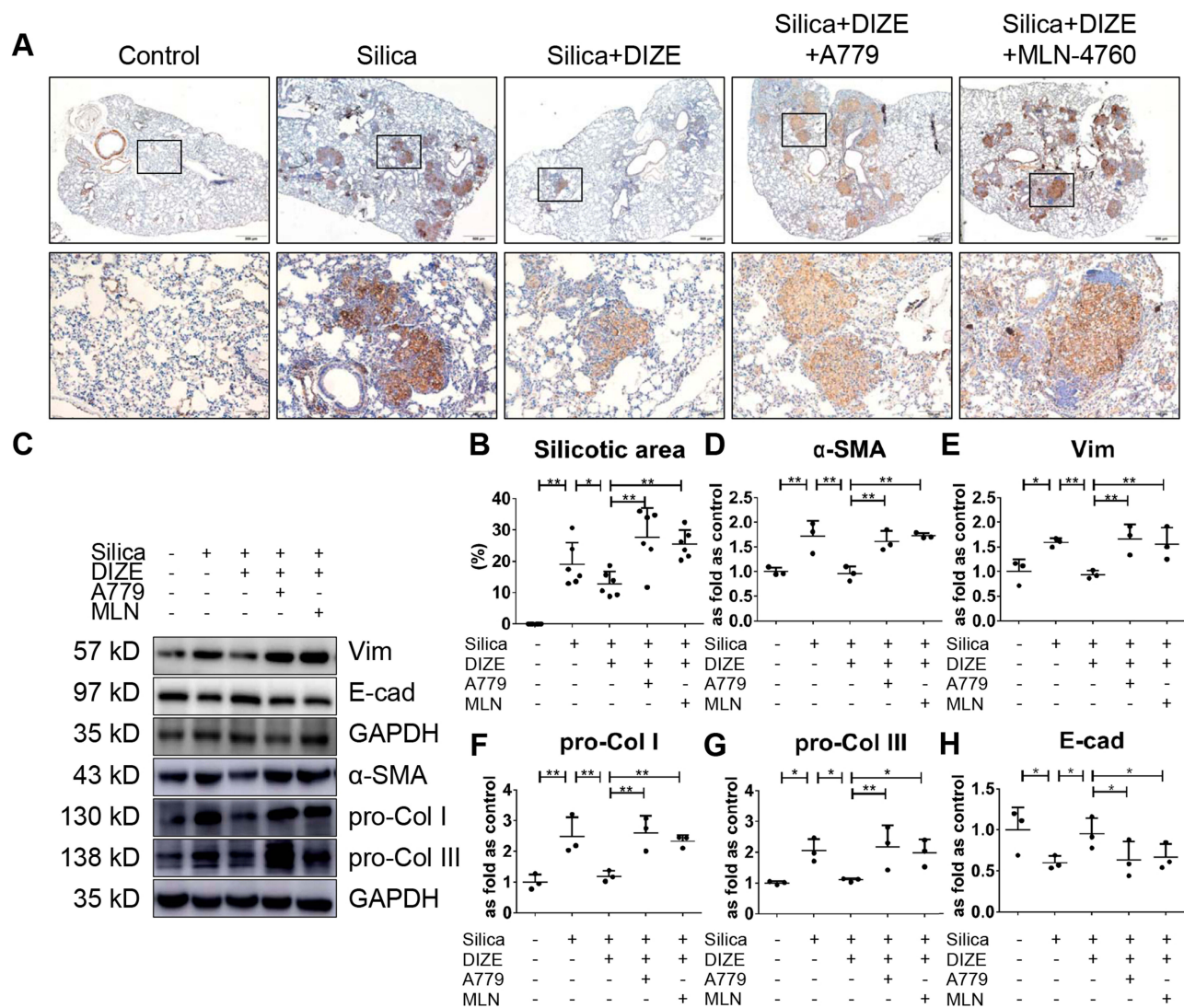


Figure 1 DIZE inhibits SiO₂-induced EMT and collagen deposition in wild-type silicotic mice. **(A)** Immunohistochemistry staining of α -SMA expression in the lungs of wild-type mice, n = 6 in each group. Magnification, $\times 40$ (top panel) and $\times 200$ (bottom panel). Silicotic mice underwent various treatment combinations with DIZE (ACE2 activator), A779 (Mas receptor blocker), and MLN-4760 (ACE2 inhibitor). **(B)** The proportion of silicotic areas in the lung samples in **(A)**. **(C)** Western blot showing the protein expression of α -SMA **(D)**, Vim **(E)**, pro-Col I **(F)**, pro-Col III **(G)**, and E-cad **(H)** in the lungs of mice from the various treatment groups. Values represent the mean \pm SD, n = 3 independent experiments, fold change is expressed relative to the control (no treatments), *P < 0.05 vs corresponding group, **P < 0.01 vs corresponding group.

blotting (Figure 7B) indicated that silica treatment resulted in increased protein expression of α -SMA, Vim, pro-Col I, and pro-Col III (Figure 7C–F), along with decreased protein expression of E-cad (Figure 7A and G). Treatment with exogenous Ang-(1–7) was able to reverse the effects of silica, while A779 treatment abrogated the beneficial effects of Ang-(1–7).

In addition, Western blotting (Figure 8A) indicated that silica treatment of the MLE-12 cells resulted in increased protein levels of ACE, Ang II, and AT1R (Figure 8B–D), while that of ACE2, Ang-(1–7), and Mas decreased (Figure 8E–G). Treatment with exogenous Ang-(1–7) reversed these changes in the RAS markers, while the

beneficial effects of Ang-(1–7) were abrogated by treatment with A779.

DIZE Attenuates SiO₂-Induced EMT in MLE-12 Cells

We further treated MLE-12 alveolar type II epithelial cells induced by SiO₂ with DIZE to investigate its anti-fibrotic effect. Immunohistochemistry staining (Figure 9A) and Western blotting (Figure 9B) showed that treatment with DIZE reversed the increased protein expression of α -SMA, Vim, pro-Col I, and pro-Col III (Figure 9C–F) and reversed the reduced protein expression of E-cad (Figure 9A and G), thereby mitigating the pro-fibrotic effect of SiO₂ in MLE-12

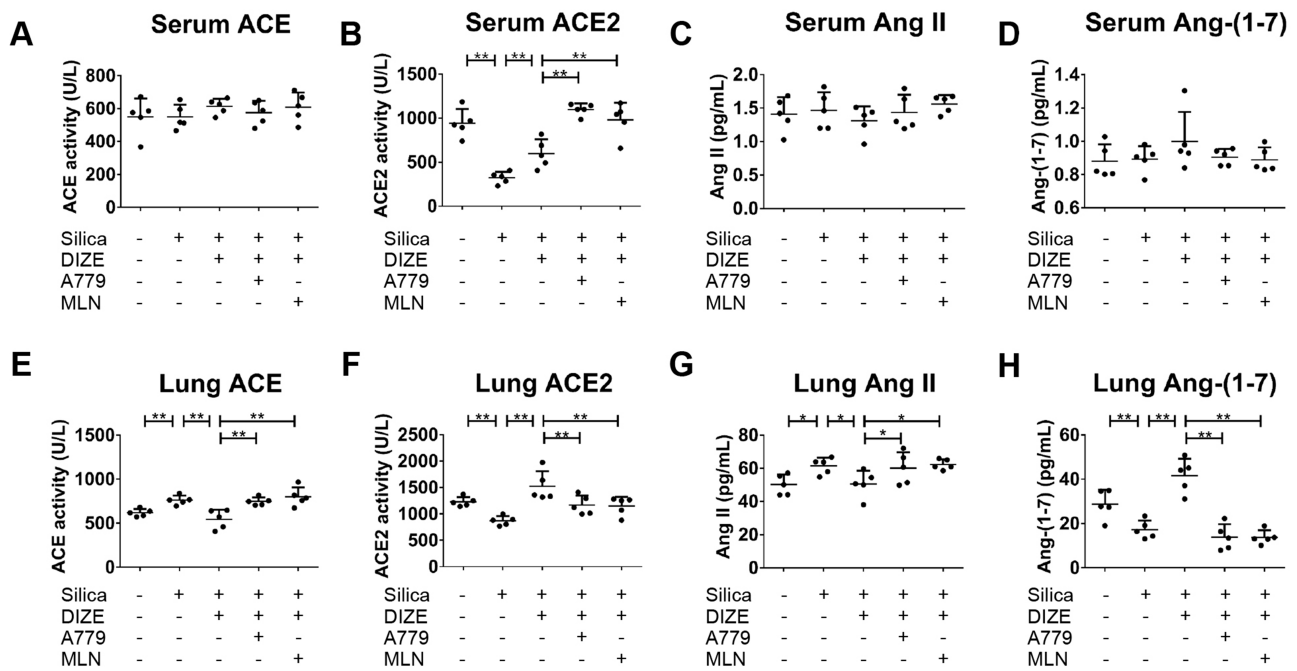


Figure 2 DIZE regulates the activity of ACE/ACE2 and the concentration of Ang II/Ang-(1-7) in wild-type silicotic mice. (A, B) The activity of ACE/ACE2 was measured in the serum samples of silicotic mice, along with (C, D) the concentration of Ang II/Ang-(1-7), following various treatment combinations with DIZE, A779, and MLN-4760. (E, F) The activity of ACE/ACE2 was measured in lung tissue samples from silicotic mice, along with (G, H) the concentration of Ang II/Ang-(1-7). ELISA was used for all experiments. Values represent the mean ± SD, n = 5 independent experiments, *P < 0.05 vs corresponding group, **P < 0.01 vs corresponding group.

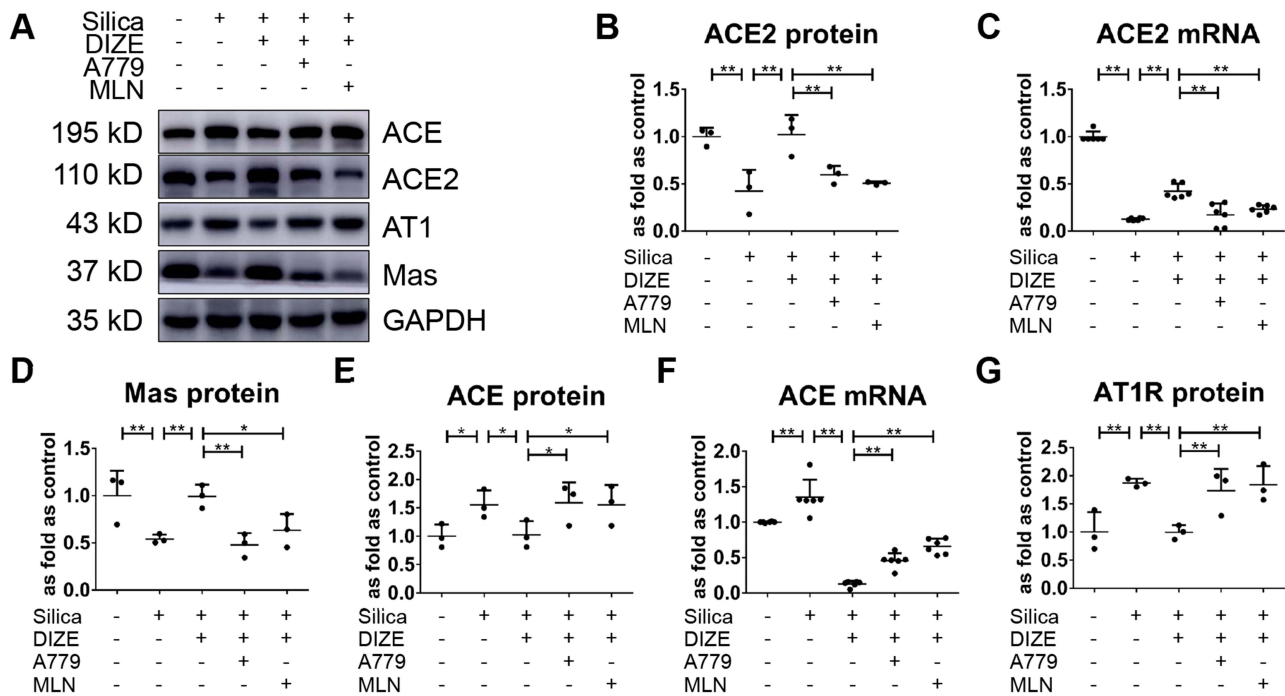


Figure 3 DIZE regulates lung RAS in wild-type silicotic mice. (A) Western blot showing the protein expression of various components of lung RAS in the lungs of wild-type silicotic mice. (B, C) The protein and mRNA expression levels of ACE2, respectively. (D) The protein expression levels of Mas. (E, F) The protein and mRNA expression levels of ACE, respectively. (G) The protein expression level of AT1R. Protein and mRNA expression levels were quantified based on the results of the Western blot in (A) and RT-qPCR, respectively. Values represent the mean ± SD, n = 3 and n = 6 independent experiments for protein and mRNA detection, respectively, fold change is expressed relative to the control (no treatments), *P < 0.05 vs corresponding group, **P < 0.01 vs corresponding group.

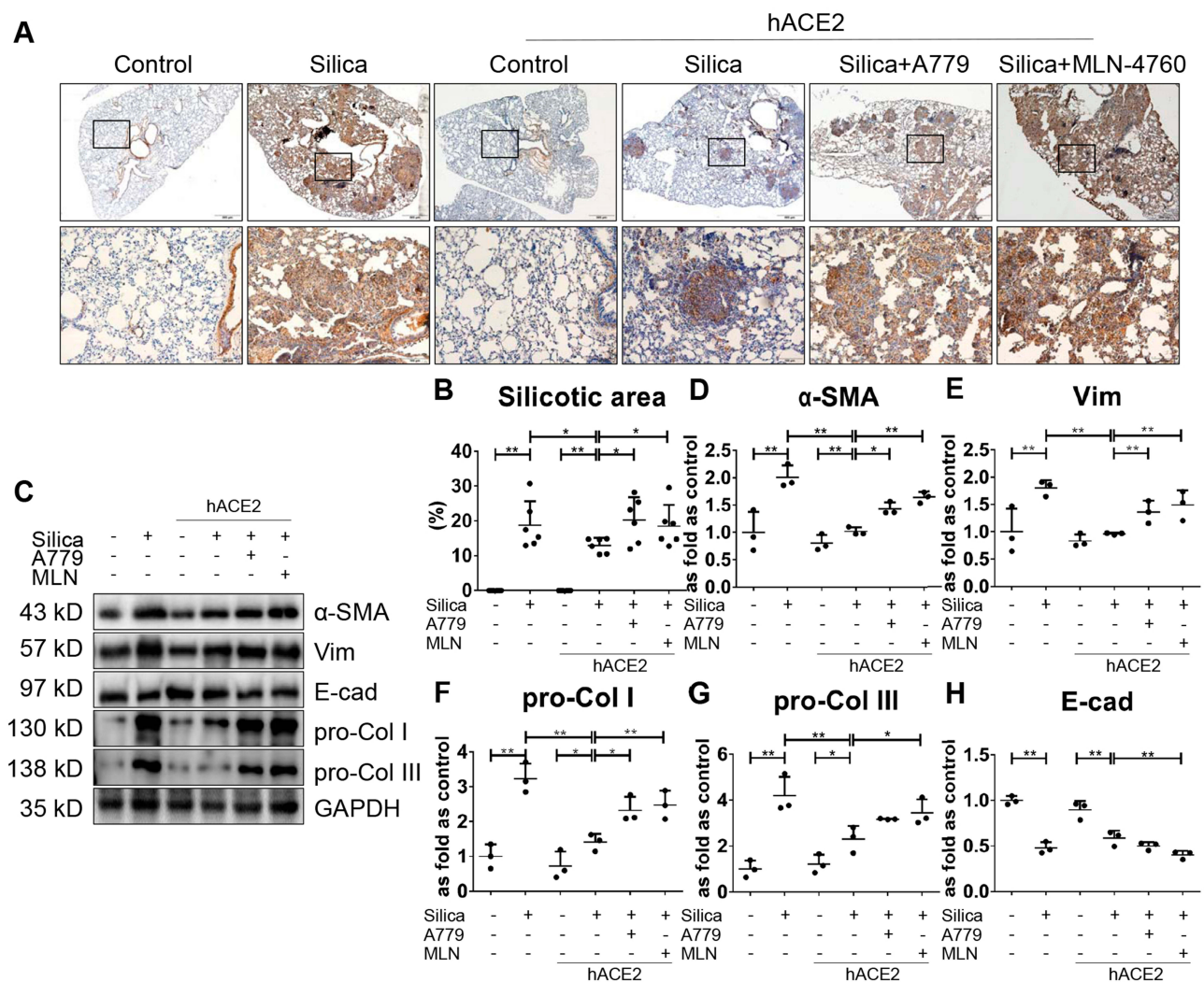


Figure 4 Overexpression of hACE2 inhibits SiO₂-induced EMT and collagen deposition in hACE2-transgenic silicotic mice. **(A)** Immunohistochemistry staining of α -SMA expression in the lungs of hACE2-transgenic mice, n = 6 in each group. Magnification, $\times 40$ (top panel) and $\times 200$ (bottom panel). Silicotic mice underwent various treatment combinations with DIZE (ACE2 activator), A779 (Mas receptor blocker), and MLN-4760 (ACE2 inhibitor). **(B)** The proportion of silicotic areas in the lung samples in **(A)**. **(C)** Western blot showing the protein expression of α -SMA **(D)**, Vim **(E)**, pro-Col I **(F)**, pro-Col III **(G)**, and E-cad **(H)** in the lungs of mice from the various treatment groups. Values represent the mean \pm SD, n = 3 independent experiments, fold change is expressed relative to the control (wild-type mice with no treatments), *P < 0.05 vs corresponding group, **P < 0.01 vs corresponding group.

cells. Treatment with either A779 or MLN-4760 abrogated the beneficial effects of DIZE in silica-treated MLE-12 cells.

Western blotting results (Figure 10A) further indicated that DIZE treatment of MLE-12 cells exposed to silica reversed the elevated protein expression of ACE, Ang II, and AT1R (Figure 10B–D) while reversing the suppressed protein expression of ACE2, Ang-(1–7), and Mas (Figure 10E–G), which are all changes associated with silica treatment. Treatment with MLN-4760 and A779 abrogated the beneficial effects of DIZE. In short, these observations indicated that DIZE treatment of MLE-12 cells promoted the expression of ACE2, thereby inhibiting the EMT induced by silica.

Discussion

Studies have shown that the overexpression of ACE2 inhibits inflammatory injury and cell proliferation, which protects the lung, heart, and kidney from the effects of injury.^{24–27} Knocking out ACE2 in mice leads to an imbalance between ACE/ACE2 and Ang II/Ang-(1–7), which aggravates lung injury,²² whereas exogenous ACE2 significantly reduces the injury lesions.²⁸ The dysregulation of RAS may trigger this pathological pulmonary process.

As previously reported, we found that EMT induced by silica has an important role in the progression of silicosis.²⁹ The epithelial to mesenchymal or myofibroblast transition results in extracellular matrix (ECM) deposition

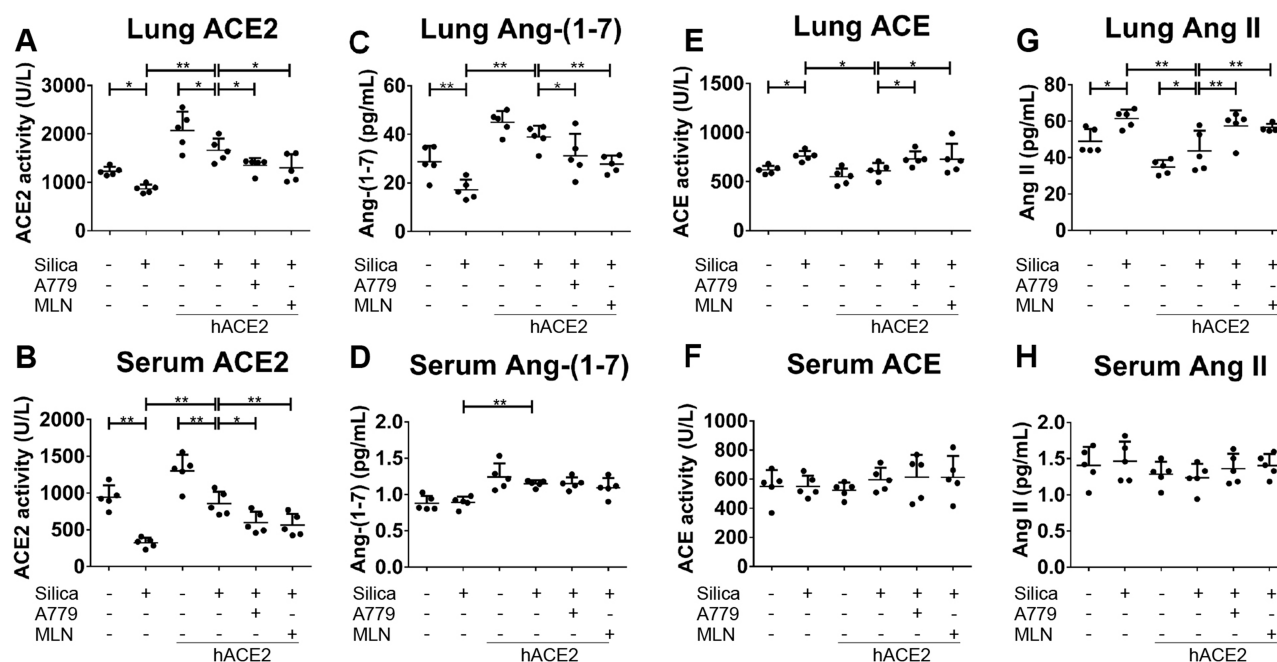


Figure 5 Overexpression of hACE2 regulates the activity of ACE/ACE2 and the concentration of Ang II/Ang(1-7) in hACE2-transgenic silicotic mice. (A, B) The activity of ACE2 in the lung tissue and serum, respectively. (C, D) The concentration of Ang(1-7) in the lung tissue and serum, respectively. (E, F) The activity of ACE in the lung tissue and serum, respectively. (G, H) The concentration of Ang II in the lung tissue and serum, respectively. ELISA was used for all experiments. Values represent the mean \pm SD, n = 5 independent experiments, * $P < 0.05$ vs corresponding group, ** $P < 0.01$ vs corresponding group.

and decreasing lung compliance. In the present study, we found that exogenous activation (DIZE intervention) and endogenous overexpression of ACE2 (*hACE2*-transgenic mice) could increase the level of E-cadherin and inhibit the markers of EMT and myofibroblast. We also observed that DIZE and Ang (1-7) could attenuate EMT induced by silica in MLE-12 cells. These results provide evidence that ACE2 exerts a protective effect against pulmonary fibrosis through the inhibition of EMT induced by silica.

In recent years, studies have found that DIZE, a traditional anti-trypanosomiasis drug without major toxicity³⁰ and approved by the US Food and Drug Administration,¹¹ can increase ACE2 activity, thereby promoting Ang(1-7) production through the degradation of Ang II. DIZE intervention for 4 weeks improves cardiac remodeling in myocardial infarction rats, and it is associated with increased mRNA expression and enzyme activity of ACE2. Moreover, these effects are blocked by the inhibition of either ACE2 with MLN-4760³¹ or the Mas receptor with A779.³² Recently, researchers have shed light on the applications of DIZE in the treatment of fibrotic diseases, indicating that it exerts protective effects against lung, myocardial, liver, and biliary fibrosis.¹¹⁻¹⁴ As expected, DIZE effectively mitigated pathological changes in the lung tissue of silicotic mice and restored the RAS imbalance in the present study. Thus, the mechanism underlying the effect of DIZE on

ACE2 activity is probably based, at least in part, on the upregulation of ACE2 mRNA and protein expression.

We employed transgenic mice overexpressing the human *ACE2* gene as a model for our investigations. The mouse *Ace2* gene has high homology with human *ACE2*. The model was prepared using the endogenous mouse *Ace2* promoter; under the control of this promoter, the *ACE2* gene is widely expressed in the lung, kidney, heart, and small intestine of mice.³³ Previous studies have confirmed that ACE2 mRNA and protein levels in the lung tissue of *hACE2*-transgenic mice are higher than those in wild-type mice.²² In the present study, we also found that exogenous and endogenous overexpression of ACE2 protects the lung from exposure to silica, in particular by reversing the reductions in ACE2/Ang(1-7)/Mas observed in silicotic mice.

In the current study, serum ACE/ACE2 activity and Ang II/Ang(1-7) levels were also measured, but the observed changes were not completely consistent with those in the lung tissue. This suggests that the activation mechanism and the role of circulatory and tissue RAS are different. Circulatory Ang II mainly originates in the lung tissue, while Ang(1-7) is a derivative of Ang II, formed when ACE2 cleaves the amino acid, phenylalanine, from the C-terminus.³⁴ In short, circulatory and tissue RAS responds differently in the mouse silicosis model. Circulatory RAS

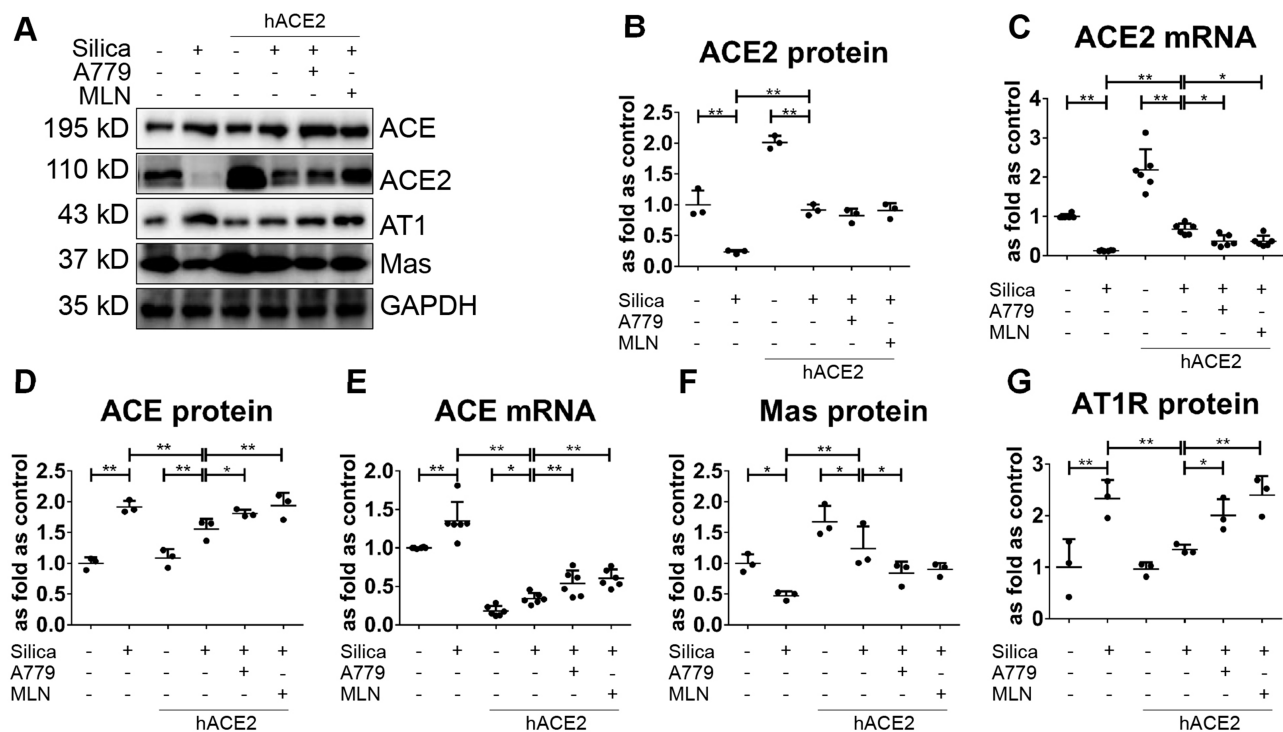


Figure 6 Overexpression of hACE2 regulates lung RAS in hACE2-transgenic silicotic mice. (A) Western blot showing the protein expression of various components of lung RAS in the hACE2-transgenic silicotic mice. (B, C) The protein and mRNA expression level of ACE2, respectively. (D, E) The protein and mRNA expression levels of ACE, respectively. (F) The protein expression levels of Mas. (G) The protein expression level of AT1R. Protein and mRNA expression levels were quantified based on the results of the Western blot in (A) and RT-qPCR, respectively. Values represent the mean \pm SD, n = 3 and n = 6 independent experiments for protein and mRNA detection, respectively, fold change is expressed relative to the control (wild-type mice with no treatments), *P < 0.05 vs corresponding group, **P < 0.01 vs corresponding group.

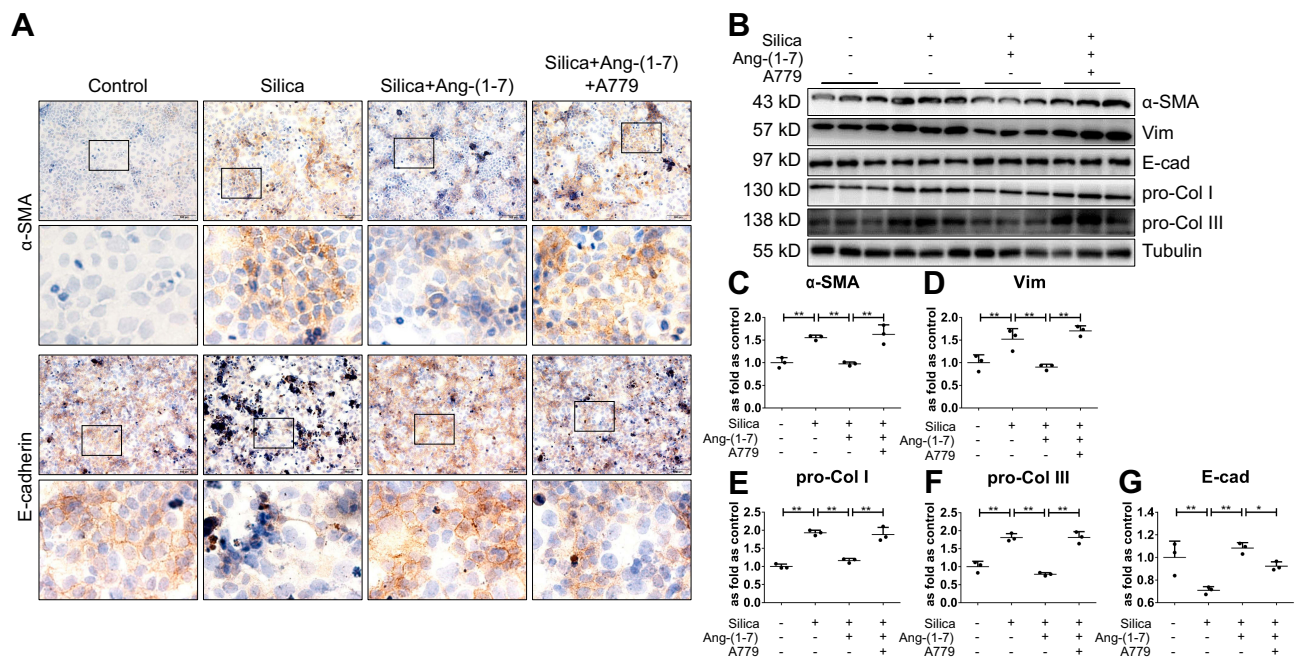


Figure 7 Ang-(1-7) inhibits SiO₂-induced EMT in MLE-12 alveolar type II epithelial cells. (A) Immunohistochemistry staining of α -SMA and E-cad expression in silica-treated MLE-12 cells. Magnification, $\times 40$ (top panels) and $\times 200$ (bottom panels). (B) Western blot showing the protein expression of α -SMA (C), Vim (D), pro-Col I (E), pro-Col III (F), and E-cad (G) in these cells. Silica-treated MLE-12 underwent various treatment combinations with Ang-(1-7), and A779 (Mas receptor blocker). Values represent the mean \pm SD, n = 3 independent experiments, fold change is expressed relative to the control (no treatments), *P < 0.05 vs corresponding group, **P < 0.01 vs corresponding group.

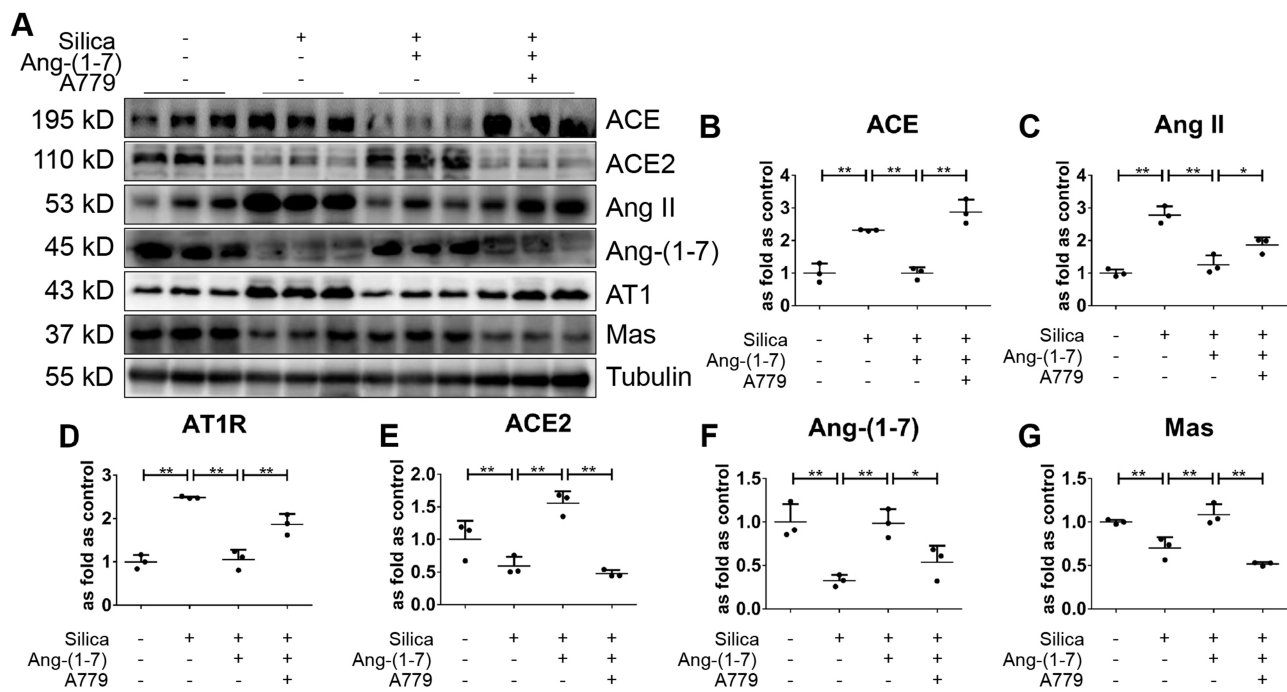


Figure 8 Ang-(1-7) regulates RAS in SiO₂-treated MLE-12 alveolar type II epithelial cells. (A) Western blot showing the protein expression of ACE (B), Ang II (C), AT1R (D), ACE2 (E), Ang-(1-7) (F), and Mas (G) in silica-treated MLE-12 cells subjected to various treatment combinations with Ang-(1-7), and A779. Values represent the mean \pm SD, n = 3 independent experiments, fold change is expressed relative to the control (no treatments), *P < 0.05 vs corresponding group, **P < 0.01 vs corresponding group.

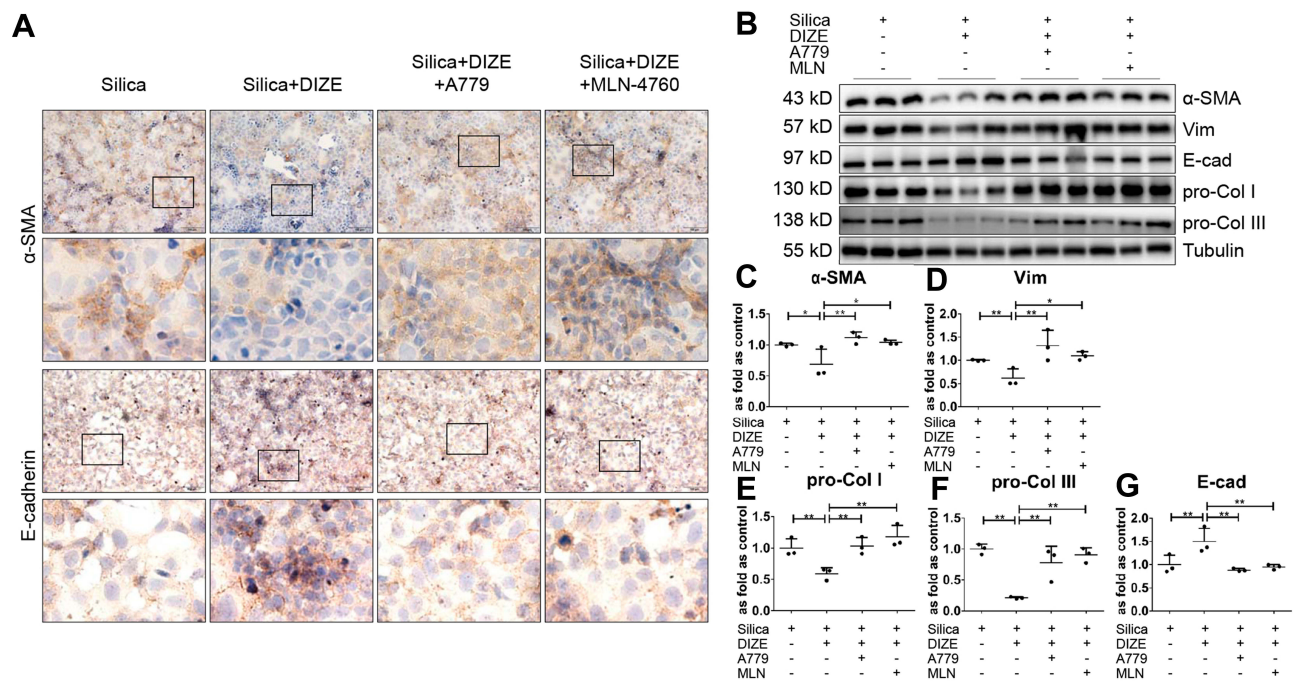


Figure 9 DIZE inhibits SiO₂-induced EMT in MLE-12 alveolar type II epithelial cells. (A) Immunohistochemistry staining of α -SMA and E-cad expression in silica-treated MLE-12 cells subjected to various treatment combinations with DIZE, A779, and MLN-4760. Magnification, $\times 40$ (top panels) and $\times 200$ (bottom panels). (B) Western blot showing the protein expression of α -SMA (C), Vim (D), pro-Col I (E), pro-Col III (F), and E-cad (G) in these cells. Values represent the mean \pm SD, n = 3 independent experiments, fold change is expressed relative to the control (silica only), *P < 0.05 vs corresponding group, **P < 0.01 vs corresponding group.

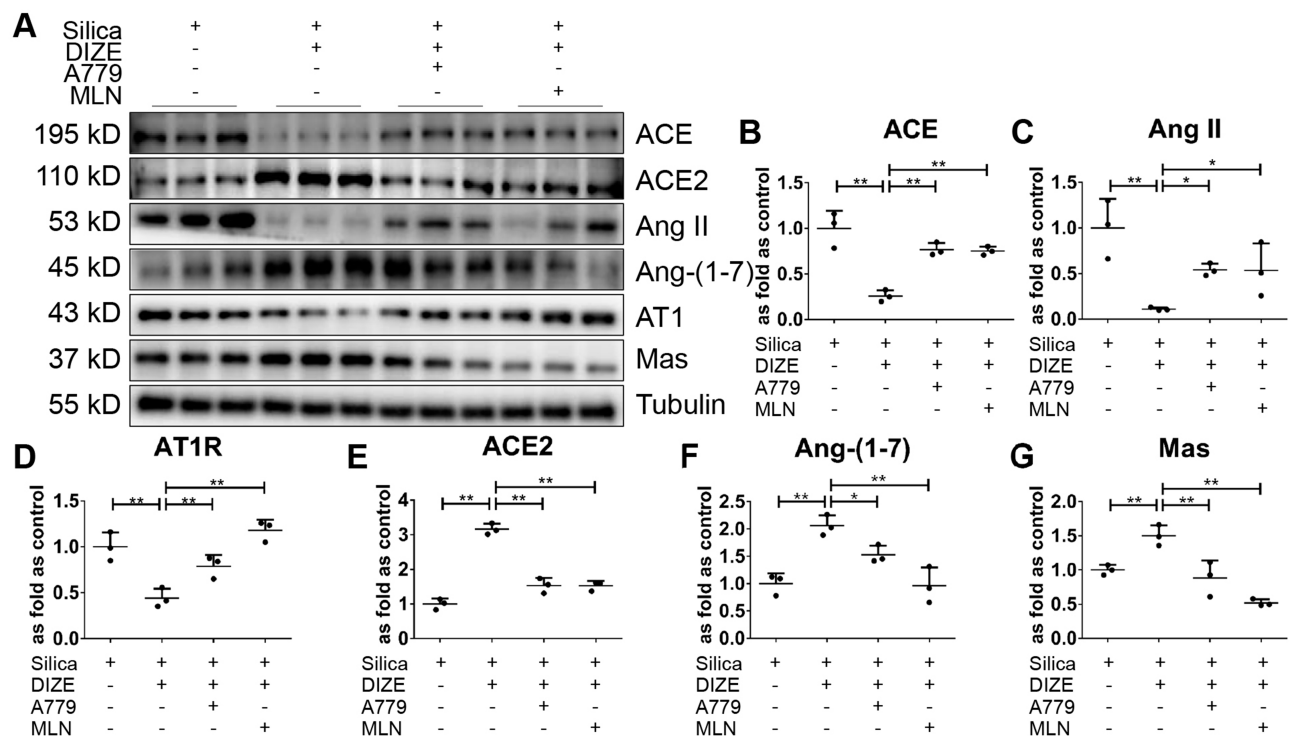


Figure 10 DIZE regulates RAS in SiO_2 -treated MLE-12 alveolar type II epithelial cells. **(A)** Western blot showing the protein expression of ACE **(B)**, Ang II **(C)**, AT1R **(D)**, ACE2 **(E)**, Ang-(1-7) **(F)**, and Mas **(G)** in silica-treated MLE-12 cells subjected to various treatment combinations with DIZE, A779, and MLN-4760. Values represent the mean \pm SD, $n = 3$ independent experiments, fold change is expressed relative to the control (silica only), * $P < 0.05$ vs corresponding group, ** $P < 0.01$ vs corresponding group.

plays an important role in maintaining blood pressure and body fluid balance, which may ensure blood supply to critical organs. Tissue RAS, however, may be necessary for maintaining local blood perfusion and for its roles in such processes as antioxidation and antiproliferation.²²

Conclusion

In summary, unbalanced RAS homeostasis in the lung tissue plays an important role in the occurrence and development of lung fibrosis in silicotic mice. The current work provided evidence that both the overexpression of ACE2 and treatment with DIZE could activate the ACE2/Ang-(1-7)/Mas receptor axis to inhibit EMT, thereby exerting anti-fibrotic effects in silicotic mice, and this was accompanied by suppression of the ACE/Ang II/AT1 receptor axis. The regulation of RAS homeostasis, which is focused on ACE2, may provide a promising treatment approach for pulmonary fibrosis; however, the specific underlying mechanisms remain to be determined.

Abbreviations

SiO_2 , silicon dioxide; RAS, renin-angiotensin system; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Ang II, angiotensin II; Ang-(1-7), angiotensin-(1-7); AT1, angiotensin II receptor 1; DIZE,

diminazene aceturate; EMT, epithelial-mesenchymal transition; ECM, extracellular matrix.

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Author Contributions

Shumin Li and Hong Xu designed the study. Shumin Li, Yaqian Li, Zhongqiu Wei, Yi Yang, Fuyu Jin, Min Zhang, Chen Wang, Wenxiong Song, Jingchen Huo and Jingyuan Zhao carried out the experimental work, analyzed the data, and drafted the manuscript. Fang Yang and Xiuhong Yang participated in the design of the study, critically reviewed the manuscript, and provided intellectual input. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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