GREM1/PPP2R3A expression in heterogeneous fibroblasts initiates pulmonary fibrosis

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Abstract

**Background:** Fibroblasts have important roles in the synthesis and remodeling of extracellular matrix (ECM) proteins during pulmonary fibrosis. However, the spatiotemporal distribution of heterogeneous fibroblasts during disease progression remains unknown.

**Results:** In the current study, silica was used to generate a mouse model of pathological changes in the lung, and single-cell sequencing, spatial transcriptome sequencing and an analysis of markers of cell subtypes were performed to identify fibroblast subtypes. A group of heterogeneous fibroblasts that played an important role in the early pathological stage were identified, characterized by the expression of inflammatory and proliferation genes (termed infla-pro fibroblasts) and concentrated in the lesion area. GREM1/protein phosphatase 2 regulatory subunit B"alpha (PPP2R3A) expression in infla-pro fibroblasts was found to initiate early pulmonary pathological changes by increasing cell viability, proliferation and migration.

**Conclusions:** Infla-pro fibroblasts play a key role in the early pathological changes of silicosis, in which GREM1 is the driving factor that targets PPP2R3A and initiates the inflammatory response, followed by irreversible fibrosis induced by SiO$_2$. The GREM1/PPP2R3A pathway may be a potential target for the early treatment of silicosis.

Introduction

The inhalation of free crystalline silica or silica occurs in many industries[1] and may lead to fibroblast activation and the excessive accumulation and deposition of extracellular matrix (ECM)[2]. Lung function impairment increases as the disease progresses and becomes progressively worse even when the patients are no longer exposed. The diagnosis of pulmonary fibrosis caused by silica inhalation is usually based on a high level of silica dust exposure and radiological characteristics; similar diseases[3], such as powdery tuberculosis, idiopathic pulmonary fibrosis (IPF), other lung interstitial diseases and cancers, must be excluded. Effective treatments for the disease are lacking, and the exact pathogenesis remains unclear.

Pulmonary fibroblasts (PFBs) are the main pulmonary interstitial cells[4]. These important effector cells are involved in damage and repair in the body and are maintained in a resting state under physiological conditions (resting) but respond rapidly under pathological conditions. The proliferation of these cells completes the repair of cellular damage and restores the characteristics of the resting state. These cells participate in fibrosis through proliferation and migration and the synthesis and release of matrix materials such as collagen. Cell injury, infection or other stimuli promote the differentiation of mesenchymal cells into activated or pathological fibroblasts, and these cells induce inflammation[5]. The heterogeneity of lung fibroblasts indicates that these cells may be derived from different cell types, may represent different activation stages, or may be affected by the microenvironment[6]. The different responses of heterogeneous fibroblasts to the internal environment may be the main cause of pulmonary
fibrosis, as well as the main cause of lung cancer and other cancers[7], and related studies have shown that the physiological and pathological types of fibroblasts display differences in gene expression and cell surface markers[8–12].

In this research, we generated all major cell types based on single-cell RNA sequencing (scRNA-Seq) and developed a molecular map of fibroblasts to comprehensively elucidate the changes in fibroblast types and spatial locations during pulmonary fibrosis. Then, through spatial transcriptomics combined with new calculation methods, we identified the localization of the different cell types. A quasichronological analysis was used to determine the source and localization of the cells. Based on these results, the newly emerging heterogeneous fibroblasts express GREM1, and PPP2R3A modulates GREM1 to participate in early pathological changes in the lung, potentially leading to the activation, proliferation and migration of fibroblasts.

**Results**

**scRNA-seq classification of mouse lung fibroblasts**

According to recent reports, heterogeneous lung fibroblasts may be the main cause of lung fibrosis and early pathological changes[6, 13]. Before a comprehensive identification and definition of the fibroblast subpopulations between normal lung tissue and fibrotic lung tissue, we first grouped all cells in the lung tissue. C57BL/6 mice (20–25 g) were treated with silica, the lung tissue was removed after the model was successfully established, and the cells extracted from the lung tissue homogenate were subjected to single-cell sequencing using the 10x Genomics Chromium platform. Through t-distribution stochastic neighborhood embedding (t-SNE) projection, the cells were visualized in two dimensions according to the expression profile, and all cells in the lung tissue were divided into 24 types according to the surface markers expressed on different cells (Figure 1A). Among them, fibroblasts were separated by their specific markers Col3a1 and Col1a1[14, 15] (Figure 1B), and then we subdivided the fibroblasts into subtypes using different known fibroblast markers[13, 16-19]. Cluster 6 was defined by a GO enrichment analysis of the molecular functions and biological processes of the top 50 genes in this subtype. (Figure 1C-D and S1A-C). The cells were defined as different subtypes, but Cluster 5 was unable to be defined accurately using existing gene markers. Due to its characteristic expression of the proliferation-related gene *gremlin* and the highest expression level of *gremlin* in this subtype, it was temporarily defined as *gremlin*^high^ fibroblasts.

**Analysis of fibroblast subtypes**

Based on the aforementioned subtype definitions and the single-cell sequencing results of the lung tissues from mice in the saline group and silica group at 7 and 56 days obtained in the laboratory, the cell percentage of different groups and different subgroups were analyzed (Figure 2A). The percentage of subtype fibroblasts in each group is shown in Table 1. The *gremlin*^high^ fibroblasts appeared specifically in the silica group, and the proportion detected at 7 days was higher than that detected at 56 days (Figure
2B and C), suggesting that this subtype of cells plays a vital role in the early stage of pathology. Then, we analyzed the source and destination of \textit{grem1}^{\text{high}} fibroblasts through a pseudochronological sequence and found that these special heterogeneous fibroblasts originate from resting fibroblasts; after performing their functions, they transdifferentiate into myofibroblasts and inflammatory fibroblasts (Figure 2D and E). We found that the \textit{grem1}^{\text{high}} fibroblasts were primarily expressed in the focus area in the spatial map (Figure 2F). We constructed a bubble chart to further determine whether the top 10 genes expressed by cells in Cluster 5 are also expressed at high levels in other subtypes and found that most genes are specifically expressed in the Cluster 5 subtype, but \textit{grem1} is only expressed at high levels in this type (Figure 2G). Then, by constructing a Venn diagram, we found that the top 50 genes expressed in the \textit{grem1}^{\text{high}} fibroblasts overlapped with those of inflammatory fibroblasts, ECM fibroblasts, and myofibroblasts (Figure 2H). Thus, we termed this subtype of fibroblasts inflammatory proliferative fibroblasts (infla-pro fibroblasts).

**Infla-pro fibroblast-related bioinformatics analysis and scRNA-Seq verification**

Based on the analysis described above, we focused on infla-pro fibroblasts and the characteristic expression of \textit{grem1}. By enriching the top 50 genes in this group and analyzing their functions (Figure 3A), these genes were shown to play an important role in regulating biological processes, cell growth and proliferation. Through the GO enrichment analysis, we found that the genes were enriched in cell adhesion, inflammation, cell death, myofibroblast differentiation and other pathways. (Figure 3B). Then, the scRNA-Seq results showed that \textit{grem1} was a characteristic gene expressed in the special subtype (Figure 3C and D) and was typically expressed in the lesion area (Figure 3E). At the same time, the combination of the single-cell sequencing and spatial transcriptome sequencing results obtained on the 7th day and the 56th day revealed that \textit{grem1} may play an important role in the early stage of pathology as the initiating factor (Figure S2A and B). The authenticity and reliability of the results were verified by histochemical staining of mouse lung tissue sections. GREM1 was expressed at high levels at 7 days and colocalized with fibroblast markers (Figure 3F). The changes in \textit{Grem1} mRNA expression levels in the process of human idiopathic pulmonary fibrosis (IPF) were analyzed using the Gene Expression Omnibus (GEO) database, in which the expression of \textit{Grem1} in the patient group with IPF was significantly higher than that in the healthy group (Figure 3G), indicating a role for \textit{Grem1} in pulmonary fibrosis. Furthermore, we stimulated HPF-a cells with the optimal concentration of TGF-β1, 5 ng/ml (Figure S3A-D), for verification in vitro and verified that GREM1 expression first increased and then decreased in a time-dependent manner, peaking at 1 h (Figure 3H and I). Recent studies have examined the function of GREM1 in fibrosis[20-24], but its mechanism remains unclear. Using the signaling pathways related to the occurrence and development of inflammation and fibrosis (Figure 3J), we analyzed the protein-protein interaction (PPI) network of the genes in these signaling pathways to analyze the downstream targets of GREM1. GREM1 was correlated with bone morphogenetic protein (BMP) and PPP2R3A (Figure 3K and S4A). GREM1 alters the pathological course of the disease through the BMP signaling pathway[25-27], but the mechanism by which GREM1 promotes the occurrence and development of disease through TGF-β1/PPP2R3A signaling is not clear. Studies have reported that PPP2R3A mainly regulates the cell cycle
by targeting cell cycle regulators and apoptosis inhibitors[28]. Because of its involvement in the regulation of important tumor signaling pathways, developmental processes and the cell cycle, this molecule has received extensive attention. We concluded that it might be another downstream target of GREM1 that causes early pathological changes.

**PPP2R3A expression is induced in HPF-a cells after exposure to TGF-β1**

Follow-up studies were conducted by analyzing protein interactions, as shown in Figure 4A. Protein phosphatase 2A (PP2A) is a cellular serine/threonine protein phosphatase involved in various cellular processes and plays an important regulatory role in cell proliferation, differentiation and death[29]. Normally, the structural core subunit PP2Aa (PPP2R1A/PPP2R1B) interacts with the catalytic subunit PP2Ac (PPP2CA/PPP2CB) to form the core of the enzyme, and the broad range of regulatory B subunits (15 genes) interact with the core enzyme. The tissue specificity and substrate specificity of the PP2A holoenzyme complex were determined (Figure 4B and C)[30]. PPP2R1A of the A subunit of PP2A promotes cell proliferation and migration and is a key fibrogenic factor[31], but the role of PPP2R3A of the B subunit in pathological processes remains unclear. Here, we mainly explored the role of PPP2R3A in the early stage of disease pathology and the mechanism by which GREM1 promotes pathological progression through TGF-β1/PPP2R3A signaling. HPF-a cells were treated with TGF-β1, and the results showed that PPP2R3A expression first increased and then decreased in a time-dependent manner, peaking at 6 h (Figure 4D and E). The immunofluorescence and qRT–PCR results were consistent with the western blot (WB) results (Figure 4F and G). The immunofluorescence results also showed that the morphology of the fibroblasts changed from the original spindle shape to an amoeba-like morphology after TGF-β1 stimulation (Figure 4F). PPP2R3A expression was detected after siRNA-mediated knockdown of Grem1 (Figure S4B and C), and Grem1 knockdown partially reversed the increase in PPP2R3A expression induced by TGF-β1 (Figure 4H and I), suggesting that PPP2R3A is a downstream target of GREM1.

**PPP2R3A mediates the TGF-β1-induce proliferation and activation of HPF-a cells**

Based on accumulating evidence, the migration, proliferation and activation of lung fibroblasts are the main causes of pulmonary fibrosis[32, 33], and thus the effect of PPP2R3A on these cellular functions was investigated in HPF-a cells. TGF-β1 increased the expression of marker proteins related to fibrosis (Figure S5A-D), induced fibroblast migration and increased fibroblast viability (Figure S5E-G). However, specific knockdown of Ppp2r3a (Figure S6A-C) partially reversed the increases in cell viability, migration and proliferation (Figure 5A-E) and specifically reversed the TGF-β1-induced increase in FN1 expression (Figure 5F and G), but had little effect on the expression of COL1 and α-SMA (Figure 5H-J).

**Upregulation of PPP2R3A in the lungs of mice exposed to SiO₂**

Mice were exposed to SiO₂ for 7 days to verify the in vitro observations based upon the scRNA-Seq results and the initiating role of PPP2R3A. Sirius red staining showed obvious collagen deposition with no typical formation of silicon nodules in the SiO₂ group, indicating the successful establishment
of a mouse silicosis model and the initiation of fibrosis in the lung (Figure 6A). The WB results showed higher expression of PPP2R3A in the SiO\textsubscript{2} group than that in the control group (Figure 6B and C). Immunohistochemistry showed an increase in the level of Vimentin, a specific fibroblast marker, which indicated the proliferation of fibroblasts in the lung, and PPP2R3A expression was consistent with the WB (Figure 6D). Moreover, ppp2r3a was mainly expressed in resting fibroblasts in the control group (Figure 6E). During the transformation from resting fibroblasts to infla-pro fibroblasts after SiO\textsubscript{2} treatment, ppp2r3a and vimentin were expressed in infla-pro fibroblasts in the SiO\textsubscript{2} group (Figure 6E and F).

Discussion

Pulmonary fibrosis caused by silica inhalation is a major challenge for clinicians and a major problem in the field of public health due to the lack of specific targets for screening and diagnosis in the early stage and the lack of specific treatment measures in the later stage. In our research, we used single-cell transcriptome sequencing to analyze and classify the subtypes of fibroblasts in the lung tissues of normal saline- and silica-treated mice. We identified a heterogeneous subtype of fibroblasts only in the silica group. Since the genes expressed in this subtype overlap with inflammatory fibroblasts, ECM fibroblasts and myofibroblasts, we defined them as infla-pro fibroblasts. According to recent studies, the occurrence of pulmonary fibrosis is due to the direct transdifferentiation of resting fibroblasts into inflammatory fibroblasts or ECM fibroblasts. In our research, we found that cells in the resting state partially transdifferentiated into infla-pro fibroblasts. Intermediate fibroblasts transdifferentiated into inflammatory fibroblasts and ECM fibroblasts, which has not been reported in previous studies. Blocking the conversion of resting fibroblasts into infla-pro fibroblasts may slow or even reverse the early, progressive development of pathology.

We compared our scRNA-Seq data with recently published analyses of mouse and human lung fibroblast subtypes[6, 17], which mainly described myofibroblasts, resting fibroblasts, adipose fibroblasts, inflammatory fibroblasts and ECM fibroblasts. The resting fibroblast body is small and fusiform. Upon stimulation with inflammation and other factors, these cells transform into other types of fibroblasts and participate in repair after injury. Myofibroblasts express α-SMA and participate in the occurrence of fibrotic diseases[6]. Increased expression of α-SMA indicates fibroblast activation. The activation of resting fibroblasts is one of the main sources of myofibroblasts. Lipofibroblasts contain large cytoplasmic lipid droplet inclusions and unrestricted biofilms or lipid vacuoles and play important roles in lung development, surfactant synthesis and retinoic acid metabolism[34]. These cells generally do not change significantly during the onset of pulmonary fibrosis. Inflammatory fibroblasts and ECM fibroblasts are representative heterogeneous fibroblasts detected under pathological conditions and mainly induce inflammation, proliferation and migration. However, infla-pro fibroblasts, which are cells in an intermediate state, were identified in this study and have not been previously reported. We found that this subtype plays an important role in the process of transdifferentiation and promotes cell proliferation and migration.
Newly emerged heterogeneous fibroblasts feature the expression of *grem1*, which has been reported to promote the migration and proliferation of normal lung cells[35, 36] and the epithelial-mesenchymal transition (EMT)[37, 38] and regulate the endothelial-mesenchymal transition (EndMT)[21]. However, the mechanism by which it initiates early pathological changes is unclear. Bioinformatics analysis showed that GREM1 was related to BMP and PP2A; in addition, GREM1, PPP2R3A, BMP2, TGF-β1 and FN1 were enriched, as shown in Table 2. BMP is a member of the TGF-β family. It was first discovered because of its ability to induce bone formation. Defects in the BMP signaling pathway or its regulation are the basis of various human diseases. It regulates cell proliferation, differentiation, migration, apoptosis and chemotaxis under different pathological conditions[39]. Existing studies have clearly shown that the BMP signaling pathway affects the occurrence and development of fibrosis[25, 40], mainly through the classic Smad-dependent pathway and the Smad-independent pathway[41]. Therefore, this study mainly explored the mechanism by which GREM1 promotes early pathological changes through PPP2R3A, a subunit of PP2A. PP2A is a major cellular serine-threonine phosphatase that has attracted attention because of its involvement in the regulation of important tumor signaling pathways, developmental processes, and the cell cycle[29, 30, 42]. Studies have reported that specific knock out of the *ppp2r1a* gene (encoding the PP2A Aα subunit) in mice promotes inflammation and liver fibrosis[31]. By analyzing a database, we found that GREM1 is related to PPP2R3A in addition to PPP2R1A. PPP2R3A is a subunit of PP2A regulatory subunit B, also known as PR72/PR130. This molecule regulates the cell cycle mainly by targeting cell cycle regulators and apoptosis inhibitors and is the main regulator of cell proliferation. The scRNA-Seq results showed a slight increase in *ppp2r3a* expression, potentially due to increased expression in single cells rather than an increased number cells expressing *ppp2r3a*. This conclusion is based on the number of dark red dots shown in Figure 6E. The scRNA-Seq results showed that this molecule was expressed mainly in resting fibroblasts among normal mouse lung fibroblasts. In an early pathological model of tracheal silica instillation, *ppp2r3a* was expressed in infla-pro fibroblasts. We postulate that although its overall level did not show major changes, this molecule still plays an important role in the fibroblast transdifferentiation process. Most researchers agree that a change in mRNA expression precedes a change in protein levels. However, in our research, we found that a change in the expression of the PPP2R3A protein preceded the change in mRNA expression. Many levels of regulation of gene expression may exist, and regulation at the transcriptional level is only one mechanism. Posttranscriptional, translational and posttranslational regulation may contribute to these findings. The results may be due to posttranscriptional regulation or positive regulation of transcriptional mechanisms. Because TGF-β and BMP play cross regulatory, synergistic or antagonistic roles in multiple signaling pathways[43], we cannot exclude the possibility that the TGF-β1/GREM1/PPP2R3A pathway promotes pathological changes, while the TGF-β1/GREM1/BMP pathway can hinder the occurrence and development of fibrosis. However, the mechanism of action between the two pathways has not been discussed in this paper and will be analyzed and studied in the future.

FN1, COL1, and α-SMA are three markers of fibrosis. Previous studies in the literature have shown that FN1 mainly affects cell migration and proliferation [44]. COL1 is the main component of ECM. Excess accumulation of ECM may impair lung function. COL1 mainly affects cell adhesion and migration [45]. α-
SMA is the most commonly used molecular marker of smooth muscle cells and myofibroblasts. Myofibroblasts play an important role in fibrosis, and α-SMA is activated in these cells. This indicator is involved in both cell migration and proliferation [46-48]. However, we found that Ppp2r3a knockdown specifically affected the expression of the fibrotic marker FN1 but had little effect on COL1 and α-SMA expression, suggesting that PPP2R3A may specifically alter FN1 expression to cause early pathological changes. One study reported that the expression of the target protein specifically affects FN1 but has almost no effect on COL1 [34], similar to our findings. We performed a GO enrichment analysis of genes expressed in infla-pro fibroblasts to further analyze the mechanisms and found that at the early pathological stage, FN1 was differentially expressed and was mainly enriched in infla-pro pathways (Table 3), which may explain why PPP2R3A only affected FN1 but did not affect other fibrosis markers.

Conclusions

In summary, in the early pathological model of tracheal silica infusion, infla-pro fibroblasts indicated the occurrence and progression of the disease, and this type of heterogeneous fibroblast was characterized by the expression of grem1, which may serve as a predictive biomarker of early pathology. Moreover, we found that PPP2R3A was a downstream target of GREM1, and its expression was related to early pathological regulation, suggesting that it may become a potential target for early blockade of disease development.

Materials And Methods

Reagents

SiO₂ particles were purchased from Sigma–Aldrich Company (S5631; Billerica, MA, USA), and approximately 80% had a diameter of 1–5 μm. According to Stokes' law, precipitation selection, acid hydrolysis, and baking were performed at 200°C for at least 16 h. The silica sample was dissolved in normal saline and used to generate the mouse model of pulmonary fibrosis. The recombinant TGF-β1 protein was purchased from Nanjing GenScript Biotechnology Co., Ltd. and used to construct a cell fibrosis model. Primary antibodies against PPP2R3A (rabbit polyclonal antibody) were purchased from Proteintech, and the GREM1 antibody (rabbit polyclonal antibody) was purchased from Shanghai Shenggong Biotechnology. Antibodies against GAPDH (mouse monoclonal antibody) were obtained from Bioworld, Inc. A calcium ion fluorescence probe (Fluo-4 AM) was purchased from Beyotime Biotechnology Company (China).

Establishment of a mouse model of silicosis

Male C57BL/6 mice weighing 20–25 g were purchased from the Experimental Center of Nanjing Medical University. Mice were housed under a constant temperature (23°C) and humidity (50%), allowed to eat and drink freely, and the light/dark cycle was 12:12 h. Animals of an appropriate age were anesthetized with an intraperitoneal injection of pentobarbital sodium, the trachea was surgically exposed, and a
prepared silica suspension (0.2 g/kg in 50 mg/ml saline) was injected into the trachea in a single dose. Animals in the control group were administered the same volume of sterile normal saline. The lung tissues were collected 7 days after modeling. The lungs used for immunohistochemistry were first perfused with PBS, treated with 4% formalin, dehydrated with a 30% sucrose solution, sectioned and frozen for staining. All animal operations were performed in strict accordance with the ARRIVE guidelines, and the animal procedures were approved by the Institutional Animal Care and Use Committee of Southeast University School of Medicine.

**Single-cell RNA library construction and sequencing**

We used Cell Ranger software (10x Genomics) for the alignment of scRNA-Seq reads, collapsing reads to unique molecular identifier (UMI) counts, cell calling, and depth normalization of the transcriptome libraries. We used the Chromium instrument and the Single Cell 3 Reagent kit (V1) to prepare individually barcoded scRNA-Seq libraries according to the manufacturer’s protocol (10X Genomics). Then, the cells were clustered according to the surface markers expressed on different cells and were divided into different subgroups. Gene comparison and naming were performed, the names were defined for each subgroup, and Loupe Browser 5.0 was used to view and analyze the obtained data.

**Spatial transcriptome sequencing**

Adult C57BL/6 mice were anesthetized, and lung tissues were removed. The samples were frozen and stored until sectioning. The tissue section was adhered to the surface of the glass slide and stained with standard hematoxylin and eosin after fixation. Subsequently, the lung tissue sample was permeabilized and prepared into an information library containing barcodes. Then, the cells were clustered, and the locations of different cells in different original spatial positions were determined.

**Cell culture**

Adult lung fibroblasts (HPF-a) were purchased from ScienCell and cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-GlutMAX (Gibco). Cells were placed in a cell culture incubator with 5% CO₂ and a temperature of 37°C. For the experiment, we seeded the cells in a 24-well plate at a concentration of 1×10⁵ cells/ml and performed further processing after the cell status stabilized. The cell concentration was adjusted for different experiments according to the requirements of specific experiments.

**Western blotting**

Western blotting was used to detect protein levels in HPF-a cells and mouse lung tissues, and the experimental results were imaged with a Tanon scanner. Briefly, HPF-a cells were cultured in a 24-well plate. After the cells were treated with TGF-β1, they were washed twice with PBS, and proteins were extracted with a cell lysis solution (100:1) containing protease inhibitors. Protein extraction from tissues was performed in a similar manner. After the tissues were ground, cell lysis buffer containing
protease inhibitors was added, and the samples were incubated at -80°C for lysis overnight. According to the reagent manufacturer’s protocol (Beyotime), the concentration of the extracted protein was determined using the BCA assay, the concentration was adjusted, loading buffer was added and the sample was boiled at 100°C for five minutes to denature the protein and successfully prepare the protein sample. The protein sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to PVDF membranes, and blocked with Tris-buffered saline containing 5% skim milk powder in Tween 20 (TBST) for 1 h at room temperature. The PVDF membrane was incubated with the primary antibody in a chromatography cabinet overnight (at least 16 h) at 4°C. The next day, the PVDF membrane was washed four times with TBST and then incubated with the secondary antibody for 1 h at room temperature. The membrane was washed 3 times, covered with a luminescent solution and imaged with a Tanon scanner.

**Real-time quantitative PCR**

Real-time quantitative PCR (qRT–PCR) was used to detect the relative mRNA expression of *Grem1* and *Ppp2r3a*. HPF-a cells were plated according to the required density of the experiment, and the corresponding treatments were administered after 24 h. After treatment, cells were washed 3 times with RNase-free PBS, and total RNA was extracted from HPF-a cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. After the total RNA was extracted, the RNA concentration was measured with a NanoDrop One spectrophotometer (Thermo Fisher Scientific). Samples at different concentrations were normalized to contain approximately 400 ng of RNA and reverse transcribed into cDNAs. The cDNA samples were used as a template for qRT–PCR, and the cycle threshold (Ct) and ΔCt value were analyzed. Opticon monitoring software (Bio-Rad) was used for ΔΔCt quantification. The relative quantitation of mRNA expression was normalized to that of the endogenous reference (*Gapdh*).

**Immunofluorescence staining**

Before the experiment, the cover glass was pretreated with polylysine, and then, the cells were seeded in a 24-well plate containing the cover glass. After the experimental treatment, the medium in the 24-well plate was removed, and the cells were washed 3 times with PBS and fixed with 4% paraformaldehyde overnight at 4°C. The next day, the paraformaldehyde was discarded, and the cells were washed with PBS 3 times, treated with 0.3% Triton X-100, blocked with goat serum at room temperature for 2 h, and incubated with the primary antibody overnight. On the third day, cells were incubated with an appropriate fluorescent dye-conjugated secondary antibody (Alexa Fluor, Thermo Fisher Scientific) in the dark, the nucleus was stained with 4,6-diamino-2-phenylindole (DAPI), and images of cells were captured using a fluorescence microscope. Immunofluorescence staining of mouse lung tissue sections was performed with the same steps as the cellular immunofluorescence staining protocol after the paraformaldehyde was discarded.

**Wound healing experiment**

A wound healing test was performed to detect cell migration. Specifically, HPF-a-GFP cells were seeded in a 24-well plate and cultured in a cell incubator until the cell density reached approximately 80%. Then, a
straight line of medium width was drawn with the tip of a sterile 200 μl pipette tip. Similarly, a straight line was drawn perpendicular to the first line in each well to create a cross-shaped space. The medium was discarded, the wells were rinsed with sterile PBS 3 times to remove cell debris, and fresh standard medium was added to each well to ensure cell growth. Then, the experimental group of cells was treated with 5 ng/ml TGF-β1, and we immediately collected digital images of the scratch gap (0 h) and then collected digital images at 12, 24, 36, and 48 h. We used ImageJ software to measure the area of the cell gap.

**CCK-8 assay**

Cell viability was measured using the CCK-8 method (Dojindo, Tokyo, Japan) according to the manufacturer's protocol. Briefly, after cells were treated, 10 μl of CCK-8 solution were added to each well of a 96-well plate, the plate was incubated at 37°C for 1 h in the dark, and the absorbance was measured at 450 nm with a spectrophotometer. Cell viability was determined by calculating the survival of the experimental group relative to the control group. The percentage is shown.

**Bromodeoxyuridine labeling**

The cells were plated on glass slides treated with polylysine, and TGF-β1 was added to the cells after they had grown to an appropriate density. Then, bromodeoxyuridine (BrdU) (Yeasen, 40204ES60) reagent was dissolved in PBS, and medium (1:1000) was added. After 4 h of incubation, cells were fixed with 4% paraformaldehyde at 4°C. Then, the cells were washed 3 times with PBS, denatured with 2 N HCl/0.3% Triton X-100 at room temperature for 30 minutes, incubated with 0.1 M boric acid buffer (pH 8.0) for 10 minutes and blocked with goat serum at room temperature for 2 h. The cells were incubated with a BrdU antibody (1:100; SC-32323, Santa Cruz) at 4°C overnight. After washing with PBS, cells were incubated with appropriate fluorescent dye-conjugated secondary antibodies (Alexa Fluor, Thermo Fisher Scientific) in the dark for 2 h. The cells were then washed 3 times with PBS and mounted with mounting medium (Prolong Gold antifade reagent with DAPI; P36931, Life Technologies). The slides were imaged using a fluorescence microscope (Olympus IX70, Olympus America, Inc., Center Valley, PA, USA).

**Sirius red staining**

After the mouse model was successfully generated, the lung tissues were fixed, removed and incubated with 4% paraformaldehyde. After sedimentation was completed, the lung tissues were sliced for use. The lung tissue sections were rinsed with PBS 3 times, incubated with Picrosirius red for 60 minutes at room temperature, quickly rinsed with an acetic acid solution 2 times, rinsed with absolute ethanol, soaked and dehydrated, mounted with neutral gum and stored at 4°C. A microscope was used to capture bright-field images.

**RNA interference**

Small interfering RNAs (siRNAs) were used to knock down the expression of proteins of interest. The siRNAs were purchased from Shanghai Jima Pharmaceutical Technology Co., Ltd., and the transfection
reagent Lipofectamine 3000 was purchased from Thermo Fisher Scientific. We inoculated the cells in a 24-well plate and started transfection when the cell density reached 60%–80%. At the beginning of the transfection experiment, we added the siRNA to one tube of serum-free medium and added transfection reagent to the other tube of serum-free medium. The samples were incubated for 5 minutes, and then, the two solutions were mixed and incubated for 15 minutes. The solution was added to the wells, incubated for at least 12 h, and the standard medium was replaced. The cells were placed in a 37°C incubator for 24–72 h and used in subsequent experiments.

**Statistical analysis**

The data are presented as the means ± standard deviations (SD). Statistical analyses were performed with Student's t test or one-way analysis of variance (ANOVA). P<0.05 was defined as significant.

**Declarations**

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**Authors’ Contributions:** S.X. performed the experiments, interpreted the data, prepared the figures, and wrote the manuscript. Y.S., L.W., W.S., H.J., C.M. and W.J. performed the experiments and interpreted the data. C.Y. and Z.X. designed the experiments, interpreted the data, and wrote the manuscript. J.C. provided laboratory space and funding, designed the experiments, interpreted the data, wrote the manuscript, and directed the project. All authors read, discussed, and approved the final manuscript.

**Conflict of interest**

The authors have no conflicts of interest to declare.

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**Availability of data and materials**

All of the relevant raw data and materials are freely available to any investigator upon request.

**Ethical approval and consent to participate**

Not applicable

**Consent for publication**
Not applicable

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Tables

| Table 1. Statistical analysis of cell numbers for different subpopulations of fibroblasts. |
|---------------------------------|------|------|-------|------|-------|------|
| NS-7 days                       | 26.12| 55.22| 7.46  | 0.75 | 5.22  | 5.22 |
| SiO2-7 days                     | 34.63| 14.79| 10.89 | 32.68| 5.06  | 1.95 |
| NS-56 days                      | 21.92| 52.97| 12.10 | 0.46 | 7.76  | 4.79 |
| SiO2-56 days                    | 26.20| 27.51| 15.72 | 11.79| 14.41 | 4.37 |
Table 1 shows the numbers of 6 subgroups of fibroblasts in different groups at different time points (two groups of resting fibroblasts are combined into one group).

Table 2. The relationship between GREM1/BMP and TGF-β1/PPP2R3A

| Pathway                                           | Gene                      |
|---------------------------------------------------|---------------------------|
| Mesenchymal cell differentiation                  | Bmp2|Fn1|Tgfβ1|Grem1 |
| Regulation of the cellular response to growth factor stimulus | Bmp2|Tgfβ1|Grem1 |
| Extracellular matrix organization                 | Bmp2|Fn1|Tgfβ1|Grem1 |
| Regulation of the Wnt signaling pathway           | Bmp2|Tgfβ1|Grem1|Ppp2r3a |
| Wnt signaling pathway                             | Bmp2|Tgfβ1|Grem1|Ppp2r3a |
| Cell–cell signaling by Wnt                         | Bmp2|Tgfβ1|Grem1|Ppp2r3a |
| Positive regulation of the Wnt signaling pathway  | Bmp2|Tgfβ1|Ppp2r3a |
| Cell surface receptor signaling pathway            | Bmp2|Tgfβ1|Grem1|Ppp2r3a |
| involved in cell–cell signaling                   | Bmp2|Fn1|Tgfβ1|Grem1 |
| Regulation of the MAPK cascade                    | Bmp2|Fn1|Tgfβ1|Grem1 |
| Negative regulation of cell population            | Bmp2|Tgfβ1|Grem1 |
| proliferation                                     | Bmp2|Fn1|Tgfβ1|Grem1 |

Table 2 shows the related signaling pathways in which GREM1, BMP, TGF-β1, PPP2R3A and FN1 were enriched.

Table 3. Signaling pathways involving FN1 that contribute to the process of inflammation

| Pathway                                           | Gene                                      |
|---------------------------------------------------|-------------------------------------------|
| Positive regulation of cell adhesion              | Fn1|Hsp90aa1|Cc1n1|Cd74|Lgals1|Ccl2|Spp1|Thbs1 |
| Inflammatory response                             | C3|Cebpb|Fn1|Hpl|Ier3|Saa3|Ccl2|Ccl7|Serpina3n|Thbs1|Timp1 |
| Response to wounding                              | C3|Fn1|Cc1n1|Ccl2|Sod2|Serpine2|Thbs1|Timp1|Tnc |
| Regulation of fibroblast proliferation            | Fn1|Fth1|Cd74|Sod2 |
| Regulation of collagen biosynthesis               | Fn1|Ccl2|Prdx5 |
Table 3 shows the signaling pathways that were enriched in the top 50 genes of the infla-pro fibroblasts, including FN1 and inflammation-related signaling genes.

**Figures**

**Figure 1**

**Classification of fibroblasts using single-cell transcriptome sequencing.** (A) Single-cell sequencing divided all cells in the lung tissue into 24 subtypes. (B) Fibroblasts were separated based on Col1a1 and Col3a1 expression. (C) A marker gene used for the subtyping of fibroblasts. (D) Fibroblasts were subdivided into 7 subtypes.

**Figure 2**

**Correlation analysis of fibroblast subtypes.** (A) The percentage of each fibroblast subtype among the total fibroblasts in different groups (saline group and silica group) at different time points (7 days and 56 days). (B) The proportion of \(grem1^{\text{high}}\) fibroblasts in different groups at different time points. (C) The distribution of each subtype of fibroblasts in the saline group and the silica group at 7 days and 56 days. (D) The quasichronological analysis shows the status of the whole fibroblast population at each node of transdifferentiation. (E) The status of each subtype of fibroblasts at the node of transdifferentiation. (F) The lesion area contains a large number of \(grem1^{\text{high}}\) fibroblasts. (G) In terms of the expression of the top 10 genes with multiple changes in Cluster 5 compared with other subtypes, the numbers 1-10 correspond to the 10 genes in the left column, and the bubble size represents the size of multiple changes. \(grem1\) is only expressed in Cluster 5. (H) Venn diagram showing the number of identical genes expressed between different subtypes.

**Figure 3**

**The results of biochemical analyses and validation of the scRNA-Seq analysis of infla-pro fibroblasts.** (A) GO enrichment analysis of the molecular functions of the top 50 genes in infla-pro fibroblasts. (B) GO enrichment analysis of signaling pathways related to the top 50 genes in infla-pro fibroblasts. (C) The expression of \(grem1\) in 7 subtypes. (D) Gene heatmap of the 7 subtypes. (E) The results of spatial
transcriptomic sequencing show that *grem1* is expressed at high levels in the lesion area. (F) Immunohistochemical staining showing that GREM1 is expressed on fibroblasts, and the expression level of the experimental group was higher than that of the control group; the scale bar represents 20 μm. (G) In the GEO database, the expression of *Grem1* in the lung tissue of patients with IPF was significantly higher than that of the healthy group (*p<0.05). (H, I) In HPF-a cells, GREM1 expression first increased and then decreased in a time-dependent manner, and the expression levels at 1 h and 3 h were significantly different from those at 0 h (*p<0.05). (J) The bubble chart shows the pathways of interest among the related pathways identified in infla-pro fibroblasts. (K) Interaction map between proteins enriched in signaling pathways examined in this study.

**Figure 4**

The effect of TGF-β1 on PPP2R3A expression in HPF-a cells and the relationship between GREM1 and PPP2R3A. (A) Protein interaction map of the main components investigated in our research. (B) Three-dimensional structure of the PP2A protein. (C) The location of each subunit of PP2A. (D, E) In HPF-a cells, the expression of PPP2R3A first increased and then decreased in a time-dependent manner. The expression levels at 1 h, 3 h, and 6 h were significantly different compared with those at 0 h (*p<0.05). (F) Representative images of immunofluorescence staining show that after TGF-β1 treatment, the expression of PPP2R3A protein in HPF-a cells increased; scale bar = 20 μm. (G) The *Ppp2r3a* mRNA level was upregulated in a time-dependent manner, and the expression levels at 12 h and 24 h were significantly different from those at 0 h (*p<0.05). (H, I) Representative Western blot results showing that *Grem1* knockdown partially reversed the upregulation of PPP2R3A induced by TGF-β1. *p<0.05 indicated that the expression level of PPP2R3A in the si-Con group was higher than that in the control group after TGF-β1 treatment, indicating successful establishment of the cell model. #p<0.05 indicated that PPP2R3A was expressed at a lower level in the si-*Grem1* group than that in the si-Con group after TGF-β1 treatment.

**Figure 5**

PPP2R3A mediates TGF-β1 signaling to induce the proliferation and activation of HPF-a cells. (A) CCK-8 assays show that *Ppp2r3a* knockdown partially reversed the increase in the viability of HPF-a cells induced by TGF-β1. *p<0.05 indicates that the cell viability of the si-Con group after TGF-β1 treatment was higher than that in the control group and the model was successfully established. #p<0.05 indicates that the cell viability of the si-*Ppp2r3a* group was lower than that of the si-Con group after TGF-β1 treatment. (B, C) Wound healing experiments show that the downregulation of *Ppp2r3a* expression
attenuated cell migration induced by TGF-β1. *p<0.05 indicates that the cell migration in the si-Con group after TGF-β1 treatment was higher than that in the control group, and the model was successfully established. #p<0.05 indicates that the cell migration of the si-\textit{Ppp2r3a} group was lower than that of the si-Con group after TGF-β1 treatment. (D) The combined immunofluorescence images of BrdU (green) and DAPI (blue) show that the downregulation of \textit{Ppp2r3a} expression attenuated cell proliferation induced by TGF-β1. (E) Percentage of BrdU-positive cells in five independent experiments. *p<0.05 indicates that the cell proliferation of the si-Con group after TGF-β1 treatment was higher than that of the control group, and the model was successfully established. #p<0.05 indicates that the cell proliferation of the si-\textit{Ppp2r3a} group was lower than that of the si-Con group after TGF-β1 treatment. (F, G) Downregulation of \textit{Ppp2r3a} expression partially reversed the increase in FN1 expression induced by TGF-β1. *p<0.05 indicates that the expression of FN1 in the si-Con group after TGF-β1 treatment was higher than that in the control group, and the model was successfully established. #p<0.05 indicates that the expression of FN1 in the si-\textit{Ppp2r3a} group was lower than that in the si-Con group after TGF-β1 treatment. (H) Downregulation of \textit{Ppp2r3a} expression had little effect on the increase in Col1 and α-SMA expression induced by TGF-β1. (I, J) *p<0.05 indicates that the expression of Col1 and α-SMA in the si-Con group after TGF-β1 treatment was higher than that in the control group, and the model was successfully established.

**Figure 6**

**Early pathological model of mouse lung silicosis and the expression of PPP2R3A in mouse lung tissue.** (A) Sirius Red staining shows significantly greater collagen deposition in the lung tissue of the silica group than that in the saline group, and the model was successfully established. (B) Representative WB showing higher PPP2R3A expression in the lung tissue of the silica group than that of the saline group. (C) *p<0.05 indicates that the difference between the two groups is significant. (D) Immunohistochemical staining showing that PPP2R3A is expressed on fibroblasts, and the expression level in the experimental group was higher than that in the control group. (E) The scRNA-Seq results show increased \textit{ppp2r3a} expression in infla-pro fibroblasts. (F) The scRNA-Seq results show that \textit{vimentin} expression increased in infla-pro fibroblasts.

**Figure 7**

**PPP2R3A affects the function and mechanism of heterogeneous fibroblasts (GREM1) during early pathological changes in the lung.**
Supplementary Files

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