PPP2R3A Affects the Function and Mechanism of Heterogeneous Fibroblasts in Pulmonary Fibrosis

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Research

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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.

**Methods:** Physiological saline and silica were used to generate a chronic pulmonary fibrosis model in mice, and single-cell sequencing, spatial transcriptome sequencing, real-time fluorescent quantitative PCR, immunohistochemistry and immunofluorescence were performed to identify fibroblast subtypes. Small interfering RNA was used to specifically knockdown the target protein, and western blotting, bromodeoxyuridine (BrdU), Cell Counting Kit-8 (CCK-8) and wound healing assays were used to detect the role of GREM1/PPP2R3A in a newly identified fibroblast subtype.

**Results:** Fibroblasts of the new subtype were mainly located in the lesion area and coexpressed inflammation- and proliferation-related genes; they were termed inflammatory-proliferation fibroblasts. Grem1 was the most highly expressed gene in this subtype, as confirmed in HPF-a cells after TGF-β1 treatment. We characterized the downstream mechanism of GREM1/PPP2R3A: these factors mediated the increases in cell viability, proliferation and migration induced by TGF-β1 in fibroblasts.

**Conclusion:** This new subtype of inflammatory, proliferative fibroblasts plays a pivotal role during pulmonary fibrosis, and PPP2R3A, as a downstream regulatory target of GREM1, is involved in pulmonary fibrosis, providing new insights for the prevention and treatment of silicosis.

Introduction

The inhalation of free crystalline silica or silica occurs in many industries [1] and can pulmonary fibrosis characterized by fibroblast activation and 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 inhalation of silica is usually based on a high level of silica dust exposure and radiological characteristics, and similar diseases [3], such as powdery tuberculosis, idiopathic pulmonary fibrosis (IPF), other lung interstitial diseases and cancers, need to be excluded. Effective treatments for the disease are lacking, and the exact pathogenesis is still unclear.

Pulmonary fibroblasts (PFBs) are the main components of pulmonary interstitial cells [4]. These important effector cells are involved in damage and repair in the body and can maintain a resting state under physiological conditions (resting) but respond rapidly under pathological conditions. 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 will promote the differentiation of mesenchymal cells into activated or pathological fibroblasts, and these cells will induce inflammation [5]. The heterogeneity of lung fibroblasts indicates that these cells may be derived 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, 9, 10, 11, 12].

In this research, to fully elucidate the changes in fibroblast types and spatial locations during pulmonary fibrosis, we generated all major cell types based on single-cell RNA sequencing (scRNA-Seq) and developed a molecular map of fibroblasts. Then, through spatial transcriptomics combined with new calculation methods, we identified the localization of the different cell types. Quasi-chronological analysis was used to determine the source and localization of the cells. These results suggest that the newly emerging heterogeneous fibroblasts express GREM1 and that PPP2R3A affects GREM1 to participate in pulmonary fibrosis, which can lead to the activation, proliferation and cell migration of fibroblasts.

Materials And Methods

Reagents

SiO$_2$ particles were purchased from Sigma-Aldrich Company (S5631; Billerica, MA, USA), and approximately 80% had a diameter of 1–5 $\mu$m. 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 TGF-β1 recombinant protein was purchased from Nanjing GenScript Biotechnology Co., Ltd., and used for the construction of a cell fibrosis model. Primary antibodies against PPP2R3A (rabbit polyclonal antibody) were purchased from Proteintech, and 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. Under a constant temperature (23°C) and humidity (50%), the mice were allowed to eat and drink freely, and the light/dark cycle was 12:12 h. At an appropriate age, the animals were anesthetized with pentobarbital sodium intraperitoneal injection, the trachea was surgically exposed, and a prepared silica suspension (0.2 g/kg in 50 mg/ml saline) was injected into the trachea at a single dose. Animals in the same group were given the same volume of sterile normal saline. The lung tissues were collected 7 days after modeling. The lungs prepared for immunohistochemistry were first perfused with PBS, treated with 4% formalin, dehydrated with 30% sucrose solution, and then 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 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 following the manufacturer's protocol (10X Genomics). Then, the cells were clustered according to the surface markers of different cells and were divided into different subgroups. Gene comparison and naming was 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 different cells were located in different original spatial positions.

**Cell culture**

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

**Western blotting**

Western blotting was used to detect the 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 the cell protein was collected with cell lysis solution (100:1) containing protease inhibitors. Protein extraction from tissues as 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 by the BCA assay, the concentration was adjusted, loading buffer was added and boiled at 100°C for five minutes to denature the protein, and the protein sample was successfully prepared. 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 and primary antibody were incubated overnight (at least 16 h) in a chromatography cabinet 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 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. The HPF-a cells were plated according to the required density of the experiment, and the corresponding treatments were given after 24 h. After the treatment, the cells were washed 3 times with RNase-free PBS, and the total RNA was extracted from HPF-a cells using TRIzol reagent (Invitrogen) according to the instructions. After the total RNA was extracted, the RNA concentration was measured with a NanoDrop One (Thermo Fisher Scientific). Samples at different concentrations were normalized to contain approximately 400 ng of RNA and reverse transcribed into cDNA. The cDNA sample was 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 quantitative expression of RNA was normalized to that of the endogenous reference (GAPDH).

**Immunofluorescence experiment**

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 primary antibody overnight. On the third day, the cells were incubated with an appropriate fluorescent secondary antibody (Alexa Fluor, Thermo Fisher Scientific) in the dark, the nucleus was stained with 4,6-diamino-2-phenylindole (DAPI), and the cell image was captured using a fluorescence microscope. Immunofluorescence analysis of mouse lung tissue sections was performed with the steps after the paraformaldehyde was discarded in the cell immunofluorescence protocol.

**Wound healing experiment**

A wound healing test was used 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 wash away 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 the cell treatment, 10 μl of CCK-8 solution was 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 the survival of the experimental group compared 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, the 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 BrdU antibody (1:100; SC-32323, Santa Cruz) at 4°C overnight. After the PBS washes, the cells were incubated with appropriate fluorescent secondary antibodies (Alexa Fluor, Thermo Fisher Scientific) in the dark for 2 h. The cells were then washed 3 times with PBS and fixed with a fixative (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 and collected 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 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 RNA (siRNA) was used to knock down the expression of proteins of interest. siRNA was 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 siRNA to one tube of serum-free medium and added transfection reagent to the other tube of serum-free medium. The samples were allowed to stand for 5 minutes, and then, the two solutions were mixed and incubated for 15 minutes. The solution was added to the wells,
the samples were 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 the subsequent experiments.

Detection of intracellular calcium ion concentration

HPF-a cells were inoculated in a 24-well plate and cultured in a cell incubator. After the cell density reached the appropriate level, the medium was discarded, the cells were washed 3 times with sterile PBS, Fluo-4 AM working solution was added, and the cells were fully covered. The cells were incubated at 37°C for 30 minutes and then washed with sterile PBS 3 times. After the cells were washed, they were incubated for 20–30 minutes to ensure that Fluo-4 AM was completely converted into Fluo-4 in the cells. Next, TGF-β1 was added to the experimental group of cells, photos were taken with a fluorescence microscope to semiquantitate the intracellular calcium ion concentration, and the intracellular calcium ion concentration was quantitatively detected with a fluorescence microplate reader.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). Statistical analysis was performed with Student’s t test or one-way analysis of variance (ANOVA). p<0.05 was defined as significant.

Results

scRNA-seq classification of mouse lung fibroblasts

According to recent reports, heterogeneous lung fibroblasts may be the main cause of lung fibrosis [6, 13]. Before 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 the cells in the lung tissue were divided into 24 types according to the surface markers of different cells (Figure 1A). Among them, fibroblasts were separated by their specific markers Col3a1 [14] and Col1a1 [15] (Figure 1B), and then, we subdivided the fibroblasts into subtypes using different known fibroblast markers. The cells were defined as different subtypes (Figure 1C and D), but we found that existing gene markers could not accurately define Cluster 5 because of its characteristic expression of the proliferation-related gene grem1; the expression degree of grem1 was the highest in this group. Thus, we defined this group as grem1high fibroblasts temporarily.

Analysis of fibroblast subtypes

Based on the above definition of subtypes, we further analyzed the number of fibroblasts in different subtypes in different groups (Figure 2A). The number of cells in each group is shown in Table 1. We
found that the \textit{grem}^{\text{high}} fibroblasts appeared specifically in the silica group, and the proportion at 7 days was higher than that at 56 days (Figure 2B and C), suggesting that this group of cells plays a vital role in the inflammatory phase of silicosis. Then, we analyzed the source and destination of \textit{grem}^{\text{high}} fibroblasts through a pseudochronological sequence and found that these special heterogeneous fibroblasts originate from resting fibroblasts, and after mediating their functions, they transdifferentiate into myofibroblasts and inflammatory fibroblasts (Figure 2D and E). We found that the \textit{grem}^{\text{high}} fibroblasts were primarily expressed in the focus area in spatial mapping (Figure 2F). To further determine whether the top 10 genes expressed by the cluster 5 type are also highly expressed in other subtypes, through the bubble chart, we found that most genes are specifically expressed in the cluster 5 type, and \textit{grem} is only highly expressed in this type (Figure 2G), which provides ideas and a basis for our next experiment. Then, through a Venn diagram, we found that the top 50 genes of the \textit{grem}^{\text{high}} fibroblasts overlapped with those of inflammatory fibroblasts, ECM fibroblasts, and myofibroblasts (Figure 2H). Thus, we named the fibroblasts inflammatory, proliferative (infla-pro) fibroblasts.

\textbf{Infla-pro fibroblast-related bioinformatics analysis and scRNA-Seq verification}

Through the above analysis, we mainly focused on the specific and characteristic expression of the \textit{grem}1 subtype. To further understand the function of infla-pro fibroblasts in fibrosis, we conducted an enrichment analysis of the functions of the top 50 genes of this type (Figure 3A) and found that these genes play an important role in regulating biological processes, cell growth and proliferation. We further performed GO enrichment analysis on the signaling pathways involving these genes and found that the genes were enriched in pathways such as cell adhesion, inflammation, cell death, and myofibroblast differentiation (Figure 3B). Then, the scRNA-Seq results showed that \textit{grem}1 was characteristic of the special subtype (Figure 3C and D) and was typically expressed in the lesion area (Figure 3E). To verify the authenticity and reliability of the scRNA-Seq results, we performed histochemistry of mouse lung tissue sections and found that \textit{grem}1 was highly expressed at 7 days and colocalized with fibroblasts (Figures 3F and S1A). What role does \textit{grem}1 play in the occurrence and development of pulmonary fibrosis? A search and analysis of the Gene Expression Omnibus (GEO) database showed that the expression of \textit{Grem1} in the IPF patient group was significantly higher than that in the healthy group, and the difference was significant (Figure 3G). \textit{Grem1} has similar expression in mice and patients; thus, we performed in vitro cell experiments to determine whether the expression was similar to that in mice and humans. Then, we used TGF-\(\beta1\) at the optimal concentration of 5 ng/ml (Figure S2A-D) to stimulate HPF-a cells and verified that GREM1 expression first increased and then decreased in a time-dependent manner, reaching a peak at 1 h (Figure 3H and I). Many studies have examined the function of \textit{Grem1} in fibrosis [16, 17, 18, 19], but its mechanism is still unclear, and we will mainly discuss the mechanism by which \textit{Grem1} causes pulmonary fibrosis. We generated a bubble chart of the signaling pathways related to the occurrence and development of fibrosis (Figure 3J) and performed protein network interaction analysis (PPI) on the genes in these signaling pathways to identify specific targets of fibrosis (Figure 3K). From the mapping, literature review and Kyoto Encyclopedia of Genes and Genomes (KEGG) database analysis, we found that Ppp2r3a is related to \textit{Grem1} (Figures S3A and Figure 4A), and studies have reported that PPP2R3A
mainly regulates the cell cycle by targeting cell cycle regulators and apoptosis inhibitors [20]. Because of its involvement in the regulation of important tumor signaling pathways, developmental processes and regulation of the cell cycle, this molecule has received extensive attention. We hypothesized that it may be the downstream target of Grem1 leading to fibrosis.

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

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 [21]. 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 broadly varied B regulatory subunits (15 genes) combine with the core enzyme. The tissue specificity and substrate specificity of the PP2A holoenzyme complex were determined (Figure 4B and C) [22]. PPP2R1A of the A subunit of PP2A can promote cell proliferation and migration and is a key fibrogenic factor [23], but the role of PPP2R3A of the B subunit in the progression of fibrosis is still unclear. Here, we mainly explored the role of PPP2R3A in the process of fibrosis and the relationship between GREM1 and PPP2R3A. 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, reaching a peak at 6 h (Figure 4D and E). The immunofluorescence and RT-PCR results were consistent with the western blot (WB) results (Figure 4F and G). The immunofluorescence results also showed that after TGF-β1 stimulation of the cells, the morphology of the fibroblasts changed from the original spindle shape to an amoeba-like morphology (Figure 4F). To further verify the relationship between PPP2R3A and GREM1, we used siRNA to knock down GREM1 expression (Figure S3B and C) and detected the expression changes in PPP2R3A. We found that knocking down GREM1 partially reversed the increase in PPP2R3A expression induced by TGF-β1 (Figure 4H and I), suggesting that PPP23RA is the downstream target of GREM1.

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

Many studies have shown that the migration, proliferation and activation of lung fibroblasts are the main causes of pulmonary fibrosis [24, 25]; thus, we explored the role of PPP2R3A in the migration, proliferation and activation of fibroblasts mediated by TGF-β1. TGF-β1 increased the expression of marker proteins related to fibrosis (Figure S4A-D), induced fibroblast migration and increased fibroblast viability (Figure S5A-C). We used siRNA to knock down PPP2R3A (Figure S6A-C); this treatment partially reversed the increase in cell viability, cell migration and cell proliferation induced by TGF-β1 (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).

**PPP2R3A promotes pulmonary fibrosis via the p53 pathway**

Various reports have shown that PP2A induces fibrosis through the WNT signaling pathway [26], PI3K/AKT signaling pathway, NFκB signaling pathway, calcium ion-related signaling pathway [27], p53 signaling pathway [28], etc (Figure 6A). To further explore the mechanism by which PPP2R3A causes
pulmonary fibrosis, we first investigated whether PPP2R3A causes pulmonary fibrosis through a mechanism involving calcium overload (Figure S7A and B). The scRNA-Seq results showed that PPP2R3A-related Camk2a was barely expressed in fibroblasts (Figure S7C). Using TGF-β1 to stimulate HPF-a cells, we found that the calcium ion concentration did not change significantly (Figure S7D and E). Furthermore, we verified the classic p53 signaling pathway (Figure S8). Through scRNA-Seq results, we found that the expression of p53 and Akt increased in the silica group (Figure 6A-C). After TGF-β1 stimulation of HPF-a cells, the PUMA and p53 levels first increased and then decreased in a time-dependent manner. PUMA expression peaked at 1 h, and p53 expression peaked at 6 h (Figure 6D-F). Then, to determine whether PPP2R3A causes fibrosis through the p53 signaling pathway, we knocked down PPP2R3A, which partially reversed the TGF-β1-induced increase in p53 (Figure 6G and H), suggesting that PPP2R3A promotes pulmonary fibrosis through the p53 pathway.

**Mouse lung fibrosis model and the expression of PPP2R3A in mouse lungs**

To verify whether the overall level and the cell level of PPP2R3A had the same changes, we used silica tracheal instillation to create a mouse pulmonary fibrosis model. As shown by Sirius red staining (Figure 7A), collagen deposition in the SiO2 group was obvious, and the model was successfully generated. In the mouse model of silicosis, the WB results showed that the expression of PPP2R3A in the model group was higher than that in the control group (Figure 7B and C). Immunohistochemistry showed an increase in vimentin, a specific fibroblast marker, which indicated an increase in fibroblasts in the lung, and the expression of PPP2R3A was consistent with the WB results (Figure 7D). All the above results indicate a high degree of consistency in the expression of PPP2R3A at the overall level and at the cellular level. Furthermore, we verified the results from scRNA-Seq and found that PPP2R3A and Vimentin are widely expressed in fibroblasts. In the saline group, these genes were mainly expressed in resting fibroblasts, and in the SiO2 group, resting fibroblasts transformed into infla-pro fibroblasts. PPP2R3A and Vimentin were mainly expressed in infla-pro fibroblasts in the SiO2 group (Figure 7E and F).

**Discussion**

Pulmonary fibrosis caused by inhalation of silica 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 group of special heterogenous fibroblasts that only appeared in the silica group. Since the genes expressed by this subtype intersect with those of inflammatory fibroblasts, ECM fibroblasts and myofibroblasts, we defined them as infla-pro fibroblasts. Current studies have reported that 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 transdifferentiate into inflammatory fibroblasts and ECM fibroblasts, which has
not been reported in previous studies. Blocking the conversion process of resting fibroblasts into infla-pro fibroblasts may reverse the progressive development of pulmonary fibrosis.

We compared our scRNA-Seq data with recently published analyses of mouse and human lung fibroblast populations [6, 29]; the literature reports mainly described myofibroblasts, resting fibroblasts, adipose fibroblasts, inflammatory fibroblasts, and ECM fibroblasts. The resting fibroblast body is small and fusiform. Under the stimulation of 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]. The increased expression of α-SMA indicates the activation of fibroblasts. The activation of resting fibroblasts is one of the main sources of myofibroblasts. Lipofibroblasts have large cytoplasmic lipid droplet inclusions and unrestricted biofilms or lipid vacuoles and play important roles in lung development, surfactant synthesis, and retinoic acid metabolism [30]. These cells generally do not change significantly during the onset of pulmonary fibrosis. Inflammatory fibroblasts and ECM fibroblasts are representative heterogeneous fibroblasts under pathological conditions and mainly induce inflammation and promote proliferation and migration. However, infla-pro fibroblasts, 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 can also promote cell proliferation and migration.

In this new type of heterogeneous fibroblasts, the characteristic gene expressed is Grem1, and related literature has shown that it can promote the migration and proliferation of normal lung cells [31, 32] and epithelial-mesenchymal transition (EMT) [33, 34]. Grem1 can also regulate endothelial-mesenchymal transition (EndMT) [17], but the mechanism that leads to fibrogenesis is still unclear. We found that GREM1 is related to PP2A through bioinformatic analysis. 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 [21, 22, 35]. Studies have reported that specifically knocking out the Ppp2r1a gene (encoding the PP2A Aα subunit) in mice can promote inflammation and liver fibrosis [23]. Through database analysis, 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 that the expression level of PPP2R3A slightly increased, which may be due to the increased expression of single cells rather than the increased number of PPP2R3A. This conclusion is based on the number of dark red dots in Figure 7E. The scRNA-Seq results showed that this molecule was expressed mainly in resting fibroblasts in normal mouse lung fibroblasts. After exposure to silica, PPP2R3A was expressed in infla-pro fibroblasts. We believe that although its overall level did not show major changes, this molecule still plays an important role in the transdifferentiation process of fibroblasts. Most researchers believe that a change in mRNA precedes a change in protein. However, in our research, we found that a change in the protein expression of PPP2R3A precedes the change in mRNA expression. There may be many levels of regulation of gene expression, and regulation at the transcriptional level is only one mechanism. Post-transcriptional, translational and post-translational regulation may be involved in these findings. The
results may be due to post-translational regulation or positive regulation of transcriptional mechanisms. This issue should be further studied.

FN1, COL1, and α-SMA are the three markers of fibrosis. Previous studies in the literature have shown that FN1 mainly affects cell migration and proliferation [36]. COL1 is the main component of ECM. Excessive accumulation of ECM can impair lung function. COL1 mainly affects cell adhesion and migration [37]. α-SMA is the most commonly used molecular marker for 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 [38, 39, 40]. However, we found that PPP2R3A knockdown specifically affected the fibrotic marker FN1 but had little effect on COL1 and α-SMA, suggesting that PPP2R3A may specifically affect FN1 to cause fibrosis. One study reported that the expression of the target protein specifically affects FN1 but has almost no effect on COL1 [30], which is similar to our findings. To further analyze the cause of these findings, we performed GO enrichment analysis of genes in infla-pro fibroblasts and found that in the silicosis stage (model mice at 7 days), FN1 is differentially expressed and is enriched in inflammation. Regarding proliferation-related pathways (Table 2), the cell model simulates the early stages of pulmonary fibrosis, which may be the reason why PPP2R3A affects FN1.

The mechanism by which PPP2R3A leads to fibrosis is currently unclear. The literature has shown that PPP2R3A can play a role in fibrosis through calcium ion (Ca\textsuperscript{2+}) channels, the p53 signaling pathway, the WNT signaling pathway, etc. Ca\textsuperscript{2+} is an important second messenger in cells. Major changes in intracellular Ca\textsuperscript{2+} concentration regulate various biological processes, such as muscle excitation and contraction coupling, cell proliferation and division, gene transcription and apoptosis [41], and intracellular calcium overload can regulate apoptosis [42]. PPP2R3A is an EF-hand-type "classic" Ca\textsuperscript{2+}-binding protein that contains two conserved EF-hand motifs, EF-hand1 and EF-hand2, which show different affinities for Ca\textsuperscript{2+} [43]. Ca\textsuperscript{2+} can regulate the expression and activity of PPP2R3A [44], leading to the occurrence of fibrosis. However, in our research, after treatment with TGF-β1, the intracellular Ca\textsuperscript{2+} concentration changed little. We speculate that organs and muscles containing more Ca\textsuperscript{2+}, such as heart, muscle [45], and pulmonary arteriole smooth muscle cells [46], are mainly regulated by Ca\textsuperscript{2+}. Lung fibroblasts are also regulated by Ca\textsuperscript{2+}, but the regulation is weak and does not result in calcium overload. For the other mechanisms by which PPP2R3A leads to fibrosis, we verified the classic p53 signaling pathway. Our research found that PPP2R3A can promote cell migration and proliferation, enhance cell viability and promote fibrosis through the p53 signaling pathway instead of Ca\textsuperscript{2+}.

In summary, our research shows that during the occurrence and development of fibrosis, there are a type of special heterogeneous fibroblast that can indicate its progression, and this type of fibroblast expresses Grem1, which may be a predictive biomarker of silicosis. Moreover, we found that PPP2R3A is a downstream target of GREM1, and its expression is related to the regulation of pulmonary fibrosis and may become a potential target for treatment of silicosis.
Declarations

Ethical Approval and Consent to participate

The use of human tissue slide was performed in accordance with the approved guidelines of the Research and Development Committee of Nanjing Chest Hospital.

Consent for publication

Not applicable.

Availability of data and materials

All relevant raw data and materials are freely available to any scientist wishing to use them.

Competing of Interest

The authors declare no competing financial interests.

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Author 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. 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.

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Authors' information

Not applicable.

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**Tables**

**Table 1.** Cell number statistics for different subpopulations of fibroblasts.
Table 1 shows the cell numbers of 6 subgroups of fibroblasts in different groups at different time points (1 two groups of resting fibroblasts are combined into a group).

Table 2. Signaling pathways involving FN1 in the process of inflammation.

| Pathway                               | Gene                                      |
|---------------------------------------|-------------------------------------------|
| Positive regulation of cell adhesion  | Fn1|Hsp90aa1|Ccn1|Cd74|Lgals1|Ccl2|Spp1|Thbs1 |
| Inflammatory response                 | C3|Cebpb|Fn1|Hp|ler3|Saa3|Ccl2|Ccl7|Serpina3n|Thbs1|Timp1 |
| Response to wounding                  | C3|Fn1|Ccn1|Ccl2|Sod2|Serpine2|Thbs1|Timp1|Tnc   |
| Regulation of fibroblast proliferation| Fn1|Fth1|Cd74|Sod2 |
| Regulation of collagen biosynthesis   | Fn1|Ccl2|Prdx5 |

Table 2 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 of the lung tissue into 24 subtypes. (B) Fibroblasts were separated with Col1a1 and Col3a1. (C) A marker gene used for the subtype of fibroblasts. (D) Fibroblasts were subdivided into 7 subtypes.
Correlation analysis of fibroblast subtypes. (A) The percentage of the number of fibroblasts in each subtype of fibroblasts in different groups (saline group and silica group) at different time points (7 days and 56 days). (B) The proportion of grem1high fibroblasts in different groups at different time points. (C) The distribution of each subtype of fibroblasts at 7 days and 56 days in the saline group and the silica group. (D) Quasi-chronological analysis shows the status of the whole fibroblast at each node of
transdifferentiation. (E) The status of each subtype of fibroblasts at the node of transdifferentiation. (F) Grem1high fibroblasts are highly expressed in the lesion area. (G) The expression of the top 10 genes of the grem1high fibroblasts in each subtype. (H) Venn diagram shows the number of identical genes expressed between different subtypes.

Figure 3
The results of biochemical analysis and validation of scRNA-Seq of infa-pro fibroblasts. (A) GO enrichment analysis of the function of the top 50 genes in infa-pro fibroblasts. (B) GO enrichment analysis of signaling pathways related to the top 50 genes in infa-pro fibroblasts. (C) The expression of grem1 in 7 subtypes. (D) Gene heat map of 7 subtypes. (E) The results of spatial transcriptomic sequencing show that grem1 is highly expressed in the lesion area. (F) The immunohistochemical results show that Grem1 is expressed on fibroblasts, the expression level of the experimental group was higher than that of the control group, and the scale bar is 20 μm. (G) In the GEO database, the expression of Grem1 in the lung tissue of patients with IPF was higher than that of the healthy group, and the difference was significant (*p<0.05). (H, I) In HPF-a cells, the expression of GREM1 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 of infa-pro fibroblasts. (K) Interaction mapping between proteins enriched in signaling pathways examined in this study.
Figure 4

The effect of TGF-β1 on the expression of PPP2R3A in HPF-a cells and the relationship between GREM1 and PPP2R3A. (A) Protein interaction mapping of the main components of our research. (B) Three-dimensional structure of the PPP2R3A protein. (C) The location of each subunit of PPP2R3A. (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 mRNA level of Ppp2r3a 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).

Figure 5
PPP2R3A mediates TGF-β1 to induce the proliferation and activation of HPF-a. (A) CCK-8 assays show that knocking down PPP2R3A can partially reverse the increase in the viability of HPF-a cells induced by TGF-β1. * p<0.05 indicates that the cell viability after TGF-β1 treatment in the si-Con group 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 downregulation of PPP2R3A expression can attenuate the cell migration induced by TGF-β1. * p<0.05 indicates that the cell migration after TGF-β1 treatment in the si-Con group 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-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 downregulation of PPP2R3A expression can attenuate 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-PPP2R3A group was lower than that of the si-Con group after TGF-β1 treatment. (F, G) Downregulation of PPP2R3A expression partially reversed the increase in FN1 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-PPP2R3A group was lower than that in the si-Con group after TGF-β1 treatment. (H) Downregulation of PPP2R3A expression has little effect on the increase in Col1 and α-SMA 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 is higher than that in the control group, and the model was successfully established.
Figure 6

PPP2R3A promotes pulmonary fibrosis via the p53 pathway. (A) Protein network interaction mapping of the signaling pathways downstream of PPP2R3A. (B) The results of scRNA-Seq show that the expression of p53 increased in infla-pro fibroblasts. (C) The results of scRNA-Seq show that Akt expression increased in infla-pro fibroblasts. (D) The representative WB shows that in HPF-a cells, the expression of p53 and PUMA first increased and then decreased in a time-dependent manner. (E) The expression of p53 was
significantly different at 3 h and 6 h compared with that at 0 h (*p<0.05). (F) The expression of PUMA was significantly different between the 1 h and 0 h time points (*p<0.05). (G) Downregulation of PPP2R3A expression partially reversed the increase in p53 induced by TGF-β1. (H) *p<0.05 indicates that the expression of p53 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 p53 in the si-PPP2R3A group was lower than that in the si-Con group after TGF-β1 treatment.

Figure 7
The success of the mouse lung fibrosis model and the expression of PPP2R3A in mouse lung tissue. (A) The Sirius Red staining results show that collagen deposition in the lung tissue of the silica group was significantly greater than that in the saline group, and the model was successfully established. (B) The representative WB shows that the expression of PPP2R3A in the lung tissue of the silica group was higher than that of the saline group. (C) *p<0.05 indicates that the difference between the two groups is significant. (D) The immunohistochemical results show that PPP2R3A is expressed on fibroblasts, and the expression level of the experimental group was higher than that of the control group. (E) The scRNA-Seq results show that the expression of PPP2R3A increased in infla-pro fibroblasts. (F) The scRNA-Seq results show that Vim expression increased in infla-pro fibroblasts.

Figure 8

PPP2R3A affects the function and mechanism of heterogeneous fibroblasts (GREM1) during pulmonary fibrosis.

Supplementary Files
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