Research Square

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# Expression, localization and anti-fibrotic effect of HSPG2 in idiopathic pulmonary fibrosis

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**Research Article**

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## Abstract

# Background

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease characterized by excessive pulmonary tissue fibrosis, and the role of pulmonary epithelial cells in its pathological process has attracted increasing attention. The heparan sulfate proteoglycan 2 (HSPG2, also known as perlecan) gene is involved in the regulation of various cellular functions, but its mechanism of action in IPF remains unclear. This study aimed to systematically investigate the expression characteristics, cellular localization and functional effects of HSPG2 in IPF.

# Methods

Lung tissue samples from 8 clinical IPF patients were collected, and the expression of HSPG2 in epithelial cells was detected by immunohistochemistry and immunofluorescence. A bleomycin-induced mouse model of pulmonary fibrosis was established, and an in vitro fibrosis model was constructed by stimulating human pulmonary epithelial cells (A549, Beas-2b) with transforming growth factor beta 1 (TGF-β1).

# Results

HSPG2 expression was significantly upregulated in lung tissues of IPF patients, lung tissues of fibrotic mice and human pulmonary epithelial cells stimulated with TGF-β1. TGF-β1 could induce the upregulation of fibronectin 1 (FN1) and alpha-smooth muscle actin (α-SMA) in human pulmonary epithelial cells, while HSPG2 silencing effectively inhibited the above effects and attenuated TGF-β1-induced cell proliferation and migration.

# Conclusions

HSPG2 is highly expressed during pulmonary fibrosis, and its silencing can inhibit fibrotic activation, proliferation and migration of epithelial cells, suggesting that HSPG2 may play an important profibrotic role in IPF.

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and irreversible interstitial lung disease of unknown etiology[1], whose core pathological features are the abnormal activation of fibroblasts and myofibroblasts, and excessive deposition of extracellular matrix, ultimately leading to pulmonary structural destruction and respiratory failure. Although anti-fibrotic drugs such as nintedanib and pirfenidone can slow disease progression, they cannot reverse established fibrotic lesions, resulting in

poor prognosis for patients with a median survival of only 3–5 years after diagnosis[2]. Therefore, in-depth exploration of the pathogenesis of IPF and identification of new therapeutic targets are of great significance.

Recent studies have indicated that pulmonary epithelial cells play a pivotal role in the development of IPF. As the barrier cells of pulmonary tissue, pulmonary epithelial cells are essential for maintaining lung homeostasis and mediating injury repair, and their dysfunction and abnormal transformation are one of the core initiating factors in the occurrence and progression of IPF[3]. In the fibrotic microenvironment, pulmonary epithelial cells can transdifferentiate into myofibroblasts through epithelial-mesenchymal transition (EMT), directly participating in the fibrotic process. Transforming growth factor-β (TGF-β) is the core factor driving EMT, which promotes the expression of mesenchymal markers such as α-SMA and FN1 by activating the Smad pathway, thus accelerating fibrotic progression[4].

Heparan sulfate proteoglycan 2 (HSPG2, also known as perlecan) is a large multi-domain glycoprotein widely expressed in the extracellular matrix and on the surface of epithelial cells, which is involved in the regulation of various physiological processes including cell adhesion, signal transduction and matrix homeostasis[5, 6]. Existing studies have suggested its regulatory role in tissue injury repair and fibrosis-related diseases, but the role of HSPG2 in IPF remains incompletely elucidated, especially the regulatory mechanism of HSPG2 in pulmonary epithelial cell EMT and the fibrotic process that needs further investigation[7, 8].

Based on this, the present study aimed to systematically investigate the expression and function of HSPG2 in pulmonary epithelial cells of IPF through clinical samples, animal models and cellular experiments. First, we detected the expression and localization of HSPG2 in lung tissues from IPF patients and bleomycin-induced mice. Subsequently, using a TGF-β1-induced pulmonary epithelial cell fibrosis model, we analyzed the expression changes of HSPG2 and fibrotic markers. Finally, by overexpressing HSPG2 combined with wound healing assay, CCK-8 proliferation assay and Transwell invasion assay, we clarified the effects of HSPG2 on the proliferation, migration and EMT of pulmonary epithelial cells. This study is intended to reveal the role of HSPG2 in pulmonary epithelial cell fibrosis, provide new insights into the pathogenesis of IPF, and identify a potential target for anti-fibrotic therapy.

# 1 Materials and Methods

Mouse Model Establishment

Specific pathogen-free (SPF) C57BL/6 mice purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. were used in this study, and all experimental procedures were strictly performed in accordance with the institutional animal ethics guidelines. Mice were randomly divided into a normal control group, an IPF single-dose group and an IPF three-dose group. Pulmonary fibrosis was induced in IPF groups by intratracheal instillation of bleomycin at a dose of 4 mg/kg as a single injection or 2 mg/kg for three separate injections, while the normal control group received an equal volume of normal saline. The modeling period was set to 21 days, during which the general condition of mice was monitored

continuously. After modeling, lung tissues were harvested, and pathological analysis was performed using hematoxylin-eosin (HE) staining and Masson's trichrome staining. The successful establishment of the pulmonary fibrosis model was verified by evaluating the degree of inflammatory infiltration and collagen deposition.

Masson's Trichrome Staining

The staining procedure was as follows: sections were first deparaffinized to water, and cell nuclei were stained with hematoxylin followed by differentiation and bluing. The cytoplasm and collagen were stained with ponceau-acid fuchsin staining solution in sequence, differentiated with phosphomolybdic acid, and counterstained with aniline blue. Finally, the sections were dehydrated, cleared and mounted. After staining, collagen was stained blue, cytoplasm red, and cell nuclei blue-black.

Immunohistochemistry

# 1 Materials and Methods (Continued)

Experimental samples were obtained from surgically resected lung tissues of 8 patients with idiopathic pulmonary fibrosis (IPF) and 8 cases of paracancerous normal lung tissues, all collected by the Department of Thoracic Surgery, the Second Affiliated Hospital of Harbin Medical University. All patients received no radiotherapy or chemotherapy before surgery and were pathologically diagnosed after operation. Paraffin sections were deparaffinized and hydrated, followed by antigen retrieval using the high-pressure cooker heating method (citrate buffer, pH 6.0). Endogenous peroxidase blocking and blocking with 5% goat serum at room temperature were then performed sequentially. Primary antibody was added dropwise to the sections and incubated overnight at 4°C. After washing with PBS, HRP-conjugated secondary antibody was added, followed by DAB color development and hematoxylin counterstaining. Finally, dehydration, clearing and mounting were completed.

Western Blot Analysis

Tissues or cells were lysed with RIPA lysis buffer (Beyotime, Shanghai) containing PMSF (Solarbio, Beijing, dilution ratio 1:100) to extract total protein. Protein concentration was determined by the BCA method (Biosharp, Beijing). Equal amounts of protein were loaded for SDS-PAGE electrophoresis to separate proteins, followed by membrane transfer and blocking. After sequential incubation with primary and secondary antibodies, ECL chemiluminescence was used for development. Gray value analysis of protein bands was performed using ImageJ 2.90 software, with GAPDH as the internal reference to calculate the relative expression level of target proteins.

Cell Culture

Beas-2b and A549 cell lines were stimulated with TGF-β1 and cultured in the corresponding medium in a constant temperature incubator at 37°C with 5% $CO_2$.

Cell Transfection

siRNAs (small interfering RNAs) were provided by Jiangsu Saifei Biotechnology Co., Ltd., including HSPG2-siRNA (experimental group, for knocking down HSPG2 gene expression) and negative control-siRNA (control group). The above siRNAs were transfected into A549 and Beas-2b cell lines respectively to achieve targeted silencing of the HSPG2 gene.

CCK-8 Proliferation Assay

After transfection, cells were seeded in 96-well plates at a density of $1\times10^3$ cells per well. CCK-8 solution was added at 24 h, 48 h, 72 h and 96 h of culture respectively, and the plates were incubated at 37°C for 2 h. The absorbance value at 450 nm was detected using a microplate reader to evaluate cell proliferation capacity.

Wound Healing Assay

Horizontal lines were marked on the back of 6-well plates, and cells were seeded until the confluency reached approximately 90%. Wounds were scratched vertically to the marked lines with a 200 μL sterile pipette tip. After washing with PBS to remove detached cells, the cells were cultured continuously in low-serum medium containing 1.5% fetal bovine serum (FBS). Cell migration was observed and photographed under an inverted microscope at 0 h, 24 h and 48 h after wounding.

Transwell Invasion Assay

The upper chambers of Transwell inserts were coated with 100 μL of Matrigel (Beyotime) at a concentration of 1 mg/mL. $4\times10^4$ cell suspensions were added to the upper chambers, and medium containing 10% FBS was added to the lower chambers, followed by 24 h of culture. Unmigrated cells in the upper chambers were removed, and the migrated cells were fixed and stained with crystal violet. The number of migrated cells was counted in 3 randomly selected fields under a microscope to evaluate cell invasion capacity.

# Statistical Analysis

GraphPad Prism 8.0 software was used for data analysis and graph plotting. Measurement data were expressed as mean ± standard deviation ($x \pm s$). Paired t-test or one-way/two-way analysis of variance (ANOVA) was used for comparison between groups. A P value $< 0.05$ was considered statistically significant.

## 2 Results

# 2.1 Bioinformatics Analysis

Bioinformatics analysis of HSPG2 was performed using the GEO database. (A) The volcano plot showed the expression changes and significance of genes in the two groups of samples, reflecting the up- and down-regulation amplitude of gene expression; the Y-axis represented the statistical significance of differences (the higher the value, the stronger the significance); red dots represented significantly up-regulated genes, blue dots represented significantly down-regulated genes, and gray dots (ns) represented genes with no significant difference. HSPG2 marked in the plot was a significantly up-regulated gene with $Log_2FoldChange > 1$ (more than 2-fold up-regulation), with extremely strong statistical significance of the difference. (B) The swarm plot showed the expression distribution of the HSPG2 gene in 12 cell types (e.g., macrophages, T cells, neutrophils). The Y-axis represented the gene expression level (value range 1 ~ 100+), and the X-axis represented cell types. Epithelial cells and macrophages showed high HSPG2 expression (Fig. 1).

## 2.2 HSPG2 Expression was Significantly Higher in Human Pulmonary Fibrotic Tissues than in Normal Lung Tissues

Dual detection by immunohistochemical staining (Fig. 2A) and immunofluorescence staining (Fig. 2B) clearly showed that the expression level of HSPG2 was significantly higher in human pulmonary fibrotic lesions than in normal lung tissues, and the gene was specifically localized in pulmonary epithelial cells. Meanwhile, pathological morphological analysis of lung tissues confirmed the successful establishment of the mouse model of pulmonary fibrosis in this study. The results of Masson's trichrome staining (100×, Fig. 2D) further confirmed that compared with the normal control group, significant collagen fiber deposition was observed in the peribronchial areas and alveolar septa of lung tissues in the two fibrosis-induced mouse groups, with a marked increase in collagen content. Consistent immunohistochemical detection results in the successfully constructed mouse pulmonary fibrosis model tissues and clinical IPF samples both revealed a significant up-regulation of HSPG2 (Fig. 2C). Specifically, the expression level of HSPG2 in pulmonary epithelial cells of fibrotic model mice and human IPF lesions was significantly higher than that in the corresponding normal lung tissue control samples (Fig. 2).

## 2.3 Low HSPG2 Expression Attenuated TGF-β-Induced Fibrotic Response and Cell Proliferation in Pulmonary Epithelial Cells

Western blot results clearly showed that the protein expression levels of pulmonary fibrosis-related markers were significantly up-regulated after TGF-β induction, indicating that TGF-β could effectively trigger the regulatory expression of fibrosis-related molecules and exert a significant profibrotic effect (Fig. 3A). Furthermore, intervention to down-regulate HSPG2 expression on the basis of TGF-β induction significantly reversed the TGF-β-mediated profibrotic effect. Specifically, the protein expressions of fibronectin 1 (FN1) and alpha-smooth muscle actin (α-SMA), the core markers of pulmonary fibrosis, were significantly inhibited and their expression levels were markedly lower than those in the TGF-β alone group (Fig. 3C). Corresponding to this, the results of the CCK-8 proliferation assay further verified the above molecular changes. Experimental data showed that the cell proliferation capacity was

significantly decreased in the experimental group with HSPG2 down-regulation combined with TGF-β treatment, which was significantly lower than that in the negative control (NC) group, with a statistically significant difference between groups ($P < 0.05$) (Fig. 3B).

## 2.4 Low HSPG2 Expression Inhibited TGF-β-Induced Migration, Invasion and Colony-Forming Ability of Epithelial Cells

Combined detection results of the wound healing assay, Transwell invasion assay and colony formation assay showed that compared with the NC group, the experimental group with HSPG2 down-regulation after TGF-β induction exhibited a significant decrease in cell migration, invasion and colony-forming ability, with statistically significant differences between the two groups ($P < 0.05$) (Figs. 4 and 5).

# 3 Discussion

This study is the first to systematically reveal the upregulated expression of HSPG2 in pulmonary epithelial cells of patients with idiopathic pulmonary fibrosis (IPF). HSPG2 expression was significantly increased in pulmonary epithelial cells both in IPF patients and bleomycin-induced mouse models of pulmonary fibrosis. In the TGF-β1-induced cellular model, the downregulation of HSPG2 was positively correlated with the upregulation of fibrotic markers FN1 and α-SMA. Functional experiments further confirmed that HSPG2 overexpression could significantly inhibit TGF-β1-induced proliferation, migration and fibrotic phenotypic transformation of epithelial cells.

This study innovatively links HSPG2 to the abnormalities of pulmonary epithelial cells in IPF. Injury, apoptosis and epithelial-mesenchymal transition (EMT) of pulmonary epithelial cells are the core initiating steps in IPF progression, and the TGF-β/Smad pathway is the key regulatory mechanism driving EMT [3, 4]. As a core component of the extracellular matrix, HSPG2 not only provides physical support for cells, but also regulates cytoskeletal rearrangement and adhesion dynamics through its interaction with cell adhesion molecules (e.g., integrin β1, fibronectin), thereby affecting cell migration [9]. Existing studies have confirmed that during pulmonary fibrosis, HSPG2 can enhance the adhesive stability between cells and the matrix by promoting the deposition and cross-linking of collagen in the extracellular matrix, and simultaneously activate the RhoA/ROCK signaling pathway to regulate the assembly of actin stress fibers, which provides the driving force for cell migration [5, 7]. In contrast, when HSPG2 expression is downregulated, the extracellular matrix structure becomes loose, cell adhesion ability decreases, the activation of the RhoA/ROCK signaling pathway is blocked, and cytoskeletal rearrangement is impaired, ultimately leading to a slowdown in migration rate [10, 11]. In addition, scholars have found that the high expression of HSPG2 in the lung tissues of patients with pulmonary fibrosis is accompanied by the increased expression of matrix metalloproteinases (MMP-2, MMP-9) [5], which promotes extracellular matrix degradation and creates conditions for epithelial cell migration and mesenchymal transition [7]. Methodologically, this study verified the findings at multiple levels using clinical samples, animal models and cellular experiments, which enhanced the reliability of the

conclusions. Wound healing assay and Transwell assay were simultaneously used to detect migration ability, simulating injury-repair migration and pathological chemotactic migration of epithelial cells, respectively. The consistent results supported the inhibitory effect of HSPG2 on abnormal migration of epithelial cells, indicating the stability of its anti-fibrotic effect.

This study also has several limitations. First, the current research focuses on pulmonary epithelial cells, and the role of HSPG2 in other IPF-related cells (e.g., pulmonary fibroblasts, pulmonary vascular endothelial cells) remains unclear, which needs to be further analyzed using cell-specific models. Second, the downstream molecular targets and regulatory signaling network of HSPG2 have not been elucidated; multi-omics techniques can be used in subsequent studies to screen its directly regulated target genes and interacting proteins. Finally, there is a lack of in vivo functional verification, and epithelial cell-specific interventions (e.g., epithelial cell-specific overexpression or knockdown of HSPG2) in animal models are required to confirm its alleviating effect on pulmonary fibrosis.

In summary, this study found that HSPG2 exerts an anti-fibrotic function in pulmonary epithelial cells of IPF, which provides a new perspective for understanding the core mechanism of epithelial injury and interstitial fibrosis progression in IPF, and also identifies a potential molecular target for the targeted therapy of IPF. Future studies need to further reveal its cell-specific mode of action and detailed molecular mechanisms, and carry out systematic in vivo verification experiments to promote the clinical translation of HSPG2-related research.

## 4 Conclusions

In conclusion, this study confirmed that HSPG2 expression is upregulated in pulmonary fibrosis, and its downregulation attenuates cell proliferation and migration abilities, suggesting that HSPG2 may be involved in the pathological process of IPF by regulating epithelial cell functions. These findings provide new experimental evidence for understanding the pathogenesis of IPF and a potential target for developing anti-fibrotic therapeutic strategies targeting the pulmonary vascular system.

## Declarations

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# Ethics Approval

This study was approved by the Ethics Committee of The Second Affiliated Hospital of Harbin Medical University (Approval No. YJSKY2024-147).

# Consent for Publication:

All participants provided written informed consent for the publication of the relevant data and clinical information involved in this study.

**Competing Interests**

Competing Interests: The authors declare that they have no competing interests.

# Author Contribution

Author Contributions: CHEN Ying (first author) and LAN Rui (second author) performed the experiments, collected experimental data, and wrote the original draft; QIN Luyao (third author) and XING Chen (fourth author) collected and organized clinical cases, and assisted with data checking and verification; HAN Guiping (corresponding author) contributed to conceptualization, supervision, project administration, and manuscript review and editing. All authors have read and approved the final manuscript.

# Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Consent for Participation: Written informed consent was obtained from all individual participants included in the study prior to their participation in this research.

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# Figures

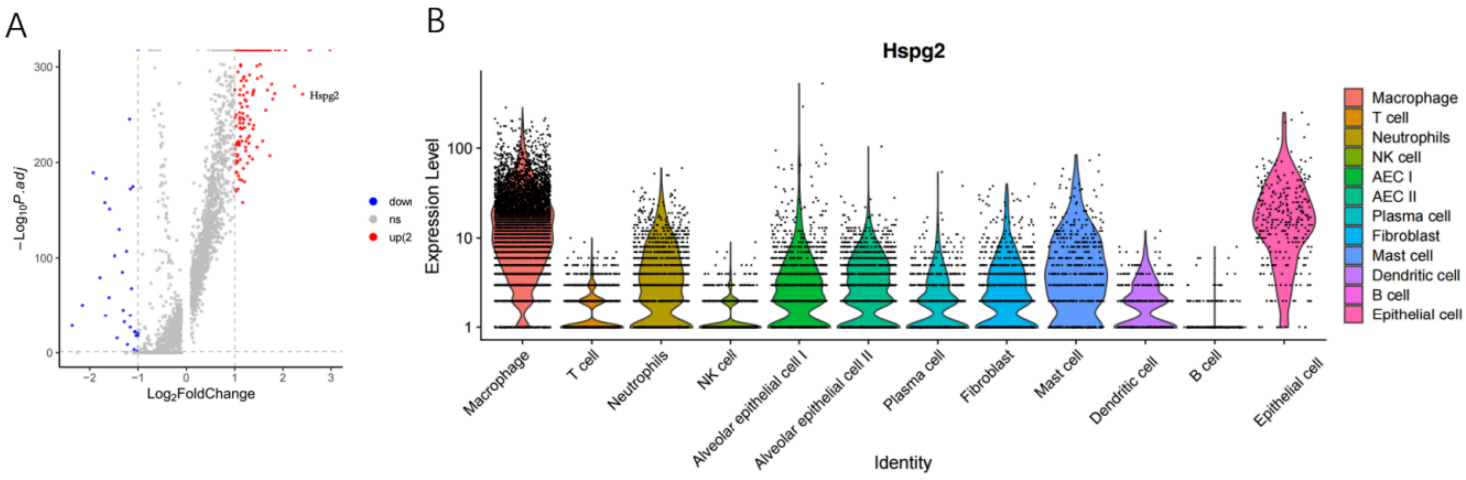
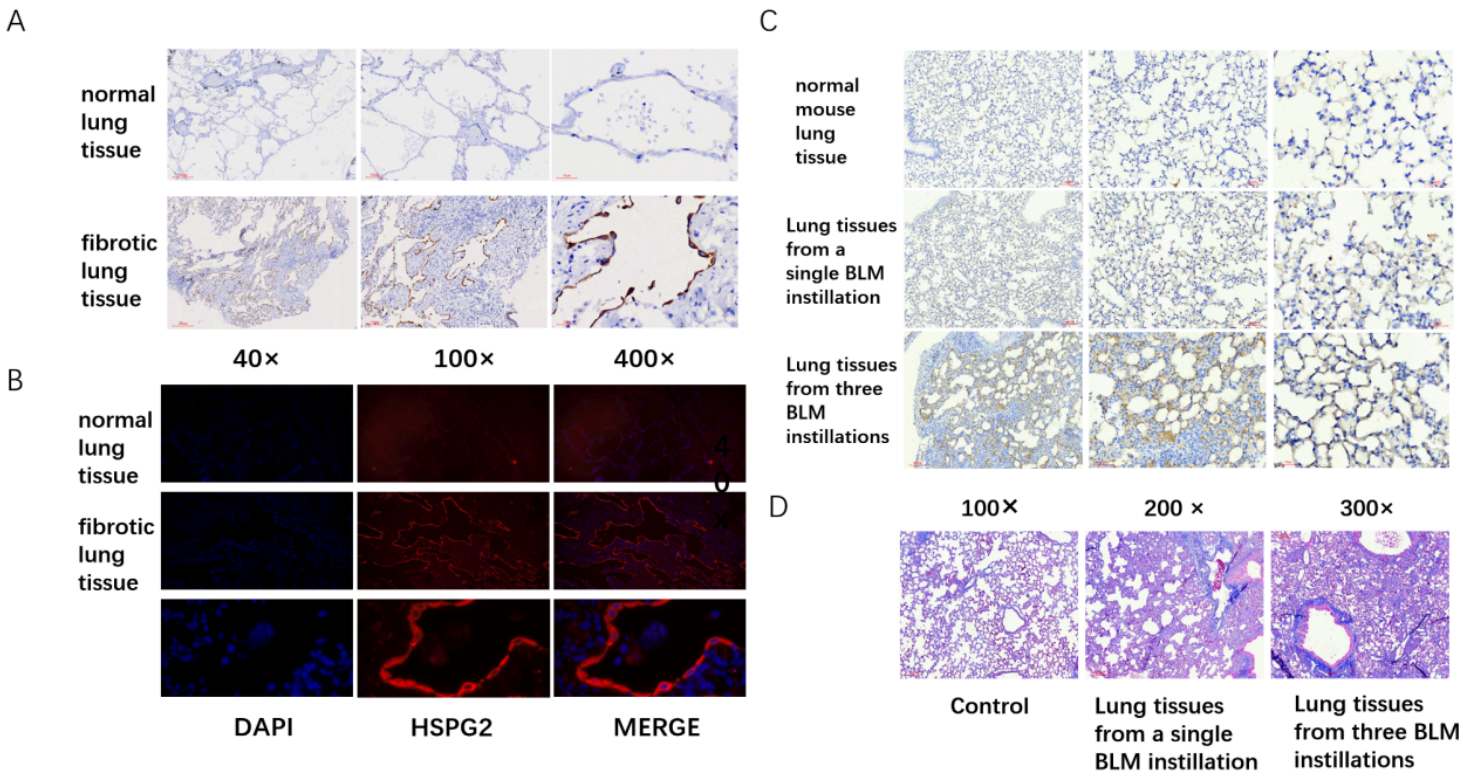


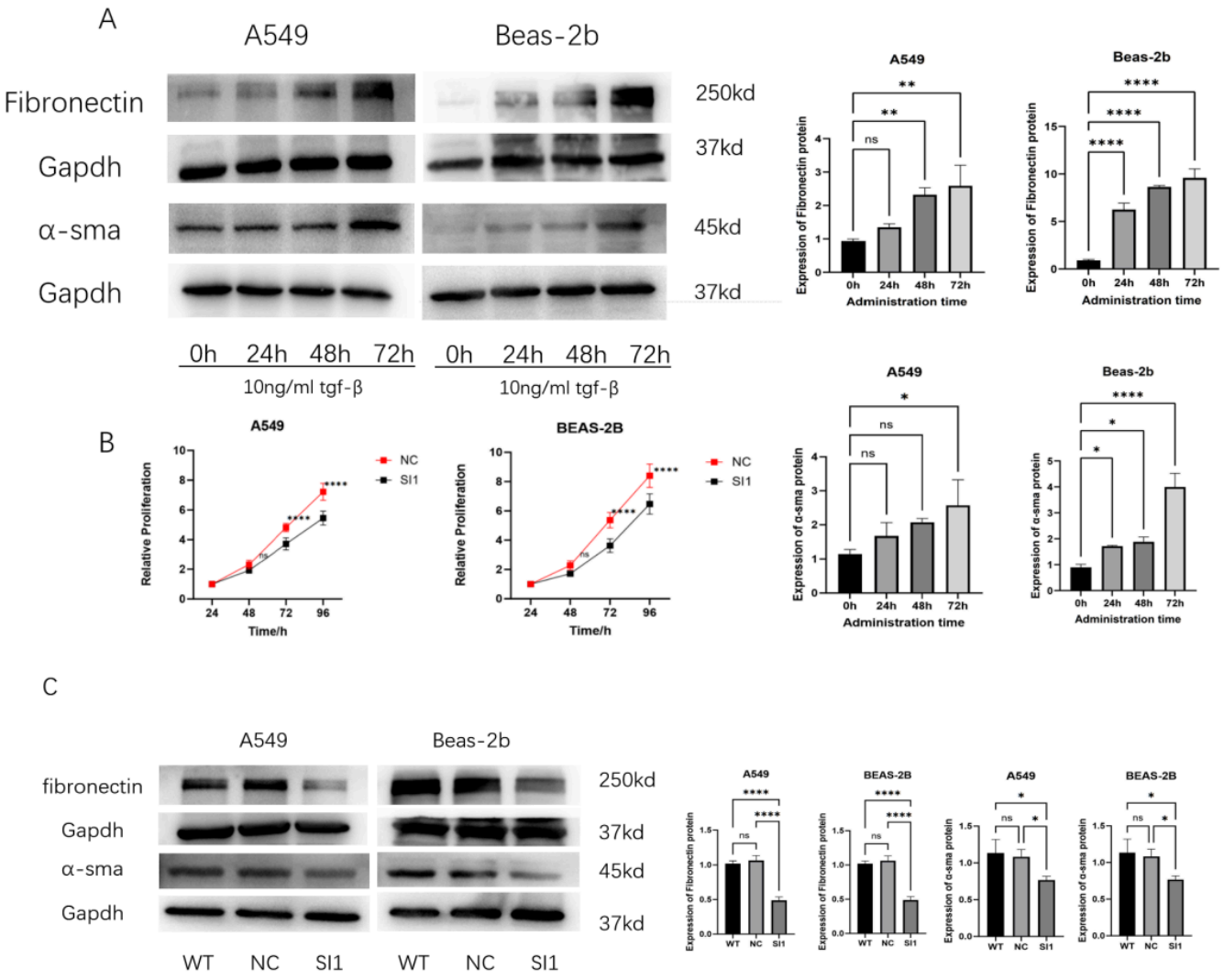

**Figure 1**

Bioinformatic analysis of HSPG2 expression differences between normal and IPF lung tissues(A) Volcano plot of differential gene analysis showing the expression changes and significance of genes in the two groups of samples (B) HSPG2 expression distribution across 12 cell types




## Figure 2

HSPG2 expression in IPF lung tissues and mouse model establishment(A) Immunohistochemical expression of HSPG2 in fibrotic and normal lung tissues (B) Immunofluorescent expression of HSPG2 in fibrotic and normal lung tissues (C) Immunohistochemical expression of HSPG2 in fibrotic and normal mouse lung tissues (D) Masson's trichrome staining of fibrotic mouse lung tissues. *P < 0.05, **P < 0.01, ***P < 0.001.




## Figure 3

Fibrotic markers after TGF-β induction and the effect of HSPG2 downregulation on cell proliferation capacity(A) Expression of Fibronectin and α-SMA in A549 and Beas-2b cells at different time points after TGF-β induction(B) HSPG2 downregulation attenuated the proliferative capacity of A549 and Beas-2b pulmonary epithelial cells(C) Further HSPG2 downregulation on the basis of TGF-β

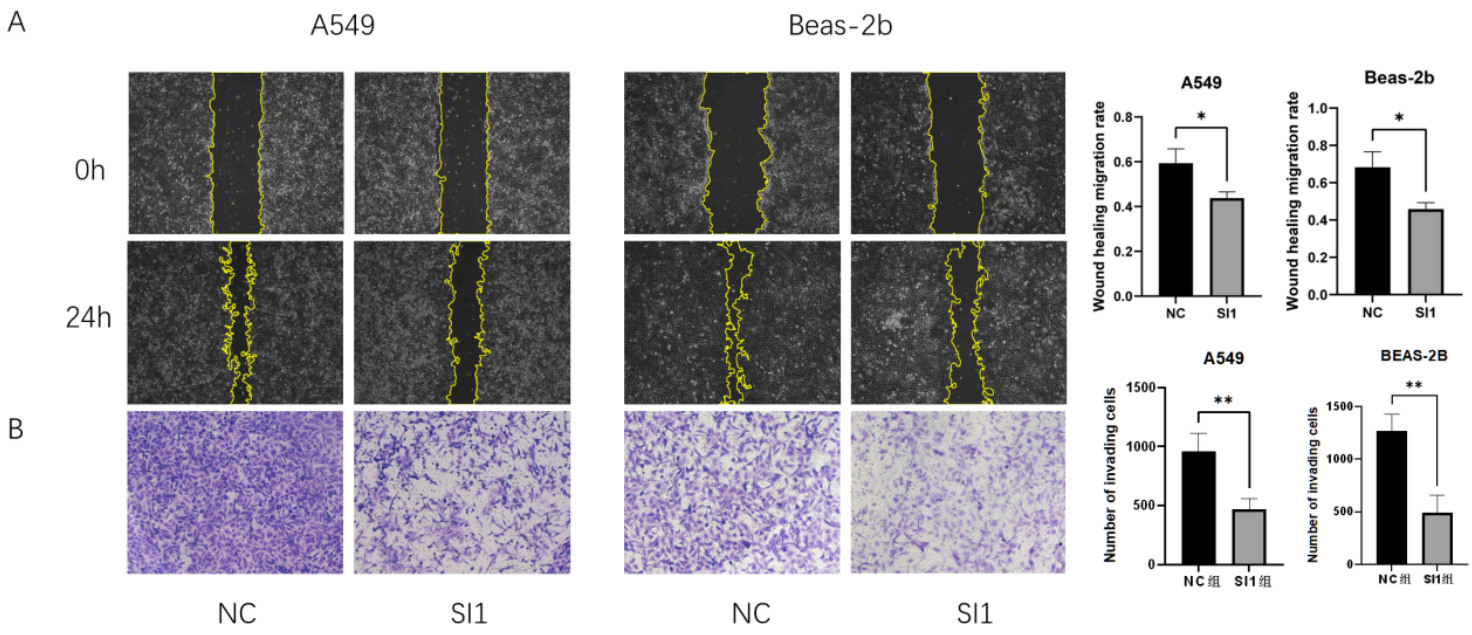



**Figure 4**

HSPG2 attenuates TGF-β-enhanced migration and invasion abilities of epithelial cells(A) Effect of HSPG2 downregulation on the migration ability of A549 and Beas-2b pulmonary epithelial cells (*, P < 0.05)(B) Effect of HSPG2 downregulation on the invasion ability of A549 and Beas-2b pulmonary epithelial cells (**, P < 0.01)

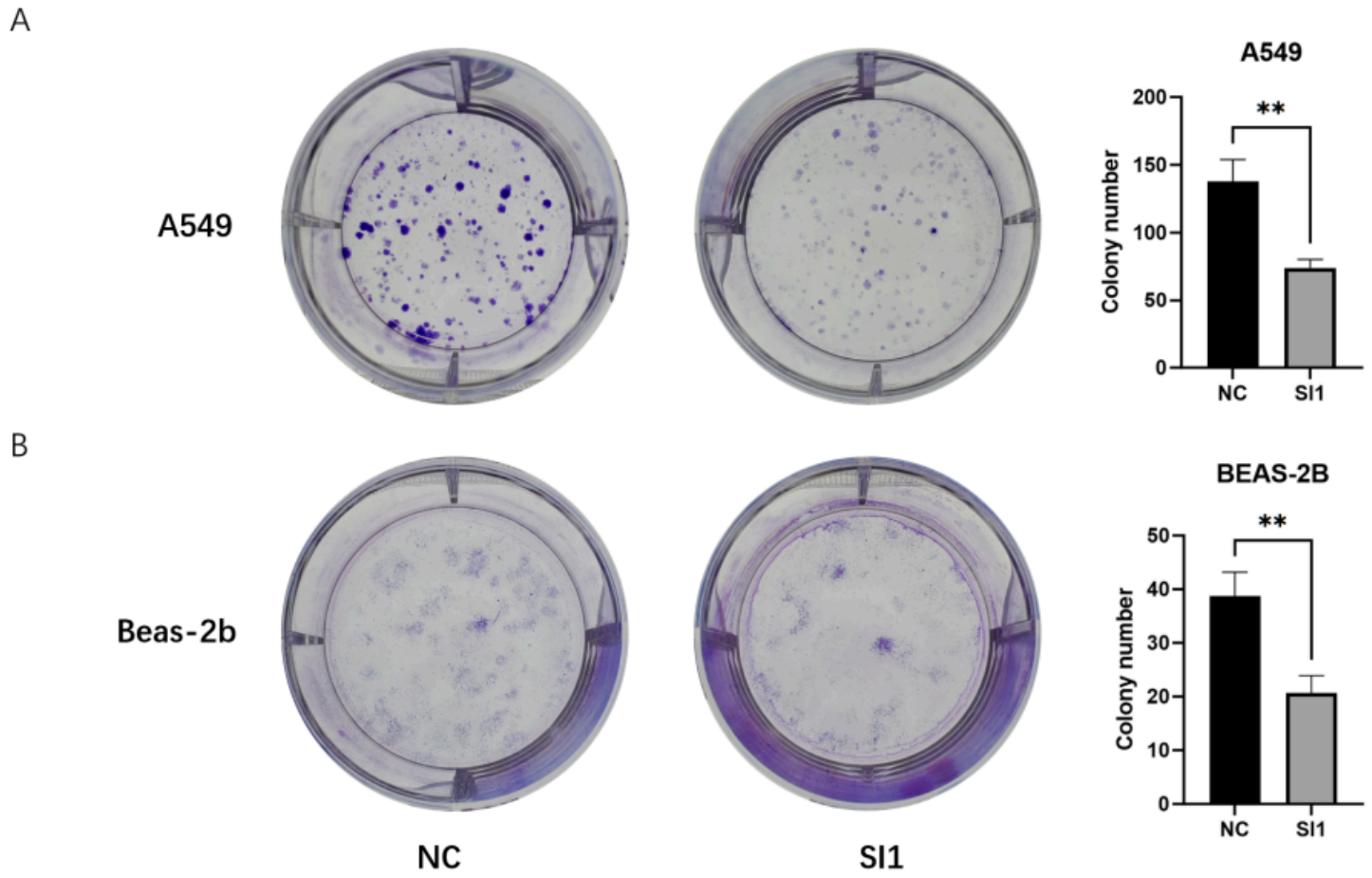



**Figure 5**

HSPG2 attenuates TGF-β-enhanced colony-forming ability of epithelial cells(A) Effect of HSPG2 downregulation on the colony-forming ability of A549 and Beas-2b pulmonary epithelial cells (, P < 0.01) (B) Effect of HSPG2 downregulation on the colony-forming ability of A549 and Beas-2b pulmonary epithelial cells (, P < 0.01)

## Supplementary Files

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