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SPP1 induces idiopathic pulmonary fibrosis and NSCLC progression via the PI3K/Akt/mTOR pathway

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Abstract

Background The prevalence of non-small cell lung cancer (NSCLC) is notably elevated in individuals diagnosed with idiopathic pulmonary fibrosis (IPF). Secreted phosphoprotein 1 (SPP1), known for its involvement in diverse physiological processes, including oncogenesis and organ fibrosis, has an ambiguous role at the intersection of IPF and NSCLC. Our study sought to elucidate the function of SPP1 within the pathogenesis of IPF and its subsequent impact on NSCLC progression.

Methods Four GEO datasets was analyzed for common differential genes and TCGA database was used to analyze the prognosis. The immune infiltration was analyzed by TIMER database. SPP1 expression was examined in human lung tissues, the IPF fibroblasts and the BLM-induced mouse lung fibrosis model. Combined with SPP1 gene gain- and loss-of-function, qRT-PCR, Western blot, EdU and CCK-8 experiments were performed to evaluate the effects and mechanisms of SPP1 in IPF progression. Effect of SPP1 on NSCLC was detected by co-cultured IPF fibroblasts and NSCLC cells.

Results Through bioinformatics analysis, we observed a significant overexpression of SPP1 in both IPF and NSCLC patient datasets, correlating with enhanced immune infiltration of cancer-associated fibroblasts in NSCLC. Elevated levels of SPP1 were detected in lung tissue samples from IPF patients and bleomycin-induced mouse models, with partial colocalization observed with α -smooth muscle actin. Knockdown of SPP1 inhibits TGF- β 1-induced differentiation of fibroblasts to myofibroblasts and the proliferation of IPF fibroblasts. Conversely, SPP1 overexpression promoted IPF fibroblast proliferation via PI3K/Akt/mTOR pathway. Furthermore, IPF fibroblasts promoted NSCLC cell proliferation and activated the PI3K/Akt/mTOR pathway; these effects were attenuated by SPP1 knockdown in IPF fibroblasts.

Conclusions Our findings suggest that SPP1 functions as a molecule promoting both fibrosis and tumorigenesis, positioning it as a prospective therapeutic target for managing the co-occurrence of IPF and NSCLC.

Keywords Fibroblasts, Idiopathic pulmonary fibrosis, Secreted phosphoprotein 1, PI3K/Akt/mTOR pathway, Non-small cell lung cancer

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Introduction

Idiopathic pulmonary fibrosis (IPF) represents an unknown and progressive interstitial lung disease, hallmarked by escalating dyspnea and a continuous decline in pulmonary function. It often culminates in respiratory failure, leading to fatality within 3–5 years post-diagnosis [1, 2]. Recent epidemiological data underscore a rising IPF incidence, with current figures in the United States approximating 14.6 cases per 100,000 individuals [3]. The pathological characteristics of IPF mainly manifest as extensive extracellular matrix deposition and the appearance of fibroblast foci [4]. Despite ongoing research, the etiology of IPF remains enigmatic, likely entailing the activation of myriad cytokines. Current therapeutic interventions for IPF, including pirfenidone and nintedanib, aim to decelerate disease progression and mitigate pulmonary function decline. However, they cannot reverse pulmonary fibrosis [5, 6]. Consequently, lung transplantation is the solitary efficacious treatment for patients with end-stage IPF, significantly enhancing life expectancy [7].

Remarkably, the incidence of lung cancer in the general populace ranges between 2% and 6.4%; yet, in the context of IPF, this prevalence escalates dramatically to 2.7–48% [8]. Studies have shown that pulmonary fibrosis is an independent carcinogenic risk factor, with pronounced fibrotic regions exhibiting a heightened propensity for lung cancer development, especially the non-small-cell lung cancer (NSCLC) [9, 10]. At present, the mechanism of IPF in combination with NSCLC remains unclear; however, research has shown that many genes, cytokines, and pathways related to NSCLC are activated in IPF [11]. For example, sustained fibroblast activation, extracellular matrix remodeling, telomere and telomerase abnormalities, and *TP53* gene mutations may induce IPF in combination with lung cancer [12, 13].

Fibroblasts, pivotal components within the tumor microenvironment, undergo activation and transformation into cancer-associated fibroblasts (CAFs) in tumoral extracellular matrices [13]. Compared to normal tumor cells, CAFs secrete various metabolites and interact with tumor cells, playing important roles in various mechanisms, such as tumor proliferation, invasion, metastasis, and angiogenesis [14]. Moreover, transforming growth factor-beta (TGF- β), a critical element within the tumor environment, facilitates CAF activation and extracellular matrix production, thereby cultivating a conducive tumor microenvironment [15, 16]. A previous study has reported that elevated TGF- β expression in lung cancer patients portends a grim prognosis [17]. Inhibiting the activation and proliferation of fibroblasts might be a promising anti-tumor and anti-fibrotic treatment strategy.

Secreted phosphoprotein 1 (SPP1) is known as a multifaceted glycoprotein encoded on human chromosome 4 (4q13.22). It belongs to the integrin small-binding N-glycoprotein family and is ubiquitously expressed in bones and various tissues [18]. Its molecular functionality, spanning 41 to 75 kDa, engages with several cell surface receptors, influencing bone metabolism, immune modulation, wound healing, and tumor progression [19]. Recent studies have highlighted the involvement of SPP1 in tumoral activities, including growth, invasion, migration, and drug resistance, with a notable correlation with tumor prognosis. Liu et al. found that SPP1 expression is elevated in head and neck squamous cell carcinoma, and that SPP1 overexpression promotes tumor proliferation, migration, and invasion; inhibits cell apoptosis; and develops resistance to cetuximab by activating the KRAS/MEK pathway [20]. Furthermore, SPP1 is also involved in fibrosis development. Lin et al. reported that SPP1 activates the Akt/GSK-3 β / β -catenin pathway and that the inhibition of autophagy promotes atrial fibrosis [21]. However, the role of SPP1 in the interplay of IPF and NSCLC remains underexplored, with its mechanistic contributions to IPF still veiled in ambiguity.

In the present study, we identified SPP1 as a critical molecular target interlinking IPF and NSCLC. SPP1 was overexpressed in lung tissues from both IPF and NSCLC patients, and associated with the prognosis of NSCLC. Furthermore, our findings demonstrate that SPP1 knock-down impedes TGF- β 1-induced activation in normal fibroblasts and curtails the proliferative capacity of IPF fibroblasts via PI3K/Akt/mTOR pathway. Additionally, SPP1 suppression in IPF fibroblasts resulted in a diminished proliferation of NSCLC cells and reduced PI3K/Akt/mTOR pathway activation. These discoveries reveal the pivotal role of SPP1 in the pathogenesis of IPF co-occurring with NSCLC.

Materials and methods

Collection of patient samples and tissue specimens

We collected pulmonary tissue samples from patients with a confirmed diagnosis of IPF during lung transplantation surgery at Wuxi People's Hospital. The inclusion criteria for selecting IPF patients were as follows: Male patients aged over 50 years with diagnostic imaging of the chest via high-resolution computed tomography (HRCT) showing usual interstitial pneumonia (UIP) patterns. These patterns were primarily characterized by subpleural and basal predominant reticular abnormalities and honeycombing, with or without associated traction bronchiectasis, and minimal ground-glass opacities. Additionally, post-operative histopathological confirmation of UIP was required, including evidence of patchy pulmonary fibrosis and visible foci of fibroblast proliferation. Patients with other known causes of interstitial lung

disease (ILD), such as familial or occupational exposure, connective tissue disease, or drug toxicity, were excluded. For comparison, we obtained normal lung tissues from two sources: donor lungs during transplant procedures and non-tumorous regions of lungs excised during lung cancer surgeries. The study was approved by the Ethics Committee of Wuxi People's Hospital (No. KY22060) and all participants provided their informed consent.

Cell culture and primary fibroblasts isolation

A549 cells and the HFL1 (human fetal lung fibroblast 1) cell lines were obtained from iCell Bioscience in Shanghai. We isolated primary fibroblasts from both IPF and healthy lung tissues. These tissues were finely chopped into segments of 1–2 mm, homogenized, and then cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA), enriched with 10% fetal bovine serum (FBS) and 1% antibiotic mix. Following cell adhesion, we removed the non-adherent cells. The HFL1 cells were grown in F12K medium (Meilunbio, Dalian, China), fortified with 10% FBS (Gibco), all within an environment maintained at 37 °C with a 5% CO₂ atmosphere.

Reagents

Transforming Growth Factor-beta (TGF-β) was purchased from Abcam (UK), preserved at –20 °C within a 10 mM citrate buffer. The PI3K inhibitor, LY294002, was acquired from Sigma and similarly conserved at –20 °C, but in dimethylsulfoxide (DMSO). Bortezomib, sourced from MedChemExpress (MCE, USA), was also stored at –20 °C, but dissolved in 0.9% saline solution.

Quantitative RT-PCR analysis

The total RNA was extracted from both lung tissue and fibroblast samples using the Animal RNA Isolation Kit (Beyotime, China) and then reverse transcribed into cDNA with the PrimeScript RT Master Mix (Takara, Japan). Quantitative real-time PCR was performed in the Applied Biosystems 7500 Real-Time PCR System (ABI, USA) using the SYBR qPCR master mix (Vazyme, Nanjing, China). In this process, GAPDH served as the reference gene, and the data were analyzed using the $2^{-\Delta\Delta C_t}$ method.

Western blotting

Total protein from lung tissues and fibroblasts were prepared using a lysis buffer. These proteins were then separated via gel electrophoresis and subsequently transferred to membranes. After blocking these membranes with milk, they were incubated overnight with primary antibodies at 4 °C. These antibodies targeted SPP1, α-SMA, COL1A1, Akt, pAkt, mTOR, pmTOR, and GAPDH. Post-incubation, the membranes were treated with secondary

antibodies and the protein bands were visualized using enhanced chemiluminescence in a gel imaging system.

Tissue histological analysis

Lung tissue samples were processed through fixation, embedding, and sectioning. These sections underwent deparaffinization and rehydration, followed by staining with hematoxylin and eosin (H&E) for lung pathology assessment. Masson's trichrome staining was used for assessing the degree of fibrosis, employing a specific staining kit (Sigma). Immunohistochemical staining on these samples was performed to identify target proteins, using 3,3'-diaminobenzidine and subsequent microscopic examination for colorimetric analysis.

Immunofluorescence staining

For immunofluorescence staining, cells and tissue sections were prepared through fixation, permeabilization, and blocking processes. These were incubated with primary antibodies targeting α-SMA and SPP1 at 4 °C overnight. Subsequently, the samples were washed and treated with secondary antibodies, specifically goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 550, for 1 h in a light-shielded environment. DAPI staining was performed for nuclear visualization for 10 min. The final step involved capturing the stained samples' images using a Leica confocal microscope.

Lentivirus-mediated transfection

Lentiviruses were purchased from Heyuan Company (Shanghai, China), designed to express shRNA targeting SPP1 or vector-SPP1 plasmids. Fibroblasts were cultured in 24-well plates, subsequently introducing a complete medium enriched with 5 µg/mL polybrene and the respective viruses. After a period of 12–24 h, the medium was replaced and the transfection efficiency was assessed through microscopic examination of cellular fluorescence at 72 h. Following this, the infected cells were selected using puromycin in the culture medium. The success of SPP1 knockdown or overexpression in these lines was verified by qRT-PCR and western blot analysis.

Cell counting kit-8 (CCK-8) assay

The CCK-8 assay provided by Vazyme (China) was used to measure the growth rate of fibroblasts and A549 cells. In this assay, the fibroblasts and A549 cells were seeded into a 96-well plate, with seeding densities of 4,000 and 1,000 cells per well, respectively. The assay was conducted daily from day 1 to day 5. The old culture medium was removed, and 90 µL of fresh medium along with 10 µL of CCK-8 solution was added to each well. After incubation, the absorbance was measured in each well to create proliferation curves, using the absorbance data as a reference.

EdU cell proliferation assay

To further analyze cell growth, an EdU (5-ethynyl-2'-deoxyuridine) cell proliferation assay was conducted using a kit provided by RiboBio (China). Fibroblasts and A549 cells were seeded in 96-well plates and allowed to adhere. After adhesion, the old culture medium was replaced with 100 μ L of EdU solution in each well and the plates were incubated for 2 h. Subsequent to this incubation, the cells were fixed and permeabilized. The cells were then stained with Apollo dye for 30 min in a light-protected environment and nuclei were highlighted using DAPI staining. Fluorescence microscope was used to examine and photograph the stained cells.

Bleomycin-induced mouse pulmonary fibrosis model

Male C57BL/6 mice, aged between 6 and 8 weeks, were procured from Changzhou Animal Company (China) for this experiment, which was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. These mice were randomly divided into two groups: a control group ($n=7$) and a bleomycin-treated group ($n=7$). The bleomycin group received a tracheal injection of 2.5 mg/kg of bleomycin via a microinjector, whereas the control group received a similar volume of saline. On the 21st day post-injection, the mice were euthanized to collect their lung tissues for analysis.

Data collection and functional analysis

This investigation utilized four datasets: two focused on non-small cell lung cancer (NSCLC) (GSE18842 and GSE74706) and two on idiopathic pulmonary fibrosis (IPF) (GSE110147 and GSE53845). We applied R (4.1.3) and the limma (3.50.3) package to analyze these datasets for genes showing more than a twofold change in expression, either up or down, with a significance threshold of $p<0.01$. To identify genes commonly affected across all datasets, an online tool for Venn diagrams (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) was utilized. Both Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the clusterProfiler (4.2.2) package in R, setting a significance cutoff at $p<0.05$. A protein–protein interaction (PPI) network was conducted through the STRING database, visualized using Cytoscape software (<https://cn.string-db.org>). Additionally, we sourced expression and clinical data on NSCLC from The Cancer Genome Atlas (TCGA), including 999 NSCLC and 103 normal samples, to conduct Kaplan–Meier survival analysis using the R package 'survival' (3.2.13) and to determine statistical significance at $p<0.05$. Analysis of immune cell infiltration was conducted using the TIMER database.

Statistical analysis

GraphPad Prism 5.0 was used for our statistical analysis. Our data were presented as the mean $\pm$ standard error of the mean (SEM). Student's t-test (2-tailed) was used to determine differences for experiments between two groups. For experiments involving more than two groups, we applied one-way ANOVA followed by Dunnett's test to adjust for multiple comparisons. A threshold for statistical significance was established at a two-tailed p -value of less than 0.05. All in vitro experiments were independently replicated at least three times.

Results

SPP1 expression is elevated in IPF and NSCLC database

Four datasets were analyzed for IPF (GSE110147 and GSE53845) and NSCLC (GSE18842 and GSE74706) (Additional file 1: Figure S1). Utilizing Venn diagram methodologies, we discerned 32 genes consistently upregulated and 80 genes downregulated across these datasets (Fig. 1A, B). *SPP1* is a coupled upregulated gene. Further, KEGG pathway enrichment analysis delineated that these differentially expressed genes predominantly clustered in pathways such as extracellular matrix–receptor interaction, protein digestion and absorption, relaxin signaling, focal adhesion, and the PI3K/Akt signaling pathway (Fig. 1C, Supplementary Table 1). *SPP1* was particularly pronounced in extracellular matrix–receptor interactions, focal adhesion, and the PI3K/Akt signaling pathway. All common differential genes were subjected to GO enrichment analysis with a significance threshold set at $P<0.05$. The results revealed that commonly upregulated genes were primarily enriched in functions such as extracellular matrix organization, extracellular structure organization, collagen metabolic process, collagen catabolic process, and extracellular matrix disassembly (Fig. 1D). Commonly downregulated genes were mainly enriched in endothelial cell differentiation, regulation of endothelial cell differentiation, endothelium development, positive regulation of endothelial cell differentiation, and regulation of epithelial cell differentiation (Fig. 1E). *SPP1* was primarily enriched in the extracellular matrix and extracellular structure organization. PPI network analysis positioned *SPP1* among the top 10 hub genes, interacting with pivotal proteins like MMP12, COL3A1, and ITGB8 (Fig. 1F, G).

SPP1 serves as a prognostic factor in NSCLC and is associated with the infiltration of cancer-associated fibroblasts (CAFs)

To further confirm the clinical significance and expression of *SPP1* in NSCLC, we accessed the expression matrix and clinical data for NSCLC from TCGA. A comparative analysis underscored a significantly higher *SPP1* expression in NSCLC samples than normal samples

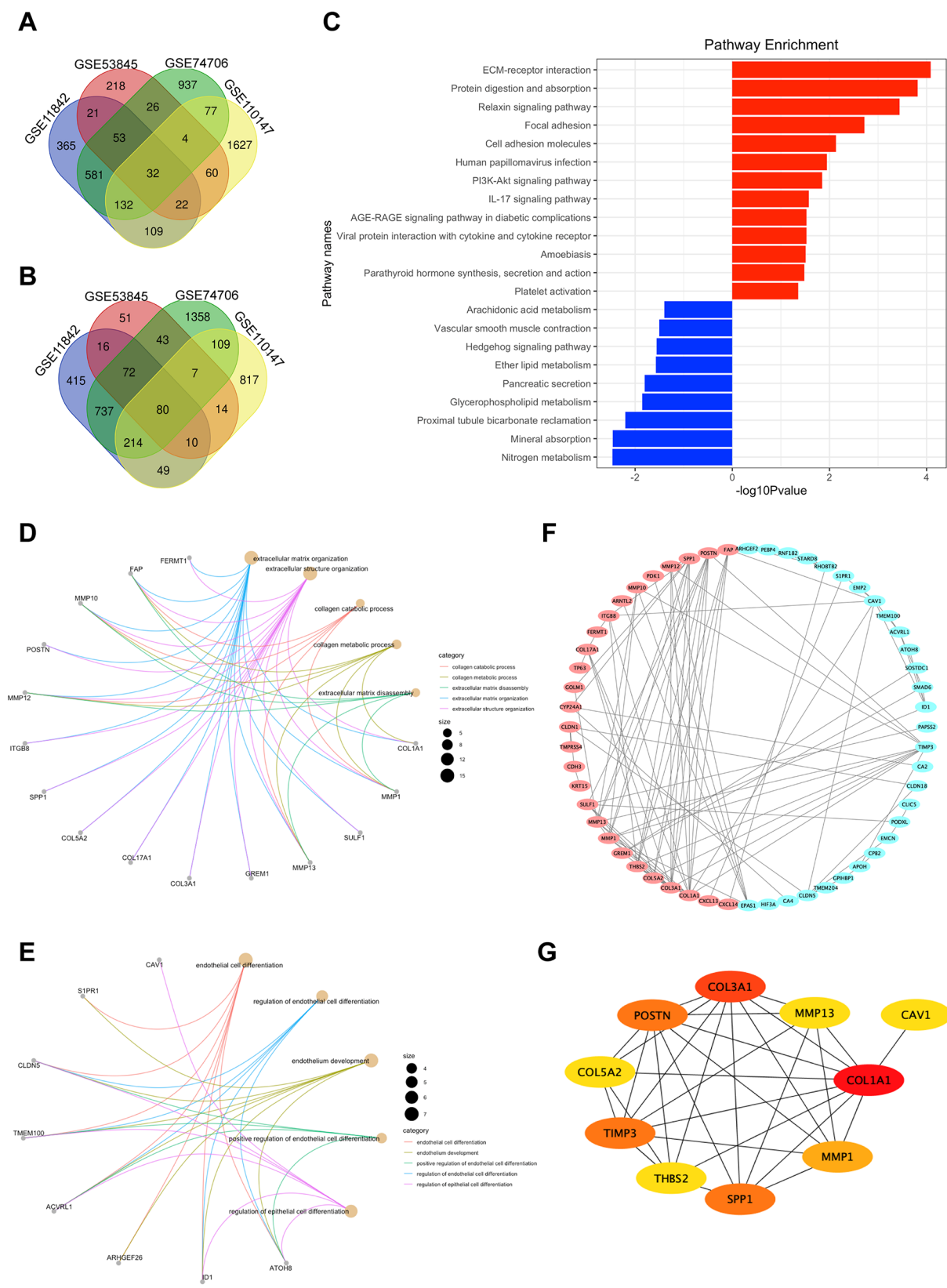


Fig. 1 Differential gene and functional analysis of IPF and NSCLC datasets in the GEO database. **A, B** Venn diagram of common upregulated (A) and downregulated (B) differential genes from the four microarray datasets. **C** KEGG pathway enrichment analysis for all common differential genes, with $p < 0.05$ as the inclusion criterion. **D, E** Gene Ontology enrichment analysis for all commonly upregulated genes (D) and downregulated differential genes (E), with $p < 0.05$ as the inclusion criterion. **F** PPI analysis for all common differential genes. **G** Top 10 hub genes in PPI analysis

($p < 0.001$; Fig. 2A). Moreover, elevated SPP1 levels in NSCLC patients were associated with a decrease in overall survival ($p = 0.013$; Fig. 2B). Additionally, we utilized the TIMER database to examine the relationship between SPP1 expression and the degree of infiltration by specific immune cell subtypes in lung squamous carcinoma and lung adenocarcinoma. The analysis reveals that SPP1 expression significantly positively correlates with CAFs immune infiltration in both lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD). Moreover, there is no significant correlation between SPP1 expression and sample purity in LUSC, and a statistically significant but weak negative correlation in LUAD. These observations led us to hypothesize a central role for SPP1 in the pathogenesis of both IPF and NSCLC, potentially modulating tumor development through fibroblast regulation.

Elevated SPP1 expression in IPF lung tissue

To elucidate the clinicopathological significance of SPP1 in IPF, we examined its expression in IPF patients. HE staining results revealed extensive destruction of alveolar structures and collagen fiber accumulation in IPF samples. Conversely, the normal samples were intact and clear, the alveolar septum was normal, and the alveolar cavity was intact without apparent exudation. Masson staining further confirmed the presence of abundant blue-stained fibrous strips in IPF samples, while the normal group displayed minimal staining (Fig. 3A). Furthermore, fibrosis markers, including α -SMA, COL1A1, and COL3A1, were notably overexpressed in IPF lung tissues (Fig. 3B). Collectively, these data suggest that the IPF lung tissue showed significant fibrosis. qRT-PCR, immunohistochemistry, and western blotting confirmed a significant upsurge of SPP1 in IPF lung tissues, with partial colocalization with α -SMA, as evident from immunofluorescence staining (Fig. 3C–F). These data suggest that increased SPP1 expression may play a crucial role in the process of pulmonary fibrosis, especially in fibroblasts. To validate our clinical findings, we conducted in vivo experiments using a mouse model of lung fibrosis.

Elevated SPP1 expression in lung tissue of BLM-induced mouse

The BLM-induced mouse lung fibrosis model is widely employed in pulmonary research to study fibrosis. Our methodology involved the intratracheal instillation of BLM, juxtaposed against a control group receiving a saline solution of equal volume. After 21 days, histological examinations, including HE and Masson staining, revealed that the alveolar architecture in the control group's lung tissue retained its normal morphology with negligible fibrotic changes in the alveolar septa. In stark contrast, the BLM-treated group exhibited pronounced

alveolar damage, characterized by the emergence of blue-stained fibrous bands within the alveolar interstitium, as depicted in Fig. 4A. Furthermore, a marked elevation in fibrotic markers, including α -SMA, COL1A1, and COL3A1, was observed in the BLM group compared to the control group, confirming the successful establishment of the BLM-induced mouse lung fibrosis model (Fig. 4B). Both qRT-PCR and immunohistochemical analyses corroborated a significant upsurge in SPP1 expression in the BLM group (Fig. 4A, B). Immunofluorescence staining elucidated the localization of SPP1, revealing its overexpression and partial co-localization with α -SMA in the BLM group (Fig. 4C). These findings not only confirm an in vivo elevation of SPP1 expression but also its pronounced presence in fibroblasts, mirroring clinical observations.

Knockdown of SPP1 inhibits TGF- β 1-Induced fibroblast-to-myofibroblast differentiation

We further explored the role of SPP1 in fibroblast activation during pulmonary fibrosis. TGF- β 1, recognized as a pivotal fibrogenic factor, induces fibroblast-to-myofibroblast differentiation in vitro. Utilizing concentrations of TGF- β 1 to stimulate primary normal fibroblasts resulted in the upregulated expression of α -SMA, COL1A1, and SPP1 at both protein and mRNA levels (Fig. 5A, B). This successful establishment of a pulmonary fibrosis cell model through TGF- β 1 induction led us to select a TGF- β 1 concentration of 10 ng/mL for subsequent experiments. This upregulation was further corroborated in HFL1 cells, which manifested elevated expression levels of α -SMA, COL1A1, and SPP1 following TGF- β 1 stimulation (Fig. 5C, D). To assess the impact of SPP1 modulation on fibroblast activation, we knocked down SPP1 expression in primary normal fibroblasts and HFL1 cells. The efficiency of SPP1 knockdown was validated using qRT-PCR and western blotting (Fig. 5E, F). After the knockdown of SPP1, the expression of α -SMA and COL1A1 were significantly diminished in normal primary fibroblasts and HFL1 cells upon TGF- β 1 stimulation (Fig. 5G–J). This underlines the critical role of SPP1 in the TGF- β 1-induced transformation of fibroblasts into myofibroblasts.

Knockdown of SPP1 suppresses the proliferation of IPF fibroblasts

To elucidate the mechanistic role of SPP1 in IPF fibroblasts, we extracted primary IPF fibroblasts from lung tissues of IPF patients. Immunofluorescence analysis distinguished between the two groups of primary fibroblasts; those derived from IPF patients exhibited significantly higher α -SMA expression compared to those from normal group (Additional file 1: Figure S2). The expression levels of α -SMA, COL1A1, and SPP1 were

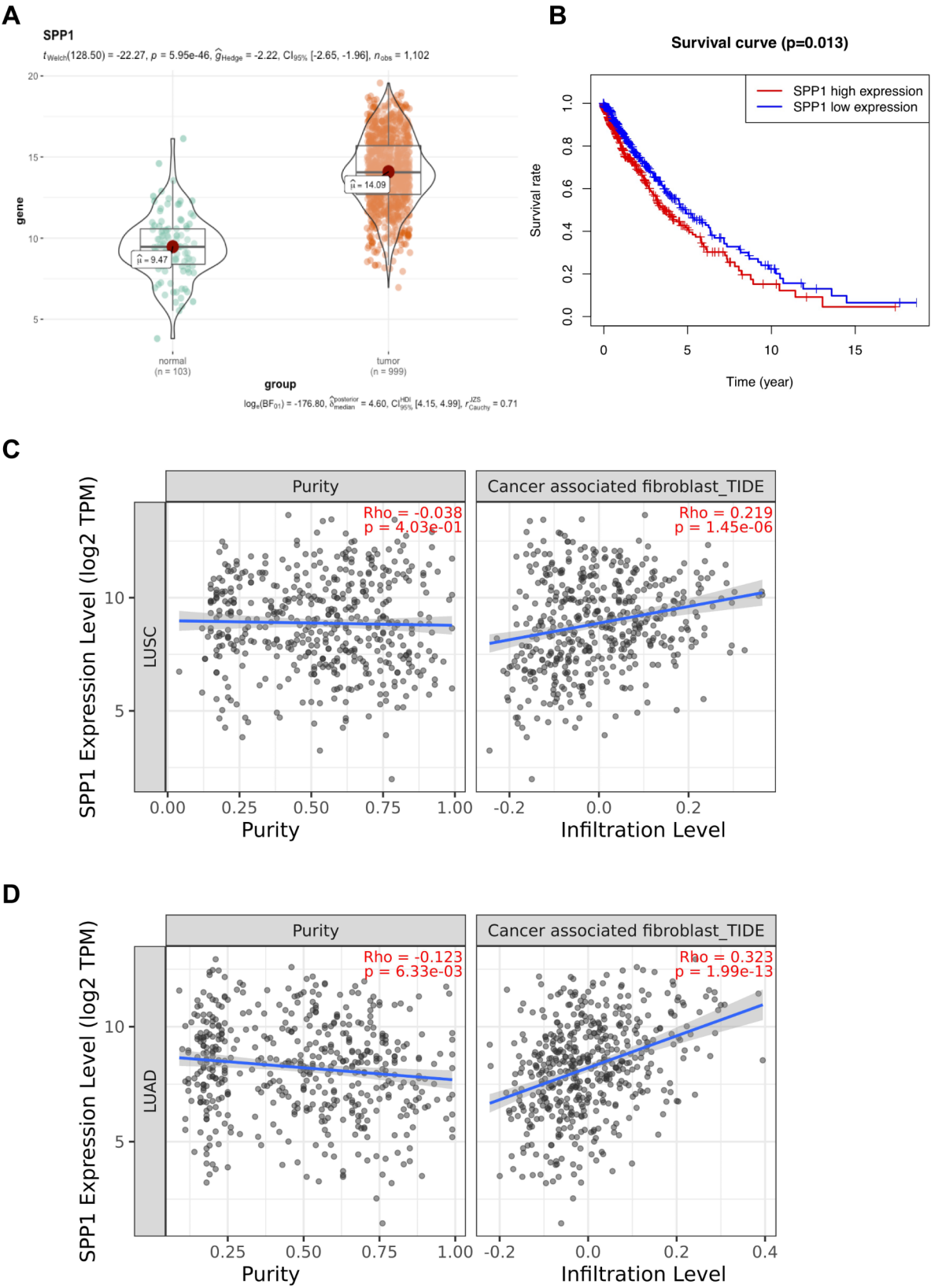


Fig. 2 Expression, survival analysis, and immune infiltration analysis of SPP1 in NSCLC from TCGA database. **A** Expression of SPP1 between NSCLC samples and normal samples in TCGA. **B** Kaplan–Meier analysis of SPP1 expression in patients with NSCLC. **C, D** Immune infiltration analysis of SPP1 in lung squamous cell carcinoma **(C)** and lung adenocarcinoma **(D)** from the TIMER database

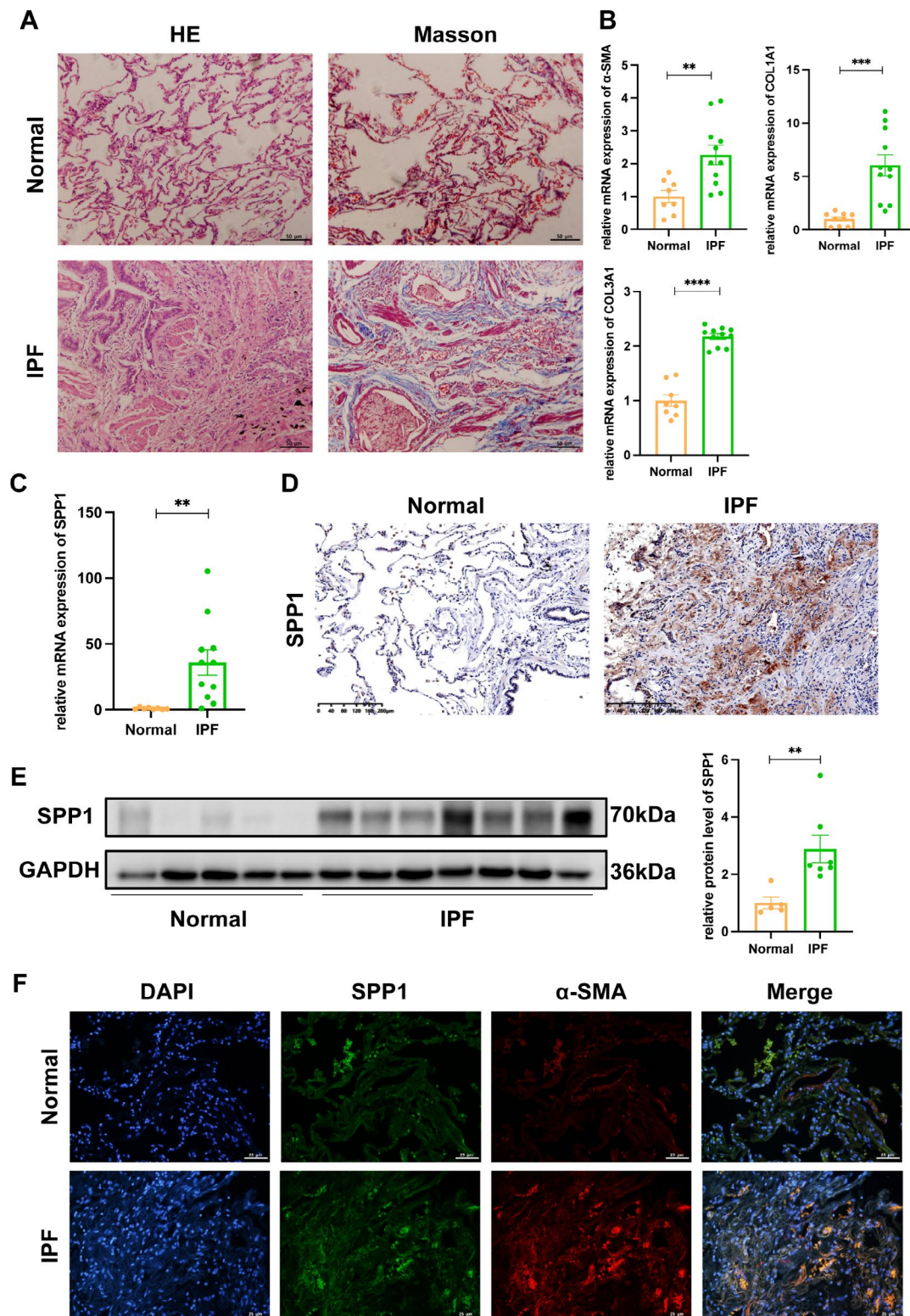


Fig. 3 SPP1 is significantly overexpressed in the lung tissues of patients with IPF. **A** Representative images of HE and Masson's trichrome-stained sections of lung tissues from patients with IPF and normal lung tissues; scale bars, 50 μ m. **B** Fibrotic markers (α -SMA, COL1A1, and COL3A1) in IPF lung tissues measured via qRT-PCR analysis. **C–E** qRT-PCR analysis (**C**), IHC staining (**D**), and western blotting (**E**) were used to detect the expression of SPP1 in lung tissues from patients with IPF and normal lung tissues; scale bars, 200 μ m. **F** Immunofluorescence staining of α -SMA (a fibroblast-specific marker, red) and SPP1 (green) was conducted to examine the spatial distribution of SPP1; scale bars, 25 μ m. Data are presented as the mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001

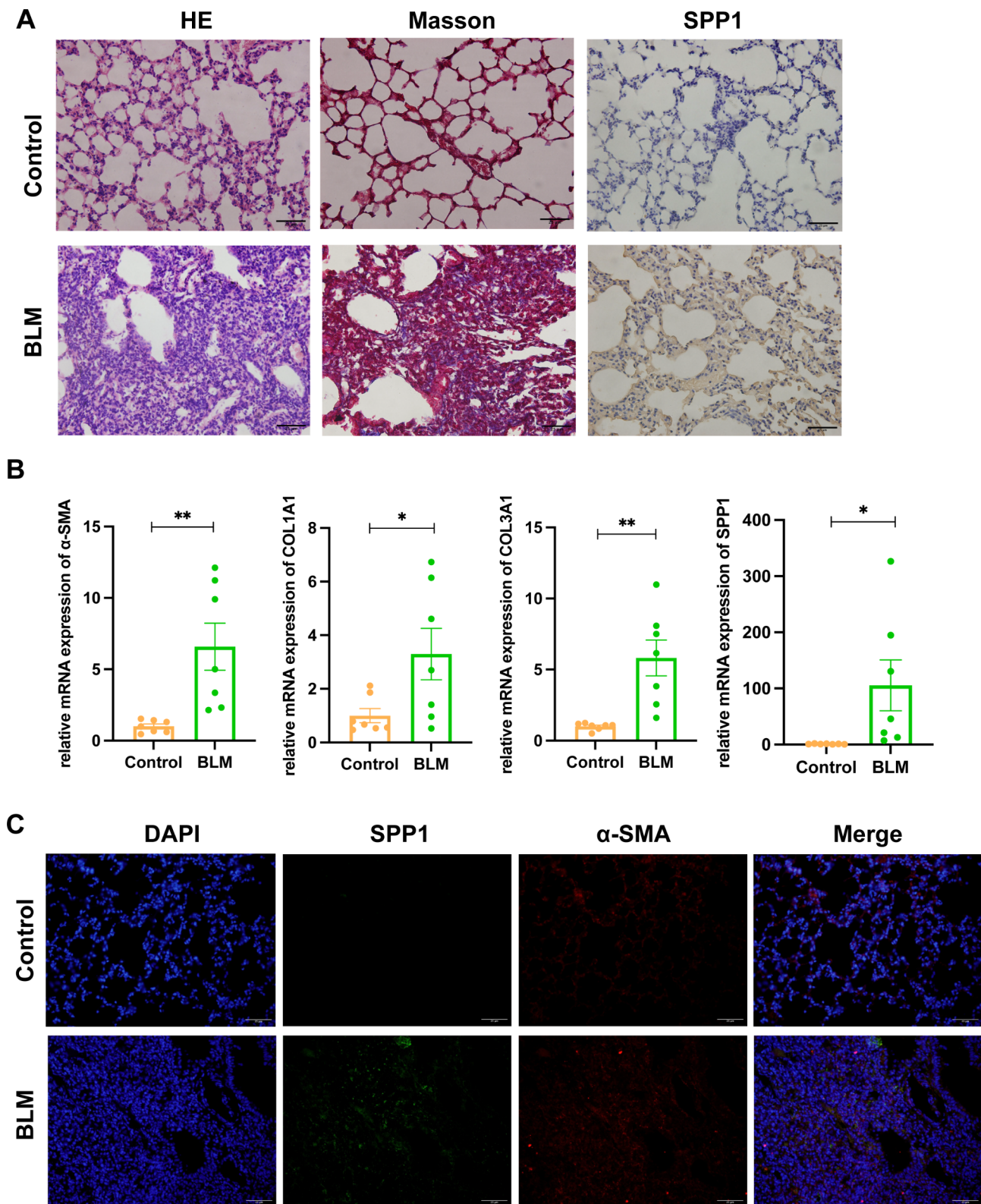


Fig. 4 Overexpression of SPP1 in a BLM-induced mouse lung fibrosis model. **A** Representative images of HE, Masson, and IHC staining of SPP1 in lung tissues from a BLM-induced mouse-lung fibrosis model and a normal mouse; scale bars, 25 μ m. **B** mRNA Expression of fibrotic markers (α -SMA, COL1A1, and COL3A1) and SPP1 in lung tissues from a BLM-induced mouse-lung fibrosis model and a normal mouse measured using qRT-PCR analysis. **C** Immunofluorescence staining of α -SMA (red) and SPP1 (green) was conducted to examine the spatial distribution of SPP1; scale bars, 25 μ m. Data are presented as the mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001

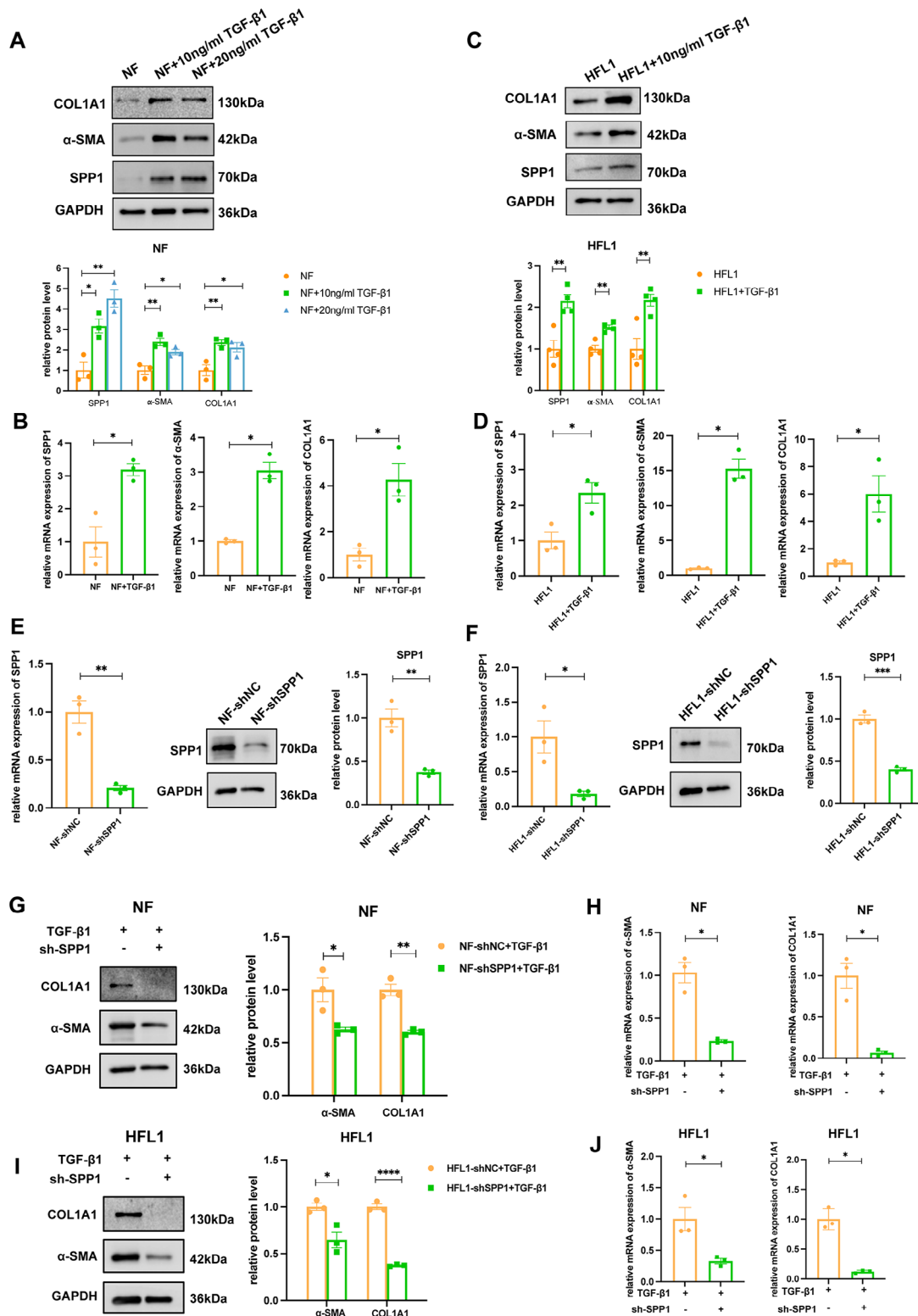


Fig. 5 Knockdown of SPP1 inhibits fibrogenesis in fibroblasts induced by TGF-β1. **A-D** Western blotting and qRT-PCR was used to detect SPP1, α-SMA, and COL1A1 expression in primary normal lung fibroblasts (**A, B**) and HFL1 cells (**C, D**) that responded to TGF-β1. **E, F** A lentiviral vector was used to knock down SPP1 expression, and western blotting and qRT-PCR were used to detect SPP1 expression in primary normal lung fibroblasts (**E**) and HFL1 cells (**F**). **G-J** Western blot and RT-PCR were used to detect α-SMA and COL1A1 expression in SPP1-depleted primary normal lung fibroblasts (**G, H**) and SPP1-depleted HFL1 cells (**I, J**) treated with TGF-β1. Data are presented as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NF, normal fibroblast

notably higher in IPF fibroblasts than in normal fibroblasts (Fig. 6A, B). Next, we knocked down SPP1 in IPF fibroblasts, aiming to determine its effect on fibroblast proliferation. The efficiency of the SPP1 knockdown was established by qRT-PCR and western blotting (Fig. 6C, D). CCK-8 and EdU assays showed that SPP1 inhibition mitigated IPF fibroblast proliferation (Fig. 6E, F). Based on prior KEGG analysis indicating SPP1 enrichment in the PI3K/Akt pathway, we examined alterations in this pathway after SPP1 knockdown in IPF fibroblasts. The results showed a remarkable downregulation in the ratios of pAkt/Akt and pmTOR/mTOR following SPP1 knockdown (Fig. 6G). These findings collectively indicate that SPP1 knockdown impedes IPF fibroblast proliferation via the PI3K/Akt/mTOR pathway.

SPP1 overexpression promotes the proliferation of IPF fibroblasts via the PI3K/Akt/mTOR pathway

Next, we explored the impact of SPP1 overexpression on IPF fibroblasts, specifically focusing on the PI3K/Akt/mTOR pathway. We transfected the vector-SPP1 plasmids into IPF fibroblasts, and overexpression efficacy was verified by western blotting and qRT-PCR (Fig. 7A, B). Utilizing LY294002, a PI3K inhibitor, we observed that while SPP1 overexpression spurred primary fibroblast proliferation, these effects were significantly attenuated by LY294002 (Fig. 7C, D). Additionally, the activation of the PI3K/Akt/mTOR pathway subsequent to SPP1 overexpression was effectively counteracted by LY294002 (Fig. 7E). These results suggest a pivotal role of SPP1 overexpression in promoting IPF fibroblast proliferation, predominantly mediated through the PI3K/Akt/mTOR pathway.

SPP1 as a regulatory factor in IPF fibroblasts influencing NSCLC cell proliferation

Our previous study showed that SPP1 is overexpressed in NSCLC and is associated with fibroblast infiltration. Given that SPP1 is a secreted protein with higher expression levels in IPF fibroblasts compared to normal fibroblasts, we collected conditioned medium from both IPF and normal fibroblasts to incubate NSCLC cells, aiming to assess its impact on NSCLC cell proliferation. A549, a commonly used NSCLC cell line, demonstrated enhanced proliferation when stimulated by supernatants in IPF fibroblasts, as evidenced by CCK-8 and EdU assays (Fig. 8A, B). In pursuit of understanding the effect of SPP1 on NSCLC cell proliferation, we knocked down SPP1 in IPF fibroblasts and gathered the conditioned medium to stimulate A549 cells. This intervention diminished the proliferative capacity of A549 cells compared to the control conditioned medium group. (Fig. 8C, D). Next, we detected changes in the PI3K/Akt/mTOR pathway in A549 cells. Western blotting showed

that conditioned medium from IPF fibroblasts activated the PI3K/Akt/mTOR pathway in A549 cells, an effect that was diminished when SPP1 was knocked down in IPF fibroblasts (Fig. 8E). These findings underscore the role of IPF fibroblasts in enhancing NSCLC cell proliferation and activating the PI3K/Akt/mTOR pathway, with these effects being negated by SPP1 knockdown.

Discussion

Our investigation provides compelling evidence that SPP1 significantly influences the pathophysiology of IPF and NSCLC. Specifically, SPP1 was notably elevated in both IPF and NSCLC groups. Crucially, SPP1 promoted the proliferation of IPF fibroblasts through PI3K/Akt/mTOR pathway, and its inhibition mitigated the TGF- β 1-induced transformation of fibroblasts-into-myofibroblasts, indicating its pivotal role in fibrotic processes. More importantly, SPP1 silencing in IPF fibroblasts reduces the proliferation of NSCLC cells. These findings underscore SPP1's dual role in pulmonary fibrosis and oncogenesis.

The etiology of IPF is multifactorial, encompassing genetic predispositions and environmental factors. These collectively contribute to the deterioration of alveolar structures, significant extracellular matrix deposition, and extensive pulmonary interstitial remodeling [22]. A notable correlation between IPF and NSCLC was first documented in 1965 [23]. Earlier research has documented the correlation between a history of smoking and the onset of IPF [24]. Smoking and age are also risk factors for lung cancer because long-term exposure of the alveolar epithelium to particulate matter leads to cell apoptosis, DNA repair defects, and atypical hyperplasia [25]. The occurrence of lung cancer, particularly NSCLC, is elevated among individuals with IPF compared to the general population [26]. Emerging research has delineated striking pathogenetic similarities between IPF and NSCLC, including mutations in surfactant proteins [27]. Furthermore, TGF- β not only fuels fibrosis but also facilitates epithelial-mesenchymal transition in tumors, thus promoting their growth and metastasis [28]. Nintedanib, an antifibrotic drug, has been recognized for its efficacy in treating both IPF and lung cancer [29]. The coexistence of IPF and NSCLC markedly diminishes patient's quality of life and escalates mortality, underscoring the urgent need for improved diagnosis and therapy. To this end, our analysis of datasets revealed elevated SPP1 expression across IPF and NSCLC samples, with a notable enrichment in the PI3K/Akt pathway. GO analysis indicated that SPP1 is primarily enriched in the regulation of the extracellular matrix and extracellular structure organization, which is crucial for understanding its role in disease mechanisms, particularly in idiopathic pulmonary fibrosis (IPF). Protein-protein interaction (PPI)

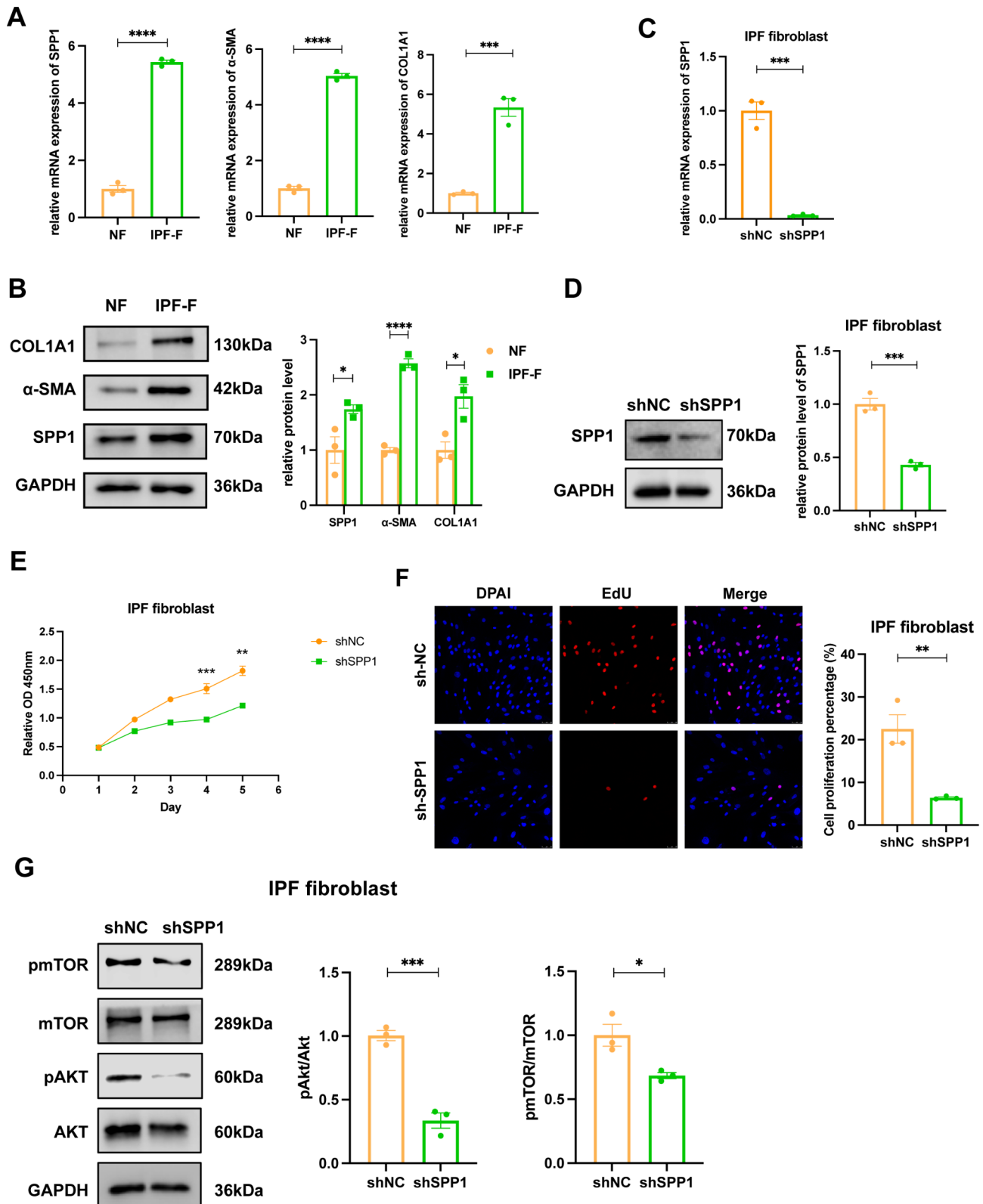


Fig. 6 Knockdown of SPP1 inhibits the proliferation of IPF fibroblasts. **A, B** SPP1, α-SMA, and COL1A1 expression between normal fibroblasts and IPF fibroblasts measured via qRT-PCR (**A**) and western blotting (**B**). **C, D** qRT-PCR (**C**) and western blotting (**D**) were used to detect SPP1 expression in IPF fibroblasts after knockdown. **E, F** CCK-8 assay (**E**) and EdU staining (**F**) revealed the effects of SPP1 inhibition on IPF fibroblast proliferation; scale bars, 50 μm. **G** Western blotting was used to detect the expression of the PI3K/Akt/mTOR pathway in IPF fibroblasts after knockdown of SPP1. Data are presented as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

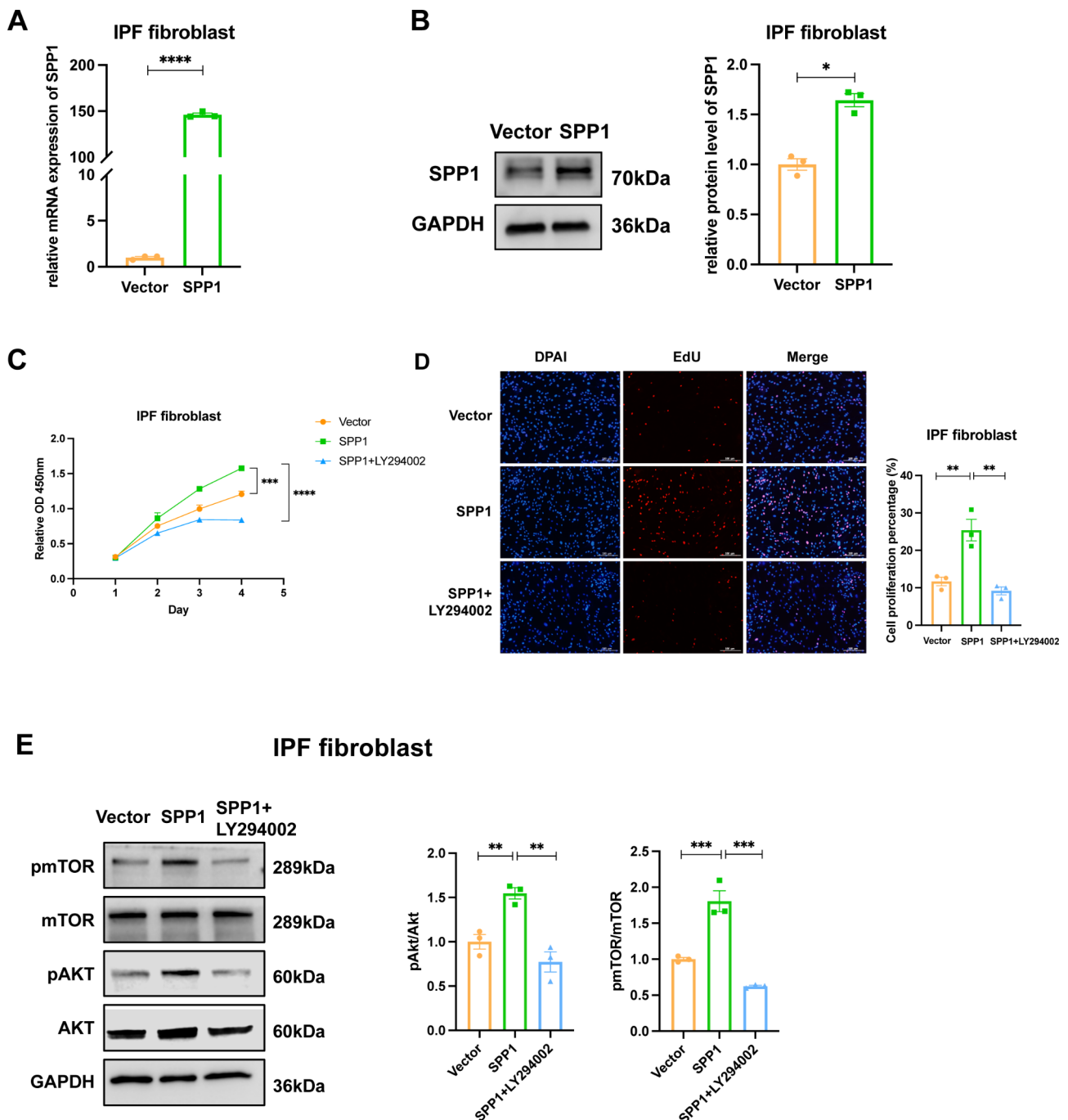


Fig. 7 Overexpression of SPP1 promotes the proliferation of IPF fibroblasts via the activation of the PI3K/Akt/mTOR pathway. **A**, **B** qRT-PCR (**A**) and western blotting (**B**) were used to detect SPP1 expression in IPF fibroblasts after transfection of SPP1. **C**, **D** CCK-8 assay and EdU staining revealed the effects of SPP1 overexpression on IPF fibroblast proliferation, and an inhibitor of the PI3K pathway (LY294002) was added; scale bars, 100 μ m. **E** Western blotting was used to detect the expression of the PI3K/Akt/mTOR pathway in IPF fibroblasts after SPP1 overexpression and inhibition of the PI3K pathway. Data are presented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

analysis revealed interactions between SPP1 and key proteins such as COL1A1, COL1A2, THBS2, MMP1, and MMP13. These proteins are closely associated with collagen formation and degradation. THBS2 plays a significant role in cell adhesion, migration, proliferation, and tissue repair. Therefore, we hypothesize that SPP1 may

be involved in collagen formation and fibroblast aggregation, suggesting a pivotal role in the pathogenesis of IPF. Further validation using the TCGA database confirmed SPP1 expression in NSCLC and its prognostic relevance. Immune infiltration analysis via the TIMER database also established a substantial positive correlation between

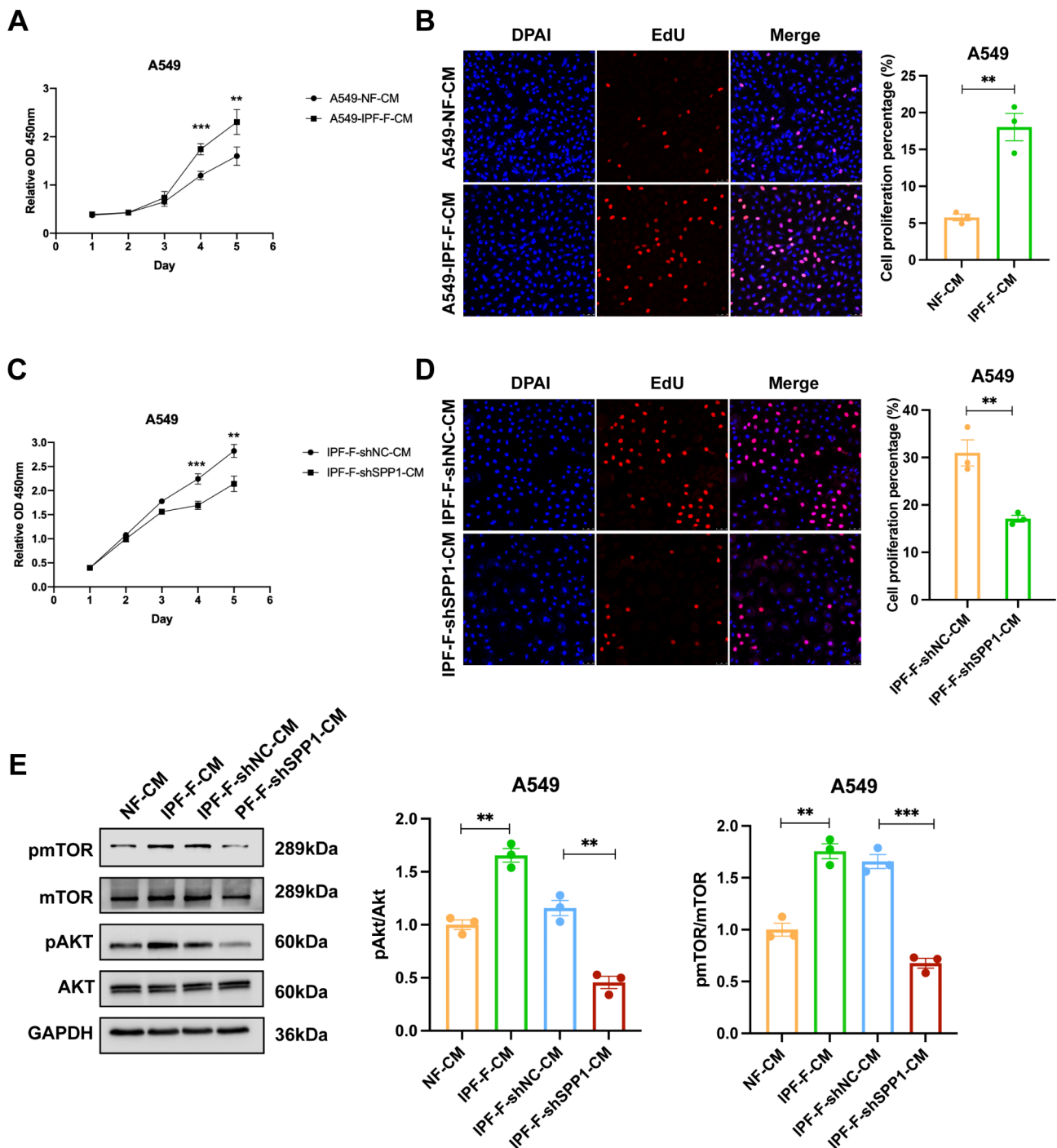


Fig. 8 Knockdown of SPP1 in IPF fibroblasts decreases the proliferation of NSCLC cells. **A, B** CCK-8 assay (**A**) and EdU staining (**B**) were used to detect the proliferation of NSCLC cells after following stimulation with CM from IPF fibroblasts and normal fibroblasts; scale bars, 50 μ m. **C, D** CCK-8 assay (**C**) and EdU staining (**D**) were used to detect the proliferation of NSCLC cells after knockdown of SPP1 in IPF fibroblasts; scale bars, 50 μ m. **E** Western blotting was used to detect the expression of the PI3K/Akt/mTOR pathway in NSCLC cells after stimulating by CM from IPF fibroblasts and SPP1-depleted IPF fibroblasts. Data are presented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. CM, conditioned medium

SPP1 expression and the immune infiltration level of CAFs in NSCLC.

SPP1, an extracellular matrix glycosylated phosphoprotein, undergoes various post-translational modifications

and interacts with integrin receptors, initiating a cascade of cellular processes, including proliferation, adhesion, and migration [30–33]. SPP1 has been demonstrated to promote tumor progression and immune regulation in

various types of cancers. Its expression levels may affect the activity of CAFs and infiltration by modulating intercellular signaling within the microenvironment [34]. Eun et al. found that SPP1 derived from CAFs activates the RAF/MAPK and PI3K/AKT/mTOR signaling pathways and promotes epithelial-mesenchymal transition, thereby enhancing resistance to tyrosine kinase inhibitors in hepatocellular carcinoma [35]. Cheng et al. found that SPP1 promotes the proliferation, migration, and invasion of colorectal cancer cells and the upregulation of cancer-like stem cells with aldehyde dehydrogenase 1 positivity [36]. Gui et al. found that its elevated serum levels in acute IPF exacerbation correlated with increased mortality [37]. Nevertheless, the specific role of SPP1 in IPF requires additional exploration. In our study, we observed a substantial increase in SPP1 expression in IPF patients compared to healthy individuals. The BLM-induced pulmonary fibrosis-mouse model was the earliest developed model and is extensively used because of its excellent replication of many IPF characteristics and ease of operation [38]. We demonstrated a marked increase in SPP1 expression in BLM mouse group following fibrosis induction. Given that fibroblast activation and proliferation are central to IPF pathology, and considering TGF β 1's role in myofibroblast activation, we established a pulmonary fibrosis cell model using TGF- β 1 in normal fibroblasts. We observed that the expression of both fibroblast activation markers and SPP1 was elevated following TGF- β 1 administration. Notably, our research showed that SPP1 knockdown curtailed the activation of normal fibroblasts induced by TGF- β 1 and the proliferation of IPF fibroblasts, underscoring SPP1's potential as a therapeutic target in IPF treatment.

Research has indicated that the PI3K/Akt pathway participates in the development of myocardial, hepatic, and interstitial kidney fibrosis [39–41]. Peng et al. found that the PI3K/Akt/mTOR pathway is closely related to the growth and activation of stellate cells and promotes the progression of hepatic fibrosis [42]. Furthermore, targeting the PI3K/Akt/mTOR pathway has emerged as a crucial therapeutic strategy in the treatment of hepatic fibrosis [43]. Research suggests that the PI3K/Akt/mTOR pathway may directly participate in IPF or fibrosis development through some interactions [44]. Our study found that SPP1 overexpression promoted the proliferation of IPF fibroblasts and activation of the PI3K/Akt/mTOR pathway, whereas these effects were abrogated by a PI3K inhibitor. These findings strongly suggest that SPP1 plays a pro-fibrotic role in IPF and may hold great promise as a target for the development of innovative therapies aimed at addressing the challenges posed by IPF.

Fibroblasts are key effector cells in IPF. Similarly, CAFs play a significant role in NSCLC development [45]. Given the high expression of SPP1 in NSCLC and its association

with CAFs, our hypothesis posits that activated fibroblasts within IPF may create a conducive microenvironment for NSCLC proliferation. In support of this, our findings indicate that IPF fibroblasts bolster NSCLC cell proliferation and activate the PI3K/Akt/mTOR pathway, an effect mitigated by SPP1 silencing in IPF fibroblasts. This suggests that targeting SPP1 may offer a novel dual therapeutic approach for managing both lung fibrosis and lung cancer.

In conclusion, our research elucidates the multifaceted role and mechanistic underpinnings of SPP1 in lung fibrosis progression, offering crucial insights into the co-occurrence of IPF and NSCLC. These discoveries not only advance our understanding of these complex conditions but also pave the way for identifying potential therapeutic interventions that could benefit patients affected by both IPF and NSCLC.

Abbreviations

NSCLC	Non-small-cell lung cancer
IPF	Idiopathic pulmonary fibrosis
SPP1	Secreted phosphoprotein 1
CAFs	Cancer-associated fibroblasts
TGF- β	Transforming growth factor-beta
KEGG	Kyoto Encyclopedia of Genes and Genomes
PPI	Protein-protein interaction
TCGA	The Cancer Genome Atlas
GEO	Gene Expression Omnibus
HE	Hematoxylin and eosin
α -SMA	α -smooth muscle actin
qRT-PCR	Real-time quantitative PCR
IHC	Immunohistochemistry

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12931-024-02989-7>.

Additional file 1: Figure S1. Volcano plot of differentially expressed genes from four GEO datasets by cutoff values ($|\log FC| > 0.5$ and $p < 0.01$).

Additional file 2: Figure S2. Identification of normal and IPF fibroblasts. A Representative images of normal and IPF fibroblasts observed under a light microscope; scale bars, 100 μ m. B Immunofluorescence staining of α -SMA expression (green) in normal and IPF fibroblasts; scale bars, 25 μ m.

Supplementary Material 3

Acknowledgements

Not applicable.

Author contributions

BQY, DX, and JYC: Conceptualization and design; BQY, DX, XCY, JZ, and JBS: Investigation and methodology; BQY, DW, and FG: Resources and data curation; BQY, JC and DX: Formal analysis and software; BQY, MH, and JYC: Writing—original draft and Writing—review & editing. All authors reviewed the manuscript.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Wuxi People's Hospital (Institutional Review Board Reference Number: KY22060).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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