Article

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USP7 Promotes TGF-β1 Signaling by De-Ubiquitinating Smad2/Smad3 in Pulmonary Fibrosis

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Background: Idiopathic pulmonary fibrosis (IPF) is a long-term, progressive, and irreversible pulmonary interstitial disease. The activation of Smad family member 2 (Smad2) and Smad3 transcription factors by transforming growth factor β-1 (TGF-β1) is a critical event in the pathogenesis of IPF. However, there is still a lack of understanding regarding the molecular mechanisms governing Smad2 and Smad3 proteins. Ubiquitin-specific protease 7 (USP7) is a deubiquitinase that plays a vital role in regulating protein stability within cells. However, its regulation of the TGF-β signaling pathway and its significance in IPF remain undiscovered. This study aims to clarify the function of USP7 in the TGF-β signaling pathway, while simultaneously exploring the specific molecular mechanisms involved. Additionally, this study seeks to evaluate the therapeutic potential of targeted USP7 inhibitors in IPF, thereby providing novel insights for the diagnosis and management of IPF.

Methods: We first detected the expression of USP7 in lung tissues of mice with Bleomycin (BLM)-induced pulmonary fibrosis and in Beas-2B cells treated with or without TGF-β1 through Western blot analysis. Subsequently, we explored the influence of USP7 on fibrotic processes and the TGF-β1 signaling pathway, utilizing *in vitro* and *in vivo* studies. Finally, we assessed the effectiveness of USP7-specific inhibitors in an IPF murine model.

Results: In the present study, USP7 was found to de-ubiquitinate Smad2 and Smad3, consequently increasing their stability and promoting the TGF-β1-induced production of profibrotic proteins including α-smooth muscle actin (α-SMA) and fibronectin 1 (FN-1). Inhibition or knockdown of USP7 resulted in decreased levels of Smad2 and Smad3 proteins, leading to reduced expression of FN-1, Collagen Type I Alpha 1 Chain (Col1A1), and α-SMA induced by TGF-β1 in human pulmonary epithelial cells. These findings demonstrate that overexpression of USP7 reduces Smad2/3 ubiquitination, whereas inhibition or knockdown of USP7 enhances their ubiquitination. USP7 is abundantly expressed in IPF lungs. The expressions of USP7, Smad2, and Smad3 were upregulated in bleomycin-induced lung injury. The USP7 inhibitor P22077 reduced the expression of FN-1 and type I collagen as well as Smad2/3 and collagen deposition in lung tissue in a model of pulmonary fibrosis induced by bleomycin.

Conclusions: This study demonstrates that USP7 promotes TGF-β1 signaling by stabilizing Smad2 and Smad3. The contribution of USP7 to the progression of IPF indicates it may be a viable treatment target.

Keywords: idiopathic pulmonary fibrosis (IPF); transforming growth factor β-1 (TGF-β1); ubiquitin-specific protease 7 (USP7); Smad2; Smad3

Introduction

Idiopathic pulmonary fibrosis (IPF) is the most common type of idiopathic interstitial lung disease, characterized by a persistent, progressive, and fatal lung condition with a high mortality rate [1]. IPF is characterized by significant fibroblast hyperplasia and the deposition of extracellular matrix (ECM) within the lung interstitial and alveolar spaces. The average survival period for IPF patients post-diagnosis is 3 to 5 years [2]. Abnormal reepithelization and healing of the damaged alveolar epithelium are considered to cause fibroblast accumulation and ECM deposition [3]. Despite extensive efforts, only two Food and Drug Administration (FDA)-approved oral preparations (Pirfenidone and Nintedanib) are available for IPF treatment. However, these medications only offer delayed disease development rather than a cure and are associated with significant adverse effects [4]. As a result, there is substantial interest in developing new, more effective medications for IPF treatment.

Epithelial-mesenchymal transition (EMT) is a primary cause of fibrosis within the alveolar and airway epithelium. This transition is accompanied by increased expression of interstitial markers like E-cadherin, alongside decreased expression of epithelial markers such as Vim, N-cadherin, α-smooth muscle actin (α-SMA), and collagen [5]. Transforming growth factor-β (TGF-β) is the main trigger for EMT, recognized for its vital role in the progression of fi-

brotic diseases [6]. Given its pathophysiological role in IPF, targeting TGF-β has been considered as a possible treatment approach. Three isoforms of TGF-β have been identified in mammals: TGF-β1, TGF-β2, and TGF-β3. Among them, TGF-β1 is the most commonly found in the lungs and is believed to cause fibrosis [7]. However, the exact etiology and pathological mechanisms of IPF aren't fully understood.

Importantly, many downstream mediators of TGF-β are usually regulated by the ubiquitin-proteasome system (UPS) [8]. Accumulating evidence suggests that ubiquitination and de-ubiquitination are central to the pathological tissue remodeling observed in IPF [9]. Deubiquitinating enzymes (DUBs) are a crucial part of UPS, engaging in various cellular processes and biological functions by removing the ubiquitin chain from substrates [10]. DUBs are involved in the modulation of several pathological mechanisms in human diseases, including IPF [9,11]. Among these, ubiquitin-specific protease 7 (USP7), also recognized as herpesvirus-associated ubiquitin-specific protease (HAUSP), is among the most investigated DUBs. USP7 is mainly found in the nucleus and plays multiple critical functions in DNA repair, epigenetics, transcription, carcinogenesis, and immunity [12]. Abnormal expression and activity of USP7 are closely linked to biological processes like apoptosis and inflammation [13]. However, to date, no studies have reported on the involvement of USP7 in IPF and its underlying molecular mechanisms.

In this study, we explore the function of USP7 and its potential involvement in IPF through its deubiquitinase activity.

Materials and Methods

Cell Culture and Reagents

The human lung epithelial cell line Beas-2B (CCTCC, GDC0139) was generously provided by the Institute of Anesthesia and Perioperative Medicine of the First Affiliated Hospital of Nanchang University. Its authenticity was confirmed by STR analysis, and the detection of mycoplasma was negative using a reagent kit (G1900, Servicebio, Wuhan, China). These cell lines were grown in 1640 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, SH30071, South Logan, UT, USA) and 1% penicillin/streptomycin. Culturing of HEK293T cells was carried out in Dulbecco's Modified Eagle Medium (DMEM) (11885099, Gibco, Grand Island, NY, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were cultured at 37 °C with 5% CO2 in a humidified incubator.

Immobilized protein A/G beads (P2108) were purchased from Beyotime Biotechnology, Shanghai, China, while the antibodies of FN (14395-1-AP, 1:2000), α-SMA (14395-1-AP, 1:1000), Hemagglutinin (HA) tag (51064-2-AP, 1:1000; 66006-2-Ig, 1:1000), Flag tag (20543-1-AP,1:1000; 66008-4-Ig, 1:1000), β-actin (66009-1-Ig, 1:5000), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (60004-1-Ig, 1:3000), and control IgG (30000-0-AP) were acquired from Proteintech® Group Inc, Wuhan, China. USP7 (A13564, 1:1000) was procured from ABclonal Technology Company, MA, USA. Phospho-Smad family member 2 (Smad2) (3108T, 1:1000), Phospho-Smad3 (9520T, 1:1000) total Smad2 (53395, 1:1000), total Smad3 (95235, 1:1000), Type I collagen (72026T, 1:1000), and ubiquitin (20326T, 1:2000) antibodies were collected from Cell Signaling Technology Company, Shanghai, China. Goat anti-Mouse IgG (H+L) Secondary Antibody (31430, 1:5000) and Goat anti-Mouse IgG (H+L) Secondary Antibody (31460, 1:5000) were acquired from Thermo Fisher Scientific Inc, Shanghai, China. Bleomycin as well as recombinant TGF-β1 were obtained from Sigma Aldrich, Shanghai, China. MG132 was procured from Calbiochem, Darmstadt, Germany. All acquired materials are of the highest grades commercially available.

Western Blot

The cells were washed with chilled PBS and then immediately lysed using 2×SDS loading buffer. Lung tissues were harvested and homogenized in RIPA lysis buffer containing protease inhibitors. Following homogenization, cell lysates underwent centrifugation at 12,000 g for 15 min at 4 °C in order to eliminate cellular debris. Protein concentrations were determined with 23227-Pierce™ BCA Protein Assay Kits (Thermo Fisher Scientific Inc, Shanghai, China) using BSA as the standard. Western blot was conducted as previously described [14]. Equal amounts of cell lysates (20 µg) were used to be loaded onto a 10% SDS-PAGE gel. Polyvinylidene difluoride (PVDF) membrane was used for transferring. Subsequently, the membrane was incubated in TBST buffer containing 5% (wt/vol) BSA, 5mM Tris·HCl (pH 7.4), 137mM NaCl, and 0.1% Tween 20. The membrane was left in the TBST buffer for 30 min. Subsequently, the membrane underwent an overnight incubation with the primary antibody in 1% (wt/vol) BSA in TBST. The following day, the membrane underwent at least three washes with TBST. Then, the membrane underwent incubation with a second antibody conjugated with mouse or rabbit horseradish peroxidase (1:2000) for 1h. Finally, protein bands were visualized utilizing an enhanced chemiluminescence detection technique. Quantification of the Western blot bands was performed using ImageJ, Version 1.52p, National Institutes of Health, Bethesda, MD, USA, and results were presented as "fold changes".

Luciferase Reporter Assays

TGF-β signaling activity was assessed utilizing a luciferase reporter assay, which quantified the luminescence generated by Smad-binding elements (SBE) luciferase (SBE-luc) activity. This reporter comprises the luciferase gene under the regulation of the SBE. Usually,

cells with an exponential growth rate of 50% confluency were transfected with SBE-luc. Following transfection for 24 hours, cells were exposed to 10 ng/mL TGF-β for 16 hours. Then, cells were collected to measure luciferase and β-galactosidase activity using the Steady-Glo Luciferase kit (E2510, Promega, Beijing, China) following the instructions outlined by the manufacturer. All measurements were made in triplicate, and all values were standardized for transfection efficiency against β-galactosidase activity.

Immunoprecipitation (IP)

Cells were disrupted utilizing a cell lysis solution supplemented with protease and phosphatase inhibitors, as previously described [15]. Immunoprecipitation (IP) was performed as outlined before. Whole-cell lysates were incubated with specific primary antibodies or normal IgG for 4 hours at 4 °C. The immune complexes were then collected by incubating with protein A/G (Beyotime) for 12 hours at 4 °C. After extensive washing, the immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting with Smad2, Smad3, and USP7 antibodies. For the ubiquitination assay, expression plasmids encoding HA-tagged USP7 and Flag-tagged Smad2 or Smad3 were co-transfected into Beas-2B cells using Lipofectamine 3000 Transfection Reagent. The cell lysate was then used with Flag antibodies or normal IgG for IP, and the corresponding protein ubiquitination was detected by immunoblotting using ubiquitin antibodies.

Lentivirus Construction

HEK293T cells were transfected with pHAGE-USP7 plasmids or empty carriers, alongside packaging carriers psPAX2 and pMD2G. After 24 hours of transfection, the culture medium was exchanged for fresh complete medium (10% FBS, 1% streptomycin–penicillin). After an additional 48 hours, the supernatant that carried lentivirus was obtained and filtered using a 0.45-µm pore size filter. Subsequently, the lentivirus was concentrated at 82,000 $\times$g for 90 min using an SW28 rotor at 4°C. The lentivirus was harvested in PBS and preserved at –80°C.

Murine Model of Pulmonary Fibrosis Induced by Bleomycin

The *in vivo* model of lung fibrosis was established as previously described [16]. C57BL/6 male mice (aged 8 weeks, and weighing approximately 20–25 g) were procured from Hunan SJA Laboratory Animal Co., China. Mice were kept in a regulated setting where they had regular access to water and food, and the environment was kept at a temperature of 22 $\pm$ 2 °C, following a 12-hour light-dark cycle. A total of 48 mice were used and randomly divided into eight groups for two separate experiments: experiment 1 (n = 24) to validate the role of USP7 overexpression in bleomycin-induced IPF in mice, and experiment 2 (n = 24) for assessing the effectiveness of the USP7 inhibitor P22077 in mitigating IPF. For experiment 1, lentivirus encoding the *USP7* gene (10^7 CFU) and control lentivirus were intranasally injected into 12 mice each in a randomized manner. After 4 days, 6 mice injected with lenti-USP7 and 6 mice injected with control lentivirus were separately treated with bleomycin (3 mg/kg, i.t.) for 21 days. Bronchoalveolar lavage fluid (BALF) was harvested to assess cell count, infiltrates, and cytokines. For experiment 2, 10mg/kg of P22077 and an equivalent volume of corn oil (vehicle) were intraperitoneally administered into 12 mice each for 14 consecutive days. Six mice treated with P22077 and six mice treated with corn oil were treated with PBS the following day as a control, while the remaining mice in each group received bleomycin from the second day post-treatment until the mice were euthanized. Mice were euthanized on the 21st day by cervical dislocation under anesthesia induced by 5% isoflurane inhalation. Before paraffin embedding and sectioning, the left lung of these mice was fixed with 4% paraformaldehyde for at least 24 hours. They were then stained with hematoxylin & eosin (H&E) and Masson's trichrome. A portion of the right lung was homogenized in lysis buffer to analyze the protein levels using specific antibodies (USP7, fibronectin 1 (FN-1), α-actin, Smad2, Smad3, and GAPDH or β-actin).

BALF Collection

Mice were humanely euthanized with an overdose of 120 mg/kg intraperitoneal sodium pentobarbital anesthesia. Following euthanasia, a cut in the middle of the neck was made and a tube was inserted into the windpipe to collect BALF, as previously mentioned [17]. The lungs underwent three rounds of rinsing with ice-cold saline, followed by centrifugation of the cells at 400 g for 10 min at 4 °C. Following that, the supernatant was kept in storage at –80 °C for subsequent analysis. After resuspending the sedimentary cells in PBS, the red blood cells were eliminated. The total cell count was determined using a hemocytometer. Additionally, BALF protein concentration was assessed by employing a bicinchoninic acid assay kit (Thermo Fisher Scientific Inc, Shanghai, China).

Enzyme-Linked Immunosorbent Assay (ELISA)

The CXC chemokine ligand 1 (CXCL1) levels in BALF from mice were assessed using the Mouse CXCL1/KC Quantikine Enzyme-Linked Immunosorbent Assay (ELISA) Kit (MKC00B) from R&D Systems, Shanghai, China. in accordance with the manufacturer's guidelines. Absorbance at a wavelength of 450 nm was quantified utilizing a Varioskan Lux Multimode Microplate Reader (Thermo Fisher Scientific Inc, Shanghai, China), and sample concentration was determined by interpolating from absorbance curves generated by recombinant protein standards.

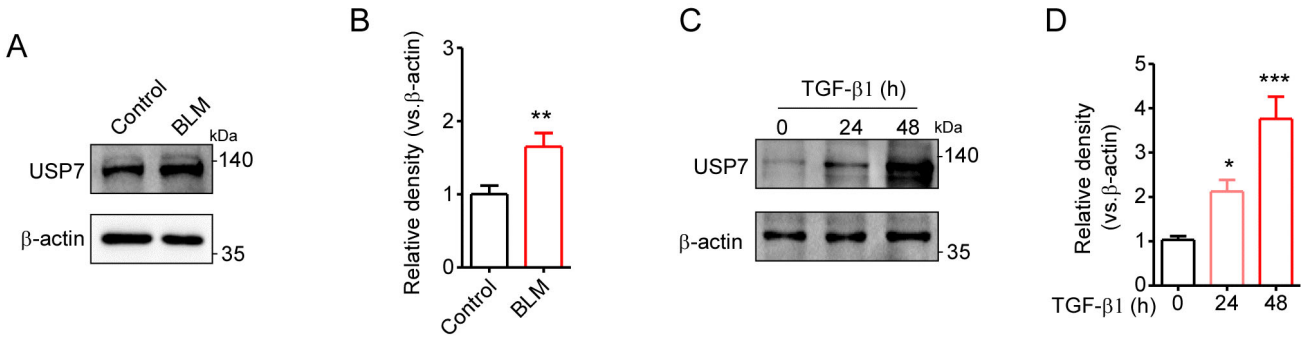

Fig. 1. USP7 expression was found to be increased in murine lung tissue induced by Bleomycin (BLM) and in Beas-2B cells stimulated with TGF-β. (A) Analysis of representative immunoblot for USP7 protein expression in BLM-induced murine lung tissue. (B) Quantitative analysis of USP7 relative protein levels, n = 6. (C) Analysis of representative immunoblot for USP7 protein level in Beas-2B cells exposed to TGF-β1 (10 ng/mL). (D) Quantification of the relative USP7 protein level, n = 3. The data are expressed as the mean $\pm$ SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. TGF-β, transforming growth factor-β; USP7, ubiquitin-specific protease 7.

H&E Staining

The fixed lung tissue was embedded in paraffin and sectioned into 3-µm thickness sections along the transverse axis. The sections were placed onto slides and immersed in filtered Harris Hematoxylin for 10 s. Following gentle washing with tap water, the sections were stained for 30 s with Eosin Y solution. The slides were then rinsed thoroughly with tap water and dehydrated in ascending alcohol solutions (50%, 70%, 80%, 95%, 95%, 100%, 100%) before being cleared with xylene. Subsequently, the serial sections were mounted, and observations were made using an optical microscope.

Masson's Trichrome Staining

The lung slice underwent deparaffinization and rehydration, followed by staining with Weigert's iron hematoxylin solution for 10 min. After washing in running tap water and rinsing in distilled water, the slide was immersed in a solution of Biebrich scarlet-acid fuchsin for 15 min and then treated with a phosphomolybdic-phosphotungstic acid solution for 10 min to achieve differentiation. Once the red color faded from collagen-rich regions, as observed with the naked eye, the section was directly immersed in aniline blue solution and stained for a duration of 5 min. The slide was subsequently washed with distilled water and subjected to differentiation in a 1% acetic acid solution for a duration of 5 min. Rapid dehydration of the slide was carried out through 95% alcohol and then absolute alcohol to remove Biebrich scarlet-acid fuchsin staining. Finally, the slide was immersed in xylene for clearing and subsequently mounted with a resinous medium.

Statistical Analysis

The data were presented in the form of means $\pm$ standard deviation. Utilizing Pearson's correlation coefficient to analyze correlations between two groups. Student's *t*-test was employed for the comparison of two groups. For the purpose of comparing multiple groups, we performed a one-way Analysis of Variance (ANOVA) followed by Tukey's test. Data were collected from a minimum of three separate experiments. A significance level at $p < 0.05$ was indicative of statistical significance.

Results

The Expression of USP7 Increases in Pulmonary Fibrosis

In this study, pulmonary fibrosis was induced in C57BL/6J male mice through tracheal instillation of Bleomycin (BLM) to establish a model. Western blot analysis was conducted to measure USP7 protein expression in lung tissue. As illustrated in Fig. 1A, the upregulation of USP7 expression was notably observed in the pulmonary tissue of mice afflicted with bleomycin-induced pulmonary fibrosis (Fig. 1A,B). Furthermore, considering the role of TGF-β1 as a master regulator of fibrosis [18], we explored the possibility of TGF-β1 inducing the expression of USP7 in lung epithelial cells. Our findings demonstrated that the expression of USP7 was upregulated in Beas-2B cells upon stimulation with TGF-β1 (Fig. 1C,D). Therefore, these results indicate that USP7 could potentially function as a regulator of TGF-β signaling or IPF.

USP7 Promotes TGF-β Signaling

Given the crucial importance of TGF-β signaling in IPF [18], we investigated whether USP7 could affect TGF-β signaling in lung epithelial cells using SBE luciferase (SBE-luc) reporter assay, a synthetic TGF-β-responsive reporter gene reliant on Smad2/3 activation. As depicted in Fig. 2A, USP7 overexpression increased SBE-luc activity upon TGF-β1 stimulation, while exhibiting no effect without TGF-β1 stimulation ($p > 0.05$). Furthermore, USP7 could promote Smad2/3 expression and phosphorylation, consequently augmenting the levels of fibrotic proteins fi-

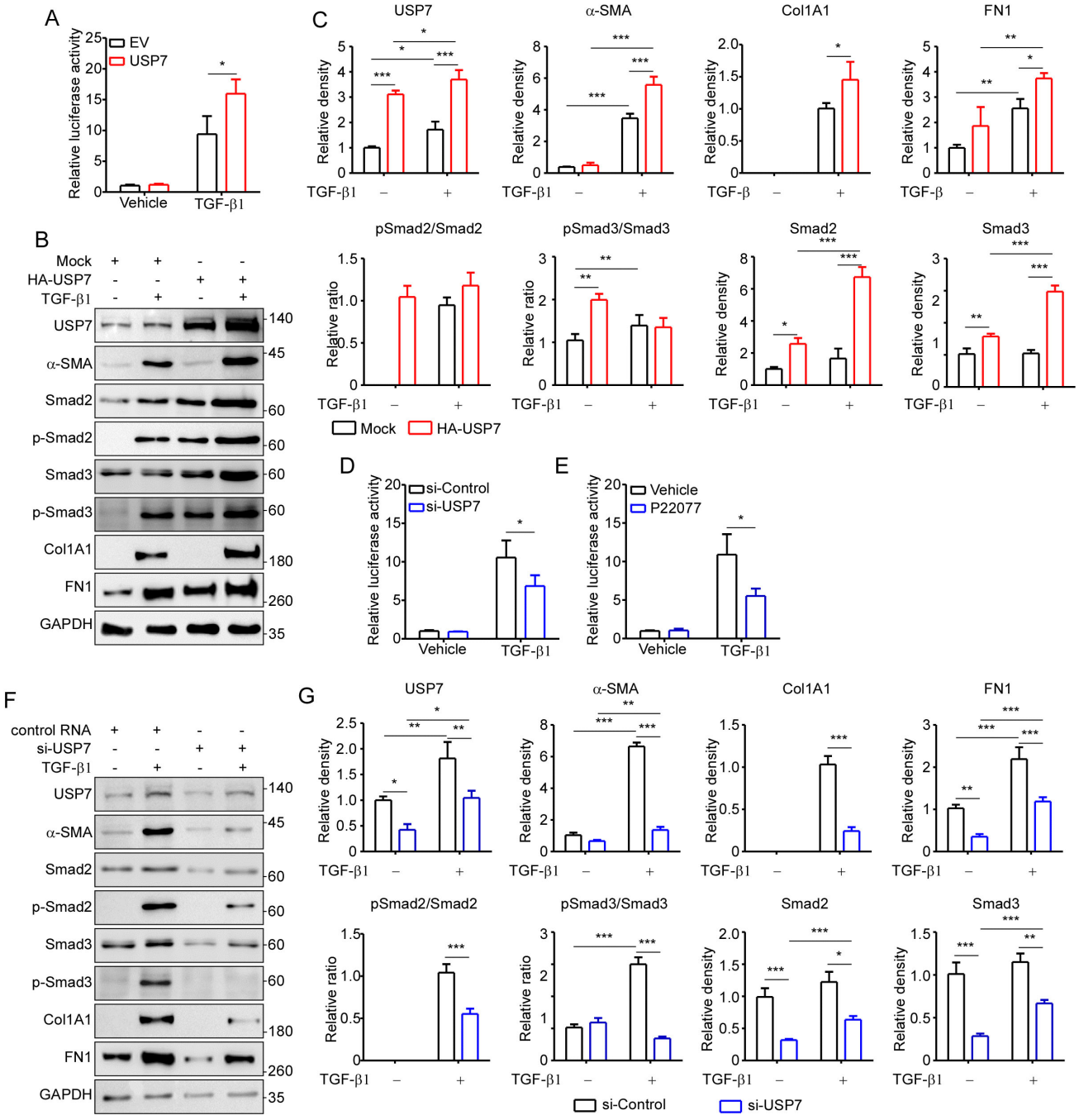

Fig. 2. USP7 positively regulates TGF-β signaling in Beas-2B cells. (A) Beas-2B cells were transfected with HA-USP7 and SBE-luc reporter. After 16 h treatment with TGF-β1 at a concentration of 10 ng/mL, luciferase activity was assessed, n = 6. (B) Representative western blot analysis of indicated protein levels in Beas-2B cells stably expressed HA-USP7 and exposed to a concentration of 10 ng/mL of TGF-β1 for a duration of 24 hours. (C) Quantification of the relative protein levels, n = 3. (D) Beas-2B cells were subjected to transfection with si-USP7 and SBE-luc reporter, luciferase activity was assessed following a 16 h of TGF-β1 (10 ng/mL) treatment, n = 6. (E) Beas-2B cells were subjected to transfection with SBE-luc reporter. After pretreatment with P22077 for 2 h, cells were induced with TGF-β1 (10 ng/mL) for 16 h, and then luciferase activity was measured, n = 6. (F) Representative western blot analysis of indicated protein levels in Beas-2B cells knocked down USP7 with or without treatment of TGF-β1. (G) Quantification of the relative protein levels, n = 3. The data are expressed as the mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. α-SMA, α-smooth muscle actin; Col1A1, Collagen Type I Alpha 1 Chain; SBE, Smad-binding elements; SBE-luc, SBE luciferase; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, Hemagglutinin; Smad2, Smad family member 2.

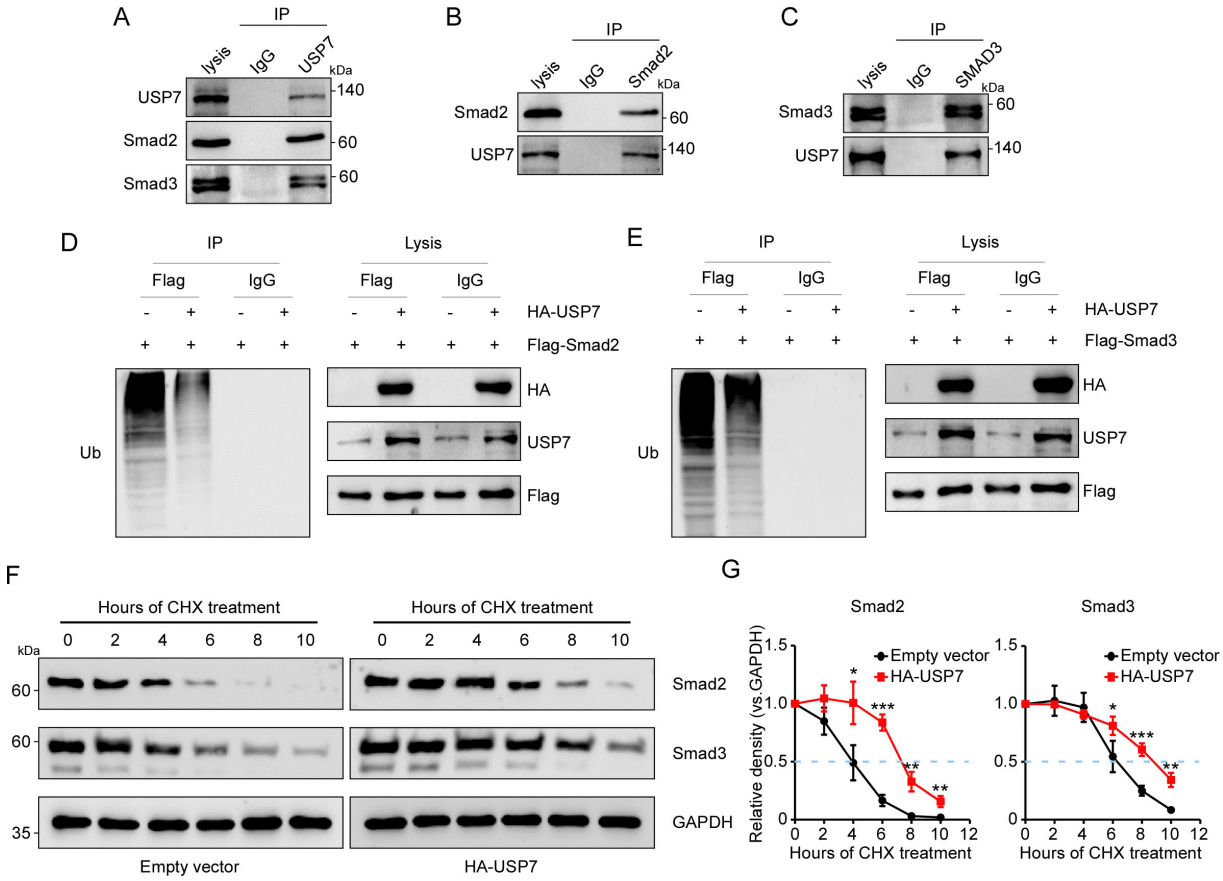

Fig. 3. USP7 interacts with and deubiquitinates Smad2/3. (A–C) Endogenous interaction among USP7, Smad2 and Smad3 was demonstrated in Beas-2B cells by performing co-immunoprecipitation (co-IP) assays with anti-USP7 or anti-Smad2 or Smad3 antibody. (D,E) HEK293T cells were transfected with both HA-USP7 and either Flag-Smad2 or Flag-Smad3 for 36 h. Following this, the cells were exposed to MG132 (20 µmol/L) for 6 hours prior to collection. Cell lysates underwent immunoprecipitation with an anti-Flag antibody, followed by analysis of Smad2/Smad3 ubiquitination through immunoblotting with a ubiquitin antibody. (F) Assessment of Smad2 and Smad3 protein stability in Beas-2B cells was carried out through Western blot analysis. Beas-2B cells were subjected to transfection with HA-USP7 or empty vector for 36 h, and then exposed to CHX (100 µg/mL) for indicated times. (G) The half-life of Smad2 and Smad3 in Beas-2B cells was quantified by density recorded via Western blot, n = 3. The data are expressed as the mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

bronectin (FN), Collagen Type I Alpha 1 Chain (Col1A1), and α-smooth muscle actin (α-SMA) in Beas-2B cells ($p < 0.05$) (Fig. 2B,C). However, TGF-β1 does not affect the expression of Smad2 or Smad3, and USP7 does not affect these profibrotic proteins without the presence of TGF-β1 stimulation. ($p > 0.05$) (Fig. 2C). After USP7 knockdown using shRNAs, with shRNA-control serving as a negative control, SBE-luc activity significantly decreased upon TGF-β1 stimulation ($p < 0.05$), while no change in activity was observed without the presence of TGF-β1 stimulation ($p > 0.05$) (Fig. 2D). P22077, a potent and selective USP7-inhibitor [19], also decreased SBE-luc activity ($p < 0.05$) (Fig. 2E). USP7 knockdown decreased Smad2/3 expression and phosphorylation (Fig. 2F), and blocked the fibrotic proteins FN, Col1A1, and α-SMA in Beas-2B cells ($p < 0.05$) (Fig. 2G). Therefore, USP7 can promote TGF-β signaling in lung epithelial cells.

USP7 Interacts with and Deubiquitinates Smad2/3

We proceeded to investigate the molecular mechanism through which USP7 promotes TGF-β/Smad signaling. Given USP7's role as a DUB and its impact on Smad2/3 protein expression, we hypothesized that USP7 might deubiquitnate Smad2/3. Initially, we examined the interaction between these proteins. As shown in Fig. 3A, both Smad2 and Smad3 were found in the immunoprecipitates of USP7 in Beas-2B cells. This finding was further validated through reciprocal immunoprecipitation experiments using anti-Smad2 or Smad3 antibodies, followed by detection of USP7 via Western blot analysis. The results showed that USP7 was also detected in the Smad2 or Smad3 interacting proteins from Beas-2B cells (Fig. 3B,C). Subsequently, we investigated whether USP7 affects the ubiquitination level of Smad2 or Smad3. As shown in Fig. 3D,E, the polyubiquitin chain attached to Smad2/3 decreased af-

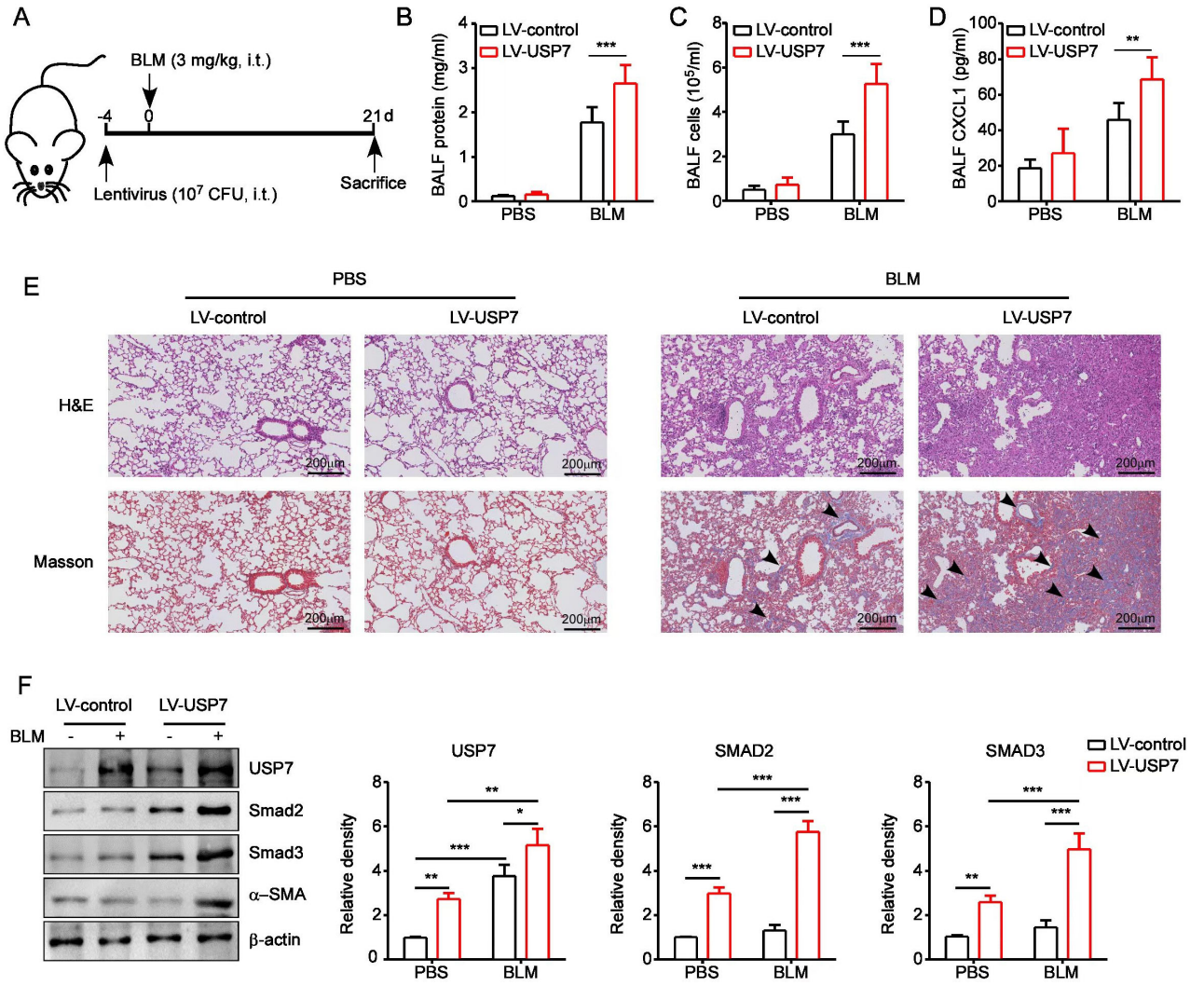

Fig. 4. Effects of USP7 on BLM-induced lung fibrotic lesions. (A) Mice were intratracheally administered lentivirus containing USP7. After 4 days, BLM was administered intratracheally to mice on day 0, and lung fibrosis was permitted to progress over the next 21 days. The preparation included bronchoalveolar lavage fluid (BALF) samples, lung lysates, and tissue sections embedded in paraffin. (B) Soluble protein concentration in BALF was quantified by BCA assay on day 21, n = 6. (C) Total cells in BALF were determined on day 21, n = 6. (D) The CXC chemokine ligand 1 (CXCL1) level in BALF was detected by Enzyme-Linked Immunosorbent Assay (ELISA) on day 21, n = 6. (E) Lung tissue samples fixed in paraffin were subjected to staining with hematoxylin & eosin (H&E) or Masson's trichrome for the assessment of injury or collagen deposition. (F) Representative immunoblotting analysis of indicated protein levels in BLM-induced murine lung tissue on day 21. ImageJ software (Version 1.52p, National Institutes of Health, Bethesda, MD, USA) was utilized to quantify the relative protein levels. The data are expressed as the mean ± SD, n = 6, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

ter USP7 overexpression, suggesting that USP7 regulates Smad2/3 deubiquitination. Smad2 and Smad3 can be modified by polyubiquitination and degraded via the proteasome pathway [8]. To assess whether USP7-mediated deubiquitination affects the stability of Smad2 and Smad3, we evaluated their half-life in Beas-2B cells exposed to the protein synthesis inhibitor CHX. Our findings indicate that USP7 significantly prolongs the half-life of Smad2 and Smad3 ($p < 0.05$) (Fig. 3F,G). In conclusion, these results indicate that USP7 interacts with Smad2/3 and reduces its polyubiquitination levels, thereby exerting an important regulatory role in protein stability.

USP7 Exacerbates BLM-Induced Pulmonary Fibrosis in Vivo

Given the ubiquitous activation of TGF-β signaling, which is crucially implicated in fibrosis induction [20], our findings suggest that USP7 may exacerbate fibrotic lung injury. To verify the role of USP7 in IPF *in vivo*, we utilized a BLM-induced pulmonary fibrosis mouse model, widely recognized as a reliable model for IPF research. Wild-type mice were injected with either control lentivirus or lentivirus encoding USP7 for 4 days before being challenged with BLM (Fig. 4A). On day 21, the mice were eu-

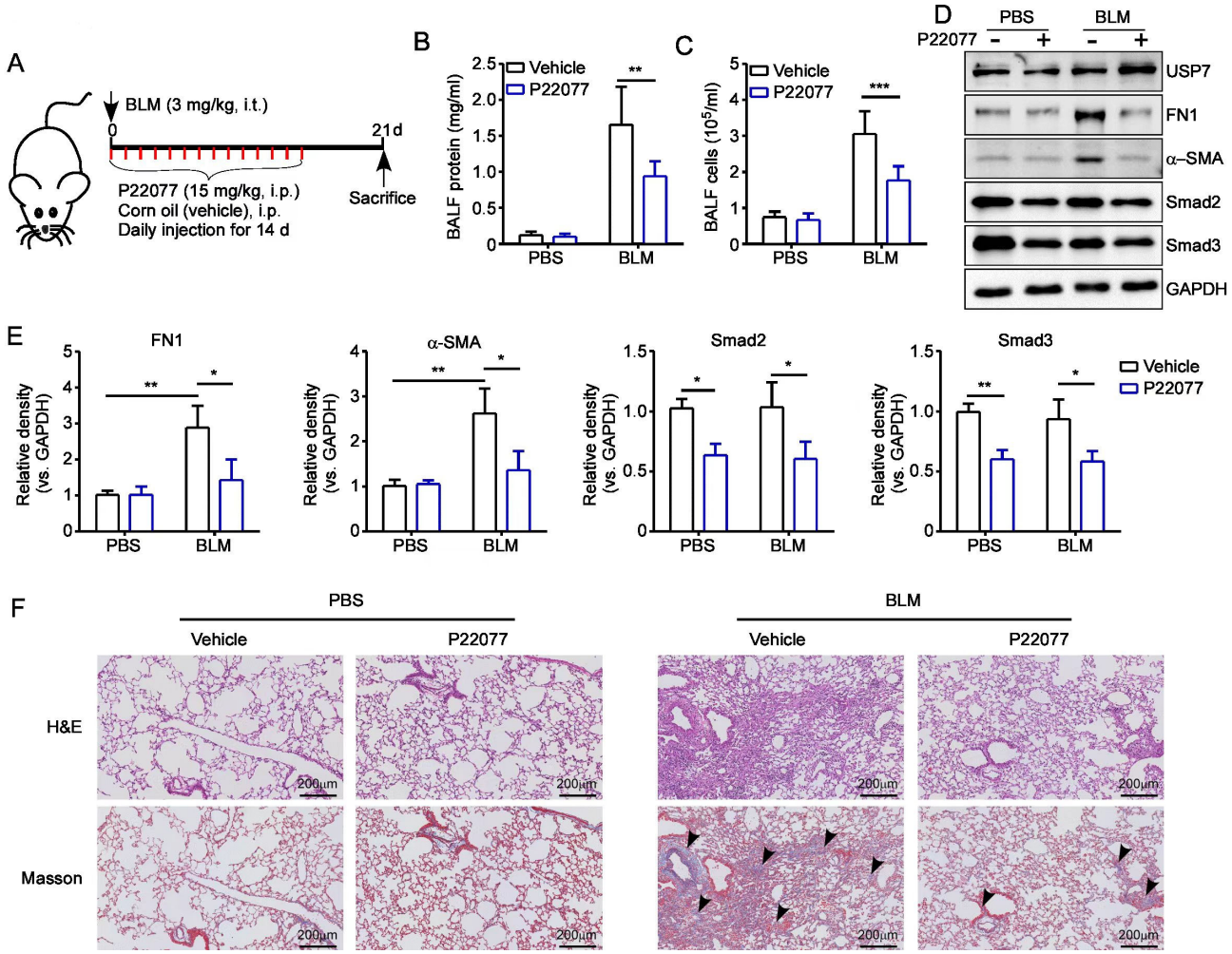

Fig. 5. Effects of P22077 on BLM-induced IPF. (A) BLM was delivered intratracheally to mice on day 0, with lung fibrosis being allowed to progress for 21 days. P22077 (15 mg/kg) or corn oil (negative control) was intraperitoneally injected (i.p.) into mice every day for 14 days. BALFs, lung lysates, and tissue paraffin sections were prepared. (B) The protein concentration and (C) total cells in BALF were quantified by BCA assay on day 21, n = 6. (D) The presentative western blot analysis of indicated protein levels in BLM-induced murine lung tissue on day 21. (E) Quantification of the relative protein levels. The data are expressed as the mean ± SD, n = 6. (F) The paraffin sections of lung tissues were stained with H&E or Masson's trichrome, and then observed by microscopy at 20× magnification. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. IPF, idiopathic pulmonary fibrosis.

thanized to analyze fibrotic factors. As shown in Fig. 4B, mice overexpressing USP7 exhibited a notably higher concentration of total protein in bronchoalveolar lavage fluid (BALF) compared to control mice following BLM-induced injury. Additionally, mice overexpressing USP7 also demonstrated significantly increased immune cell infiltration and CXC chemokine ligand 1 (CXCL1) levels in BALF ($p < 0.05$) (Fig. 4C,D). Masson's Trichrome staining further revealed enhanced lung collagen deposition, indicating that USP7 overexpression worsens bleomycin-induced lung fibrosis (Fig. 4E). Moreover, USP7 increased the expression of Smad2 and Smad3, consistent with our *in vitro* study ($p < 0.01$, $p < 0.001$) (Fig. 4F).

Inhibition of USP7 Alleviated BLM-Induced Lung Fibrosis

Given the important role of USP7 in IPF, we sought to investigate the therapeutic potential of its inhibitor. P22077 is a selective inhibitor of USP7 [19], and it has demonstrated significant anti-inflammatory effects [21]. We aimed to determine if treating mice with the inhibitor P22077 could reduce BLM-induced lung fibrosis. Wild-type mice were intratracheally infused with BLM at a dosage of 3 mg/kg. Subsequently, these mice were intraperitoneally injected with either the USP7 inhibitor P22077 (100 mg/kg) ($n = 6$) or corn oil (vehicle) ($n = 6$) as a control daily from day one to day 14 (Fig. 5A). The mice injected with corn oil exhibited higher levels of protein and cells in their lung fluid because of the lung injury caused

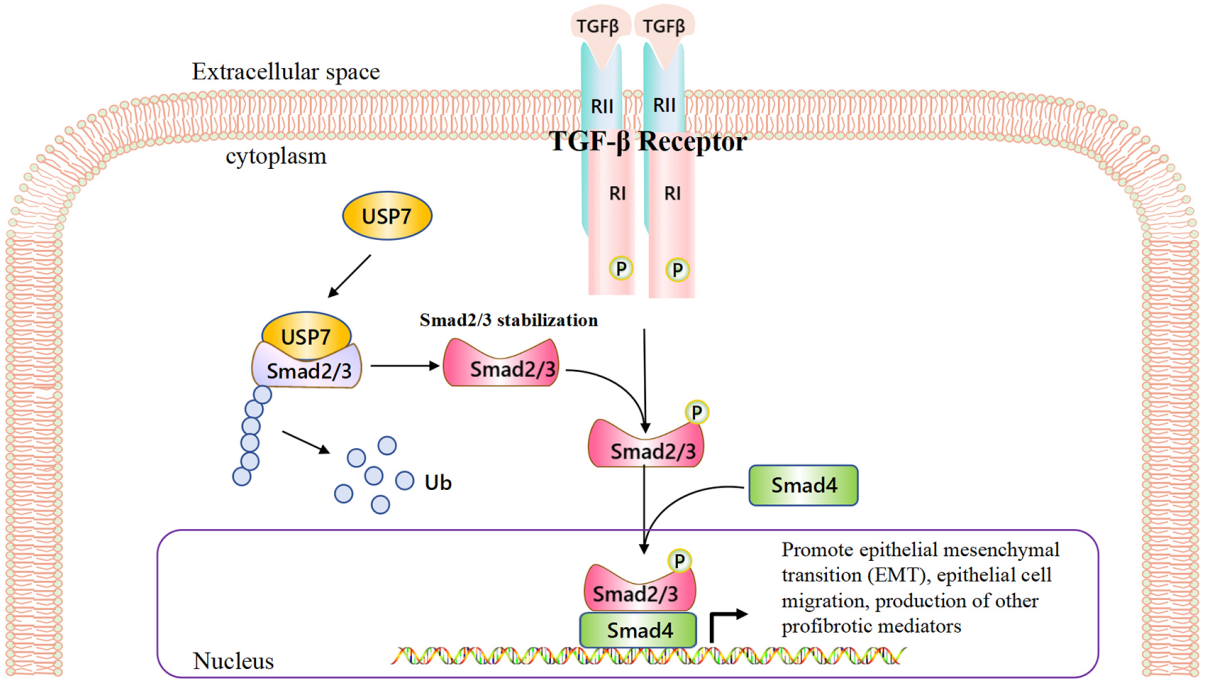

Fig. 6. A proposed model of USP7 deubiquitinating Smad2 and Smad3 in IPF. Upregulation of USP7 expression in IPF leads to the deubiquitination of Smad2 and Smad3, thereby stabilizing Smad2 and Smad3 and promoting TGF-β1 signaling pathway activation, ultimately leading to IPF progression. Microsoft PowerPoint software (Microsoft Office 2019, Microsoft, Redmond, WA, USA) was used to draw this image.

by BLM ($p < 0.001$) (Fig. 5B,C). Interestingly, the mice treated with P22077 had lower levels of protein and cells, suggesting that the treatment helped reverse the effects of BLM (Fig. 5B,C). Pulmonary epithelial cells can turn into myofibroblast cells through a process called EMT, which can contribute to pulmonary fibrosis [22]. However, when we treated the mice with P22077, the increase in FN-1 and α-SMA was reduced (Fig. 5D,E). These findings suggest that P22077 can reduce BLM-induced EMT. We also observed the expression of Smad2/3, finding that P22077 inhibited its expression, whether or not the mice were treated with BLM ($p < 0.05$) (Fig. 5D,E). This finding suggests that P22077 can prevent the abnormal activation of Smad2/3, which may be linked to the reduction in treatment-induced pulmonary fibrosis. Additionally, when we examined the lung tissue using H&E and Masson staining (Fig. 5F), we observed that P22077 helped mitigate structural damage and collagen buildup caused by BLM, supporting the conclusion that inhibiting USP7 can delay the progression of pulmonary fibrosis.

Discussion

Our research demonstrates that USP7 promotes TGF-β1 signaling by interacting with and deubiquinating Smad2/3, suggesting that USP7 may be an efficient drug target for IPF. Smad2 and Smad3 are important for canonical TGF-β1 signaling and controlling fibrosis by activating gene expression [18]. Previous studies have shown that Smad2 and Smad3 can be ubiquitinated by different ubiquitin E3 ligases [23,24]. Ubiquitination is a process that can be reversed by DUBs [25]. Lina Herhaus *et al.* [26] discovered that ovarian tumor (OTU) domain-containing ubiquitin aldehyde binding protein 1 (OTUB1) can reduce polyubiquitination of Smad2/3 by inhibiting the E2 ubiquitin-conjugating enzymes, independent of its own enzyme activity. More recently, it was found that UCHL5 can stabilize Smad2 and Smad3 by de-ubiquitination and promoting TGF-β1 signaling [27]. Our study found that USP7 levels increase within the lungs of mice following bleomycin challenge, and that it can regulate the de-ubiquitination and stability of Smad2/3. This research suggests that USP7 inhibition could be a potential treatment for lung fibrosis.

In recent years, USP7 has gained significant attention from researchers given its role in cell growth regulation, DNA damage response, tumor development, apoptosis, and inflammation by controlling specific substrates like mouse double minute 2 homolog (MDM2) /P53, forkhead box protein O4 (FOXO4), and others [28]. Recently, studies found that USP7 may be involved in cardiomyocyte injury or myocardial injury [29,30]. Moreover, USP7 directly regulates

checkpoint kinase 1 (CHK1) by cleaving the polyubiquitination chain which exerts an essential role in renal fibrosis [31]. To date, the function of USP7 in pulmonary fibrosis is not fully understood. Our data reveal that USP7 can mediate Smad2/ Smad3 deubiquitination to regulate the pathogenesis of IPF.

Both Smad2 and Smad3 are TGF-β signaling molecules. Their ubiquitylations are important for regulating the strength and duration of TGF-β signaling [32]. Degradation of polyubiquitinated Smad2/Smad3 occurs via the lysosome-autophagy pathway, which can be suppressed by ubiquitin carboxyl-terminal hydrolase-L5 (UCHL5) [27]. UCHL5 deubiquitinates Smad2/Smad3 regardless of TGF-β treatment to increase TGF-β signaling and pulmonary fibrosis [27]. Interestingly, OTUB1, another DUB, only regulates phosphorylated Smad2/Smad3 under TGF-β treatment by regulating ubiquitin E2 conjugating-enzyme activity [26]. In our investigation, we identified that USP7 interacts with and deubiquitinates Smad2/Smad3, thereby enhancing TGF-β signaling, which contributes to the regulation of IPF.

Given its involvement in various cellular pathways and the association of abnormal deubiquitination of its downstream proteins causes diseases, USP7 has been identified as an innovative biomarker and a target for drug development [33]. Although there are no USP7 inhibitors on the market currently, advancements in the development of these inhibitors for clinical purposes have been noteworthy. Notably, P22077 is a specific selective USP7 inhibitor and can inhibit the growth of many cancers [19, 34–37]. Recently, Zhao XB *et al.* [21] reported that P22077 demonstrates substantial anti-inflammatory properties by promoting K48-linked ubiquitination and subsequent degradation of TRAF6 to suppress NF-κb and MAPK pathways. Moreover, P22077 was found to attenuate cardiac hypertrophy, cardiac fibrosis, oxidase stress, and inflammation through the inhibition of various signaling pathways [38]. The present study demonstrates that P22077 can reduce Smad2/3 protein levels and alleviate pulmonary fibrosis.

This study is not without its limitations. For instance, further clarification is needed regarding the precise ubiquitination sites and types of modifications on Smad2 and Smad3 influenced by USP7. Furthermore, creating conditional knockout USP7 mice in various cell types is crucial to determine which lung cells contribute significantly to pulmonary fibrosis.

Conclusions

In summary, our findings demonstrate that USP7 plays a regulatory role in TGF-β1 signaling through binding and deubiquitinating Smad2/Smad3. Importantly, this study reveals that the regulation of TGF-β1 signaling by USP7 is critical for the pathogenesis of IPF (Fig. 6). Thus, our study may provide opportunities for potential therapeutic interventions in IPF targeting excess TGF-β1 signaling in IPF.

Availability of Data and Materials

The source data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

FT and ZL conceived and designed this project, wrote the original draft, data curation, and performed experiments. HG, TK, WY and YY contributed to the laboratory work for the study and formal analysis and have been involved in drafting the manuscript or revising it critically for important intellectual content. All authors read and approved the final version submitted. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

All animal procedures were approved by The Institutional Animal Care and Use Committee of The First Affiliated Hospital of Nanchang University (No. CDYFY-IACUC-202302QR085) and performed in accordance with the ARRIVE guideline. All methods were carried out in accordance with relevant guidelines and regulations.

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] Saha P, Talwar P. Idiopathic pulmonary fibrosis (IPF): disease pathophysiology, targets, and potential therapeutic interventions. Molecular and Cellular Biochemistry. 2023. (online ahead of print)

[2] Saito S, Alkhatib A, Kolls JK, Kondoh Y, Lasky JA. Pharmacotherapy and adjunctive treatment for idiopathic pulmonary fibrosis (IPF). Journal of Thoracic Disease. 2019; 11: S1740–S1754.

[3] Ptasinski VA, Stegmayr J, Belvisi MG, Wagner DE, Murray

LA. Targeting Alveolar Repair in Idiopathic Pulmonary Fibrosis. American Journal of Respiratory Cell and Molecular Biology. 2021; 65: 347–365.

[4] Bonella F, Spagnolo P, Ryerson C. Current and Future Treatment Landscape for Idiopathic Pulmonary Fibrosis. Drugs. 2023; 83: 1581–1593.

[5] Salton F, Volpe MC, Confalonieri M. Epithelial–Mesenchymal Transition in the Pathogenesis of Idiopathic Pulmonary Fibrosis. Medicina (Kaunas, Lithuania). 2019; 55: 83.

[6] Budi EH, Schaub JR, Decaris M, Turner S, Derynck R. TGF-β as a driver of fibrosis: physiological roles and therapeutic opportunities. The Journal of Pathology. 2021; 254: 358–373.

[7] Fernandez IE, Eickelberg O. The impact of TGF-β on lung fibrosis: from targeting to biomarkers. Proceedings of the American Thoracic Society. 2012; 9: 111–116.

[8] Imamura T, Oshima Y, Hikita A. Regulation of TGF-β family signalling by ubiquitination and deubiquitination. Journal of Biochemistry. 2013; 154: 481–489.

[9] Li S, Zhao J, Shang D, Kass DJ, Zhao Y. Ubiquitination and deubiquitination emerge as players in idiopathic pulmonary fibrosis pathogenesis and treatment. JCI Insight. 2018; 3: e120362.

[10] Lange SM, Armstrong LA, Kulathu Y. Deubiquitinases: From mechanisms to their inhibition by small molecules. Molecular Cell. 2022; 82: 15–29.

[11] Komander D, Clague MJ, Urbé S. Breaking the chains: structure and function of the deubiquitinases. Nature Reviews. Molecular Cell Biology. 2009; 10: 550–563.

[12] Pozhidaeva A, Bezsonova I. USP7: Structure, substrate specificity, and inhibition. DNA Repair. 2019; 76: 30–39.

[13] Wang Z, Kang W, You Y, Pang J, Ren H, Suo Z, *et al*. USP7: Novel Drug Target in Cancer Therapy. Frontiers in Pharmacology. 2019; 10: 427.

[14] Li Y, Zhang Q, Li N, Ding L, Yi J, Xiao Y, *et al*. Ataxin-10 Inhibits TNF-α-Induced Endothelial Inflammation via Suppressing Interferon Regulatory Factor-1. Mediators of Inflammation. 2021; 2021: 7042148.

[15] Huang Y, Chen T, Jiang M, Xiong C, Mei C, Nie J, *et al*. E3 ligase TRIM65 alleviates intestinal ischemia/reperfusion injury through inhibition of TOX4-mediated apoptosis. Cell Death & Disease. 2024; 15: 29.

[16] Walters DM, Kleeberger SR. Mouse models of bleomycin-induced pulmonary fibrosis. Current Protocols in Pharmacology. 2008; Chapter 5: Unit 5.46.

[17] Dong Y, Geng Y, Li L, Li X, Yan X, Fang Y, *et al*. Blocking follistatin-like 1 attenuates bleomycin-induced pulmonary fibrosis in mice. The Journal of Experimental Medicine. 2015; 212: 235–252.

[18] Meng XM, Nikolic-Paterson DJ, Lan HY. TGF-β: the master regulator of fibrosis. Nature Reviews. Nephrology. 2016; 12: 325–338.

[19] Altun M, Kramer HB, Willems LI, McDermott JL, Leach CA, Goldenberg SJ, *et al*. Activity-based chemical proteomics accelerates inhibitor development for deubiquitylating enzymes. Chemistry & Biology. 2011; 18: 1401–1412.

[20] Györfi AH, Matei AE, Distler JHW. Targeting TGF-β signaling for the treatment of fibrosis. Matrix Biology: Journal of the International Society for Matrix Biology. 2018; 68-69: 8–27.

[21] Zhao XB, Ji FY, Li HR, Zhu HH, Zhao ZZ, Ling J, *et al*. P22077 inhibits LPS-induced inflammatory response by promoting K48-linked ubiquitination and degradation of TRAF6. Aging. 2020; 12: 10969–10982.

[22] Ye Z, Hu Y. TGF β1: Gentlemanly orchestrator in idiopathic pulmonary fibrosis (Review). International Journal of Molecular Medicine. 2021; 48: 132.

[23] Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, *et al*. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. Molecular Cell. 2000; 6: 1365–1375.

[24] Fukuchi M, Imamura T, Chiba T, Ebisawa T, Kawabata M, Tanaka K, *et al*. Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins. Molecular Biology of the Cell. 2001; 12: 1431–1443.

[25] Wilkinson KD. Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. Seminars in Cell & Developmental Biology. 2000; 11: 141–148.

[26] Herhaus L, Al-Salihi M, Macartney T, Weidlich S, Sapkota GP. OTUB1 enhances TGFβ signalling by inhibiting the ubiquitylation and degradation of active SMAD2/3. Nature Communications. 2013; 4: 2519.

[27] Nan L, Jacko AM, Tan J, Wang D, Zhao J, Kass DJ, *et al*. Ubiquitin carboxyl-terminal hydrolase-L5 promotes TGFβ-1 signaling by de-ubiquitinating and stabilizing Smad2/Smad3 in pulmonary fibrosis. Scientific Reports. 2016; 6: 33116.

[28] Zhou L, Ouyang T, Li M, Hong T, Mhs A, Meng W, *et al*. Ubiquitin-Specific Peptidase 7: A Novel Deubiquitinase That Regulates Protein Homeostasis and Cancers. Frontiers in Oncology. 2021; 11: 784672.

[29] Xue Q, Yang D, Zhang J, Gan P, Lin C, Lu Y, *et al*. USP7, negatively regulated by miR-409-5p, aggravates hypoxia-induced cardiomyocyte injury. APMIS: Acta Pathologica, Microbiologica, et Immunologica Scandinavica. 2021; 129: 152–162.

[30] Xu Q, Liu M, Gu J, Ling S, Liu X, Luo Z, *et al*. Ubiquitin-specific protease 7 regulates myocardial ischemia/reperfusion injury by stabilizing Keap1. Cell Death Discovery. 2022; 8: 291.

[31] Shao Y, Zhang W, Du D, Yu Y, Li Q, Peng X. Ubiquitin-like protein FAT10 promotes renal fibrosis by stabilizing USP7 to prolong CHK1-mediated G2/M arrest in renal tubular epithelial cells. Aging. 2022; 14: 7527–7546.

[32] Al-Salihi MA, Herhaus L, Sapkota GP. Regulation of the transforming growth factor β pathway by reversible ubiquitylation. Open Biology. 2012; 2: 120082.

[33] Li P, Liu Y, Liu HM. A patent review of ubiquitin-specific protease 7 (USP7) inhibitors (2014-present). Expert Opinion on Therapeutic Patents. 2022; 32: 753–767.

[34] Fan YH, Cheng J, Vasudevan SA, Dou J, Zhang H, Patel RH, *et al*. USP7 inhibitor P22077 inhibits neuroblastoma growth via inducing p53-mediated apoptosis. Cell Death & Disease. 2013; 4: e867.

[35] Zhang C, Lu J, Zhang QW, Zhao W, Guo JH, Liu SL, *et al*. USP7 promotes cell proliferation through the stabilization of Ki-67 protein in non-small cell lung cancer cells. The International Journal of Biochemistry & Cell Biology. 2016; 79: 209–221.

[36] Zhang W, Zhang J, Xu C, Zhang S, Bian S, Jiang F, *et al*. Ubiquitin-specific protease 7 is a drug-able target that promotes hepatocellular carcinoma and chemoresistance. Cancer Cell International. 2020; 20: 28.

[37] Xiang M, Liang L, Kuang X, Xie Z, Liu J, Zhao S, *et al*. Pharmacological inhibition of USP7 suppresses growth and metastasis of melanoma cells in vitro and in vivo. Journal of Cellular and Molecular Medicine. 2021; 25: 9228–9240.

[38] Gu YH, Ren KW, Wang Y, Wang SH, Yu XH, Xu LW, *et al*. Administration of USP7 inhibitor P22077 inhibited cardiac hypertrophy and remodeling in Ang II-induced hypertensive mice. Frontiers in Pharmacology. 2022; 13: 1021361.