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P120-catenin regulates pulmonary fibrosis and TGF-β induced lung fibroblast differentiation☆

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ABSTRACT

P120-catenin (P120) was known to function in adhesion between cells and signal transduction in many types of cells. In this study, we investigated the expression and role of P120 in pulmonary fibrosis and transforming growth factor beta (TGF-β) mediated lung fibroblast-to-myofibroblast differentiation (fibroblast differentiation). Our data indicated that P120 expression increased in lung fibrotic foci and primary lung fibroblasts isolated from bleomycin- (BLM) challenged mice, compared to controls. In vitro, TGF-β induced P120 expression in human lung fibroblasts, and siRNA-mediated SMAD3 depletion inhibited TGF-β stimulated P120 expression. Blocking nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway through chemical inhibitor or knockdown of NF-kB p65 subunit also suppressed TGF-β induced P120 expression in human lung fibroblast. Knockdown of P120 expression inhibited TGF-β induced human lung fibroblast differentiation, as well as suppressed the activation of SMAD and ERK signaling pathways. Administration of lentivirus coding mouse P120 shRNA into mouse lung tissue dramatically attenuated the expression of P120 in lung tissue and lung fibroblast, suppressed BLM induced increase of TGF-β, alpha smooth muscle actin (α-SMA) and fibronectin (FN) expression, and decreased the deposition of collagen and pulmonary fibrosis. Collectively, these results suggested that P120 involved in lung fibroblast differentiation and pulmonary fibrosis, and inhibition of P120 expression decreased pulmonary fibrosis in BLM challenged mice. Thus, attenuation of P120 expression might be a potential therapeutic strategy for human lung fibrosis.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease, resulted in scarring (fibrosis) tissue growing inside of lung tissue with unknown reasons [1]. There are ~50,000 people in the U.S. who is suffering in IPF, and every year > 15,000 new cases of IPF are identified [2]. Although a lot of progressions have been archived in the last two decades, half of IPF patients will die in 5 years after the first time diagnosis due to unknown mechanism and limited treatments [3]. Recently, two drugs (Nintedanib and Pirfenidone) have been approved by FDA to treat IPF patients [4], which still have strong limitations due to weak effects and various side effects. Thus, it is critical to investigate the detail pathological mechanism of pulmonary fibrosis and to identify the effective therapeutic targets to IPF.

Accumulated evidences indicated that lung epithelial cell damage [5], fibroblasts differentiation and accumulation into the lung injury foci [6], involved in the development of pulmonary fibrosis in both human and animal [7,8]. Transforming growth factor-β (TGF-β), which is known to play various physiological and pathological functions in many types of cells [9–11], has been identified as the critical factor for pulmonary fibrosis mainly through regulating the differentiation and proliferation of fibroblasts [8]. During the differentiation of fibroblast

Abbreviation: NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; α-SMA, alpha smooth muscle actin; FN, fibronectin; P120, P120-catenin; TGF-β, transforming growth factor beta; BLM, bleomycin; IPF, Idiopathic pulmonary fibrosis; ECM, extracellular matrix proteins; VILI, ventilation induced lung injury; HRP, horseradish peroxidase; BSA, bovine serum albumin; FBS, fetal bovine serum; fibroblast differentiation, fibroblast-to-myofibroblast differentiation; Veh, Vehicle

☆ The authors have no conflicts of interest to declare.

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Table 1
Primers for *RT-qPCR*.

Primer	Sequence
Human SMAD3 forward	5′-TGGACGCAGGTTCTCCAAAC-3′
Human SMAD3 reverse	5′-CCGGCTCGCAGTAGGTAAC-3′
Human GAPDH forward	5′-TGTGGGCATCAATGGATTTGG-3′
Human GAPDH reverse	5′-ACACCATGTATTCCGGGTCAAT-3′
Human α-SMA forward	5′-AAAAGACAGCTACGTGGGTGA-3′
Human α-SMA reverse	5′-GCCATGTTCTATCGGGTACTTC-3′
Human FN forward	5′-TCTGTGCCTCCTATCTATGTGC-3′
Human FN reverse	5′-GAGGGACCACGACAACTCTTC-3′
Human P120 forward	5′-GTGACAACACGGACAGTACAG-3′
Human P120 reverse	5′-TTCTTGCGGAAATCACGACCC-3′
Mouse P120 forward	5′-GTGGAAACCTACACCGAGGAG-3′
Mouse P120 reverse	5′-CGTCTAGTGGTCCCATCATCTG-3′
Mouse FN forward	5′-AAGACCATACCTGCCGAATG-3′
Mouse FN reverse	5′-GAACATGACCGATTTGGACC-3′
Mouse α-SMA forward	5′-CCCAGACATCAGGGAGTAATGG-3′
Mouse α-SMA reverse	5′-TCTATCGGATACTTCAGCGTCA-3
Mouse GAPDH forward	5′-AGGTCGGTGTGAACGGATTTG-3′
Mouse GAPDH reverse	5′-GGGGTCGTTGATGGCAACA-3

to myofibroblast, TGF-β binds to its receptor complexes (type I/II receptors), localized on the cell membrane, and stimulates receptor activation resultant to the phosphorylation of SMAD-2/3 in cytosol [12]. Activated SMAD-2/3 further complexed with SMAD-4, and translocated to the nucleus where they drive the expression of target extracellular matrix proteins (ECM) during fibrogenesis via binding with SMAD binding element in the promoter [13]. It also has been proved that inhibiting NF-kB pathway or knockdown NF-kB P65 subunit also suppressed TGF-β stimulated ECM gene expression in human lung fibroblast, suggesting that TGF-β induced fibroblast differentiation also partly through NF-kB pathway [14]. TGF-β induced the expression and deposition of ECM, including collagens and fibronectin (FN), into the fibrosis foci, and continuously resulted in the destruction of alveolar units and suppress of lung functions [8]. Targeting the TGF-β induced fibroblast-to-myofibroblast differentiation (fibroblast differentiation) and accumulation of ECM proteins would be the potential therapeutic strategy to cure the pulmonary fibrosis.

P120-catenin (P120) is an adhesion junction protein, which is known to function in adhesion between cells and signal transduction in various types of cells [15,16]. P120 is highly expressed in both human and mouse lung tissues and plays various functions [17]. In the mouse model of ventilation induced lung injury (VILI), the expression of P120 was dramatically decreased in epithelial cells in a time-dependent and strain-dependent manner due to cyclic-stretch of alveolar epithelial cells [18]. In vitro studies indicated that P120 acts as a critical regulator of cell-cell adhesion by stabilizing E-cadherin through directly interaction and maintaining of E-cadherin expression at high level [19]. These investigations suggested that P120 would be a novel therapeutic target for the prevention and treatment of VILI [18]. In endothelial and macrophages, P120 was demonstrated to modulate and inhibit the development of inflammation in lung through interfering the association of TLR4 with its adaptor MyD88 resulting in the blocking of TLR4 signaling and NF-κB activation [20]. However, the expression and role

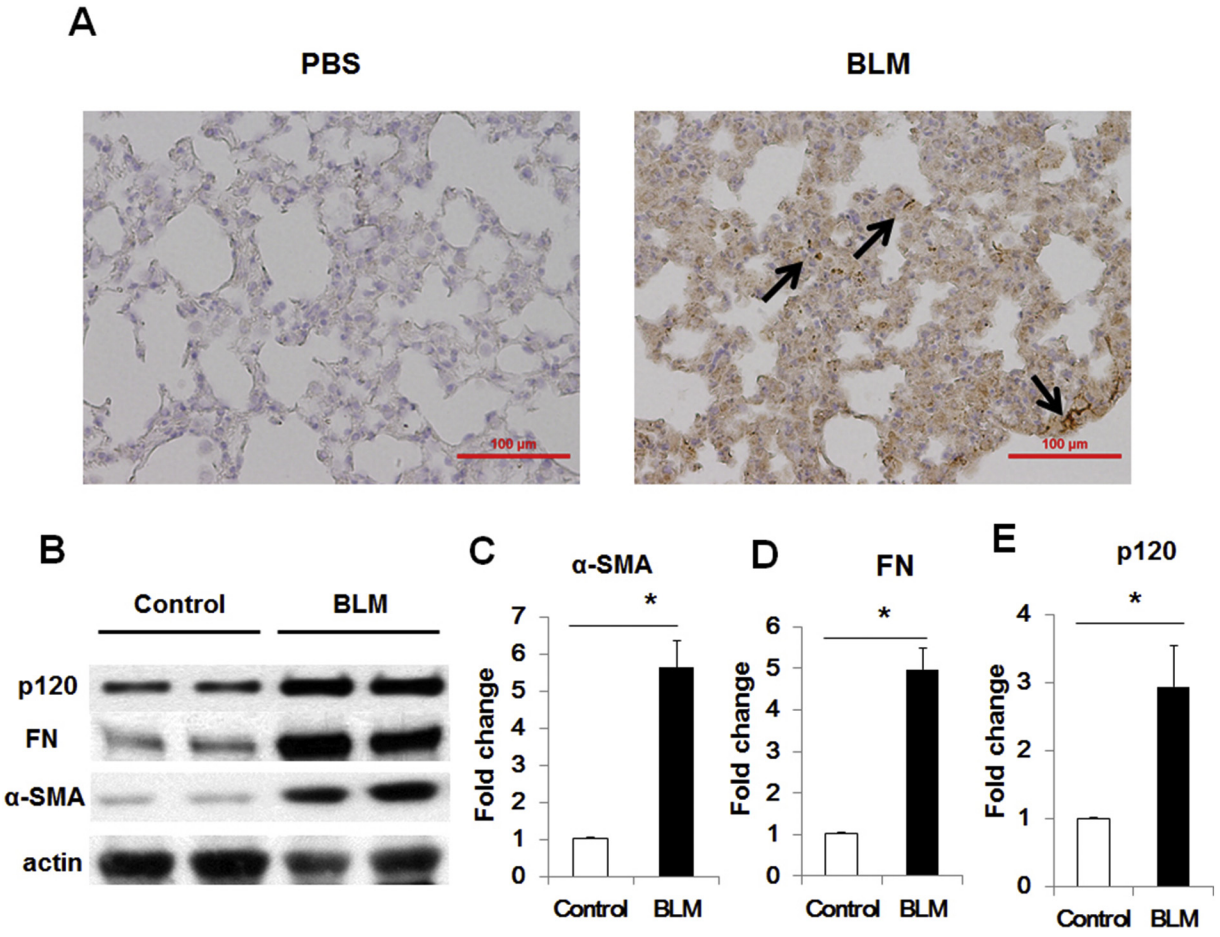

Fig. 1. P120 was up-regulated in fibrotic lung tissues from BLM challenged mice. (**A**) Representative immunohistochemical staining of P120 expression in lung tissues from mice (n = 5 per group) with or without BLM challenge (1.5 U/kg, IT, 21 days). (**B**) Representative western blot of actin, P120, FN and α-SMA expression in lung tissues from mouse with or without BLM challenge (n = 5 per group). (**C–E**) Quantification the expression of α-SMA (**C**), FN (**D**) and P120 (**E**). Protein expression was quantified and normalized with the expression of actin. Data were expressed as mean ± SEM. $^{*}P < 0.05$.

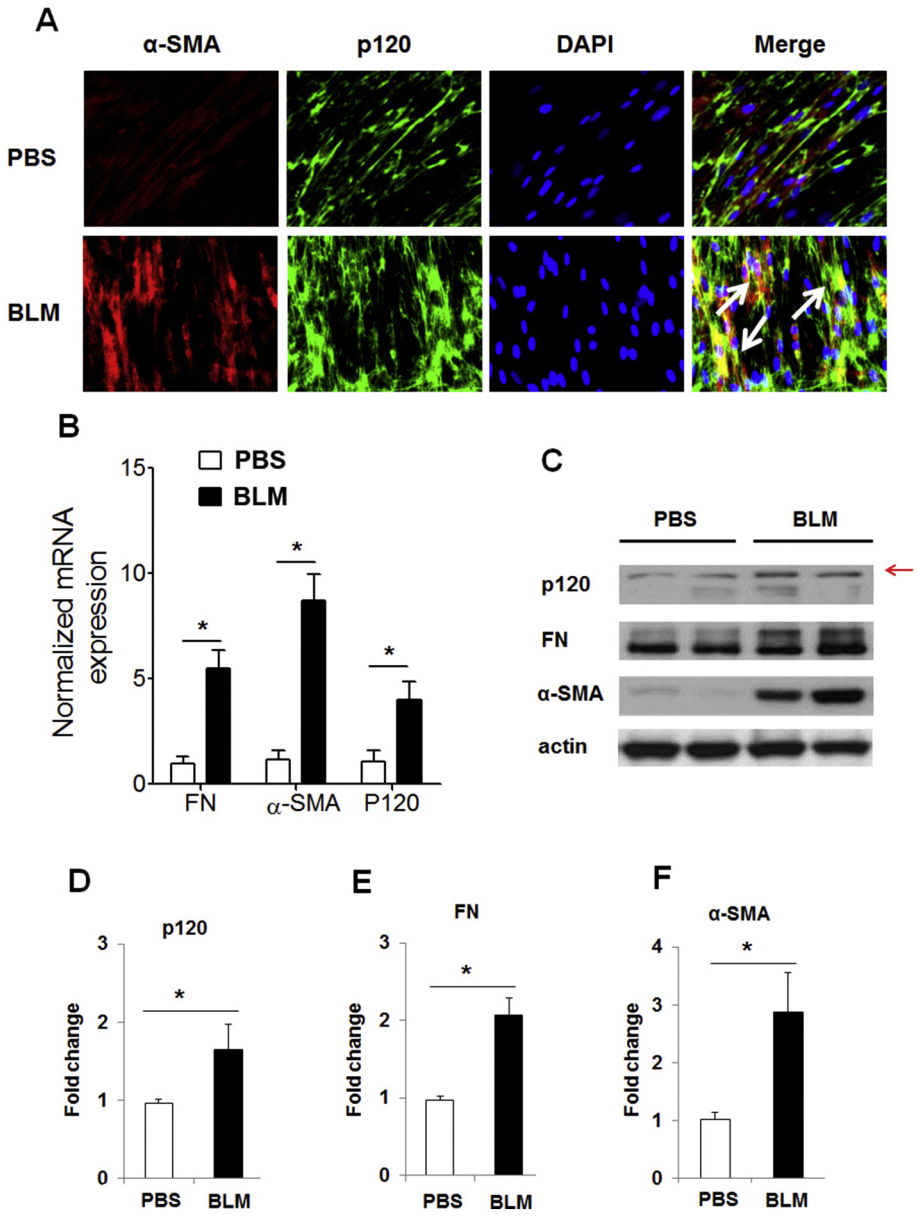

Fig. 2. P120 was up-regulated in lung fibroblast from BLM challenged mice. (**A**) Representative immunofluorescence staining of P120 and α-SMA expression in lung tissues from mice (n = 5 mice per group) with or without BLM challenge (1.5 U/kg, IT, 21 days). White arrow indicated the co-localization of α-SMA and P120 expression in fibrotic foci. (**B**) q-PCR analyzed mRNA expression of FN, α-SMA and P120 in lung fibroblasts isolated from mice with or without BLM challenge. (**C**) Representative western blot of actin, P120, FN and α-SMA expression in lung fibroblast isolated from mouse with or without BLM challenge (n = 5 mice per group). Arrow indicated the P120 band for quantification. (**D–F**) Quantification of the expression of P120 (**D**), FN (**E**) and α-SMA (**F**). Protein expression was quantified and normalized with the expression of actin. Data were expressed as mean ± SEM. $^{*}P < 0.05$.

of P120 in pulmonary fibrosis and fibroblast differentiation was still elusive. In present study, we used a bleomycin (BLM)-induced mouse model of pulmonary fibrosis to investigate the expression and role of P120 during pulmonary fibrosis. The results indicated that the expression of P120 was increased in pulmonary fibroblast during pulmonary fibrosis, and the expression of P120 in TGF-β1-challenged fibroblasts was partly depended on the SMAD and NF-κB-mediated pathway, and knockdown of P120 attenuated TGF-β1-induced lung fibroblast differentiation and BLM induced pulmonary fibrosis in mouse. Thus, P120 might be the new therapeutic target to fibroblast differentiation and pulmonary fibrosis.

2. Materials and methods

2.1. Reagents and kits

Protease inhibitor cocktail tablets (EDTA-free Complete) were from Roche Diagnostics (Indianapolis, IN, USA). Rabbit anti-SMAD3 and anti-P120 antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Bleomycin sulfate (BLM) was from Hospira, Inc. (Lakeforest, IL). Bay 11-7082 (NF-κB inhibitor; cat. no. B5556), lentivirus with control sh-RNA (sc-108080) or mouse P120 sh-RNA (sc-36140-V), mouse anti-α-smooth muscle actin (α-SMA) and anti-beta actin antibodies were from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Recombinant human TGF-β1 was obtained from Pcpro Tech, Inc. (Eocky Hill, NJ). Vehicle (Veh) si-RNA, human SMAD3, P120 and P65 si-RNA, rabbit anti-fibronectin (FN) and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-linked anti-mouse IgG and anti-rabbit IgG antibodies were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Control mouse IgG (cat. no. sc-2025) and control rabbit IgG (cat. no. sc-2051) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). TGF-β1 ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA).

2.2. BLM induced pulmonary fibrosis in mouse

30 male wild type mice (C57BL/6J, 8–10 weeks) were ordered from Vital River Lab Animal Technology (Beijing, China), which were housed under a 12 h light/dark cycle at 18–23 °C and 40–60% humidity. Mice

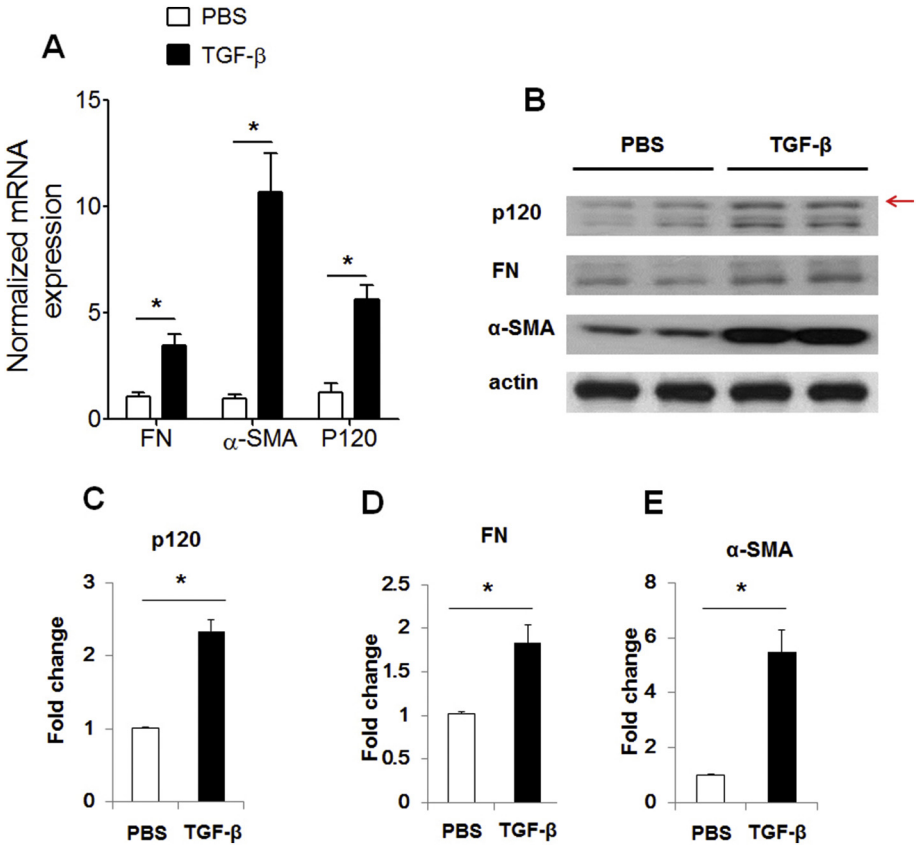

Fig. 3. TGF-β induced P120 expression in human lung fibroblasts. (**A**) TGF-β (5 ng/ml) increased the mRNA level of P120, FN and α-SMA in human lung fibroblasts. (**B**) TGF-β (5 ng/ml) increased the protein expression of P120, FN and α-SMA in human lung fibroblasts at 48 h. Arrow indicated the P120 band for quantification. (**C–E**) Quantification of protein expression (P120 (**C**); FN (**D**); and α-SMA (**E**)). Protein expression was quantified and normalized with the expression of actin. Data were expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$.

were free to access food and water. Mice were anesthetized (IP, 87.5 mg/kg of ketamine and 12.5 mg/kg of xylazine, cat. no. K113, Sigma-Aldrich, Inc., Merck KGaA, Darmstadt, Germany) and then intratracheally administrated with of BLM (1.5 U/kg, in 50 μl of saline) or saline alone [14]. At 21 days following the BLM challenge, mice were sacrificed. Lungs were lavaged with 1 ml of PBS, and the Bronchoalveolar Lavage (BAL) fluids were approached for total cell count and protein assay. Lungs were harvested and the lobes were fixed at 25 °C in 10% Formalin for 24 h and 70% EtOH for 48 h, embedded in paraffin, cut into 5-μm sections and subjected to hematoxylin and eosin (H & E) staining and trichrome staining. All animal studies were approved by the Animal Care Use Committee of Xuzhou Medical University (Xuzhou, Jiangsu, China).

2.3. Administration of lentivirus

Lentivirus with control sh-RNA (sc-108080) or mouse P120 sh-RNA (sc-36140-V) were ordered from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and diluted with sterilized PBS solution before administration. 7 days post IT administration of PBS or BLM (1.5 U/kg), the mice were administrated with 50 μl of diluted lentivirus (IT, 10^7 pfu/mouse). After lentivirus administration, animals still maintained until sacrificed at day 21 post BLM challenge.

2.4. Immunofluorescence staining

Immunofluorescence microscopy was used to assess the expression of α-SMA and P120 in lung tissue from mouse with or without BLM treatment as previously described [14]. After dewaxed, rehydrated and subjected to antigen retrieval the lung tissues sections were subsequently blocked with TBST blocking buffer (2% bovine serum albumin (BSA) and 1% fetal bovine serum (FBS); Santa Cruz Biotechnology, Inc.) for 30 min at room temperature, and incubated with primary antibodies (1:300; rabbit anti-P120, 1:500, mouse anti-α-SMA, control IgG form mouse and rabbit) for 1 h, followed by three 15-min washes with TBST. Tissue slides were then incubated with Alexa Fluor secondary antibodies (Thermo Fisher Scientific, Inc., Waltham, MA, USA; 1:250, cat. no. R37117 and A-21202) at 25 °C for 1 h, followed by washing with TBST for 15 min. After the incubation with mounting media (containing DAPI) at 25 °C for 10 min, the stained lung slides were examined under a Nikon Eclipse TE 2000-S fluorescence microscope (Nikon Corporation, Tokyo, Japan). Images were captured using a digital camera (Hamamatsu Photonics, Hamamatsu, Japan) with a 20× immersion or 4× objective lens. Fluorescence intensity was analyzed using the ImageJ analysis system v1.45 (National Institutes of Health, Bethesda, MA, USA).

2.5. Isolation and culture of mouse lung fibroblasts

Mouse lung fibroblast was isolated and cultured as described before [14]. Primary mouse lung fibroblast cells and WI-38 (human lung fibroblast cell line, purchased from ATCC) were cultured in DMEM medium containing 10% FBS. Serum starved (12 h) lung fibroblasts (~80% confluent) were treatment with TGF-β (5 ng/ml) for 0–48 h. Treated cells were harvested by using lysis buffer for protein estimation and western blot analysis.

2.6. NF-κB inhibitor treatment

Cells (human lung fibroblast, WI-38) were pretreated with 5 μM Bay 11-7082 and vehicle solution (0.05% dimethylsulfoxide), for 1 h [14,21]. The cells were subsequently challenged with TGF-β (5 ng/ml) for a subsequent 48 h prior to analysis using western blotting.

2.7. RNA isolation and RT-qPCR

Following treatment with TGF-β (5 ng/ml, 12 h), total RNA was extracted and purified from WI-38 cells by using TRIzol (Invitrogen;

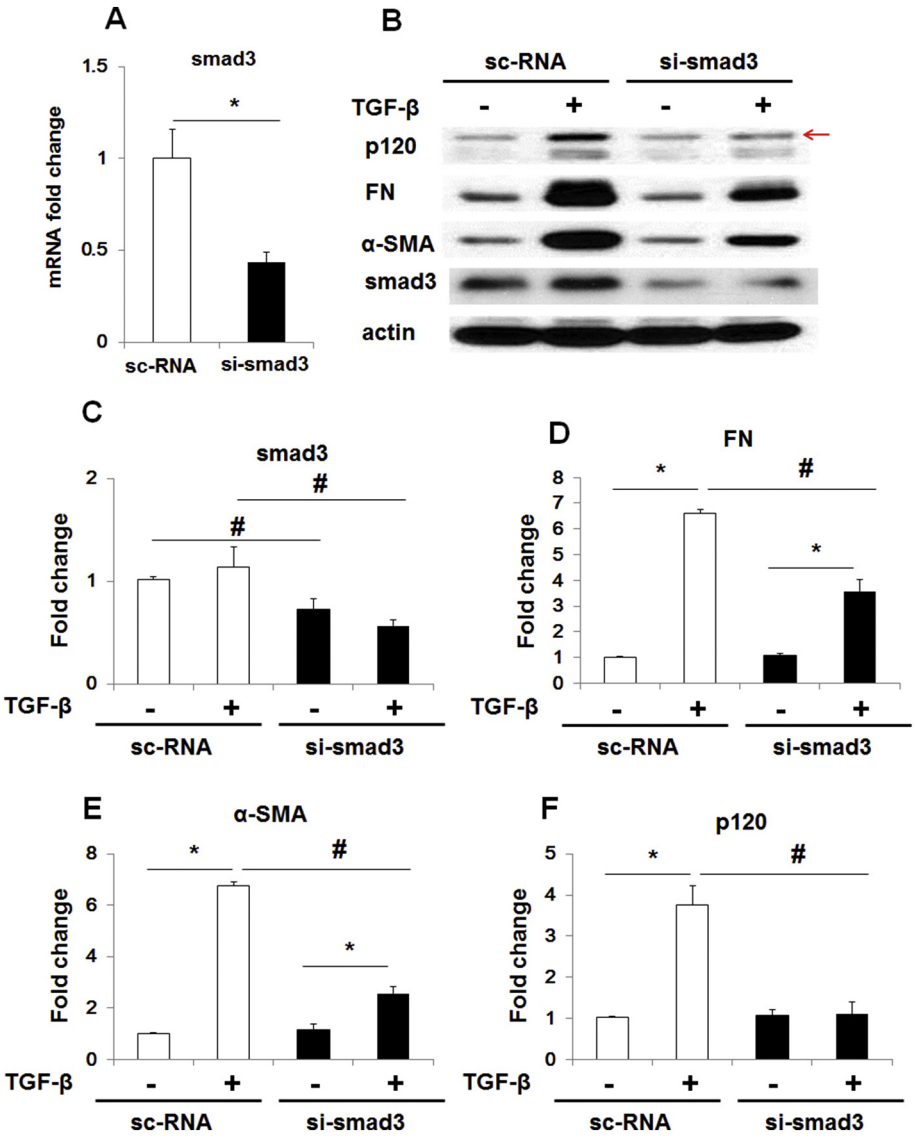

Fig. 4. Knockdown SMAD3 attenuated TGF-β induced P120 expression in human lung fibroblasts. (**A–C**) Human lung fibroblasts were transfected with scrambled or SMAD3 siRNA (100 nM) for 24 h, prior to challenge with TGF-β (5 ng/ml, 0–48 h). The mRNA level of (**A**) SMAD3 and (**B–F**) protein levels of SMAD3, FN, α-SMA and P120 (48 h post TGF-β challenge) were determined. (**B**) Representative western blot and quantification of protein expression (**C**, SMAD3; **D**, FN; **E**, α-SMA; **F**, P120) in human lung fibroblasts. Arrow indicated the P120 band for quantification. Data were expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized (46 °C, 20 min) by using a cDNA synthesis kit (iScript cDNA synthesis kit; cat. no. 1708890) from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). 1 μg of total RNA was converted to cDNA, and this was used for qPCR. A SYBR green qPCR kit and a CFX96 Touch qPCR System (both from Bio-Rad Laboratories, Inc.) were used. For real time qPCR, the amplification reactions were performed in triplicate, and the thermal cycling conditions were as follows: 10 s at 95 °C followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. GAPDH was used as the reference gene to normalize the gene expression levels. Primer information was summarized in Table 1. Gene expression was analyzed using CFX Manager software version 3.1 (Bio-Rad Laboratories, Inc.). The $2^{-\Delta\Delta Cq}$ method was used for quantification [21].

2.8. si-RNA transfection

si-RNA was transfected into WI38 cells as described before [21]. Briefly, WI38 human lung fibroblasts with 50–60% of confluence were transfected with 200 nmol/l of si-RNA by using si-RNA transfection reagent (Huperfect Transfection Reagent; Qiagen AB, Sollentuna, Sweden, Cat. no. 301704). si-RNAs mixed with the transfection reagents were diluted in 900 μl basal DMEM and incubated with the cells for at 37 °C for 6 h. Then cells were changed with DMEM with 10% FBS every 24 h. At 48 h post transfection, cells were used for TGF-β treatment and further analysis.

2.9. Ashcroft analysis for pulmonary fibrosis

Ashcroft score analysis of lung fibrosis was performed as described before [22]. Briefly, the severity of the fibrotic changes in each lung section was given a mean score from the observed microscopic fields. More than 20 fields within each lung section were observed at X100, and each field was assessed individually for severity and allotted a score from 0 (normal) to 8 (total fibrosis) [22]. The severity was then averaged for each lung section. To avoid bias, all histological specimens were evaluated in a blinded fashion. Finally, the mean of their individual scores was taken as the fibrotic score.

2.10. Collagen determination

The acid-soluble collagen was measured as described before by using Sircol Collagen Assay kit from Thermo Fisher scientific, Inc. (Hampton, NH) according to the manufacturer's instructions [23]. Firstly, mouse right lungs were homogenized in PBS solution with 0.5 M acetic acid and 0.6% pepsin. After extraction at 4 °C overnight, the collagen in lysate was pelted by centrifugation at 10,000*g* for 15 min.

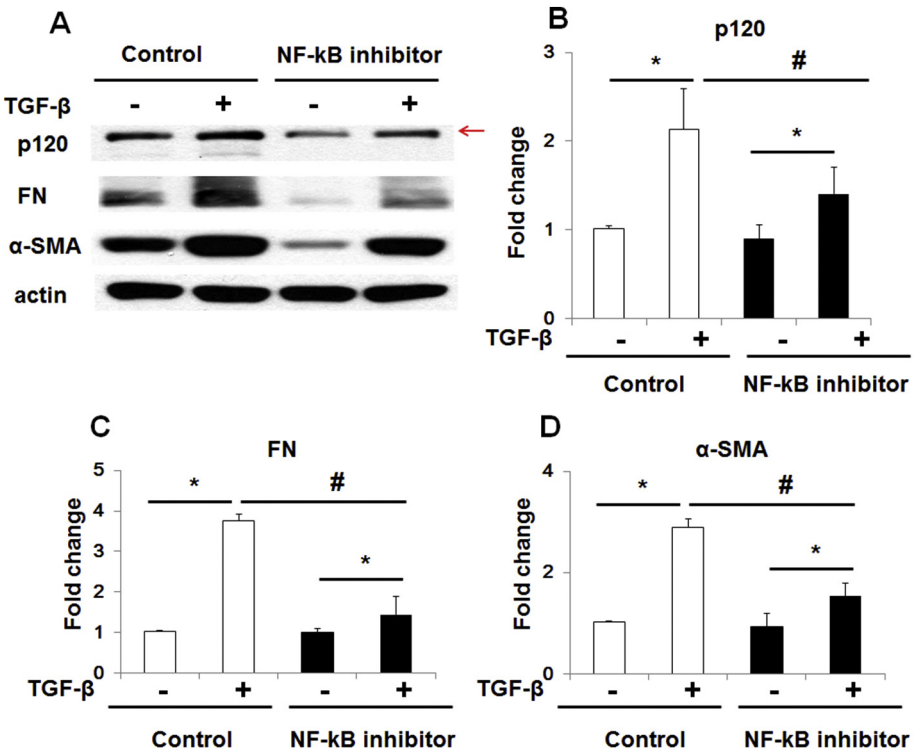

Fig. 5. NF-κB inhibitor attenuated TGF-β-induced fibroblast differentiation and P120 expression in human lung fibroblasts. WI-38 cells were treated with control solution or NF-κB Inhibitor prior to treatment with TGF-β. (**A**) Representative western blots and (**B–D**) quantification of P120 (**B**), FN (**C**) and α-SMA (**D**) expression. Arrow indicated the P120 band for quantification. Data were expressed as mean ± SEM of three independent experiments. *P < 0.05.

Collagen content was presented as micrograms of acid-soluble collagen per right lung.

2.11. Western blot analysis

Western blot was processed and analyzed as described before [21]. In general, lysate from cells or lung tissues was incubated on ice for 10 min, and followed by centrifugation (5,000 ×*g*, 15 min, 4 °C). Protein in cell lysate was evaluated by using Micro BCA protein assay (Thermo Fisher Scientific, Inc.; cat. no. 23235). 20 μg of protein in cell lysate was separated by SDS-PAGE (10 or 4–20%). Proteins were transferred onto nitrocellulose membranes (100 V, 1 h), blocked with blocking buffer (TBS solution containing 0.1% Tween 20 and 5% BSA) at 25 °C for 1 h and then incubated with primary antibodies (anti-P120, 1:1,000; anti-FN, 1:2,000; anti-GAPDH, 1:2,000; anti-α-SMA, 1:5,000) overnight at 4 °C. The membranes were subsequently incubated with secondary antibodies (1:2,000) at 25 °C for 2 h. Finally, proteins were visualized using an enhanced chemiluminescence kit (Bio-Rad Laboratories, Inc.) and analyzed by using Image Quant 5.2 software (Molecular Devices, LLC, Sunnyvale, CA, USA).

2.12. Statistical analysis

Data were analyzed using SPSS v16 statistical software (SPSS Inc., Chicago, IL, USA) [21]. Data are expressed as the mean ± standard error from at least three independent experiments. Data were also analyzed using a two-tailed Student *t*-test or two way ANOVA analysis of variance plus a multiple comparisons post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. P120 expression increased in lung fibroblastic lesions and lung fibroblasts from BLM-challenged mice

In order to check the role of P120 in pathobiology of lung fibrosis, the expression of P120 in lung tissue from BLM-challenged mice was analyzed. As shown in Fig. 1A, the immunohistochemistry staining indicated that the expression of P120 was significantly increased in lung fibroblastic lesions from BLM-challenged mice compared to controls. The western blot also showed that the expression of P120, as well as α-SMA and FN (Fig. 1B–E), was much higher in the lung tissue from BLM treated mice than that from control mice. For further study the role of P120 in lung fibroblast, we stained P120 and α-SMA, the marker of myofibroblasts, in the fibrotic and normal lung tissues. As shown in Fig. 2A, the increased expression of P120 was co-localized with α-SMA in lung fibrotic foci from BLM treated mice. Next, we isolated the primary lung fibroblast from control and BLM treated mice, and analyzed the expression of P120 in lung fibroblasts. As shown in Fig. 2B–F, the mRNA (Fig. 2B) and protein (Fig. 2C–F) expressions of P120, as well as α-SMA and FN, were significantly increased in lung fibroblasts from BLM treated mice. These data suggested that P120 expression was increased in fibrotic foci and isolated fibroblasts from BLM-challenged mice.

3.2. TGF-β induced P120 expression in human lung fibroblasts

TGF-β is a key cytokine involved in lung fibroblast differentiation and pulmonary fibrosis [24]. And the role of TGF-β in P120 expression during fibrogenesis was not known. As shown in Fig. 3, TGF-β (5 ng/ml), increased P120 mRNA level (Fig. 3A), and protein expression (Fig. 3B–E) in human lung fibroblasts during fibroblast differentiation to myofibroblast, which was characterized with the increased expression of the marker genes, fibronectin (FN) and α-SMA, (Fig. 3B, D & E).

3.3. TGF-β induced P120 expression through SMAD and NF-kB dependent pathways

We next determined the signaling pathway(s) by which TGF-β mediated P120 expression. To determine the role of SMAD2/3 in P120 expression, we treated the fibroblast with SMAD3 siRNA. As shown in Fig. 4A–C, transfection of human lung fibroblasts with si-SMAD3 (100 nM, 48 h) significantly decreased the mRNA (~50%) (Fig. 4A) and protein levels (~30%) of SMAD3 (Fig. 4B & C). Knockdown the

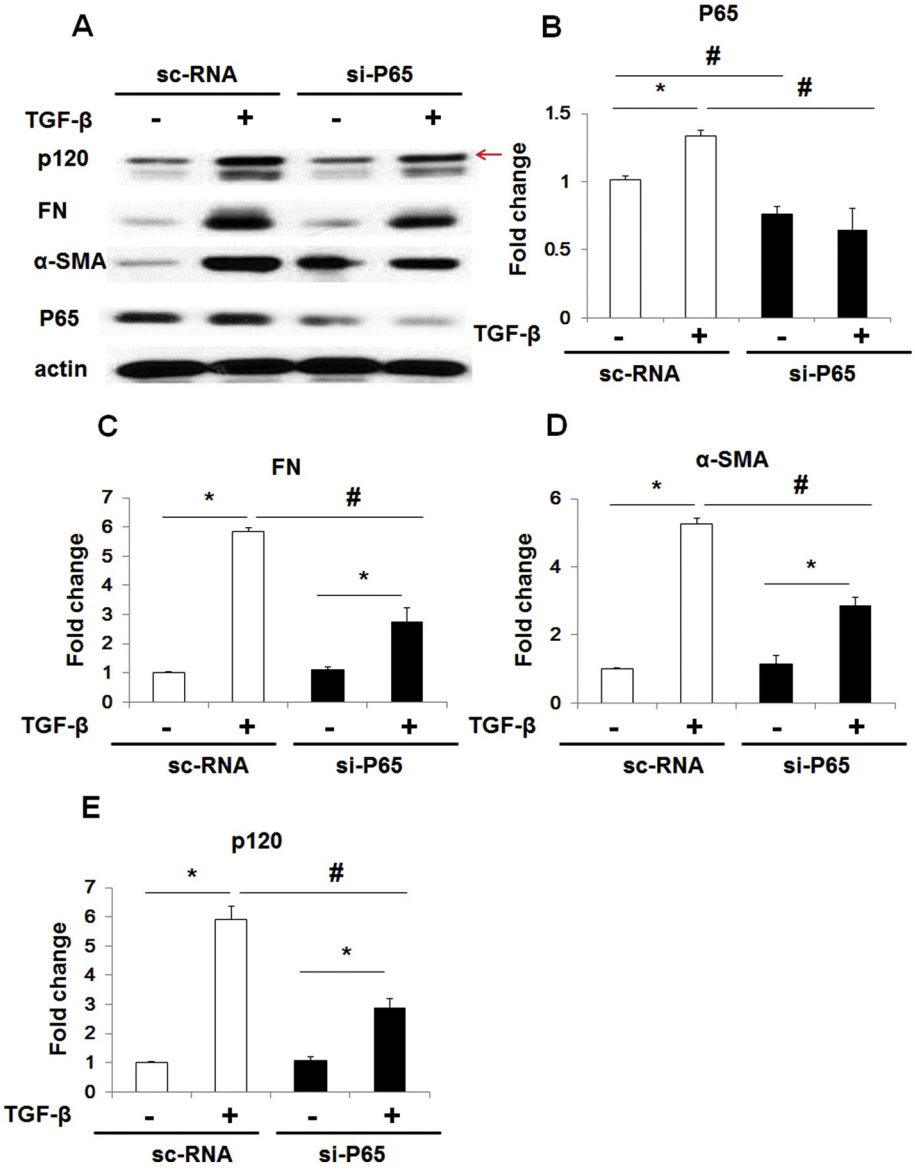

Fig. 6. Knockdown NF-kB P65 subunit attenuated TGF-β induced P120 expression in human lung fibroblasts. (**A–C**) Human lung fibroblasts were transfected with scrambled or NF-kB P65 siRNA (100 nM) for 24 h, prior to challenge with TGF-β (5 ng/ml, 0–48 h). The protein levels of P65, FN, α-SMA and P120 (48 h post TGF-β challenge) were determined. (**A**) Representative western blot and (**B–E**) quantification of protein expression (**B**, P65; **C**, FN; **D**, α-SMA; **E**, P120) in human lung fibroblasts. Arrow indicated the P120 band for quantification. Data were expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

expression of SMAD3 by siRNA significantly attenuated TGF-β-induced P120, FN and α-SMA expressions in human lung fibroblasts (Fig. 4B, D–F).

Our previous investigations indicated that NF-κB pathway also involved in the TGF-β-induced fibroblast differentiation and gene expression in lung fibroblasts [25]. Thus, we also investigated that whether NF-kB pathways affected TGF-β induced P120 expression. Human lung fibroblasts was treated with Bay 11-7082 (5 μM), the NF-kB signaling pathway inhibitor, then TGF-β induced gene expression was assessed by using western blotting. Treatment of Bay 11-7082, TGF-β-induced fibroblast differentiation, and P120 expression were significantly blocked (Fig. 5A–D). Similarly, knockdown the expression of P65 (Fig. 6A), the key factor of NF-kB signaling pathway, also suppressed TGF-β-induced expression of P120, FN and α-SMA in human lung fibroblasts (Fig. 6A, C–E). These data suggested that SMAD and NF-κB dependent pathways regulated TGF-β induced P120 expression in human lung fibroblasts.

3.4. P120 regulated TGF-β induced human lung fibroblast differentiation via SMAD and ERK dependent pathways

To study the role of P120 in TGF-β-induced fibroblast differentiation, human lung fibroblasts were transfected with si-RNA targeting human P120 protein. siRNA transfection decreased the expression of P120 protein in human lung fibroblasts (Fig. 7A & B), and further significantly inhibited the TGF-β-induced lung fibroblast differentiation by compared with that in control cells (Fig. 7A, C & D).

It was known that SMAD and ERK activation were critical for TGF-β induced fibroblast differentiation and fibrogenesis [8,25]. Thus, we further investigated whether P120 regulated fibroblast differentiation through these signaling pathways. As shown in Fig. 8, knockdown of P120, significantly attenuated TGF-β induced activation of SMAD (Fig. 8A, C) and ERK (Fig. 8A & D). These results suggested that knockdown of P120 inhibited TGF-β mediated lung fibroblast differentiation via blocking SMAD and ERK dependent pathways.

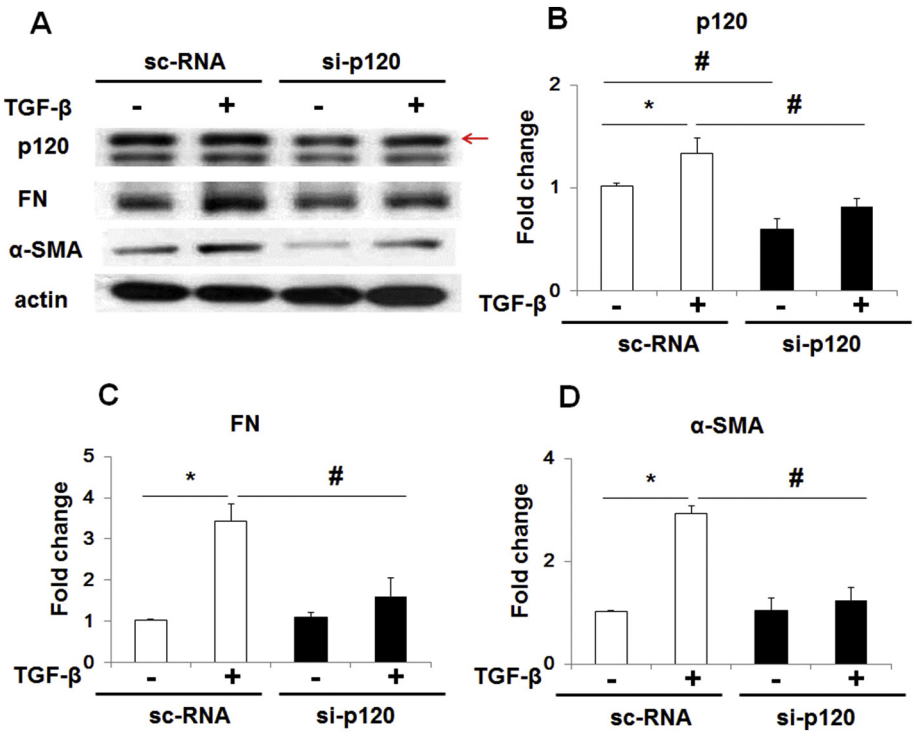

Fig. 7. Knockdown P120 attenuated TGF-β induced human lung fibroblasts differentiation. (**A–C**) Human lung fibroblasts were transfected with scrambled or P120 siRNA (100 nM) for 24 h, prior to challenge with TGF-β (5 ng/ml, 0–48 h). The protein levels of FN, α-SMA and P120 (48 h post TGF-β challenge) were determined. (**A**) Representative western blot and (**B–D**) quantification of protein expression (**B**, P120; **C**, FN; **D**, α-SMA) in human lung fibroblasts. Arrow indicated the P120 band for quantification. Data were expressed as mean ± SEM of three independent experiments. *P < 0.05; **P < 0.01.

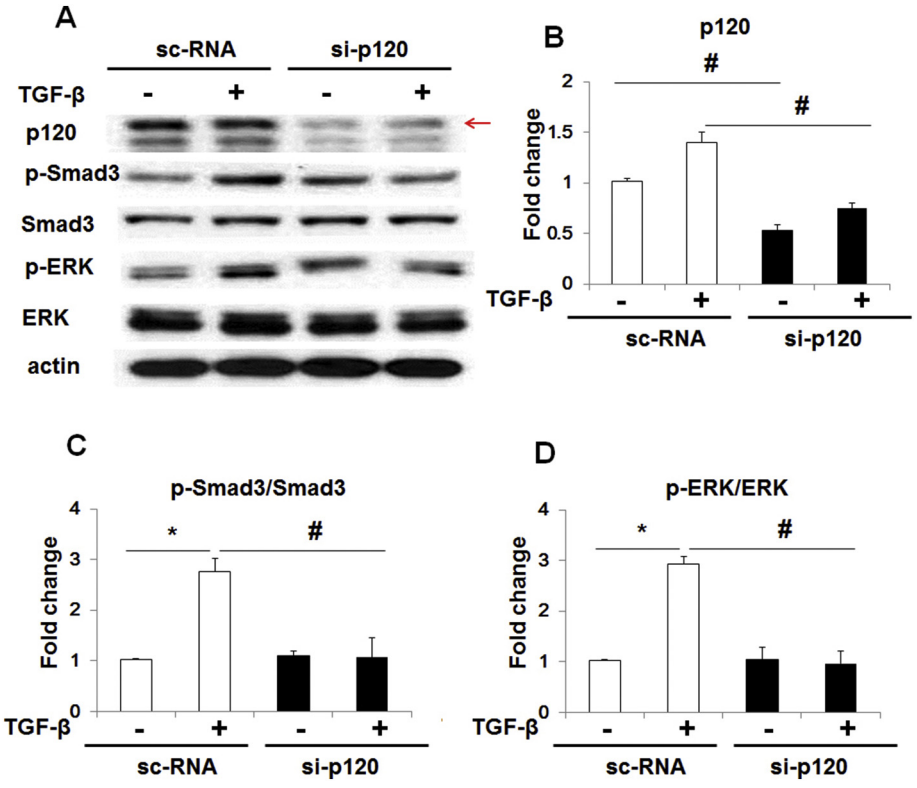

Fig. 8. Knockdown P120 attenuated TGF-β induced activation of SMAD and ERK dependent pathways in human lung fibroblasts. (**A–C**) Human lung fibroblasts were transfected with scrambled or P120 siRNA (100 nM) for 24 h, prior to challenge with TGF-β (5 ng/ml, 0–1 h). The protein levels of p-SMAD3, SMAD3, p-ERK, ERK, actin and P120 (1 h post TGF-β challenge) were determined. (A) Representative western blot and (B-D) quantification of protein expression (B, P120; C, p-SMAD3/SMAD3; D, p-ERK/ERK) in human lung fibroblasts. Arrow indicated the P120 band for quantification. Data were expressed as mean ± SEM of three independent experiments. *P < 0.05; **P < 0.01.

3.5. Knockdown P120 attenuated BLM induced pulmonary fibrosis in mice

In mouse model of BLM induced pulmonary fibrosis, the inflammation started to slowdown and the fibrogenesis start to speed up at day 7 post BLM administration. To investigate the role of P120 in pulmonary fibrosis, we suppressed the P120 expression during the fibrogenesis stage (day 7 to 21 post BLM challenge) through intratracheal (IT) administration of lentivirus coding with shRNA targeting mouse P120 (sh-P120) gene (Fig. 9A). As shown in Fig. 9B–C, administration of P120 sh-RNA significantly attenuated the mRNA level in lung tissue (Fig. 9B) and protein (Fig. 9C) expression of P120 in mouse lung fibroblast isolated from mice by comparing with that from control mice. Interestingly, attenuation of P120 expression in fibrogenesis stage did not shown significantly inhibition of BLM increased total cell number (Fig. 9D) and protein level (Fig. 9E) in mice BAL fluid. This indicated that knockdown of P120 did not shown dramatically effect on the lung

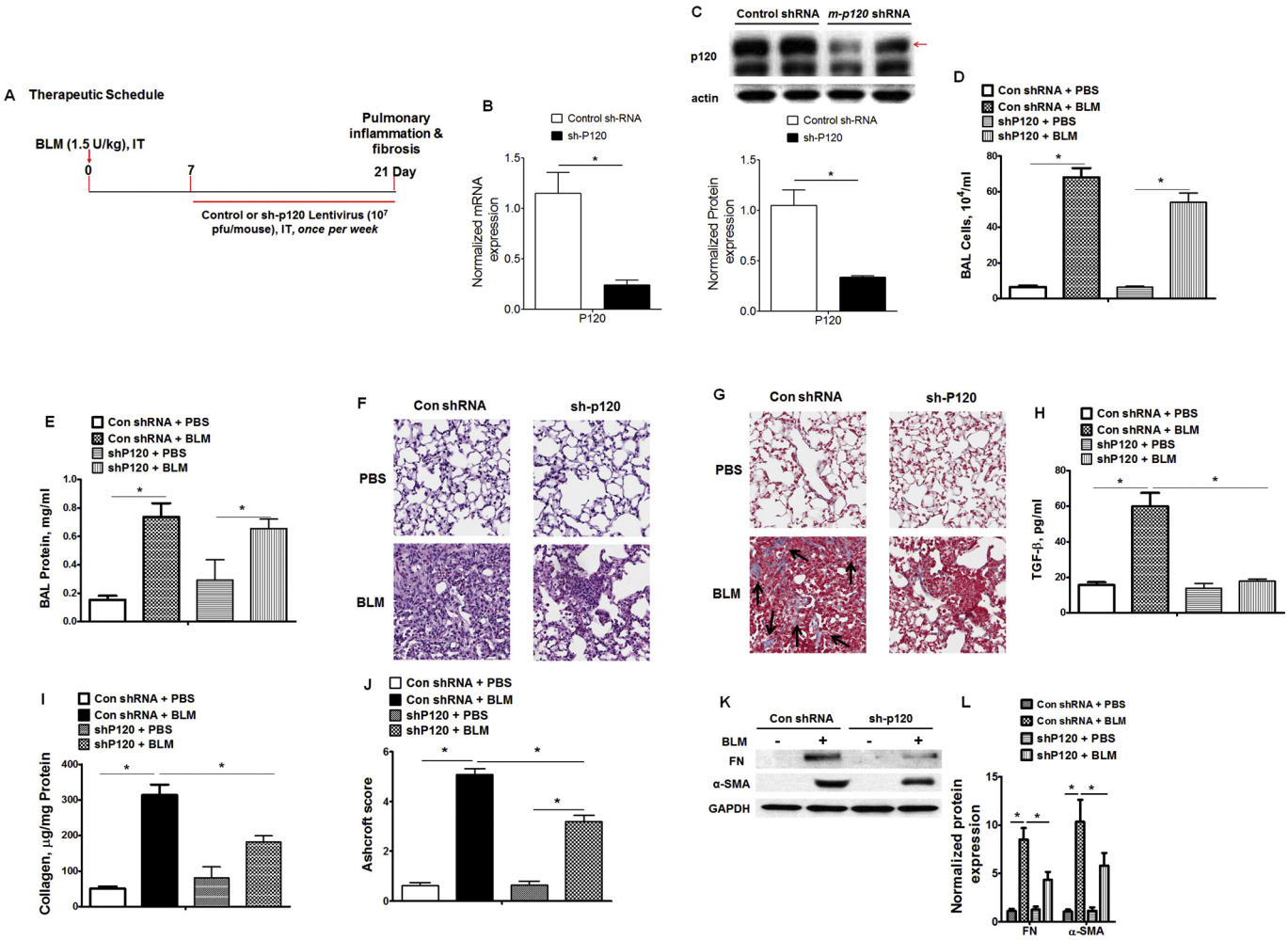

Fig. 9. Knockdown P120 attenuated BLM induced pulmonary fibrosis in mouse. (**A**) Schema of lentivirus administration. (**B**) q-PRCT analysis of p120 expression in lung tissue from mice with treatment of lentivirus (control sh-RNA Vs sh-P120). (**C**) Protein expression of P120 in mouse lung fibroblast isolated from mice with treatment of lentivirus (control sh-RNA Vs sh-P120). Arrow indicated the P120 band for quantification. Upper panel, representative western blot; lower panel, quantification of P120 expression in mouse lung fibroblasts. (**D**) Total cell number and (**E**) protein level in BAL fluids from mice with or without BLM treatment. (**G**) H &E staining and (**H**) trichome staining of lung tissue slides from mice with or without BLM treatment. Black arrow indicated the deposition of collagen in fibrotic foci. (**H**) ELISA analysis of TGF-β in BAL fluids, (**I**) quantification of collagen in lung tissue, (**J**) fibrotic score of lung tissue slides from mice with and without BLM challenge. (**K** & **L**) Expression of fibrosis marker proteins in lung tissues from mice with or without BLM challenge. (**K**) Representative western blot; (**L**) quantification of protein expression in mouse lung tissue. Data were expressed as mean ± SEM, $n = 5$ mice per group, $^{*}P < 0.05$; $^{**}P < 0.01$.

injury post BLM challenge. However, the H&E staining (Fig. 9F) and Trichome staining (Fig 9G) indicated that attenuation of P120 expression in fibrogenesis stage decreased the fibrosis foci formation and deposition of collagen in the fibrotic foci. And we further investigated the fibrosis parameters in lung tissue and BAL fluid. Knockdown of P120 significantly decreased the Ashcroft score (Fig. 9j), which was the score to evaluate the lung fibrosis; suppressed the accumulation of TGF-β in BAL fluid (Fig. 9H), and collagen in lung tissue (Fig. 9I); and inhibited the expression of fibrosis marker proteins (α-SMA and FN, Fig. 9K & L). These results showed that knockdown the expression of P120 in fibrogenesis stage blocked the BLM induced lung fibrosis in mouse.

4. Discussion

P120 catenin (P120) was known to regulate adhesion junction and signal transduction in lung epithelial [17,19], endothelial cells and macrophages [20]; while its expression and roles in lung fibroblast and pulmonary fibrosis was not investigated. β-catenin, another important catenin family member, was well investigated in pulmonary fibrosis [25,26]. β-catenin was firstly proved to regulate lung development under normal conditions [27,28]. Recent investigation from Immunohistochemistry staining of lung tissue indicated that β-catenin expression accumulated into the nuclei in the cells localized in lung fibroblastic foci from IPF patients [25,26]. Our present data demonstrated that P120 expression was also associated with pulmonary fibrosis and lung fibroblast differentiation, indicating that P120 plays important roles in pulmonary fibrosis. As the critical cofactor for Wnt signaling pathway, β-catenin was proved to regulate the hippo pathways through interaction with YAP1 [29], the key transcription factor in hippo pathways, to control the pathogenesis of various diseases, especially for pulmonary fibrosis [29,30]. Although our studies proved that P120 regulated pulmonary fibroblast differentiation at least partly through the ERK and SMAD dependent pathway, the interaction between hippo pathway and P120 was not been studied, which may need further investigation.

TGF-β was known to be a multifunctional cytokine, and critical for pulmonary fibrogenesis in both human and animals [31]. The expression of TGF-β was increased in lung tissues from IPF patients and BLM challenged animals [7,8]. TGF-β induced fibroblast differentiation to myofibroblast, epithelial-mesenchymal transition (EMT), expression and accumulation of ECM proteins mainly through SMAD dependent

canonical and other non-canonical pathways [7,8]. In this study, we proved that TGF-β increased P120 expression in lung fibroblasts through SMAD dependent pathways and NF-kB dependent pathways, and suppressing of these pathways also attenuated the expression of P120, as well as blocking the fibroblast differentiation. Our data showed that knockdown of SMAD 3 (Fig. 4) partly blocked TGF-β induced fibroblast differentiation and almost completely blocked P120 expression. However, knockdown of P65, key subunit of NF-kB pathway, or administration of NF-kB inhibitor, partly decreased fibroblast differentiation and P120 expression in TGF-β treated cells (Figs. 5 & 6). Similarly, TGF-β1 also induced synergistic NF-κB activation via Smad3/4–PKA–p300-dependent p65 acetylation in human epithelial cells [32]. And in colon cancer cell line (SW480), stable knockdown of SMAD4, cofactor of SMAD2/3 in TGF-β pathway, almost completely abolished the TGF-β induced activation of NF-kB pathway [33]. These data suggested that TGF-β mediated SMAD3 signaling may partly through NF-kB pathway during fibroblast differentiation. Interestingly, P120 was also proved to express in endothelial and epithelial cells, which were critical for lung injury and fibrogenesis. Thus, the expression and roles of P120 in epithelial and endothelial cell still needed further investigation.

In vitro knockdown the expression of P120 in lung fibroblasts dramatically attenuated the TGF-β induced fibroblast differentiation and expression of α-SMA and FN. And in vivo knockdown the expression of P120 in BLM challenged mice during the fibrogenesis stages suppressed the lung fibrosis. These data suggested the P120 played important roles in the fibroblast differentiation and pulmonary fibrosis, which may through promoting ERK and SMAD dependent pathways. Both ERK and SMAD pathways were demonstrated to regulate lung fibroblast differentiation and fibrosis [21,25]. In BLM induced mouse fibrosis, suppressing of P120 expression in fibrogenesis stages dramatically blocked pulmonary fibrosis, but had not shown significant effects on lung injury. These data suggested that the role of P120 in pulmonary fibrosis was mainly through the regulation of fibroblast differentiation and accumulation of ECM proteins, but not in the lung injury. Interestingly, P120 was proved to play important roles on embryonic epithelium and endothelium development functions by using the knockout mouse model. Conditional knockout of P120 in epithelium of intestine was lethal for mice [34]. Endothelial ablation of p120-catenin caused hemorrhages and microvascular patterning defects, and further resulted in midgestational lethality [35]. Thus, precision knockout or regulation of P120 function is important to the therapeutic of IPF, and which may need further investigation.

In summary, our results suggested that TGF-β-mediated P120 expression in lung fibroblast through NF-kB and SMAD dependent pathways, and the expression of P120 further promoted fibroblast differentiation and pulmonary fibrosis; and knockdown of P120 was beneficial to pulmonary fibrosis via suppressing pulmonary fibrogenesis and fibroblast differentiation. Our findings indicated that selectively inhibition of P120 expression in lung fibroblasts via gene therapeutic approaches would be beneficial for developing novel therapy to IPF.

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