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RS4651 suppresses lung fibroblast activation via the TGF-β1/SMAD signalling pathway

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ABSTRACT

Background: Idiopathic pulmonary fibrosis (IPF) is a progressive disease resulting in respiratory failure with no efficient treatment options. We investigated the protective effect of RS4651 on pulmonary fibrosis in mice and the mechanism.

Methods: Intratracheal injection of bleomycin (BLM) was used to induce pulmonary fibrosis in mice. RS4561 was administered intraperitoneally at different doses. Histopathological changes were observed. The level of alpha-smooth muscle actin (α-SMA) were also tested. In vitro, the proliferation and migratory effects of RS4651 treatment on MRC-5 cells pre-treated with transforming growth factor (TGF-β1) were examined. RNA-sequencing was used to detect differentially expressed target genes. Then, the expression of α-SMA, pSMAD2 and SMAD7 were analysed during RS4651 treatment of MRC-5 cells with or without silencing by SMAD7 siRNA.

Results: Histopathological staining results showed decreased collagen deposition in RS4651 administered mice. Additionally, a lower level of α-SMA was also observed compared to the BLM group. The results of in vitro studies confirmed that RS4651 can inhibit the proliferation and migration, as well as α-SMA and pSMAD2 expression in MRC-5 cells treated with TGF-β1. RNA-sequencing data identified the target gene SMAD7. We found that RS4651 could upregulate SMAD7 expression and inhibit the proliferation and migration of MRC-5 cells via SMAD7, and RS4651 inhibition of α-SMA and pSMAD2 expression was blocked in SMAD7-siRNA MRC-5 cells. In vivo studies further confirmed that RS4651 could upregulate SMAD7 expression in BLM-induced lung fibrosis in mice.

Conclusions: Our data suggest that RS4651 alleviates BLM-induced pulmonary fibrosis in mice by inhibiting the TGF-β1/SMAD signalling pathway.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal disease with high mortality, increased incidence over time and few treatment options (Hutchinson et al., 2015; Zhou et al., 2016). In recent years we have made significant progress in the pathogenesis and treatment of IPF, but there is still no way to reverse or cure the disease (Somogyi et al., 2019; Zhao et al., 2020). In late 2014, two drugs, pirfenidone and nintedanib, were approved for treatment of IPF based on their ability to slow disease progression. However, there has been no clear evidence

Abbreviations: IPF, idiopathic pulmonary fibrosis; TGF, transforming growth factor; ECM, extracellular matrix; H&E, haematoxylin and eosin; BLM, bleomycin; IOD, integrated optical density; α-SMA, alpha-smooth muscle actin; MEM, minimum essential medium; FBS, foetal bovine serum; SD, standard deviation; siSMAD7, SMAD7 -siRNA; I.T, intratracheal; MFBs, myofibroblasts; RS, RS4651; PCR, polymerase chain reaction; RT-PCR, real-time PCR; IHC, immunohistochemistry; siRNA, small interfering RNA.

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that they could prolong survival up to now (Canestaro et al., 2016). Therefore, exploring new effective strategies for pulmonary fibrosis has become the focus of current research.

Myofibroblasts differentiated from fibroblasts have long been considered the source of extracellular matrix (ECM) deposition during fibrogenesis, and they are the main cells expressing alpha-smooth muscle actin (α-SMA) (Jin et al., 2018; van der Velden et al., 2016). Transforming growth factor (TGF)-β1 is known as an effective fibrogenic cytokine, and it plays a central fibrogenic role in IFP by promoting the activation, proliferation and migration of fibroblasts, ECM deposition and the transformation from fibroblasts to myofibroblasts (Kawashima et al., 2012; van der Velden et al., 2016). SMAD family proteins perform the function of transmitting the TGF-β1 signal from the cell surface to the nucleus to regulate target gene transcription, such as α-SMA and collagen I (Hayashi et al., 1997; Hu et al., 2018). This classic pathway of pulmonary fibrosis is the TGF-β1/SMAD-dependent signalling pathway, which plays a central role in the pathogenesis of IPF (Hayashi et al., 1997; Hu et al., 2018). SMADs are transcription factors, including the stimulatory, receptor-activated SMAD1, 2, 3, 5, 8 and 9, a common co-mediator SMAD4 and inhibitory SMAD6 and 7 (Mahmood et al., 2017). Inhibitory SMAD7 act as an important brake on TGF-β1-induced α-SMA and collagen I expression (Lv et al., 2019), and additionally, SMAD 7 shows significant anti-IPF effects in vitro and in vivo (Qin et al., 2019). Therefore, TGF-β1 and its SMAD-dependent signalling pathway have been attractive therapeutic targets for the development of anti-fibrotic medications (Feng et al., 2020; Lee et al., 2014; Qin et al., 2019).

RS4651 is a molecule synthesized in the pharmaceutical laboratory of the Sapienza University of Rome, Italy. Previous studies have shown that RS4651 can inhibit the replication of HCV. And this activity of RS4651 was mediated by Cyclooxygenase-2 (COX-2) inhibition (Manvar et al., 2015). COX-2 is an important regulatory factor involved in pulmonary fibrosis (Pasini et al., 2018). However, other report has claimed that the role of COX-2 deficiency in pulmonary fibrosis is to aggravating pulmonary function impairment (Card et al., 2007). Therefore, the regulatory role of COX-2 in pulmonary fibrosis is still controversial. And the function of RS4651 has not been fully explored. In this study, we evaluate the effect of RS4651 on lung fibroblast activation and elucidate the possible mechanisms involved.

2. Material and methods

2.1. Animals and treatments

Male 6-week-old, C57BL/6 mice weighting 18–22 g were purchased from the Experimental Animal Centre of the Capital Medical University, Beijing Friendship Hospital (Beijing, China). All mice were maintained in a 12/12 h light/dark cycle at constant temperature and humidity and allowed free access to a standard rodent diet. All experimental procedures and experiments using animals were approved by the Ethical Committee and Institutional Animal Care and Use Committee of the Capital Medical University Beijing Friendship Hospital (18-2019). Mice were randomly divided into five treatment groups with 8 animals per group as follows: 1) Control group; 2) BLM; 3) BLM + RS4651 (RS) (5 mg/kg); 4) BLM + RS (10 mg/kg); and 5) BLM + RS (15 mg/kg). Mices were given one intratracheal administration of BLM (5 mg/kg, Nippon 92 Kayaku, Tokyo, Japan) in 0.1 mL of saline to induce lung fibrosis in the BLM groups and RS treatment group, while mice in the control group received an equal volume of saline. After two weeks, RS was dissolved in olive oil and the RS groups of mice were intraperitoneally injected with RS (5 mg/kg body weight, 10 mg/kg, or 15 mg/kg) twice a week; the mice in control and BLM only groups were intraperitoneally injected with the same volume of olive oil. All mice were euthanized 28 days after bleomycin administration. The right lung tissues were frozen and then stored at −80 °C for further analysis, while the left lung tissues were perfused and fixed in 4% formalin for immunohistochemical analysis.

2.2. Immunohistochemical (IHC) analysis

For histological analysis, the left lung tissues were fixed in 4% formalin, embedded in paraffin, and then sectioned and stained with Masson's trichrome or haematoxylin and eosin (H&E). The sections were immunostained with primary antibodies against α-SMA (ab5694) at 4 °C overnight, followed by incubation with an HRP-conjugated secondary antibody at room temperature for 1 h. The expression of α-SMA was visualized using DAB solution and counterstained with haematoxylin. The staining intensities were determined by measuring the integrated optical density (IOD) by light microscopy using Image-Pro v6.0.

2.3. Cell culture and reagents

Human embryonic lung fibroblasts MRC-5 cells were purchased from Fu Dan IBS Cell Centre (Shanghai, China) and cultured in Minimum Essential Medium (MEM) (Sigma) supplemented with 10% foetal bovine serum (FBS) (sigma), 100 U/mL penicillin, and 100 mg/L streptomycin at 37 °C in a 5% CO_2 relative humidified incubator. TGF-β1 (Peprotech, USA) was dissolved in citric acid (pH 3.0) to a concentration of 10 μg/mL, stored at −20 °C, and diluted in culture medium to the required concentration of 10 ng/mL. RS was dissolved in DMSO at a concentration of 20 mM, stored at room temperature, and diluted in culture medium to the required concentration (0–60 μM).

2.4. Cell viability analysis

Treated or untreated human MRC-5 cells were initially seeded at a density of 1×10^4 cells/well in a 96-well culture plate (Corning, USA) and incubated overnight to allow adhesion. Cell culture media was then replaced by complete media containing 30 μM of RS for additional 24–72 h. Following the manufacturer's instructions, 10 μL of MTS reagent (Promega, USA) mixed with 100 μL of culture medium was administered to the cells at 37 °C for 2 h. The culture medium supernatant was then collected and the absorbance at an OD of 490 nm was recorded using a 96-well plate reader.

2.5. Cell transwell migration assay

Treated or untreated human MRC-5 cells were resuspended at 5×10^5 cells/mL in MEM medium. Then, a 200 μL cell suspension was placed in the upper chamber and 1 mL of complete MEM medium was placed in the lower chamber. After culture for 24 h, cells were fixed in 4% paraformaldehyde and then stained with 0.1% crystal violet. The transferred cells were viewed and counted under a microscope (Leica, Germany).

2.6. Real time (RT)-quantitative PCR (qPCR) analysis

Total RNA was extracted from MRC-5 cells or lung tissues using Trizol Reagent (Sigma, USA). Then, a total of 2 μg of RNA were subjected to reverse transcription using the Reverse Transcription Kit (Roche, Germany) to prepare cDNA according to the manufacturer's instructions. Quantitative RT-PCR was performed with SYBR-Green Master Mix (Roche, Germany). Each sample was examined in triplicate, and GAPDH was used as an internal control. The primers for the real-time PCR were synthesized by Eurogentec, Belgium, and the primers sequences are listed in Table 1.

2.7. Western blot analysis

Total proteins were isolated from MRC-5 cells or fresh lung tissue and lysed in RIPA buffer. The centrifuged protein supernatants were collected, and their concentrations were determined using a BCA protein assay kit (Thermo, USA). Equal amounts of protein were separated by

Table 1
Primers used for PCR.

Name	Accession No.	Sequences (5′-3′)		Product size (bp)
ACTA2 for human	NM_001141945.2	Forward	TTCATCGGGATGGAGTCTGCTGG	141
		Reverse	TCGGTCGGCAATGCCAGGGT	
ACTA2 for mouse	NM_007392.3	Forward	AAGCCCAGCCAGTCGCTGTCA	115
		Reverse	GAAGCCGGCCTTACAGAGCCC	
SMAD7 for human	NM_005904.4	Forward	TTCCTCCGCTGAAACAGGG	116
		Reverse	CCTCCCAGTATGCCACCAC	
SMAD7 for mouse	NM_001042660.1	Forward	GGCCGGATCTCAGGCATTC	153
		Reverse	TTGGGTATCTGGAGTAAGGAGG	
GAPDH for human	NM_002046.7	Forward	GAGTCAACGGATTTGGTCGT	185
		Reverse	GACAAGCTTCCCGTTCTCAG	
GAPDH for mouse	NM_001289726.1	Forward	TGGCCTTCCGTGTTCCTAC	178
		Reverse	GAGTTGCTGTTGAAGTCGCA	

SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% skim milk at room temperature for 1 h. Primary antibodies against α-SMA (ab5694; Abcam), pSMAD2 (#12747; Cell Signalling Technology), tSMAD2 (#12747; Cell Signalling Technology), SMAD7 (MAB2029; R&D), and β-actin (#3700; Cell Signalling Technology) were used at 4 °C overnight. β-actin was detected as an internal control to normalize the relative expression of the target proteins. Membrane immunocomplexes were visualized using Image Lab Software (BIO-RAD) with Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA).

2.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism software. Normally distributed data are represented as the mean ± standard deviation. Statistical analyses were performed using Students t-tests or one-way ANOVA as appropriate. Nonparametric analyses were performed using Mann-Whitney tests or Kruskal-Wallis tests. $P < 0.05$ was accepted as statistically significant.

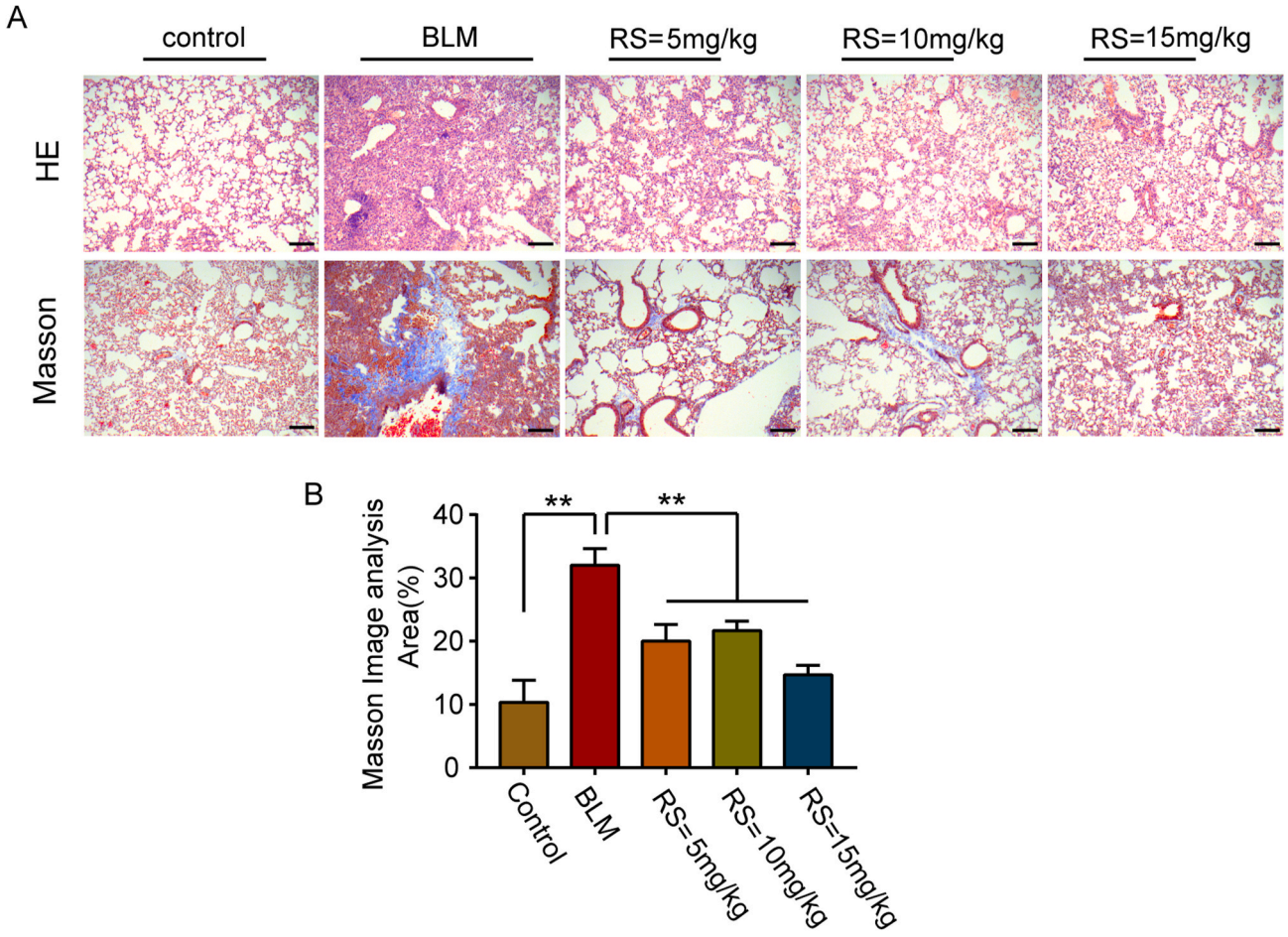

Fig. 1. RS4651 attenuated fibrosis severity in mice with BLM-induced pulmonary fibrosis. (A) Top, lung tissue specimens were stained with haematoxylin and eosin (H&E); Bottom, Masson's trichrome staining was carried out to determine the collagen levels in lung tissue specimens obtained from mice treated with various RS concentrations (original magnification: ×10; scale bars: 200 um); RS4651, RS. (B) Masson's trichrome staining score was used to evaluate the grade of the fibrotic changes in the lungs of different groups. **P<0.01 versus the control group; **P<0.01 versus the BLM group. RS4651, RS.

3. Results

3.1. RS4651 attenuated fibrosis severity in mice with BLM-induced pulmonary fibrosis

To evaluate the effect of RS4651 on lung fibrosis in vivo, we collected lung histopathologic sections and evaluated histopathological changes and collagen deposition using H&E staining and Masson's trichrome staining in different groups. The lung histopathology sections from the control group of mice showed normal pulmonary architecture and tissue structure. By contrast, in the BLM group, the alveolar epithelial cell structure collapsed and was destroyed, part of the alveolar septum was fractured, the alveolar walls were significantly thickened, a large number of fibroblasts gathered in the stroma, and the fibres were distributed. However, pulmonary lesions in the mice treated with 5 mg/kg of RS4651 showed decreased collagen deposition and improved architectural structure compared to the BLM group. Moreover, in mice treated with 15 mg/kg of RS4651, the extent of pulmonary fibrosis was further decreased; the mice exhibited repaired or restored alveolar walls and alveolar septa, as well as reduced collagen deposition (Fig. 1A). Masson's trichrome staining score was used to evaluate the grade of the fibrotic changes in the lungs of different groups. The scores of RS4651 treatment groups were obviously lower than that of the BLM group (Fig. 1B).

3.2. RS4651 reduced the BLM-induced expression of α-SMA in the mouse lung

To verify the anti-fibrosis function of RS4651 in our BLM-induced pulmonary fibrosis mouse model, α-SMA was used to monitor the degree of fibrosis in the mouse pulmonary tissue of each group. Using IHC, we found that the expression of α-SMA was dramatically higher in the lung sections of the BLM group than in the control group. By contrast, the high α-SMA expression induced by BLM was downregulated in the RS4651 treatment group, especially in the 15 mg/kg RS4651 group (Fig. 2A and B). Western blot and RT-PCR data were consistent with the IHC data (Fig. 2C and D). Those results suggested that RS4651 reduced the BLM-induced increase in α-SMA expression in the mouse lung.

3.3. RS4651 inhibited the activation of lung fibroblasts

To evaluate the function of RS4651 in lung fibrosis, the MRC-5 lung fibroblast cell line was used. First, we treated MRC-5 cells with RS4651 and investigated the effect of RS4651 on the activation of these cells. Interestingly, we found that both protein and mRNA expression of α-SMA was suppressed in a concentration-dependent manner in RS4651-treated MRC-5 cells (Fig. 3A and B). Specifically, the 30 μM RS4651 treatment led to a 50% decrease in α-SMA expression compared to the untreated group; therefore, we chose 30 μM RS4651 for the following in vitro studies. Because TGF-β1 is a key fibrogenic cytokine in lung fibrosis

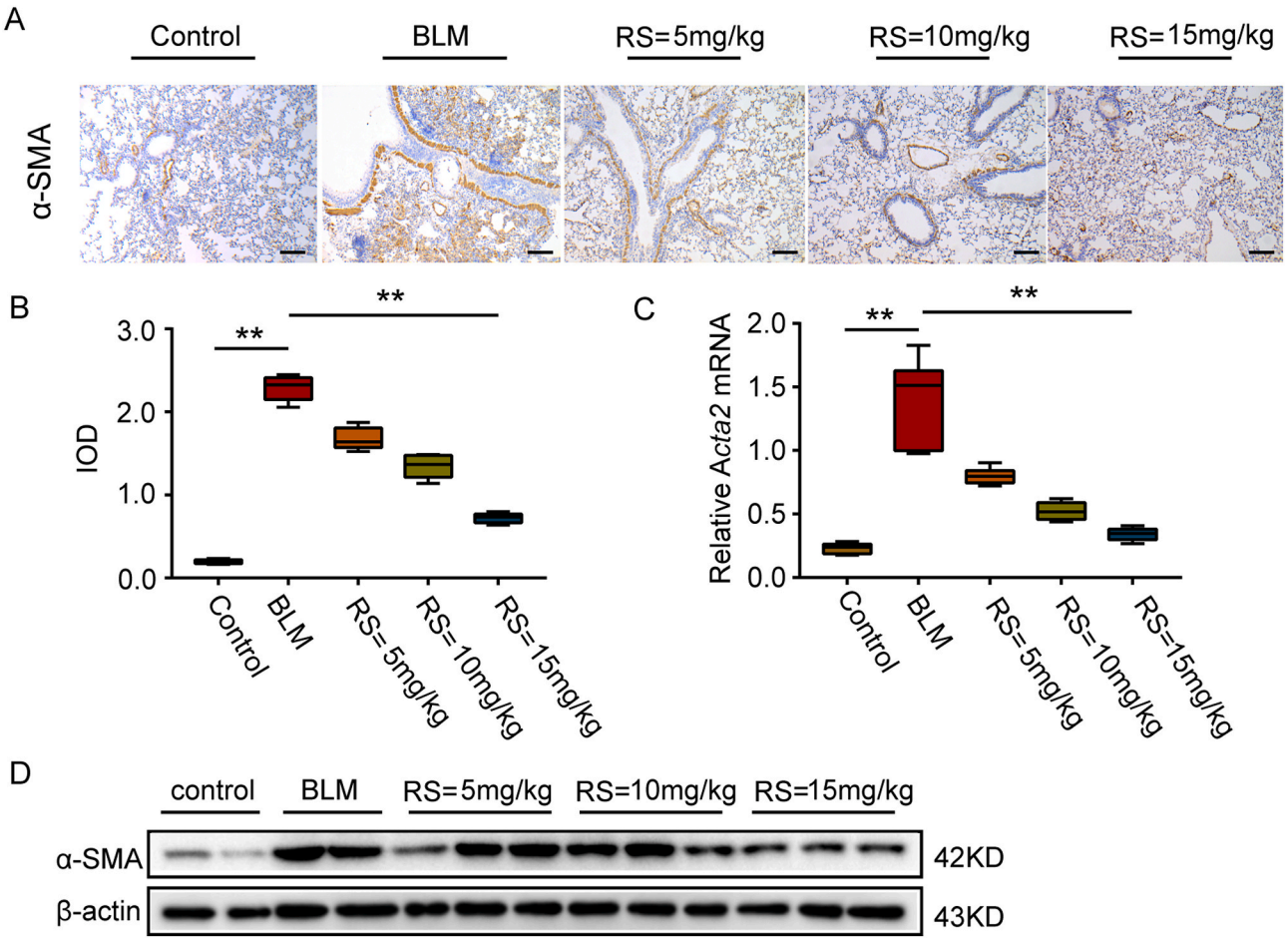

Fig. 2. RS4651 reduced the BLM-induced expression of α-SMA in the mouse lung. BLM-induced lung fibrosis mice were treated with various concentrations of RS. (A) and (B) Immunohistochemistry was performed to analyse α-SMA levels in lung tissue sections (original magnification: ×10; scale bars: 200 um). The representative images and quantified levels are displayed. (C) RT-PCR analysis was carried out to determine α-SMA mRNA levels in mouse lung samples. (D) Western blots were used to evaluate α-SMA protein expression. * $P < 0.05$ and ** $P < 0.01$ versus the control group; * $P < 0.05$ and **$P < 0.01$ versus the BLM group. RS4651, RS.

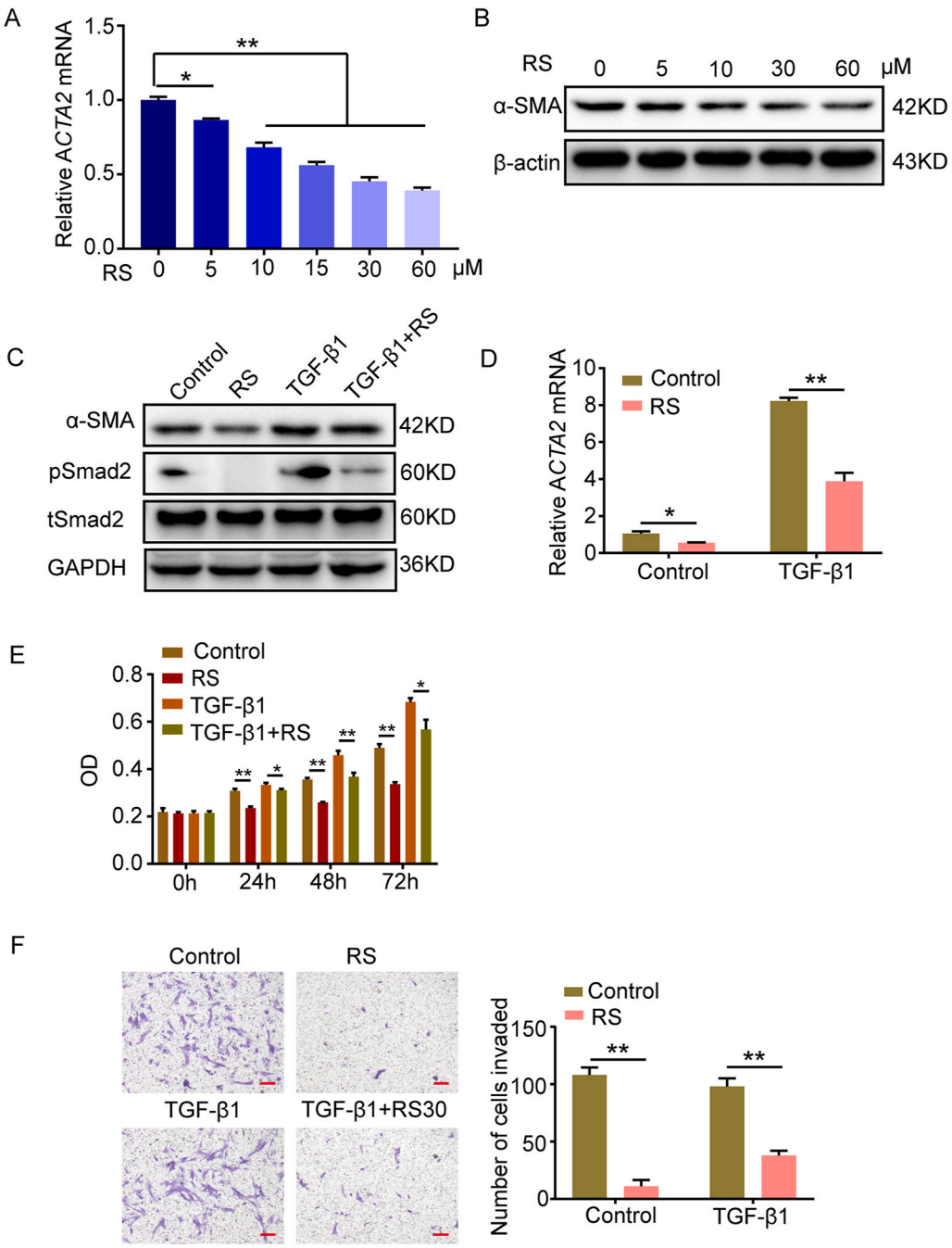

Fig. 3. RS4651 inhibited the activation of lung fibroblasts. (A) RT-PCR and (B) Western blot were used to assess the expression of α-SMA in MRC-5 cells after treatment with various concentrations of RS4651 for 48 h. MRC-5 cells were pre-treated with TGF-β1 (10 μg/mL) for 24 h before RS4651 (30 μM). (C) α-SMA, pSmad2 and tSmad2 expression levels were determined by Western blot. (D) RT-PCR analysis of α-SMA expression in MRC-5 cells. (E) An MTS assay was performed to assess cell viability for 0, 24, 48, and 72 h. (F) A transwell assay was used to assess the effect of RS4651 on MRC-5 migration (original magnification: ×10; scale bars: 100 um). Assay results are quantified. *P < 0.05 and **P < 0.01 versus the control group; *P < 0.05 and **P< 0.01 versus the TGF-β1 group.

and lung fibroblast cell activation, we next evaluated the effect of RS4651 on lung fibroblast cell activation induced by TGF-β1. Our results showed that TGF-β1 increased the level of phosphorylated SMAD2 (pSMAD2) and the expression of α-SMA, while RS4651 treatment downregulated the level of pSMAD2 and the expression of α-SMA induced by TGF-β1 (Fig. 3C and D). Furthermore, we measured the effect of RS4651 treatment on the proliferation and migration of MRC-5 cells in the presence of TGF-β1. Our results showed that RS4651 treatment could inhibit TGF-β1-induced proliferation and migration of MRC-5 cells (Fig. 3E and F). Taken together, these results suggest that RS4651 can inhibit lung fibroblast activation.

3.4. RS4651 inhibits lung fibroblast activation and is related to TGF-β1 downstream signals

To further understand the mechanism of RS4651 in lung fibroblast activation, we performed RNA-sequencing to analyse the gene expression profiles of MRC-5 cells after incubation with RS4651. Differential expression analysis resulted in 585 coding genes with FDR-corrected p values of <0.05 and fold changes of >2 (Fig. 4A and B). Specifically, the expression of fibrosis-associated genes, such as *Acta2, Col15A1, Col3A1, Col5A3, Col8A1, Col9A2 and Loxl3,* was significantly downregulated in RS4651-treated MRC-5 cells, while the fibrosis reversal associated genes MMP3 and MMP10 were significantly upregulated (Supplementary Table 2). In RS4651 treatment group, the expression of *Ptgs2*, the gene of COX-2 is also upregulated compared with control group (Supplementary Table 2). To identify the primary molecular events occurring in RS4651-

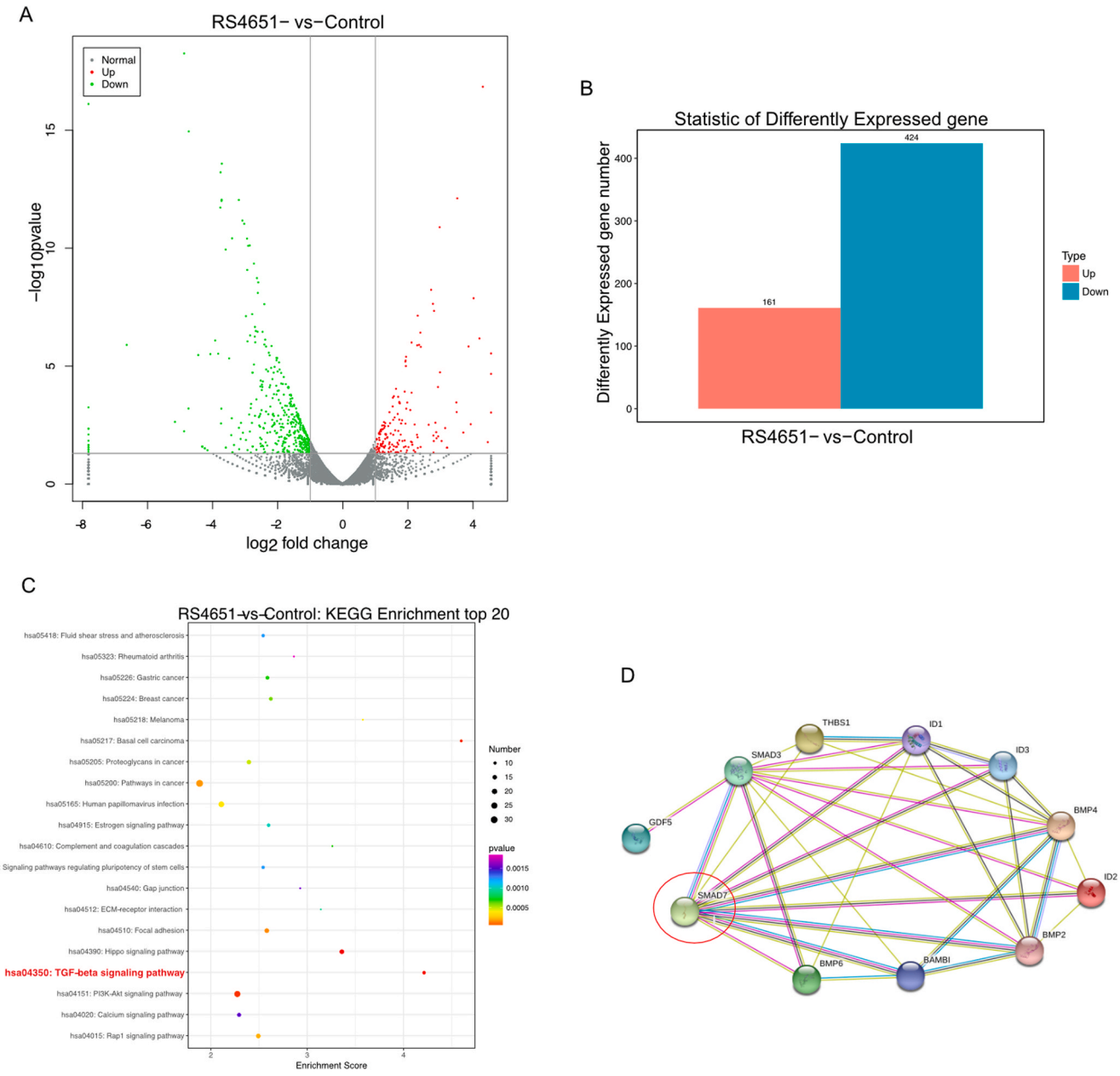

Fig. 4. RS4651 inhibits TGF-β1 downstream signalling. Gene expression analysis (RNA-sequencing) of MRC-5 cells treated with RS4651 (30 μM). (A) Volcano plot of the gene expression values. Red, gene expression upregulated in RS4651-treated MRC-5 cells. Green, gene expression downregulated in RS4651-treated MRC-5 cells. (B) The number of differentially expressed genes in A. (C) The top 20 most significant cellular component signals from the KEGG-enrichment analysis of the differentially expressed genes. (D) STRING analysis of the interaction network of 11 differentially expressed genes in the TGF-β signalling pathway.

treated MRC-5 cells, we performed a KEGG enrichment to analyse the function of the differentially expressed genes. Notably, the TGF-β1 signalling pathway was the second most significantly relevant event with 11 genes differentially expressed (Fig. 4C). To further reveal the key genes in the TGF-β1 signalling pathway that are regulated by RS4651, we used STRING software to analyse the interaction network of those 11 differentially expressed genes. As seen in Fig. 4D, we found that SMAD7 was the most important node of the whole interaction network.

3.5. RS465 inhibits the activation of lung fibroblasts via upregulating SMAD7

First, we confirmed the expression of SMAD7 in RS4651-treated MRC-5 cells. Our results showed that RS4651 treatment significantly upregulated the expression of SMAD7 and downregulated the expression of pSMAD2. To further investigate the effect of SMAD7 in the inhibition of MRC-5 cell activation by RS4651, we next constructed the SMAD7-siRNA (siSMAD7) MRC-5 cell model. We confirmed that the expression of α-SMA and the level of pSMAD2 were upregulated in SMAD7-siRNA MRC-5 cells. Moreover, the inhibitory effect of RS4651 on the expression of α-SMA and pSMAD2 was reduced in the SMAD7-siRNA MRC-5 cells (Fig. 5A and B). In addition, SMAD7 knockdown induced the proliferation and migration of MRC-5 cells and inhibited the effect of RS4651 on the proliferation and migration of MRC-5 cells (Fig. 5C and D). At the same time, we confirmed the effect of RS4651 on SMAD7 and pSMAD2 in an in vivo study (Fig. 5E and F).

4. Discussion

IPF is a chronic, progressive disease with a poor prognosis (Caminati

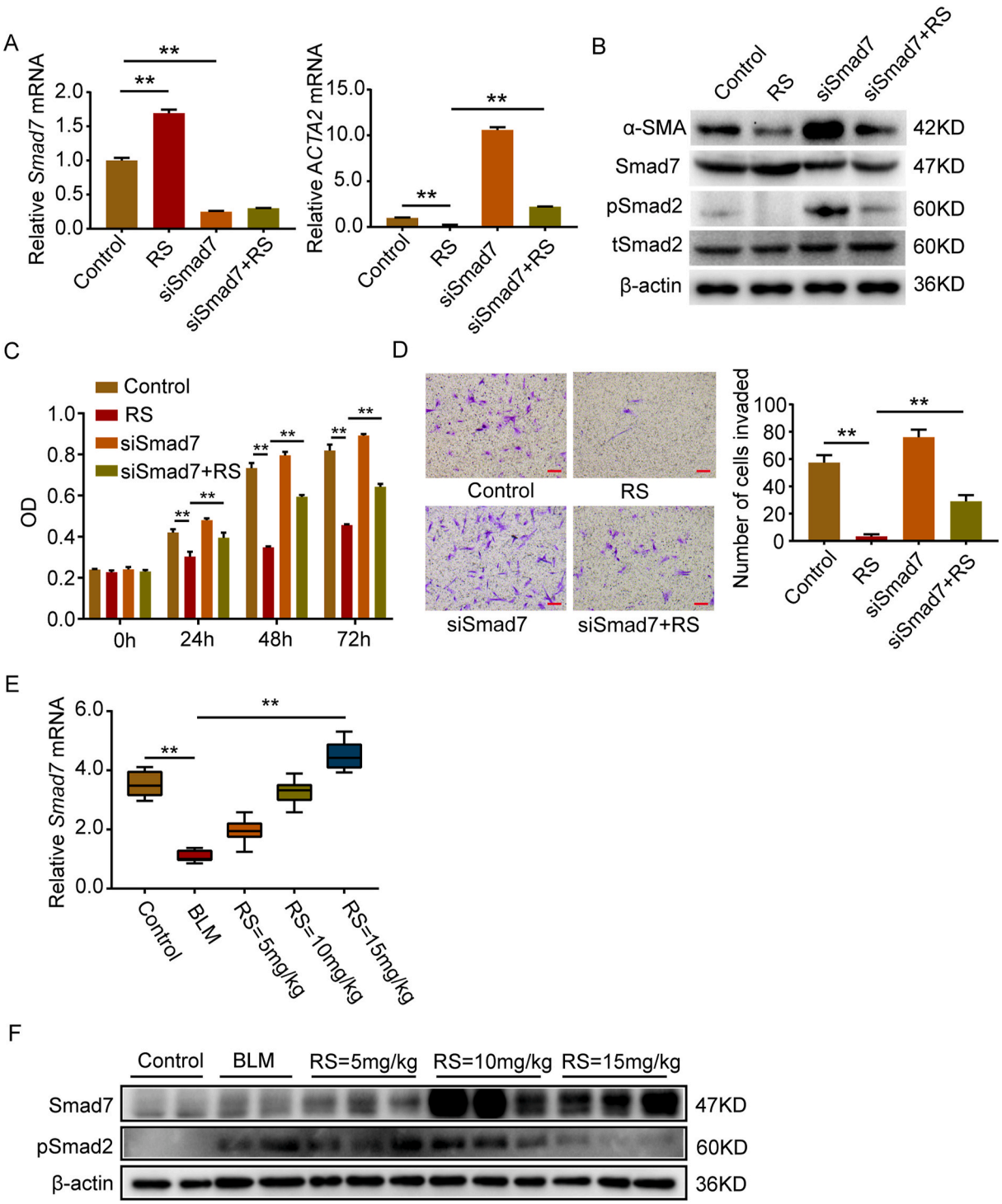

Fig. 5. RS4651 inhibits the activation of lung fibroblasts via upregulating Smad7. MRC-5 cells were treated with or without Smad7-siRNA. After incubation for 24 h, cells were stimulated with or without RS4651 for 24 h. (A) RT-PCR analysis of smad-7 and α-SMA expression. (B) Western blot assays were performed to examine the expression of α-SMA, Smad-7, pSmad2 and tSmad2. (C) MRC-5 cell proliferation was assessed by an MTS assay. (D) MRC-5 cell migration was assessed using a transwell assay (original magnification: ×10; scale bars: 100 um). (E) RT-PCR analysis was carried out to determine Smad7 mRNA levels in the lung samples obtained from mice treated with various concentrations of RS. (F) Western blot assays were performed to measure the expression of Smad7 and pSmad2.**P<0.01 versus the control group; **P<0.01 versus the BLM group. RS4651, RS.

et al., 2019) and high economical healthcare burden (Lu and El-Hashash, 2019). RS4651 (C21H18N4O; molecular weight, 342.39, Chart 1) is the 6th compound of a series of 1-phenyl-5-(1H-pyrrol-1-yl) pyrazolecarboxamide compounds synthesized by the State University of New Jersey and the Sapienza University, Department of Drug Chemistry and Technologies (Manvar et al., 2015). In this study, we found for the first time that RS4651 could alleviate BLM-induced experimental pulmonary fibrosis in mice and inhibit the activation, proliferation and migration of MRC-5 cells. This anti-fibrosis function of RS4651 was found to occur, at least in part, through the TGF-β1/SMAD signalling pathway. Our findings suggest that RS4651 may be a potentially effective treatment for IPF.

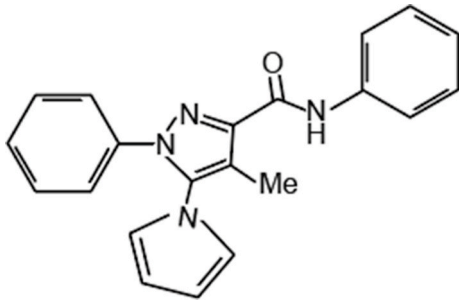

Chart 1. Structures of compounds of RS4651.

In vivo, a single intratracheal (I.T.) injection of BLM to induce progressive pulmonary fibrosis in mice is a classic and popular animal model for human pulmonary fibrosis (Tashiro et al., 2017). Because the pathophysiological characteristics of the lung in this animal model are similar to those of IPF patients (Carrington et al., 2018; Chen et al., 2013; Helms et al., 2010), it has been confirmed by an ATS workshop report as the most characteristic and first line animal model for preclinical test (Jenkins et al., 2017), and widely used to investigate the pathogenesis and therapeutic effect of IPF. We successfully established the pulmonary fibrosis model in mice using this method.

The transition from fibroblasts to myofibroblasts (MFBs) is critical to the development and progression of IPF (Scruggs et al., 2020). α-SMA is a characteristic marker of fibroblast activation and transition into MFBs, which stimulates the production of ECM components (Bollong et al., 2017). The expression level of α-SMA indirectly reflects the proliferation of MFBs. In vivo, we found that pulmonary lesions and collagen deposition were significantly reduced, and the expression of α-SMA was significantly decreased in the different RS4651 administration groups compared to the BLM group. The results showed that RS4651 can alleviate BLM-induced pulmonary fibrosis in mice in a dose-dependent manner. It was well known that excess fibroblast activation, proliferation, migration and transitions to MFBs, as well as ECM deposition, lead to pulmonary fibrosis (Vu et al., 2019). In vitro, as expected, our results found that RS4651 could inhibit the proliferation, migration and α-SMA expression of MRC-5 cells in a concentration-dependent manner.

It has been identified that RS4651 mediates its anti-HCV function through targeting COX-2 (Manvar et al., 2015). COX-2 is the rate-limiting enzyme for the synthesis of Prostaglandin E2(PGE2) expressed in alveolar epithelial cells, alveolar macrophages, fibroblasts and alveolar type II (ATII) epithelial cells (Bauer et al., 2000; Cheng et al., 2016; Wilborn et al., 1995). Quite a number of studies have shown the protective functions of COX-2 and PGE2 for pulmonary fibrosis these years especially in inhibition of fibroblast proliferation and collagen synthesis through inhibiting collagen synthesis and promote degradation (Feng et al., 2019b; Park and Christman, 2006). Failure to up-regulate COX-2 expression and reduced PGE2 production have been proved to associated with the activation, migration and proliferation of lung fibroblasts and the excessive deposition of collagen (Pasini et al., 2018). While, the role of COX-2 in pulmonary fibrosis remains controversial. Jeffrey W. Card (Card et al., 2007) found that the role of COX-2 deficiency is not to aggravate pulmonary fibrosis but to deteriorate the functional impairment associated with pulmonary fibrosis. RS4651 has different effects on COX-2 in different models, the mechanism is still unclear and need further research. Our result of transcriptome sequencing confirmed that the expression of *Ptgs2*, the gene of COX-2 was significantly upregulated in the RS4651 treatment group compared with the untreated group. This result is inconsistent with the effect of RS4651 on COX-2 of HCV in the previous report (Manvar et al., 2015). We further explored the mechanism of RS4651's protective effect on pulmonary fibrosis.

Among the numerous pro-fibrosis factors, TGF-β1 is considered the chief regulator of α-SMA and the key cytokine controlling the activation, proliferation and migration of fibroblasts (Jin et al., 2018; Wei et al., 2019). There are three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) and correspond to three serine/threonine kinase receptors (TGF-β RI, TGF-β RII and TGF-β RIII). TGF-β1 is the critical cytokine exerts its biological effects through TGF-β1/SMAD-dependent and TGF-β1/SMAD-independent signalling pathway (Mu et al., 2012). TGF-β/SMAD signalling, known as the classical pathway, is considered to be the most important pathway for fibrogenesis (Chen et al., 2018; Feng et al., 2020; Higgins et al., 2018; Ojiaku et al., 2018). There are 3 categories SMADs involved in TGF-β1/SMAD signalling pathway: R-SMADs (SMAD2 and SMAD3), Co-SMADs (SMAD4), and I-SMADs (SMAD-7). The active form TGF-β1 first binds to the extracellular segment of TGF-β RII to phosphorylate and activate TGF-β RI located inside the membrane to form an activate complex TGF-β/TβRI/TβRII, which phosphorylates SMAD2 and SMAD3 in the cytoplasm, the actived R-SMADs subsequently bind to SMAD4 and form a SMAD complex, which transferred into the nucleus where they bind to the targets sequence to regulate specific genes' transcription (Chen et al., 2018; Feng et al., 2020; Li et al., 2019). SMAD7 competitively blocks SMAD2 and SMAD3 phosphorylation by binding to activated receptors, negatively regulating the TGF-β1/SMAD signalling pathway (Huse et al., 2012). SMAD7 acts negatively as an antagonist and protects against TGF-β1-mediated fibrosis (Hayashi et al., 1997; Hu et al., 2018). Upregulation of SMAD7 was considered to be effective in treating IPF (Feng et al., 2019a). TGF-β1/SMAD-dependent signalling pathway is widely used for pathogenic mechanism and therapeutic studies of IPF (Aschner and Downey, 2016; Hu et al., 2018). Pirfenidone and nintedanib reduce the expression of α-SMA, the proliferation of lung fibroblasts and their differentiation into MFBs by attenuating TGF-β1-induced signalling pathways (Lehtonen et al., 2016; Raghu, 2017; Wollin et al., 2014, 2019).

Our studies showed that RS4651 could inhibit the expression of α-SMA and pSMAD2 and the proliferation and migration induced by TGF-β1 in MRC-5 cells. Further research confirmed that in the RS4651 treatment group, SMAD7 was the target gene act as the most important node of the whole gene interaction network, and SMAD7 siRNA could significantly increase the expression of α-SMA and pSMAD2 in MRC-5 cells, in addition to reducing the RS4651 inhibitory effect on the expression of α-SMA and pSMAD2 and the proliferation and migration of MRC-5 cells. These data suggest that SMAD7 plays a significant role in the anti-fibrosis function of RS4651. These results are consistent with many studies on the efficacy of drugs acting on the TGF-β1/SMAD signalling pathway (Feng et al., 2020; Qin et al., 2019).

We then verified the mechanism of RS4651 in alleviating BLM-induced pulmonary fibrosis in mice. We found that in vivo, RS4651 therapy also upregulated SMAD7 expression and downregulated pSMAD2 expression. All of these results revealed that RS4651 alleviates the activation of MRC-5 cells and attenuates BLM-induced pulmonary fibrosis in mice by upregulating SMAD7 to inhibit the TGF-β1/SMAD signalling pathway.

5. Conclusion

In conclusion, our studies identified that RS4651 alleviates BLM-induced pulmonary fibrosis in mice by upregulating SMAD7 to inhibit the TGF-β1/SMAD signalling pathway. These results have been confirmed by in vitro studies. Thus, RS4651 may be a potential agent for the treatment of IPF.

Authors' contributions

HY W and WJ Q designed the study and supervised it; Silvestri R donated RS4651 and revised the study; SR L performed the most experiments, drafted the manuscript and did the bulk of the revisions; AJ X and YM L provided technique supports. CT T participated in animal model. YM L made most of the figures. WJ Q and Silvestri R revised the manuscript. La Regina G re-synthesized RS4651 for this study. All

authors have read and approved the manuscript in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.174135.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The animal study in this research was approved by the Animal Experiments and Experimental Animal Welfare Committee of Capital Medical University (18-2019).

Consent for publication

Not applicable.

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