Platyconic acid A-induced PPM1A upregulation inhibits the proliferation, inflammation and extracellular matrix deposition of TGF-β1-induced lung fibroblasts

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Abstract. Platyconic acid A (PA), the active component of Platycodi radix-derived saponin, exerts ameliorating effects on liver fibrosis. Platycodon grandiflorum is used to treat lung disease. Therefore, the present study evaluated the effects of PA on pulmonary fibrosis. Transforming growth factor-β1 (TGF-β1) was used to induce MRC-5 cells to establish an in vitro pulmonary fibrosis model. The viability of MRC-5 cells in the presence or absence of TGF-β1 induction was examined using a Cell Counting Kit-8 assay and the results demonstrated that PA markedly decreased viability of TGF-β1-induced MRC-5 cells in a dose-dependent manner. Wound healing analysis, immunofluorescent staining and western blotting were performed to determine the levels of cell migration and expression of α-smooth muscle actin and extracellular matrix (ECM)-associated proteins. The results of the present study demonstrated that PA significantly suppressed the migration and ECM deposition of TGF-β1-induced MRC-5 cells. Furthermore, results obtained from ELISA and western blotting demonstrated that PA exerted suppressive effects on the inflammation of MRC-5 cells following TGF-β1 stimulation. The mRNA and protein expression levels of protein phosphatase Mg2+/Mn2+-dependent 1A (PPM1A) before and after transfection were assessed using reverse transcription-quantitative PCR and western blotting and the results demonstrated that the mRNA and protein expression levels of PPM1A were significantly decreased following transfection with small interfering RNA targeting PPM1A. Moreover, following PPM1A knockdown, PA significantly inhibited the proliferation, migration, inflammation and ECM deposition of TGF-β1-induced MRC-5 cells via activation of the SMAD/β-catenin signaling pathway. In conclusion, PA activated PPM1A to ameliorate TGF-β1-elicited lung fibroblast injury via modulating SMAD/β-catenin signaling.

Introduction

Pulmonary fibrosis is a chronic, irreversible interstitial lung disease, primarily affecting middle-aged and elderly populations (1). The incidence of pulmonary fibrosis is increasing globally as the population ages; however, its pathogenesis is still poorly understood and there is a lack of effective therapeutic agents (2,3). Previous studies have reported the presence of a large number of lung fibroblasts that maintain the structural and functional integrity of lung tissue by synthesizing extracellular matrix (ECM) and cytokines (4,5). However, when lung tissue is damaged, fibroblasts become dysfunctional and over-proliferate or convert to ECM, ultimately leading to inflammatory and fibrotic processes in the lung (6,7). Therefore, inhibition of excessive lung fibroblast proliferation, inflammation and ECM deposition may be key for improving the treatment of pulmonary fibrosis.

Transforming growth factor-β1 (TGF-β1) is regarded as the most potent pro-fibrotic cytokine and regulates numerous cellular processes, such as proliferation, growth inhibition, migration and ECM remodeling via the SMAD/β-catenin signaling pathways (8-10). An increasing number of studies have reported that TGF-β1 signaling is involved in numerous fibrosis-associated diseases, such as renal (11) and in particular, pulmonary fibrosis (12). Furthermore, Liu et al (13) reported that inhibition of TGF-β1 protects against liver fibrosis. The results of a recent study further demonstrated that knockdown of TGF-β1 alleviates high mechanical stress-induced chondrocyte fibrosis (14). Moreover, Leask and Abraham (15) reported the involvement of TGF-β1 signaling in the development of pulmonary fibrosis. Therefore, inhibiting TGF-β1 activity may be an effective strategy for inhibiting pulmonary fibrosis.

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**Platycodon grandiflorus** (PG), a widely used edible, traditional Chinese medicinal herb, is commonly used as a pulmonary adjuvant to enhance the effect of other drugs in the management of lung disease (16,17). It was previously reported that bronchitis, asthma and tuberculosis, as well as inflammatory disease, can be treated using PG extract (18). Platycodon acid A (PA) is the active component of *Platycodon saponin*, which possess similar effects to PG in the treatment of lung disease (19). A previous study reported that PA inhibits TGF-β1-induced liver fibrosis by blocking SMAD signaling pathway and activating the peroxisome proliferator-activated receptor γ signaling pathway (20). These results suggest that PA may serve as an important factor in the pathological process of pulmonary fibrosis; however, the specific underlying mechanisms are yet to be fully elucidated.

Protein phosphatase Mg²⁺/Mn²⁺-dependent 1A (PPM1A) is a Ser/Thr protein phosphatase that belongs to the protein phosphatase 2C family, which regulates cell cycle progression, proliferation, differentiation and apoptosis (21-23). A previous study reported that PPM1A is an important inhibitory regulator in the TGF-β1 signaling pathway (24). Moreover, numerous studies have demonstrated that upregulation of PPM1A is associated with anti-pulmonary fibrosis effects (25-27). These data suggested that PPM1A may serve an inhibitory role in pulmonary fibrosis via inhibition of TGF-β1.

The present study analyzed TGF-β1-induced pulmonary fibrosis cell models; it was hypothesized that PA may be involved in TGF-β1-induced pulmonary fibrosis via effects on the SMAD/β-catenin signaling pathway and regulation of PPM1A.

**Materials and methods**

**Cell culture and transfection.** The BeNa Culture Collection (Beijing Beina Chunglian Institute of Biotechnology) supplied the human lung fibroblast MRC-5 cell line. Cells were incubated in 10% FBS (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin in DMEM (Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. To establish an in vitro cell model of pulmonary fibrosis, MRC-5 cells were induced with 10 ng/ml TGF-β1 (R&D Systems, Inc.) for 48 h at 37°C and subsequently harvested using 0.25% trypsin for further analysis, as previously described (28). Small interfering (si)RNA targeting PPM1A [si-PPM1A#1 (cat. no. siB05113142945-1-5)] and corresponding negative control (NC; siN0000001-1-5) were designed and manufactured by Guangzhou RiboBio Co., Ltd. Transfection of the aforementioned siRNAs (60 nM) into MRC-5 cells was performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) at 37°C for 24 h. At 48 h after transfection with si-PPM1A#1/2, reverse transcription-quantitative PCR (RT-qPCR) and western blotting were used to assess the mRNA and protein expression levels of PPM1A.

**Cell counting kit (CCK)-8 analysis.** To determine whether PA was toxic to MRC-5 cells, 0.5, 1.0, 2.0, 5.0 and 10.0 µM PA (Shanghai Yuanye Bio-Technology Co., Ltd.) was used to treat cells for 24 h at 37°C (19). Following TGF-β1 induction and treatment with PA, MRC-5 cells were cultured in 96-well plates (1x10⁴) for 24 h at 37°C. Each well was supplemented with 10 µl CCK-8 solution (Beijing AVIVA Systems Biology) for 4 h at 37°C. A Synergy™ 2 multifunctional microplate reader (BioTek Instruments, Inc.) was used to assess the optical density at 450 nm.

**Wound healing analysis.** Following treatment of TGF-β1 (10 ng/ml; R&D Systems, Inc.) and PA (0.5, 1.0, 2.0, 5.0 and 10.0 µm; Shanghai Yuanye Bio-Technology Co., Ltd.), transfected or untransfected MRC-5 cells were cultured at the bottom of a 60-mm culture dish. When 100% confluence was reached, a wound was created in the confluent monolayer using a sterile pipette tip (20 µl). Following rinsing with PBS, MRC-5 cells were cultured again in serum-free DMEM at 37°C for 24 h. The width of the scratch was assessed using a light microscope (CKX53; Olympus Corporation) at 0 and 24 h (magnification, x100). The wound width was visualized using ImageJ software version 1.8.0 (National Institutes of Health).

**Immunofluorescent staining.** Cells treated by TGF-β1 (10 ng/ml; R&D Systems, Inc.) for 48 h at 37°C and PA (0.5, 1.0, 2.0, 5.0 and 10.0 µm; Shanghai Yuanye Bio-Technology Co., Ltd.) for 24 h at 37°C were grown to 100% confluence and subsequently harvested using 0.5% Triton X-100 (MP Biomedicals, LLC) was added to permeabilize the cells at room temperature. Cells were incubated with primary antibody against α-SMA (1:300; cat. no. ab124964; Abcam) overnight at 4°C. Following rinsing with PBS, cells were incubated with Alexa Fluor® 488-conjugated secondary antibody (1:500; cat. no. ab150077; Abcam) at room temperature for 1 h. DAPI was used to counterstain the cell nuclei at room temperature for 15 min and cells were visualized under a fluorescence microscope (DM6000; Leica Microsystems GmbH). Images were processed by ImageJ Software version 1.52s (National Institutes of Health) to assess the fluorescence intensity.

**Western blotting.** MRC-5 cells were lysed using RIPA buffer (Beyotime Institute of Biotechnology) containing protease and phosphatase inhibitors to obtain total protein, which was quantified using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) to determine protein concentration. Following electrophoresis with 8% SDS-PAGE gels, protein samples (30 µg per lane) were transferred onto pure nitrocellulose membranes (Pall Life Sciences). Then, the membranes were washed three times with 0.1% Tween-20 TBS solution (TBST) and blocked with 5% skimmed milk (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature before incubation with primary antibodies against fibronectin 1 (Fn1; 1:1,000; cat. no. ab45688; Abcam), collagen 1 (1:1,000; cat. no. ab34710; Abcam), matrix metalloproteinase (MMP)-1 (1:1,000; cat. no. ab134184; Abcam), α-smooth muscle actin (SMA; 1:1,000; cat. no. ab124964; Abcam), phosphorylated (p)-NF-κB (1:1,000; cat. no. sc-136548; Santa Cruz Biotechnology, Inc.), NF-κB (1:1,000; cat. no. ab288751; Abcam), PPM1A (1:1,000; cat. no. ab231893; Abcam), p-SMAD-2 (1:1,000; cat. no. ab280888; Abcam), p-SMAD-3 (1:2,000; cat. no. ab52903; Abcam), β-catenin (1:10,000; cat. no. ab32572; Abcam) and SMAD-2/3 (1:1,000; cat. no. ab202445; Abcam) overnight at 4°C. Following primary incubation, membranes were incubated with HRP-conjugated
goat anti-rabbit IgG (1:2,000; cat. no. ab6721; Abcam) and goat anti-mouse IgG (1:2,000; cat. no. ab6789; Abcam) for 1 h at room temperature. Protein bands were visualized using BeyoECL Plus kit (Beyotime Institute of Biotechnology) and imaged using the Odyssey CLX Infrared Imaging System. The intensity of signals was semi-quantified using ImageJ 1.8.0 software (National Institutes of Health).

ELISA. Protein expression levels of TNF-α, IL-1β and IL-6 in MRC-5 cells were determined using human TNF-α (cat. no. DTA00D; R&D Systems, Inc.), IL-1β (cat. no. DLB50; R&D Systems, Inc.) and IL-6 ELISA kits (cat. no. D6050; R&D Systems, Inc.), according to the manufacturer’s protocol. Absorbance values were read at a wavelength of 540 nm and levels of TNF-α, IL-1β and IL-6 were calculated using the standard curve.

RT-qPCR. RT-qPCR was performed to quantify the mRNA expression levels of PPM1A in MRC-5 cells before and after transfection. RNA was isolated from MRC-5 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and RNA was reverse-transcribed into complementary (c)DNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. qPCR was performed using a Maxima SYBR Green/ROX qPCR Master Mix kit (Fermentas; Thermo Fisher Scientific, Inc.) on a StepOnePlus™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec. The specific primer sequences used were as follows: PPM1A forward (F), 5'-AGG GGC AGG GTAATGGTT-3' and reverse (R), 5'-GATCAGGCCGTATG TGCATC-3' and GAPDH F, 5'-GCACCTCAAGGCTGAGA AC-3' and R, 5'-GGATCTCGCTCTGGAAATG-3'. mRNA expression levels were quantified using the 2^ΔΔCt method (29).

Molecular target analysis. SwissTargetPrediction (swisstar-getprediction.ch/) was used to identify PPM1A as a potential target for PA.

Statistical analysis. All data are presented as the mean ± standard deviation of at least three independent experiments and analyzed using SPSS 22.0 (IBM Corp.). For comparisons between two groups, unpaired Student's t-test was used and one-way ANOVA followed by Tukey's post hoc test was used to determine differences between ≥3 groups. P<0.05 was considered to indicate a statistically significant difference.

Results

PA inhibits TGF-β1-induced over-proliferation of MRC-5 cells. The chemical structure of PA is presented in Fig. 1A. The effect of different concentrations of PA on the viability of MRC-5 cells was assessed. PA at concentrations of 0.5, 1.0 and 2.0 µM exhibited no significant effect on cell viability compared with the control, whereas a significant decrease was demonstrated in viability of MRC-5 cells treated with PA concentrations of 5 and 10 µM (Fig. 1B). These results demonstrated that 5 and 10 µM PA were toxic to MRC-5 cells. Therefore, PA at concentrations of 0.5, 1.0 and 2.0 µM was selected for use in subsequent experiments. Following the induction of MRC-5 cells with TGF-β1, the effect of PA on cell viability was evaluated. TGF-β1 significantly increased cell proliferation compared with the control and PA markedly suppressed TGF-β1-induced proliferation in MRC-5 cells (Fig. 1C). Furthermore, the higher the concentration of PA, the lower the levels of cell proliferation. And the proliferation of TGF-β1-challenged MRC-5 cells was the most significantly suppressed by 2 µM PA by contrast with the TGF-β1 group. These results supported the hypothesis that PA inhibited TGF-β1-induced over-proliferation of MRC-5 cells in a dose-dependent manner.

PA inhibits TGF-β1-induced migration and ECM deposition in MRC-5 cells. To elucidate the effect of PA on cell migration and ECM deposition, wound healing assay was. Cell migration was significantly higher following TGF-β1 induction compared with the control (Fig. 2A). However, cell migration was significantly decreased in the TGF-β1 + PA (0.5, 1.0 and 2.0 µM) groups compared with the TGF-β1 group. Furthermore, protein expression levels of the myofibroblast marker α-SMA were significantly elevated in the TGF-β1 group compared with the control and these levels declined significantly in a dose-dependent manner following treatment with different concentrations of PA (0.5, 1.0 and 2.0 µM; Fig. 2B) compared with the TGF-β1 group. Protein expression levels of ECM-associated proteins, including Collagen I, Fn1, MMP-1 and α-SMA were significantly elevated in TGF-β1-induced MRC-5 cells compared with the control and these levels were significantly decreased in a dose-dependent manner following treatment with PA at concentrations of 0.5, 1.0 and 2.0 µM compared with the TGF-β1 group (Fig. 2C). Overall, these findings demonstrated that PA exerted suppressive effects on TGF-β1-induced migration and ECM deposition in MRC-5 cells.

PA inhibits the TGF-β1-induced inflammatory response in MRC-5 cells. The present study assessed whether PA affected the TGF-β1-induced inflammatory response in MRC-5 cells. ELISA demonstrated a significant elevation in the protein expression levels of the inflammatory cytokines IL-6, IL-1β and TNF-α in the TGF-β1 group compared with the control (Fig. 3A). Moreover, significantly decreased protein expression levels of IL-6, IL-1β and TNF-α in all TGF-β1 + PA groups were observed compared with the TGF-β1 group. The results of the present study demonstrated that TGF-β1 induced a significant increase in protein expression levels of p-NF-κB in MRC-5 cells compared with the control group (Fig. 3B). Furthermore, the protein expression levels of p-NF-κB were significantly decreased in MRC-5 cells following all PA treatments compared with the control. As PA exerted a greater inhibitory effect at a concentration of 2 µM, this concentration was selected for subsequent experiments. These results demonstrated that PA notably inhibited the TGF-β1-induced inflammatory response in MRC-5 cells.

PA upregulates PPM1A expression in TGF-β1-induced MRC-5 cells and inhibits TGF-β1-induced proliferation and ECM deposition in MRC-5 cells via the SMAD/β-catenin signaling pathway. PPM1A was predicted as a potential target for PA using the SwissTargetPrediction website.
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To evaluate whether PA exerted effects on PPM1A in MRC-5 cells, mRNA and protein expression levels of PPM1A were assessed in TGF-β1-induced cells in the presence or absence of PA treatment. PPM1A expression levels were significantly decreased in TGF-β1-induced MRC-5 cells compared with those in the control group and significantly elevated following PA treatment compared with the TGF-β1 group (Fig. 4A and B). These results indicated that PA may have upregulated PPM1A expression. MRC-5 cells were transfected with si-PPM1A#1/2 for verification. It was discovered that si-PPM1A#1/2 both demonstrated significantly reduced mRNA and protein expression levels compared with si-NC. MRC-5 cells transfected with si-PPM1A#1 exhibited markedly lower PPM1A expression levels, compared with cells transfected with si-PPM1A#2 (Fig. 4C and D). Therefore, si-PPM1A#1 was selected for use in subsequent experiments and referred to as si-PPM1A. The results of the CCK-8 analysis demonstrated that the proliferation of TGF-β1-induced MRC-5 cells treated with PA was increased significantly in TGF-β1 + PA + si-PPM1A group compared with the TGF-β1 + PA + si-NC group (Fig. 4E). Moreover, migration of TGF-β1-induced MRC-5 cells treated with PA was significantly increased in TGF-β1 + PA + si-PPM1A group compared with the TGF-β1 + PA + si-NC group (Fig. 4F). A significant increase in expression of α-SMA was also demonstrated in TGF-β1-induced MRC-5 cells treated with PA following transfection with si-PPM1A compared with NC (Fig. 4G). PPM1A knockdown resulted in significantly increased protein expression levels of ECM-associated proteins, including Collagen1, Fn1, MMP-1.

Figure 1. PA inhibits TGF-β1-induced over-proliferation of MRC-5 cells. (A) Chemical structure of PA. (B) MRC-5 cell viability following treatment with PA (0.5, 1.0, 2.0, 5.0 and 10.0 µM) assessed using CCK-8 assay. (C) Viability of TGF-β1-induced MRC-5 cells treated with PA (0.5, 1.0 and 2.0 µM) assessed using CCK-8 assay. *P<0.05 and ***P<0.001. PA, platyconic acid A; CCK-8, Cell Counting Kit-8; TGF-β1, transforming growth factor-β1.
PA inhibits TGF-β1-induced inflammation in MRC-5 cells via the SMAD/β-catenin signaling pathway. To assess the effect of PA binding to PPM1A on TGF-β1-induced inflammation of MRC-5 cells, ELISA and western blotting were performed. The results of the present study demonstrated that protein
expression levels of the inflammatory cytokines IL-6, IL-1β and TNF-α were significantly elevated in MRC-5 cells transfected with si-PPM1A compared with NC (Fig. 5A). Moreover, p-NF-κB protein expression levels were also significantly increased in the TGF-β1 + PA + si-PPM1A group compared with the TGF-β1 + PA + si-NC group (Fig. 5B). Furthermore, the expression levels of SMAD/β-catenin-associated proteins, including p-SMAD-2, p-SMAD-3 and β-catenin, were significantly elevated in TGF-β1-induced MRC-5 cells compared with the control; these levels were significantly decreased following treatment with PA compared with the TGF-β1 group. However, PPM1A knockdown significantly increased the protein expression levels of p-SMAD-2, p-SMAD-3 and β-catenin compared with NC (Fig. 5C and D). These data demonstrated that the inhibitory effects of PA on TGF-β1-induced inflammation of MRC-5 cells were achieved following upregulation of PPM1A and the SMAD/β-catenin signaling pathway.

Discussion

Pulmonary fibrosis is characterized by varying degrees of inflammation and ECM deposition, often leading to lung dysfunction and death (30). To date, only a small number of drugs have been approved for the treatment of pulmonary fibrosis-associated diseases, such as idiopathic pulmonary fibrosis (IPF), which exhibits a poor prognosis (31). The results of previous studies have demonstrated the role of PG in treatment of pulmonary disease and the anti-liver fibrotic effect of the active component PA (32,33). However, the specific mechanisms involved in the inhibitory effect of PA on pulmonary fibrosis are yet to be fully elucidated. Therefore, the present study evaluated the potential mechanisms underlying PA inhibition in lung fibroblasts.

TGF-β1 is a key element in lung injury and pulmonary fibrosis that activates fibroblast proliferation and differentiation and increases accumulation of ECM in the lung, leading to development of pulmonary fibrosis (34). Therefore, the present study used TGF-β1 to induce MRC-5 cells to establish an in vitro pulmonary fibrosis model. Results of the present study demonstrated that PA exerted no effect on MRC-5 cell viability at concentrations of 0.5, 1.0 and 2.0 µM. A previous study reported that PA inhibits TGF-β1-induced proliferation of hepatic fibroblasts in a dose-dependent manner (33). The results of the present study demonstrated that TGF-β1 induction significantly increased proliferation of MRC-5 cells; however, PA treatment markedly inhibited the over-proliferation induced by TGF-β1. Furthermore, TGF-β1 is a regulator of cell migration (35). The results of the present study demonstrated that cell migration was significantly increased following TGF-β1 induction and treatment with PA significantly inhibited the increased cell migration in a dose-dependent manner.

A previous study reported that treatment with TGF-β1 leads to ECM production by increasing protein expression levels of α-SMA and Collagen I (36). The results of the present study also demonstrated a significant increase in α-SMA protein expression levels in TGF-β1-induced MRC-5 cells. Moreover, previous studies reported that aqueous extracts and saponins extracted from Platycodi radix inhibit expression levels of α-SMA and Collagen I in rat models induced by carbon tetrachloride, dimethyl-nitrosamine and a high-fat diet (37-39). The results of the present study demonstrated that PA significantly inhibited TGF-β1-induced cell migration and ECM deposition...
Figure 4. PA upregulates PPM1A expression in TGF-β1-induced MRC-5 cells and inhibits TGF-β1-induced proliferation and ECM deposition in MRC-5 cells via the SMAD/β-catenin signaling pathways. The mRNA and protein expression levels of PPM1A in TGF-β1-induced MRC-5 cells in the absence and presence of PA were assessed using (A) RT-qPCR and (B) western blotting. PPM1A mRNA and protein expression levels following its knockdown in MRC-5 cells were assessed using (C) RT-qPCR and (D) western blotting. (E) Viability of TGF-β1-induced MRC-5 cells treated with PA and transfected with si-PPM1A was assessed using via Cell Counting Kit-8 assay. (F) Migration capacity in TGF-β1-induced MRC-5 cells treated with PA following knockdown of PPM1A was assessed using wound healing. (G) Protein expression of α-SMA in TGF-β1-induced MRC-5 cells treated with PA following knockdown of PPM1A was evaluated using immunofluorescent staining. (H) Protein expression levels of ECM-associated proteins in TGF-β1-induced MRC-5 cells treated with PA following knockdown of PPM1A were semi-quantified using western blotting. Scale bar, 50 µm. *P<0.05, **P<0.01 and ***P<0.001. PA, platyconic acid A; PPM1A, protein phosphatase Mg2+/Mn2+-dependent 1A; RT-qPCR, reverse transcription-quantitative PCR; Fn1, fibronectin 1; MMP-1, matrix metalloprotease 1; TGF-β1, transforming growth factor-β1; ECM, extracellular matrix; si, small interfering; NC, negative control.
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in a dose-dependent manner by significantly suppressing the protein expression levels of α-SMA and ECM-associated markers, Collagen1, Fn1 and MMP-1.

Notably, imbalanced inflammation contributes to development of pulmonary fibrosis (40). Under normal conditions, inflammation and repair is controlled in the lung; however, when tissue injury occurs, TGF-β1 is released in large amounts and chemotactic neutrophils secrete pro-inflammatory molecules (41). The function of chemotactic neutrophils shifts to resolving inflammation and repair during the healing phase (41). The results of the present study demonstrated that protein expression levels of pro-inflammatory factors IL-6, IL-1β and TNF-α were significantly increased in TGF-β1-induced MRC-5 cells. Furthermore, the combination of PG and cisplatin has been reported to significantly decrease inflammation in lung tissue (42). The results of the present study demonstrated that PA significantly inhibited the production of pro-inflammatory factors. The NF-κB pathway is commonly regarded as a pro-inflammatory signaling pathway (43,44). Moreover, NF-κB is involved in the development and progression of fibrosis via regulation of transcription factors associated with fiber growth, such as platelet-derived growth factor and TGF-β1 (45). A previous study reported that activation of the NF-κB signaling pathway...
occurs in the lung tissue of patients with IPF and mice with pulmonary fibrosis (46). The results of the present study demonstrated that the protein expression levels of p-NF-κB were significantly increased in TGF-β1-induced MRC-5 cells, which was consistent with previous studies (45,46). Notably, PG or PG-derived components increase AMP-activated protein kinase (AMPK) signaling in numerous types of cell, such as macrophages and lung carcinoma cells, hepatocellular carcinoma cells (47-49). Moreover, AMPK activation relieves the inflammatory response via suppression of NF-κB activation and downregulation of IL-1β (50). The results of these aforementioned studies suggested that components of PG may inhibit NF-κB. The results of the present study demonstrated that PA significantly inhibited TGF-β1-induced phosphorylation of NF-κB in MRC-5 cells, demonstrating that PA successfully inhibited the TGF-β1-induced inflammatory response in MRC-5 cells.

PPM1A is a member of the protein phosphatase 2C family of Ser/Thr protein phosphatases (23). SwissTargetPrediction database demonstrated that PPM1A may be a potential target for PA. A previous study reported that PPM1A protein expression is decreased in bleomycin-induced pulmonary fibrosis in rats (25). The present study demonstrated that PPM1A mRNA and protein expression levels were significantly decreased in TGF-β1-induced MRC-5 cells. However, the mRNA and protein expression levels of PPM1A were significantly increased in TGF-β1 + PA group in MRC-5 cells compared with TGF-β1 group, demonstrating that PA may upregulate PPM1A expression. Previous studies reported that upregulation of PPM1A is associated with anti-inflammatory effects (23-25) and it could be hypothesized that knockdown of PPM1A may reverse these effects. Therefore, PPM1A knockdown was performed in the present study and the results demonstrated that PPM1A knockdown led to significantly increased cell viability and migration, ECM deposition and protein expression levels of pro-inflammatory factors compared with NC, which suggested that the inhibition of pulmonary fibrosis by PA was mediated by PPM1A.

A previous study reported that the pro-fibrotic process of TGF-β1 is mediated by SMAD-2/3 and the β-catenin signaling pathway (10). Activation of β-catenin-dependent genes including cyclin D1 and c-Myc leads to fibroblast activation and fibrogenesis (10,51,52). The results of the present study demonstrated that the protein expression levels of p-SMAD2, p-SMAD3 and β-catenin were significantly elevated following TGF-β1 induction compared with the control. Furthermore, previous studies reported that PPM1A promotes its nuclear export via dephosphorylation of SMAD2/3, leading to termination of the TGF-β1/SMAD signaling cascade (24,53). However, the present study demonstrated that PPM1A knockdown significantly increased expression levels of SMAD/β-catenin signaling pathway-associated proteins. These data suggested that inhibition of TGF-β1 via PPM1A was achieved via inhibition of the SMAD/β-catenin signaling pathways.

The present study had limitations. Only one cell line was used and other cell lines need to be included to confirm the results in future investigations. The function of PPM1A in TGF-β1 induced pulmonary fibrosis model needs to be confirmed by overexpressing PPM1A to determine whether this reverses the effect induced by TGF-β1. There was no reliable method for cell-to-animal dose conversion and acute toxicity testing should be used to confirm the appropriate concentration of PA in vivo. Whether PA treatment decreases both mRNA and protein levels of PPM1A and whether knockdown of PPM1A without TGF-β1 treatment affects cell migration needs to be assessed.

In conclusion, the results of the present study demonstrated that PA inhibited TGF-β1-induced proliferation, inflammation and ECM of lung fibroblasts via upregulation of PPM1A via the SMAD/β-catenin signaling pathways. The present study therefore provides a novel theoretical basis for the treatment of pulmonary fibrosis.

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Availability of data and materials

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

Authors’ contributions

HHo and CS designed and conceived the study. CS and YT performed the experiments. CS, YT, CW and HHu analyzed the data. CS drafted the manuscript. All authors have read and approved the final manuscript. CS and YT confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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