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Triptolide protects against TGF-β1-induced pulmonary fibrosis by regulating FAK/calpain signaling

PINGHENG ZHANG[1], JIAN LIU[2] and RUIKAI ZONG[2]

[1]Traditional Chinese Medicine Department, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong 510282; [2]Rheumatism Department, Anhui University of Chinese Medicine, Hefei, Anhui 230031, P.R. China

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Abstract. The present study aimed to investigate the mechanism of anti-proliferative, anti-inflammatory and anti-fibrotic effects of triptolide (TPL) on activated lung fibroblasts by regulating the focal adhesion kinase (FAK) and calpain signaling pathways. The HFL-1 human foetal lung fibroblast cell line was cultured *in vitro* and treated with 50 ng/ml transforming growth factor (TGF)-β1 for 48 h to establish the model of pulmonary fibrosis. Subsequently, the cells were divided into five groups, including a control, model, TPL, FAK inhibitor and calpeptin group. Subsequently, the proliferation of lung fibroblasts was detected using the Cell Counting Kit-8 assay. The concentration of interleukin (IL)-6 in the cell culture supernatant was examined by ELISA and the mRNA expression levels of collagen type I (ColI)α and ColIII in lung fibroblasts were quantified by reverse transcription-quantitative PCR. The protein levels of FAK, phosphorylated (p)-FAK, calpain 1 and calpain 2 were detected by western blot analysis. TGF-β1 induced the proliferation of lung fibroblasts, whereas TPL inhibited this proliferation in a dose-dependent manner. TPL also decreased the TGF-β1-induced production of IL-6 and reduced the upregulation of ColIα, ColIII, FAK, p-FAK, and inhibited the decrease of calpain 1 and calpain 2 induced by TGF-β1. In addition, the FAK inhibitor acted synergistically with TPL to decrease TGF-β1-induced production of IL-6 and attenuate TGF-β1-induced synthesis of ColIα and ColIII, while calpeptin had an antagonistic effect on the function of TPL. Furthermore, treatment with the FAK inhibitor and TPL markedly decreased the protein levels of FAK and p-FAK, and increased the protein expression of calpain 1 and calpain 2 in lung fibroblasts stimulated by TGF-β1 to a greater extent than TPL alone, while calpeptin had an antagonistic effect on the action of TPL. In conclusion, the present study indicated that TPL protected against TGF-β1-induced proliferation, inflammation and fibrosis by regulating the FAK and calpain signaling pathways.

Correspondence to: Professor Jian Liu, Rheumatism Department, Anhui University of Chinese Medicine, 117 Meishan Road, Hefei, Anhui 230031, P.R. China
E-mail: liujianahzy@126.com

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Introduction

Pulmonary fibrosis is a progressive, eventually fatal disease, which is characterized by excessive accumulation of extracellular matrix (ECM) in the alveolar parenchyma and progressive lung scarring (1). Recently, considerable research efforts have been devoted to its pathogenesis (2,3); however, the complete details thereof have remained elusive. It is well known that pulmonary fibrosis does not only occur as a primary condition, but also secondary to other diseases, including rheumatoid arthritis (4) and vasculitis (5). The lung is frequently affected by other diseases, as it has abundant blood supply and connective tissues (6), leading to secondary lung diseases, including pulmonary fibrosis (7), pulmonary arterial hypertension (8) and pulmonary nodules (9). Of note, pulmonary fibrosis, as an important complication of other diseases, has a crucial role in the progression of lung disease.

It must also be mentioned that the increment of lung fibroblasts is the major cause of pulmonary fibrosis. In the process, lung fibroblasts, together with a number of other immune cells, including T and B lymphocytes, monocytes, macrophages and neutrophils, create an inflammatory environment in the lung tissue, which recruits an increasing number of immune cells and results in pulmonary destruction and functional deficiency (10,11). In addition, when lung fibroblasts are stimulated by pro-inflammatory cytokines, including transforming growth factor (TGF)-β1 (12), the release of a number of other pro-inflammatory cytokines (13), including interleukin (IL)-6, may be induced, which contributes to the further amplification of inflammatory processes.

Triptolide (TPL), a terpene compound contained in the root, leaf, flower and fruit of *Tripterygium wilfordii*, is a bioactive component used in traditional Chinese medicine known for its anti-inflammatory and immunosuppressive effects (14,15). It has therapeutic effects against a multitude of autoimmune diseases. For instance, TPL was able to reduce hippocampal Aβ deposition in a rat model of vascular dementia and exert anti-inflammatory functions (16). Furthermore, it was reported that TPL restrained the expression of C-X-C motif chemokine receptor 4, thrombin, tumor necrosis factor-α and TGF-β receptor in colon cancer cells to exert an anti-cancer effect (17).

In addition, a previous study further demonstrated that TPL downregulates the expression of focal adhesion kinase (FAK), which leads to the imbalance of lung cancer cell migration and inhibits the ability of lung cancer cells to migrate and invade *in vitro* (18). It was also demonstrated that TPL inhibits the TGF-β1/extracellular signal-regulated kinase/mothers against decapentaplegic homolog 3 signaling pathway to reduce myofibroblast activation in the lung, thus inhibiting the progression of radioactive pulmonary fibrosis (19). However, the molecular mechanisms underlying the therapeutic effects of TPL, particularly regarding the proliferation of lung fibroblasts and the molecular mechanisms of its effects to suppress the inflammatory response have remained elusive.

FAK is a signaling molecule that mediates the conglutination of the cell and the ECM, and it is an intersection of numerous signaling pathways involved in the regulation of a variety of physiological and pathological processes, including cell metabolism, invasion, migration, adhesion, proliferation and cytoskeletal reorganization (20,21). Previous studies have conveyed that FAK is closely connected with fibrosis, including hepatic (22), myocardial (23), vascular (24) and pulmonary fibrosis (25). Calpain is a calcium-dependent protease and it has a critical role in adhesion disassembly in fibroblasts (26). To date, it has been confirmed that calpain 2-mediated proteolysis of FAK regulates adhesion dynamics in motile cells and the calpain cleavage site of FAK has been identified (27).

However, whether the possible involvement of the FAK/calpain pathway in the anti-inflammatory and anti-fibrotic properties of TPL during pulmonary fibrosis and whether this potential mechanism is involved in the proliferation of lung fibroblasts, has remained elusive.

Therefore, in the present study, the effects of TPL on TGF-β1-induced proliferation and cytokine release of lung fibroblasts were assessed with the aim of assessing the potential functional roles of the FAK/calpain pathway in these effects.

Materials and methods

Chemicals and drugs. TPL was purchased from Sigma-Aldrich (Merck KGaA). The compound was dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution with a concentration of 250 μM. This stock solution was then diluted with incubation medium. The final DMSO concentration did not exceed 0.05% (v/v).

The ELISA kit for IL-6 was purchased from Beijing Li Ke Co., Ltd., (cat. no. XL-EH0196). Anti-FAK (cat. no. CA36131), anti-phospho-(p)-FAK (cat. no. CN893300), anti-calpain 2 (cat. no. BS3696) and anti-β-actin (cat. no. 17AV0303) antibodies were obtained from Bioworld Technology, Inc. Anti-calpain 1 (cat. no. 00016377) was obtained from ProteinTech Group, Inc. Penicillin/streptomycin solution (X100), 0.05% trypsin-EDTA and DMSO were purchased from Sigma-Aldrich (Merck KGaA). The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies, Inc. Ham's F12-K medium and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc.). Radioimmunoprecipitation assay lysis and extraction buffer, horseradish peroxidase (HRP)-conjugated AffiniPure goat anti-mouse IgG, anti-rabbit IgG antibodies (cat. nos. anti-mouse 127655 and anti-rabbit 125946) and D-glucose were purchased from OriGene Technologies, Inc. PCR primers were obtained from Western Biotech. Co., Ltd. Calpeptin (calpain inhibitor) and FAK inhibitor were purchased from Sigma-Aldrich (Merck KGaA).

Cell culture and treatment. The HFL-1 human foetal lung fibroblast cell line was obtained from the cell bank of the Chinese Academy of Sciences and cultured in Ham's F12-K medium supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). Cells were cultured in an incubator with a humidified atmosphere containing 5% CO_2 at 37˚C. TGF-β1 at doses of 25, 50 and 100 ng/ml was added to HFL-1s, which were cultured for 24, 48 and 72 h. A CCK-8 assay was used to determine the growth of the cells. Finally, the most appropriate concentration and stimulation time of TGF-β1 regarding their effect of lung fibroblasts proliferation were determined by the growth of the HFL-1s and applied in the subsequent experiments.

Experimental design. The first series of experiments were designed to establish the pulmonary fibrosis model and determine the optimal concentration of TPL to inhibit cell proliferation. TGF-β1 at doses of 25, 50 and 100 ng/ml was added to HFL-1s, which were cultured for 24, 48 and 72 h. TPL at doses of 5, 10, 15 and 20 nmol/l was added to HFL-1s, which were cultured in the presence of 50 ng/ml TGF-β1 for 48 h.

The second series of experiments was designed to examine the inhibitory effect of TPL and investigate the possible mechanism. TPL, calpeptin (a calpain inhibitor) and the FAK inhibitor were, respectively diluted in DMSO. They were then added to the growth medium to yield the final concentrations with a DMSO solvent concentration of <0.05% (v/v). Cells were divided into five groups: i) Control group: Cells were treated with an equal concentration of DMSO for 48 h, so that all cultures in the present study had the same final concentration of DMSO. ii) Model group: Cells were treated with 50 ng/ml TGF-β1 for 48 h. iii) TPL group: Cells were cultured in 50 ng/ml TGF-β1 and 5 nmol/l TPL for 48 h. iv) FAK inhibitor group: Cells were cultured in 50 ng/ml TGF-β1 and 5 nmol/l TPL for 48 h, and addition of 20 μM FAK inhibitor for 24 h. v) Calpeptin group: Cells were cultured in 50 ng/ml TGF-β1 and 5 nmol/l TPL for 48 h, and addition of 50 μm calpeptin for 24 h.

CCK-8 assay. HFL-1s were seeded into 96-well plates, the count was adjusted to 5 10^4/ml, 100 μl per well and incubated with 5% CO_2 and 37˚C. Subsequently, some treatments were then performed as mentioned above. A total of 10 μl CCK-8 solution was added to each well, followed by incubation at 37˚C for 4 h. Finally, the optical density of the resulting solution in the wells was determined using an ELISA reader (Thermo Fisher Scientific, Inc.) at a wavelength of 450 nm.

ELISA. The level of IL-6 in the culture supernatant was determined using a commercial ELISA kit according to the manufacturer's protocol and evaluated by measuring the absorption at a 450-nm wavelength using a microplate reader (Thermo Fisher Scientific, Inc.).

Table I. Primers used for quantitative PCR.

Gene	Primer sequence	Length of the amplicon (bp)
β-actin	F: 5'-TGACGTGGACATCCGCAAAG-3'	205
	R: 5'-CTGGAAGGTGGACAGCGAGG-3'	
ColIα	F: 5'-GTGCGATGACGTGATCTGTGA-3'	114
	R: 5'-GTTTCTTGGTCGGTGGGTG-3'	
ColIII	F: 5'-TGCTCGGGGTAATGACGG-3'	138
	R: 5'-GCACCATTTGAACCAGGAGAC-3'	

F, forward; R, reverse; Col, collagen.

Reverse transcription-quantitative PCR (RT-qPCR). To determine the expression levels of collagen type I (ColI)α mRNA and ColIII mRNA, the total RNA extracted from the lung fibroblasts with TRIzol reagent was reverse-transcribed, the amplification conditions were as follows: Denaturation for 10 min at 65˚C, annealing for 10 min at 25˚C, cDNA extension at 42˚C for 50 min and inactivation at 85˚C for 5 min, using Thermo Reverse Transcription kits (Western Biotech. Co., Ltd.). qPCR was performed using Quanti Nova SYBR Green PCR kit (Qiagen GmbH). The sequences of the primers used for PCR are provided in Table I. The amplification conditions were as follows: 4 min at 94˚C, followed by 35 cycles at 94˚C for 20 sec, 60˚C for 30 sec and 72˚C for 30 sec. The relative expression of ColIα mRNA and ColIII mRNA was evaluated using the $2^{-\Delta\Delta Cq}$ method (28). β-actin was used as a control for normalization.

Western blot analysis. The protein levels of FAK, p-FAK, calpain 1 and calpain 2 in lung fibroblasts was detected by western blot analysis. Total protein was extracted with radioimmunoprecipitation assay lysis buffer. Proteins from each experimental group were quantified using the bicinchoninic acid assay (BestBio). An equal amount of protein (30 μg) was loaded and subjected to 10% SDS-PAGE. Total protein was separated by SDS-PAGE at 120 V and then transferred to polyvinylidene fluoride membranes (EMD Millipore) at 200 mA for 120 min. Membranes were blocked with 5% non-fat milk powder for 2 h at room temperature and then incubated with primary antibodies against anti-β-actin (1:1,000 dilution), FAK (1:1,000 of rabbit monoclonal antibody), p-FAK (1:500), calpain 1 (1:1,000) and calpain 2 (1:500) at 4˚C overnight. The membranes were then incubated with goat anti-rabbit secondary antibody labeled with HRP (1:15,000) at room temperature for 1.5 h. The signal was detected using ECL hypersensitive luminescence substrate kit (GE Healthcare). Densitometric analysis was performed using Image-Pro Plus V software (version 7.0; Media cybernetics, Inc.).

Statistical analysis. All data were analyzed using SPSS 17.0 (SPSS Inc.). Values are expressed as the mean ± standard error of the mean. Multiple comparisons were achieved using one-way analysis of variance with the Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference. Each experiment was repeated three times.

Results

TPL inhibits TGF-β1-induced proliferation and inflammation of lung fibroblasts. In order to observe the effect of TPL on HFL-1 cell proliferation, cells were first treated with different doses (25, 50 and 100 ng/ml) of TGF-β1 for 48 h. The result indicated that treatment with 50 ng/ml TGF-β1 for 48 h were the optimal conditions for inducing lung fibroblast proliferation. Subsequently, cells were pre-treated with 50 ng/ml TGF-β1 for 48 h and then treated with TPL at different doses (5, 10, 15 and 20 nmol/l) for another 48 h (Fig. 1D). The proliferation of HFL-1 cells was then determined using a CCK-8 assay. The results indicated that relative to the control group, treatment with TGF-β1 significantly induced the proliferation of lung fibroblasts (P<0.01; Fig. 1A). However, compared with the TGF-β1 treatment group, subsequent treatment with TPL significantly inhibited the proliferation of HFL-1 cells in a dose-dependent manner (P<0.001). The minimum TPL concentration required to achieve an significant suppressive effect on HFL-1 proliferation was 5 ng/ml (P<0.001; Fig. 1B).

Previous studies have reported that HFL-1 cells may secrete inflammatory cytokines, including IL-6 (29,30). Therefore, the effect of TPL on the expression of IL-6 was also investigated in the present study. HFL-1 cells were treated with 50 ng/ml TGF-β1 prior to the addition of 5 nmol/l TPL and incubation for another 48 h. Subsequently, the concentration of IL-6 in the cell culture supernatant was determined by ELISA. The results demonstrated that TPL significantly inhibited TGF-β1-induced production of IL-6 compared with TGF-β1 alone (P<0.001; Fig. 1C).

TPL regulates TGF-β1-induced expression of FAK/calpain in lung fibroblasts. After treatment with TPL for 48 h, the protein levels of FAK, p-FAK, calpain 1 and calpain 2 in HFL-1 cells were assessed using western blot analysis. The results indicated that, compared with those in the control group, the levels of FAK and p-FAK were significantly increased in the model group, the expression of calpain 1 and calpain 2 was significantly decreased (P<0.001; Fig. 2). However, compared with those in the model group, the levels of FAK and p-FAK were significantly decreased and the expression of calpain 1 and calpain 2 was significantly increased in the TPL group (P<0.001; Fig. 2).

TPL inhibits TGF-β1-induced ColIα and ColIII synthesis in lung fibroblasts. In order to determine the collagen levels, the

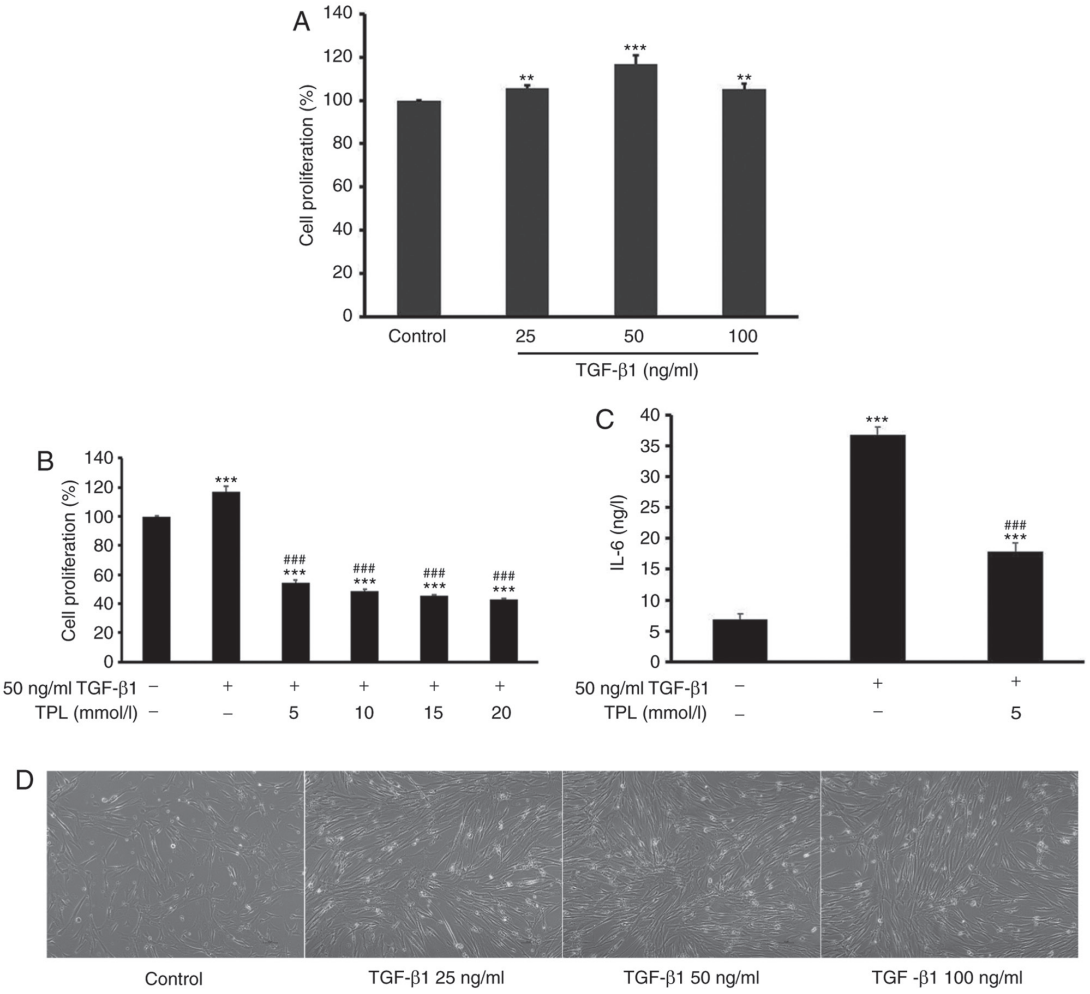

Figure 1. TPL inhibits TGF-β1-induced proliferation of lung fibroblasts. (A) Lung fibroblasts were treated with different doses (25, 50 and 100 ng/ml) of TGF-β1 for 48 h and the proliferation of lung fibroblasts was detected using the CCK-8 assay. (B) Lung fibroblasts were treated with 50 ng/ml TGF-β1 for 48 h and addition of TPL at different doses (5, 10, 15 and 20 nmol/l) for 48 h. Untreated cells served as a control. The proliferation of lung fibroblasts was detected using the CCK-8 assay. TGF-β1 induced the proliferation of lung fibroblasts, whereas TPL inhibited this proliferation in a dose-dependent manner. (C) The concentration of IL-6 in the cell culture supernatant was detected by ELISA at 48 h after TPL treatment. TPL markedly attenuated the level of IL-6. (D) Morphology of lung fibroblasts treated with different doses (25, 50 and 100 ng/ml) of TGF-β1 for 48 h (magnification, x400). Values are expressed as the mean ± standard error of the mean (n=5/group). **P<0.01 and ***P<0.001 vs. the control group; $^{###}$P<0.001 vs. the model group. TGF-β1, transforming growth factor-β1; TPL, triptolide; CCK-8, Cell Counting Kit-8; IL-6, interleukin-6.

expression of ColIα mRNA and ColIII mRNA was determined by RT-qPCR. The results indicated that, compared with those in the control group, the expression levels of ColIα mRNA and ColIII mRNA were significantly increased in the model group (P<0.001; Fig. 3). Furthermore, compared with those in the model group, the expression levels of ColIα mRNA and ColIII mRNA were significantly decreased in the TPL group (P<0.001; Fig. 3).

TPL inhibits cytokine release by TGF-β1-induced lung fibroblasts by downregulating FAK and upregulating calpain. In order to determine the possible involvement of FAK/calpain signaling in TPL-induced cytokine release, HFL-1 cells were treated with 50 ng/ml TGF-β1 for 48 h followed by 5 nmol/l TPL for 48 h and addition of 20 μM FAK inhibitor or 50 μM calpeptin for 24 h. Subsequently, IL-6 in the supernatant was determined by ELISA and the levels of FAK, p-FAK, calpain 1 and calpain 2 in the cells were assessed using western blot analysis. The results suggested that in the TPL group, the level of IL-6 was significantly decreased compared with those in the model group (P<0.001; Fig. 4). However, compared with those in the TPL group, the level of IL-6 was significantly decreased in the FAK inhibitor group (P<0.001). Furthermore, the level of IL-6 was significantly increased in the calpeptin group (P<0.001). Western blot analysis indicated that in the TPL group, the protein levels of FAK and p-FAK were significantly decreased and the protein expression levels of calpain 1 and calpain 2 were increased compared with those in the model group (P<0.001). However, compared with those in the TPL group, the protein expression levels of calpain 1 and calpain 2 were increased in the FAK inhibitor group. Furthermore, the protein levels of FAK and p-FAK were increased in the calpeptin group (Fig. 4).

TPL inhibits TGF-β1-induced pulmonary fibrosis by downregulation of ColIα and ColIII via regulation of FAK/calpain. In order to determine the possible involvement of FAK/calpain signaling in TPL-induced collagen

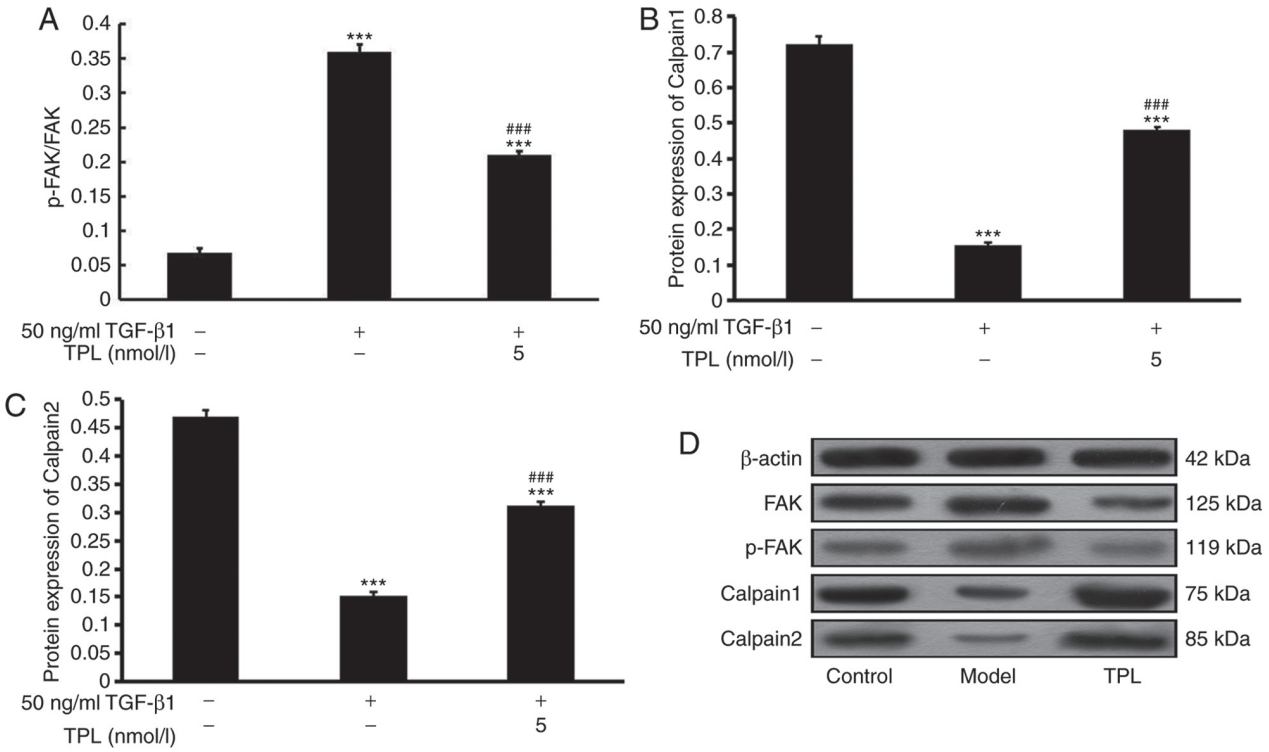

Figure 2. TPL regulates TGF-β1-induced expression of FAK/calpain in lung fibroblasts. Lung fibroblasts were treated with 50 ng/ml TGF-β1 for 48 h and addition of 5 nmol/l TPL and incubation for 48 h. Cells that were untreated served as a control. The protein levels of p-FAK/FAK, calpain 1 and calpain 2 in lung fibroblasts was determined using western blot analysis. Protein levels of (A) p-FAK/FAK, (B) calpain 1 and (C) calpain 2. Values are expressed as the mean ± standard error of the mean (n=3/group). (D) Western blot analysis of FAK, p-FAK, calpain 1 and calpain 2. ***P<0.001 vs. the control group; $^{###}$P<0.001 vs. the model group. TGF-β1, transforming growth factor-β1; TPL, triptolide; p-FAK, phosphorylated-focal adhesion kinase.

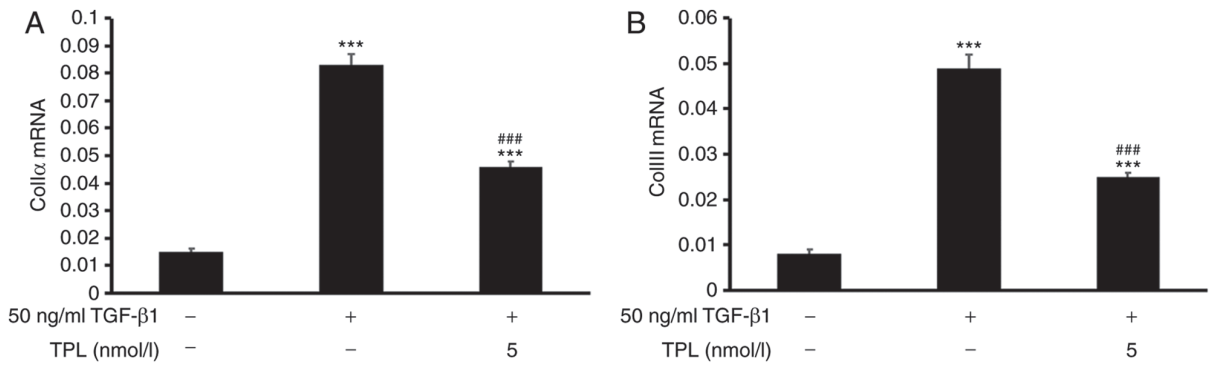

Figure 3. TPL inhibits TGF-β1-induced ColIα and ColIII synthesis by lung fibroblasts. Lung fibroblasts were treated with 50 ng/ml TGF-β1 for 48 h and addition of 5 nmol/l TPL and incubation for 48 h. Cells that were untreated served as a control. The expression of ColIα mRNA and ColIII mRNA in lung fibroblasts was detected using reverse transcription-quantitative PCR. (A) ColIα mRNA and (B) ColIII mRNA. Values are expressed as the mean ± standard error of the mean (n=5/group). ***P<0.001 vs. the control group; $^{###}$P<0.001 vs. the model group. TGF-β1, transforming growth factor-β1; TPL, triptolide; Col, collagen.

synthesis. HFL-1 cells were treated with 50 ng/ml TGF-β1 and 5 nmol/l TPL for 48 h and addition of 20 μM FAK inhibitor or 50 μM calpeptin for 24 h. The results suggested that in the TPL group, the expression of ColIα mRNA and ColIII mRNA were significantly decreased compared with those in the model group (P<0.001). However, compared with those in the TPL group, the expression of ColIα mRNA and ColIII mRNA was decreased in the FAK inhibitor group. Furthermore, the expression of ColIα mRNA and ColIII mRNA were increased in the calpeptin group (Fig. 5).

Discussion

In the present study, TGF-β1 was used to induce the proliferation of lung fibroblasts and generate an *in vitro* model of pulmonary fibrosis. In a preliminary experiment, treatment with 50 ng/ml TGF-β1 for 48 h induced the proliferation of lung fibroblasts, indicating that this was a suitable concentration to establish the *in vitro* model. This model has also been used in foreign and domestic studies (31-33). In addition, as lung fibroblasts are considered to participate in pulmonary

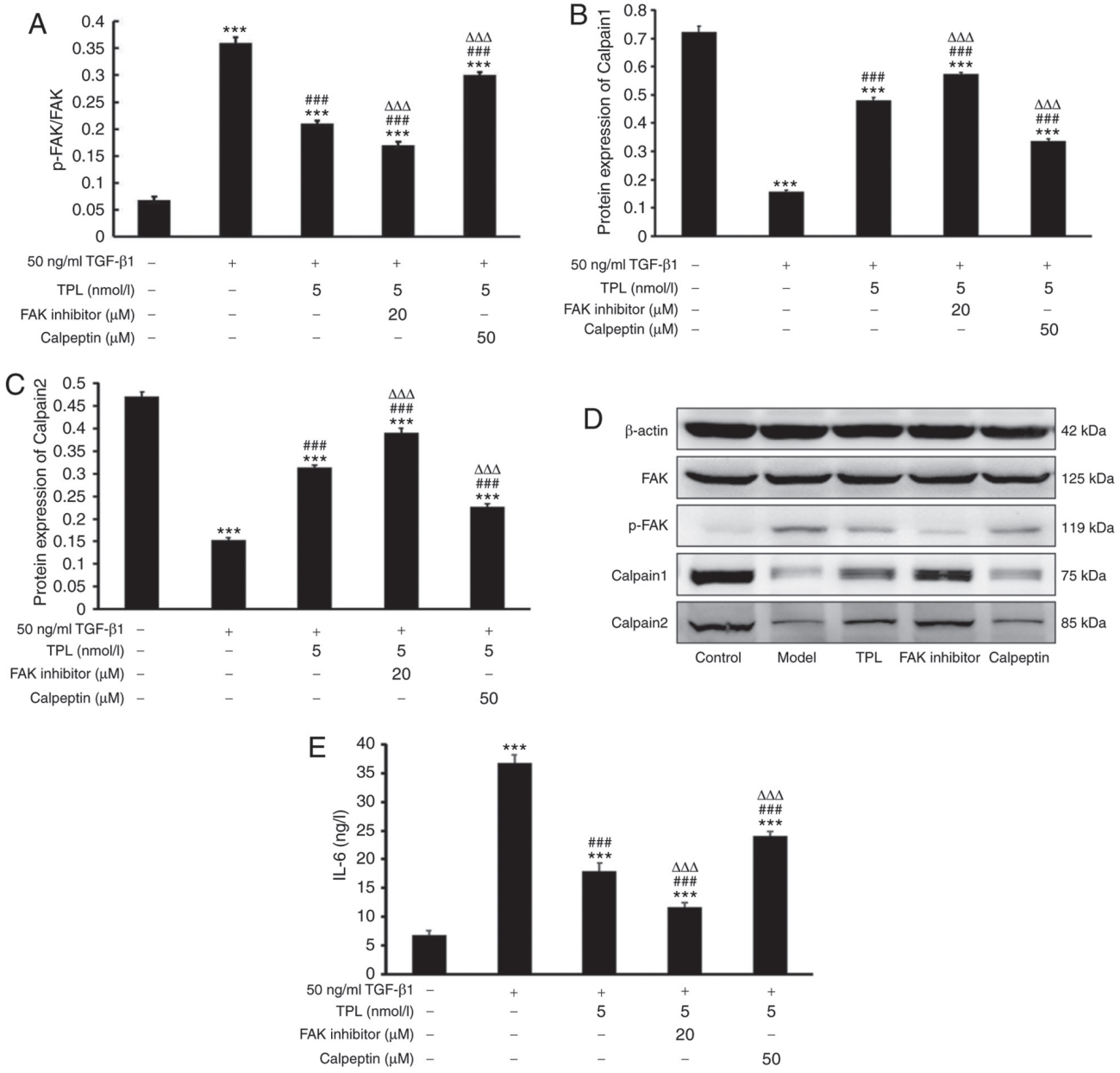

Figure 4. TPL inhibits cytokine release of TGF-β1-induced lung fibroblasts by downregulating FAK and upregulating calpain. Lung fibroblasts were treated with 50 ng/ml TGF-β1 for 48 h and addition of 5 nmol/l TPL and incubation for 48 h. In addition, cells were cultured with 50 ng/ml TGF-β1 and 5 nmol/l TPL for 48 h prior to the addition of 20 μM FAK inhibitor or 50 μM calpeptin for 24 h. Cells that were untreated served as a control. The protein levels of FAK, p-FAK, calpain 1 and calpain 2 in lung fibroblasts were determined using western blot analysis. The concentration of IL-6 in the cell culture supernatant was detected by ELISA. Quantified protein levels of (A) p-FAK/FAK, (B) calpain 1 and (C) calpain 2. Values are expressed as the mean ± standard error of the mean (n=3/group). (D) Western blotting of FAK, p-FAK, calpain 1 and calpain 2. (E) Concentration of IL-6 in the cell culture supernatant. ***P<0.001 vs. the control group; $^{###}$P<0.001 vs. model group; ^{ΔΔΔ}P<0.001 vs. TPL group. TGF-β1, transforming growth factor-β1; TPL, triptolide; p-FAK, phosphorylated focal adhesion kinase; IL, interleukin.

inflammation and fibrosis in numerous autoimmune diseases, including rheumatoid arthritis (4,34), the model has also been used to investigate pulmonary fibrosis secondary to numerous other diseases *in vitro*. The present study suggested that TGF-β1 also induced lung fibroblasts to secrete inflammatory cytokine IL-6 and synthesize ColIα and ColIII, which are among the deposited ECM materials (35). It is likely that in lung tissue affected by pulmonary fibrosis, fibroblasts were affected by inflammation for a long time and secreted inflammatory cytokine IL-6 and synthesized collagen. With the continuous accumulation of collagen and inflammatory stimuli, the lung tissue became filled with collagen and was replaced by mesenchyme tissue, which finally led to pulmonary fibrosis and lung injury.

It is well-known that pulmonary fibrosis is an important complication in numerous other diseases and seriously affects the treatment of primary diseases. At present, there are no effective therapies for pulmonary fibrosis and the development of novel drugs is urgently required (36). In the present study, TPL was confirmed to inhibit TGF-β1-induced proliferation of lung fibroblasts and decrease the expression of inflammatory cytokine IL-6, as well as ColIα and ColIII mRNA. It may

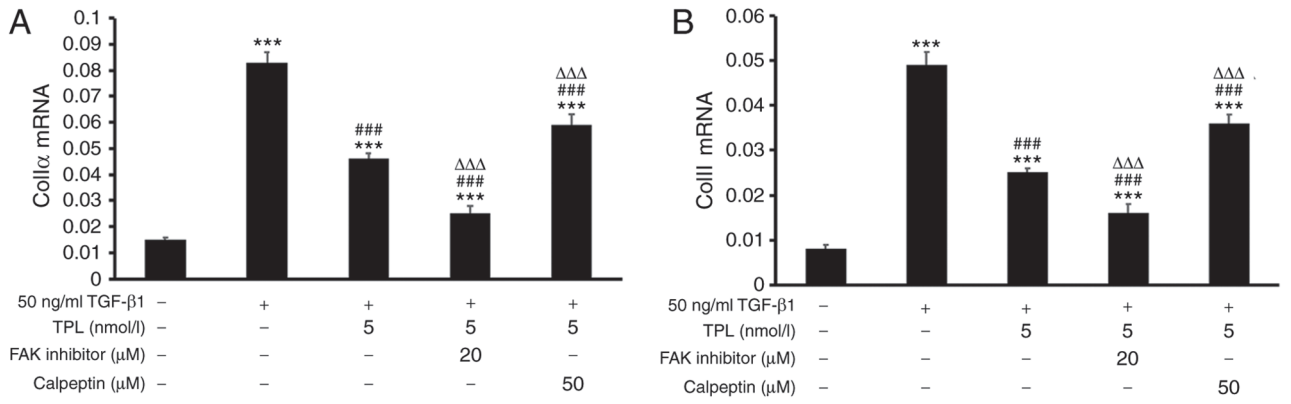

Figure 5. TPL inhibits TGF-β1-induced pulmonary fibrosis by downregulation of the expression of ColIα and ColIII via regulating of FAK/calpain. Lung fibroblasts were treated with 50 ng/ml TGF-β1 for 48 h and addition of 5 nmol/l TPL and incubation for 48 h. In addition, cells were cultured in 50 ng/ml TGF-β1 and 5 nmol/l TPL for 48 h prior to the addition of 20 μM FAK inhibitor or 50 μM calpeptin for 24 h. Cells that were untreated served as a control. The expression of ColIα mRNA and ColIII mRNA in lung fibroblasts was detected using reverse transcription-quantitative PCR. (A) ColIα mRNA and (B) ColIII mRNA. Values are expressed as the mean ± standard error of the mean (n=5/group). ***P<0.001 vs. the control group; $^{###}$P<0.001 vs. the model group; ^{ΔΔΔ}P<0.001 vs. TPL group. TGF-β1, transforming growth factor-β1; TPL, triptolide; p-FAK, phosphorylated focal adhesion kinase; Col, collagen.

therefore be implied that TPL is an effective drug candidate for treating pulmonary fibrosis. The results of the present study are consistent with those provided by Chen *et al* (37), which reported that TPL has anti-inflammatory and immune suppressive effects and even protects against radiation-induced pulmonary fibrosis.

Furthermore, in the model group, the results of the present study demonstrated that the protein levels of FAK and p-FAK were increased and the protein expression of calpain 1 and calpain 2 in lung fibroblasts was decreased compared with the control group. This indicated that the FAK signaling pathway was activated and the calpain signaling pathway was inhibited in lung fibroblasts, and it further suggested that FAK/calpain signal disorders may promote pulmonary fibrosis. Activation of the FAK signaling pathway has been reported to cause lung fibrosis and the expression of FAK was overexpressed in lung tissue (38,39). However, these results are not consistent with those of Li *et al* (40) and Chan and Mattson (41), who reported on the activation of the calpain signaling pathway in lung fibrosis. This may be mainly due to the lack of sufficient Ca^{2+} in the *in vitro* experiment to activate the calpain pathway. It is well known that calpain is a calcium-dependent intracellular cysteine protease. The excessive inflammation may inhibit the function of the calpain signaling pathway and the expression of calpain1 and 2 were decreased.

In addition, the results of the present study demonstrated that TPL inhibited activation of the FAK signaling pathway and promoted calpain signaling to restrain pulmonary fibrosis, indicating that inhibition of FAK signaling is a possible mechanism of the inhibitory effect of TPL on lung fibroblast proliferation and thereby on pulmonary fibrosis. Furthermore, in order to investigate the possible involvement of the FAK/calpain signaling pathways in the effects of TPL on pulmonary fibrosis, FAK inhibitor and calpeptin were used to treat lung fibroblasts and block the FAK and calpain signaling pathway, respectively. The results suggested that TPL and the FAK inhibitor have a synergistic effect on inhibiting the release of cytokine IL-6, by TGF-β1-induced lung fibroblasts into the cell culture supernatant, and restraining the expression of ColIα mRNA and ColIII mRNA in lung fibroblasts. Furthermore, calpeptin reversed the effect of TPL to inhibit the synthesis of collagen and the secretion of inflammatory factors. The results also suggested that phosphorylation of FAK cannot be hydrolyzed via blocking of the calpain signaling pathway. As phosphorylation of FAK may promote lung fibrosis, the condition may be aggravated via blocking of the calpain signaling pathway. A previous study also confirmed that calpain mediated the hydrolyzation of the phosphorylation of FAK and identified a calpain cleavage site on the FAK protein (27). Additionally, these studies indicated that TPL exerts its effects against pulmonary fibrosis and to reduce inflammation by downregulating the FAK signaling pathway and upregulating the calpain signaling pathway. Therefore, those studies demonstrate that TPL has a beneficial effect for treating pulmonary fibrosis occurring secondary to chronic diseases, including rheumatoid arthritis.

In conclusion, to the best of the authors' knowledge, the present study is the first to demonstrate that TPL prevented TGF-β1-induced lung fibroblast proliferation by downregulating the expression of Col α and Col III via inhibiting the activation of the FAK signal pathway and promoting the activation of the calpain signaling pathway. Furthermore, TPL may inhibit inflammatory cytokine release by downregulating the FAK signaling pathway and upregulating the calpain signaling pathway. Although further investigation is required to fully unveil the molecular mechanisms of action, the present results suggest that TPL may be suitable as a novel therapeutic drug for pulmonary fibrosis.

However, the study has some limitations. For example, the study only used one lung fibroblast cell line that was also not a primary lung fibroblast and the study about the effects of TPL on TGFß1-induced lung fibroblasts was *in vitro* rather than *in vivo*. Moreover, in this study, the result showed TPL inhibits cell proliferation and at the same time induces cell death. TPL used at 5 mmol/l may have had cytotoxic effects on the lung fibroblast cells. In the future, the authors will further optimize and explore a more appropriate TPL concentration for research. On the basis of the present study, 50 ng/ml TGF-β1 was deemed

to be the most suitable model for pulmonary fibrosis where treating cells with TGF-β1 was more appropriate. However, pulmonary fibrosis is a slow process in nature, which was not discussed in this study. The authors will make a detailed study of this point in their following work. Additionally, this study mainly investigated TPL, which is one of the active constituents in *Tripterygium wilfordii* according to Chinese herbal medicine. However, although Chinese herbal *Tripterygium wilfordii* is commonly used in clinical practice, TPL has not been used alone in clinical practice. In the future, a clinical study on TPL will need to be conducted so that TPL can be used in the clinic.

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Availability of data and materials

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

Authors' contributions

PZ, JL and RZ designed the present study and were involved in analysis and interpretation of data. All authors discussed the results and implications and commented on the manuscript at all stages, as well as in the final approval of the version to be published.

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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