Evogliptin attenuates bleomycin-induced lung fibrosis *via* inhibiting TGF-β/Smad signaling in fibroblast

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Abstract. – OBJECTIVE: Idiopathic pulmonary fibrosis (IPF) is a pulmonary interstitial fibrosis disease. Excessive activation of fibroblasts in the lung contributes to severe alveoli dysfunction and histological destruction. Evogliptin, a dipeptidyl peptidase IV inhibitor, has been widely used to reduce glucose level in type 2 diabetes, whereas evogliptin treatment to fibrosis process of lung IPF is elusive. The study aimed to investigate the mechanism of evogliptin in transforming growth factor-beta (TGF-β)-activated lung fibroblasts and evaluate the efficacy of evogliptin in lung fibrosis model.

MATERIALS AND METHODS: In lung fibroblast culture, the RNA expression of α-SMA in lung fibroblasts was detected, and α-SMA/COL-1 immunofluorescence co-staining after TGF-β stimulation and evogliptin administration was displayed. Mechanically, the phosphorylation level of Smad2/3 protein in cells was analyzed using Western blotting, and the scratch assay was used to reflect fibroblast proliferation. Furthermore, bleomycin was employed to induce lung fibrosis in mice, and IHC staining and hematoxylin and eosin (HE) & Masson staining were carried out to examine the extracellular matrix (ECM) expression and tissue fibrosis.

RESULTS: The results demonstrated that evogliptin treatment attenuated the activation of fibroblasts and collagen deposition following TGF-β stimulation. Furthermore, the extracellular matrix expression was descended via evogliptin restraining the TGF-β/Smad pathway. Besides, it was also found that evogliptin affected the proliferation degree of lung fibroblasts. In vivo, the COL-1 and α-SMA were significantly reduced through evogliptin treatment compared with the bleomycin group, and fibroblasts and collagenous fiber were remarkably decreased.

CONCLUSIONS: Evogliptin exerts an anti-fibrosis effect on TGF-β induced lung fibroblast activation, which restrains ECM formation and decreases cell proliferation level in fibroblasts. Moreover, the fibroblast infiltration and collagen deposition were ameliorated following evogliptin administration. Therefore, evogliptin serves as a potential implication to protect lung fibrosis.

Introduction

Fibrosis is a chronically hyperplastic pathological process contributing to organic parenchyma loss and dysfunction[1,2]. Idiopathic pulmonary fibrosis (IPF) is an excessive fibrosis disease that presents as agnogenic alveolar fibrosis and diffuse interstitial fibrosis[3,4]. Owing to unfavorable development in the pathophysiological cognization of IPF, there remains vague therapy of attenuating IPF. IPF, characterized by lung parenchymal tissue replaced by extensive fibrous connective tissue, provokes organic structural destruction, lung hypofunction and even respiratory failure[5,6]. Overwhelming fibroblasts activation in multiple fibrotic diseases aggravated fibrous degeneration in lesion[7], indicating that the modulation of fibroblasts activation in compensatory repair may inhibit the development of fibrotic diseases. As a physiopathological process, fibrosis participates in tissue injury repair because of insufficient primary cell proliferation. When severe damage occurs in the lung[8], it triggers a mass of fibroblasts activation and accumulation to repair histological destruction, but over-activated fibroblasts ultimately cause pathological fibrosis especially in bronchia and alveolus tissue[9,10]. Given the fibrosis process in IPF, how to decrease fibroblasts activation is deemed to be a promising regulatory aspect in anti-fibrotic therapy. Fibroblasts, differentiated from mesenchymal cells during embryonic period, serve as a main cellular component in loose connective tissue[11], which synthesize and secret collagens and matrix components when activated[12]. However, over-activated fibroblasts can

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over-proliferate and secret large amounts of extracellular matrix (ECM) such as collagen I, collagen III and fibronectin *via* multiple growth factors stimuli[13,14]. Transforming growth factor-beta (TGF-β), recognized tissue factor that stimulates fibroblast differentiation, has been generally employed to activate fibroblasts *in vitro* culture[15,16]. Moreover, the induction of TGF-β/Smad pathway is related to fibroblast activation and fibrosis process[17-19]. Emphasis of research has been put on the regulation of TGF-β pathway in an expectation that more effective therapy reduces fibrosis progression. Evogliptin, a novel dipeptidyl peptidase (DDP) IV inhibitor with high potency and selectivity, modulates type 2 diabetes by decreasing the degradation of glucagon-like peptide-1[20]. DPP-IV inhibitor plays an anti-fibrotic role in various organ including heart, liver, and kidney[21-23]. Recently, DPP-IV inhibitor attenuates renal fibrosis caused by unilateral ureteral obstruction in mice[24]. However, the treatment of evogliptin on lung fibrosis has rarely been researched. According to the past studies, it was suspected that evogliptin may change fibrosis process of IPF through modulating several pathogenic factors. Considering that the effect and mechanism of evogliptin on lung fibroblasts activation *via* TGF-β induction are unclear, whether evogliptin mediates TGF-β pathway and cell proliferation or apoptosis to play an anti-fibrosis role in lung fibrosis was explored in this study. Here, evogliptin was utilized in TGF-β-induced lung fibroblasts and bleomycin-induced lung fibrosis to investigate the effect and mechanism of evogliptin on fibrotic regulation.

Materials and Methods

Cell Culture and Treatment

Human lung fibroblasts were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Primary fibroblasts were digested with 0.25% trypsin (Gibco, Rockville, MD, USA) inoculated in a 75 cm^2 cell flask with Dulbecco's Modified Eagle's Medium containing 1% penicillin/streptomycin (DMEM, Gibco, Rockville, MD, USA) appended 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). When cells grew to satisfactory confluence, they were divided into four 25 cm^2 flasks. After starvation treatment with free serum DMEM overnight, evogliptin (10 nM, 20 nM, Standards, China) was employed to pretreat fibroblasts for 24 h,

and then TGF-β (10 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) were employed to activate fibroblasts for 24 h.

Animal Studies

C57/BL mice (male, 20-25 g) were utilized to construct models of lung fibrosis. All animals were purchased from Dongying People's Hospital Animal Center and housed in Dongying People's Hospital Animal Center. Mice were divided into three experimental groups), namely, negative control group (NC, n=6, with no treatment), bleomycin group (BLM, n=6, bleomycin-treated treatment) and bleomycin+evogliptin group (BLM+EVP, n=6, evogliptin/bleomycin co-treatment). For the bleomycin model, the mice were anesthetized *via* pentobarbital, then tracheotomy was conducted, and 70 μL bleomycin (3.5 mg/kg) was conveyed *via* transtracheal injection. The BLM+EVP group was administrated with evogliptin (300 mg/kg/day, po.) for 7 days. All mice were fed for 21 days, then anesthetized with pentobarbital and perfused with phosphate-buffered saline (PBS) for RNA isolation or 4% paraformaldehyde (PFA) for staining. This investigation was approved by the Animal Ethics Committee of Dongying People's Hospital Animal Center.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from fibroblasts and lung tissue homogenate using a RNA extraction reagent (YiFeiXue, Nanjing, China) according to the manufacturer's protocol. Synthesis of complementary deoxyribose nucleic acid (cDNA) was conducted using a qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Shanghai, China). A total of 20 μL system was reacted for RNAs quantification using a perfecta SYBR Green Supermix (Quanta Biosciences, Shanghai, China). Melting curve analysis was determined the specificity of every RNA reaction. Next, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization, and relative mRNA levels were quantified by the $2^{-\Delta\Delta Ct}$ methods. All reactions were operated in triplicate. Full list of primers used is provided as follows: COL-1 (Human), Forward Primer: GAGGGCCAAGACGAAGACATC, and Reverse Primer: CAGATCACGTCATCGCACAAC. COL-1 (Mouse), Forward Primer: GCTCCTCTTAGGGGCCACT and Reverse Primer: ATTGGGGACCCTTAGGCCAT. COL-3 (Human), Forward Primer: GGAGCTGGCTACTTCTCGC and Reverse Primer: GG-

GAACATCCTCCTTCAACAG. COL-3 (Mouse), Forward Primer: CTGTAACATGGAAACTG-GGGAAA and Reverse Primer: CCATAGCT-GAACTGAAAACCACC. FB (Human), Forward Primer: CGGTGGCTGTCAGTCAAAG and Reverse Primer: AAACCTCGGCTTCCTC-CATAA. FB (Mouse), Forward Primer: ATGT-GGACCCCTCCTGATAGT and Reverse Primer: GCCCAGTGATTTCAGCAAAGG. GAPDH (Human), Forward Primer: CTGGGCTACACT-GAGCACC and Reverse Primer: AAGTG-GTCGTTGAGGGCAATG. GAPDH (Mouse), Forward Primer: TGACCTCAACTACATGGTC-TACA and Reverse Primer: CTTCCCATTCTC-GGCCTTG.

Western Blot (WB) Analysis

Proteins were extracted from lung fibroblasts using a Total Protein Extraction Kit supplemented phenylmethylsulfonyl fluoride (PMSF), phosphatase inhibitors and proteinase inhibitor (KeyGEN, Nanjing, China). After shaken and centrifuged, the protein concentration was measured using an enhanced bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Shanghai, China). Electrophoretic separation, transferring and blocking were performed procedures in sequence, and then the proteins were incubated overnight with anti-α-SMA (Abcam, Cambridge, MA, USA, 1:1000), anti-p-Smad2/3 (Abcam, Cambridge, MA, USA, 1:1000), anti- Smad2/3 (Abcam, Cambridge, MA, USA, 1:1000), and anti-GAPDH (Proteintech, Rosemont, IL, USA, 1:10000). Washed by Tris-Buffered Saline and Tween-20 (TBST), the proteins were incubated with secondary antibody (YiFeiXue, Nanjing, China, 1:10000) for 1 h at room temperature. Proteins were visualized using an enhanced chemiluminescence (ECL) system (Thermo Fisher Scientific, Waltham, MA, USA).

Immunofluorescence (IF) Staining

Fibroblasts were fixed with 4% paraformaldehyde (PFA) for 15 minutes and blocked with an immunostaining blocking fluid (Beyotime, Shanghai, China) for 1h at room temperature. Next, IF staining was conducted with α-SMA (Abcam, Cambridge, MA, USA, 1:200), COL-1 (Abcam, Cambridge, MA, USA, 1:200) and p-Smad2/3(Abcam, Cambridge, MA, USA, 1:100) overnight at 4°C. After washed with phosphate-buffered saline (PBS), cells were incubated with an Alexa Fluor 488 or 594-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Rabbit or Mouse IgG (H+L) (Jackson, West Grove, PA, USA, 1:500) for 1 h at room temperature. Nucleus counterstaining and mounting was conducted using a 4',6-diamidino-2-phenylindole (DAPI)-Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Then, the immunofluorescent images of fibroblasts were collected using an immunofluorescent microscope.

Immunohistochemical Staining (IHC) and Histological Staining

Mouse lung tissues were fixed with 4% PFA for 24 h and underwent dehydration by alcohol of different concentration. Tissues were embedded into paraffin and placed at room temperature for 24 h. The tissues were cut into 5 μm section and placed into a 55°C oven for 1 h. Then, after deparaffinization and hydration, Hematein & Eosin staining and Masson staining were performed using a Hematein & Eosin staining Kit (Beyotime, Shanghai, China) and a Trichrome Stain (Masson) Kit (Servicebio, Wuhan, China) respectively. For IHC, the sections received antigen retrieval in citrate buffer at 95°C for 15 minutes. After blocked with 0.5% BSA-PBS containing 10% goat serum for 1 h, the sections were incubated with α-SMA (Abcam, Cambridge, MA, USA, 1:100), TGF-β (Abcam, Cambridge, MA, USA, 1:200) and COL-1 (Abcam, Cambridge, MA, USA, 1:100) overnight. Then, IHC staining was conducted using a diaminobenzidine (DAB) Rabbit & Mouse horseradish peroxidase (HRP) Kit (Biolab, Shanghai, China) following manufacturer's instruction. Finally, the images were collected using a microscope.

Scratch Assay

Fibroblasts were seeded into a 12 well plate and underwent starvation treatment overnight. Then, 2 mL of DMEM with 10% FBS was added into each well, and 10 nM and 20 nM evogliptin were used to pretreat cells for 24 h. After TGF-β (10 ng/mL) treatment for 24 h, a 10 μL pipette tip was used to scratch cells. After 24 h, images were collected using a microscope.

Statistical Analysis

Data were described as the means ± SD (standard deviations). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Data were collected and assessed using Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Ar-

monk, NY, USA). *p*<0.05 was considered statistically significant.

Results

EVP Inhibited Fibroblast Activation and Reduced ECM Expression

First, to verify whether evogliptin (EVP) inhibits fibrosis activation in human lung fibroblasts, the expression of biomarker α-Smooth muscle actin (α-SMA) was examined following TGF-β stimuli and EVP treatment. Western blotting displayed that TGF-β stimuli significantly increased α-SMA protein level in fibroblasts, while EVP pretreatment decreased α-SMA level after TGF-β

employment especially at 20 nM (Figure 1A). Hence, the RNA levels of collagen I (COL-1), collagen III (COL-3) and fibronectin (FB) were detected, and it was discovered that the RNA levels of COL-1, COL-3 and FB were decreased with EVP treatment following TGF-β stimuli (Figure 1B-1D). Furthermore, the IF of α-SMA and COL-1 co-staining was utilized after TGF-β and EVP treatment, exhibiting that TGF-β significantly increased the α-SMA (red) and COL-1 (green) expressions in cells, but 10 nM and 20 nM EVP administration reduced α-SMA and COL-1 expressions after TGF-β activation (Figure 1E). Hence, EVP inhibited fibroblast activation and alleviated ECM level.
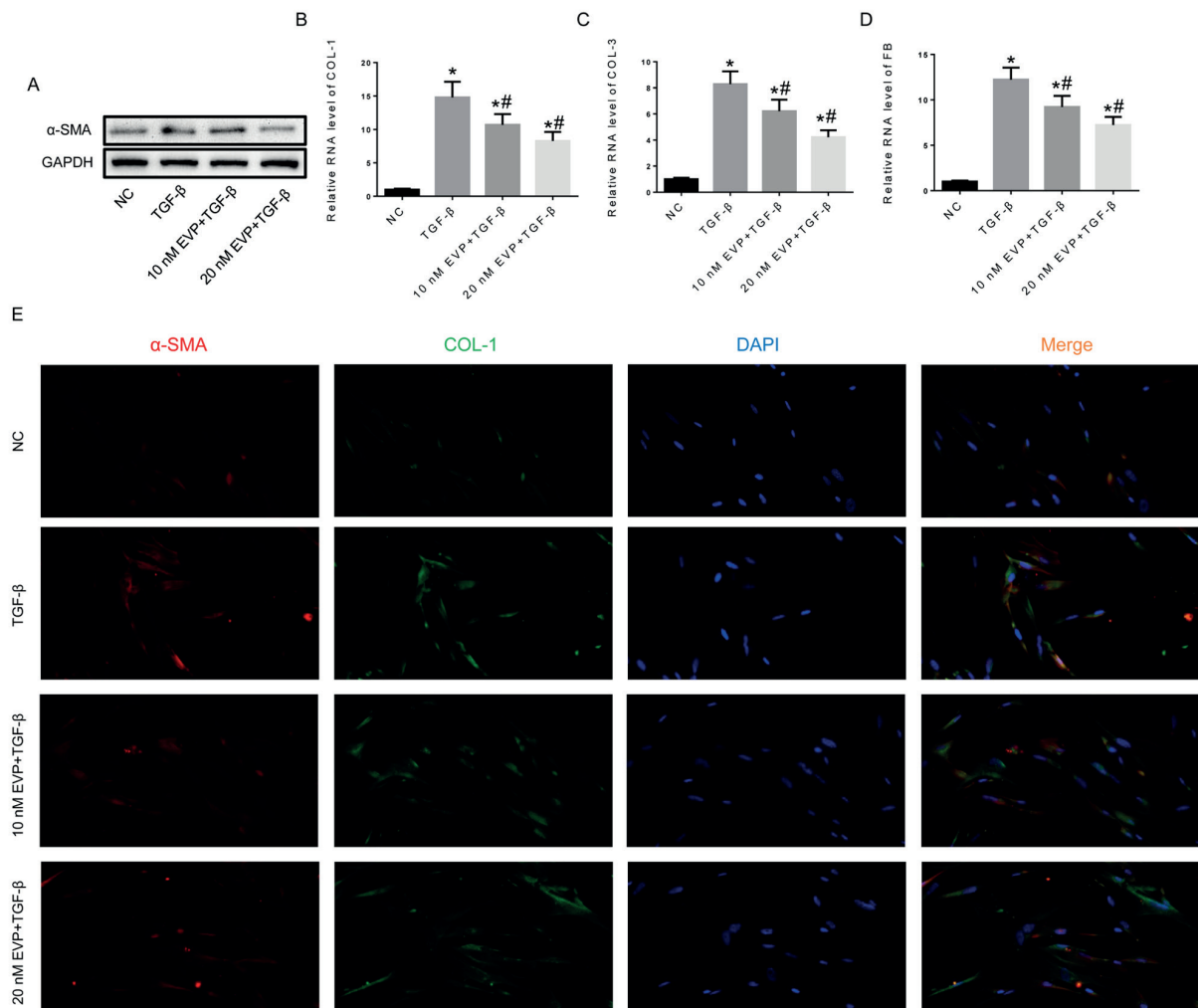

Figure 1. EVP inhibited fibroblast activation and reduced ECM expression. **A,** Representative α-SMA protein level in NC, TGF-β, 10 nM EVP+TGF-β and 20 nM EVP+TGF-β group. **B-D,** Representative RNA level of COL-1, COL-3 and FB in NC, TGF-β, 10 nM EVP+TGF-β and 20 nM EVP+TGF-β group. **E,** Representative immunofluorescence of α-SMA (red) and COL-1 (green) in NC, TGF-β, 10 nM EVP+TGF-β and 20 nM EVP+TGF-β group (magnification: 200×). "*" means *vs*. NC group, "#" means *vs*. TGF-β group with statistical significance.
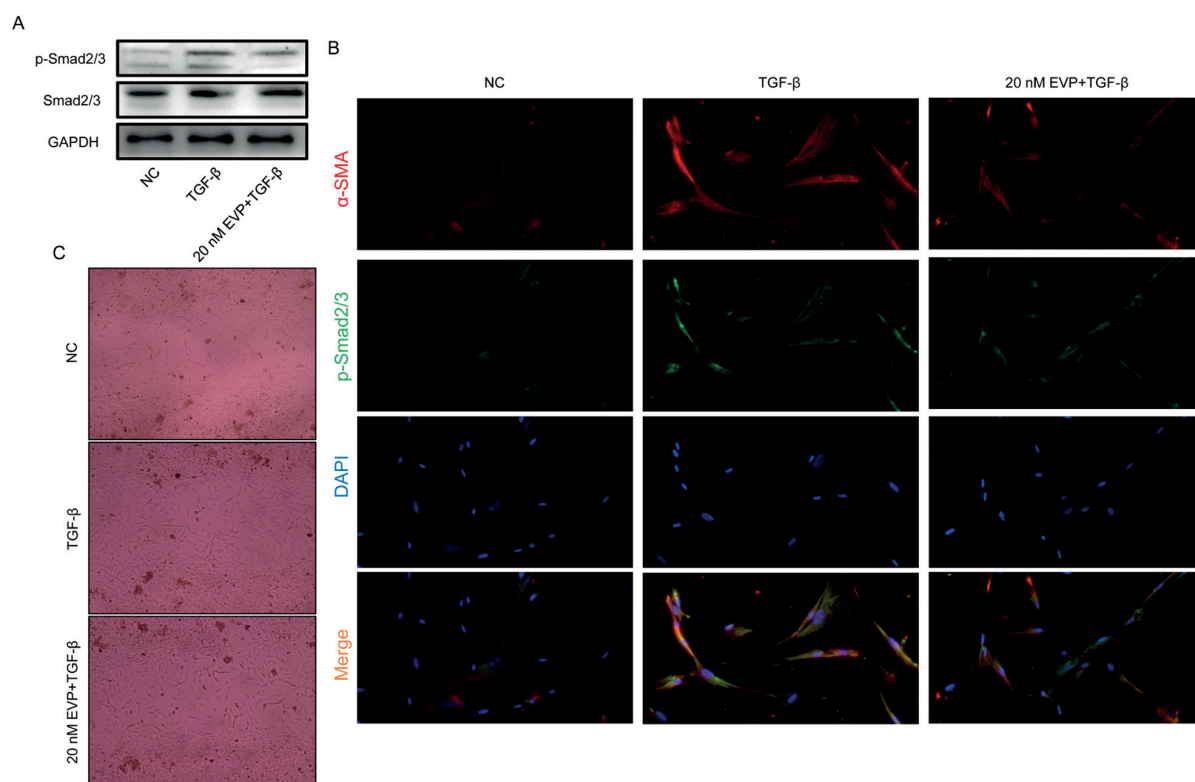
Figure 2. Administration of EVP suppressed fibroblasts activation *via* regulation of Smad2/3 signaling and reduced cell proliferation. **A,** Representative Western blotting of p-Smad2/3 and Smad2/3 in NC, TGF-β and 20 nM EVP+TGF-β group. **B,** Representative immunofluorescence of α-SMA (red) and p-Smad2/3 (green) in NC, TGF-β and 20 nM EVP+TGF-β group (magnification: 200×). **C,** Representative scratch assay at 24h in NC, TGF-β and 20 nM EVP+TGF-β group (magnification: 40×).

Administration of EVP Suppressed Fibroblasts Activation Via Regulation of Smad2/3 Signaling and Reduced Cell Proliferation

To explore the mechanism of EVP concerning anti-fibrotic effect on the lung fibroblasts, TGF-β and 20 nM EVP were further employed to treat the fibroblasts, and phosphorylated Smad2/3 (p-Smad2/3) and total Smad2/3 were measured using Western blotting (Figure 2A). It was found that p-Smad2/3 was increased remarkably in TGF-β group, but EVP decreased p-Smad2/3 protein level after TGF-β treatment. TGF-β and EVP did not affect total Smad2/3 expression. Moreover, the α-SMA (red) and p-Smad2/3 (green) co-staining showed that TGF-β provoked high expressions of α-SMA and p-Smad2/3 while EVP reduced α-SMA and p-Smad2/3 level simultaneously (Figure 2B). We also detected the cell proliferation using scratch assay, showing that TGF-β stimulation accelerated fibroblasts proliferation; hence, the amount of cell in the scratch is more than that in control group. EVP administration reduced cells in the scratch compared with the TGF-β group (Figure 2C). Thereof, the results suggested that EVP decreased fibroblast activation and proliferation through restraining TGF-β/Smad2/3 pathway.

EVP Treatment Reduced Fibroblasts Activation Level and Protected Lung Tissue Integrity

Mice with lung fibrosis were treated with BLM. After EVP treatment, the lung tissues were removed in 21 days for IHC staining of α-SMA and TGF-β, displaying that α-SMA and TGF-β expressions in BLM group were remarkably higher than those in NC group, while EVP treatment effectively inhibited α-SMA and TGF-β levels in lung tissues (Figure 3A). Moreover, the injury degree of lung was reflected using HE staining (Figure 3B). The images displayed that EVP administration reduced infiltration and proliferation of fibroblasts in lung. Hence, it could be concluded
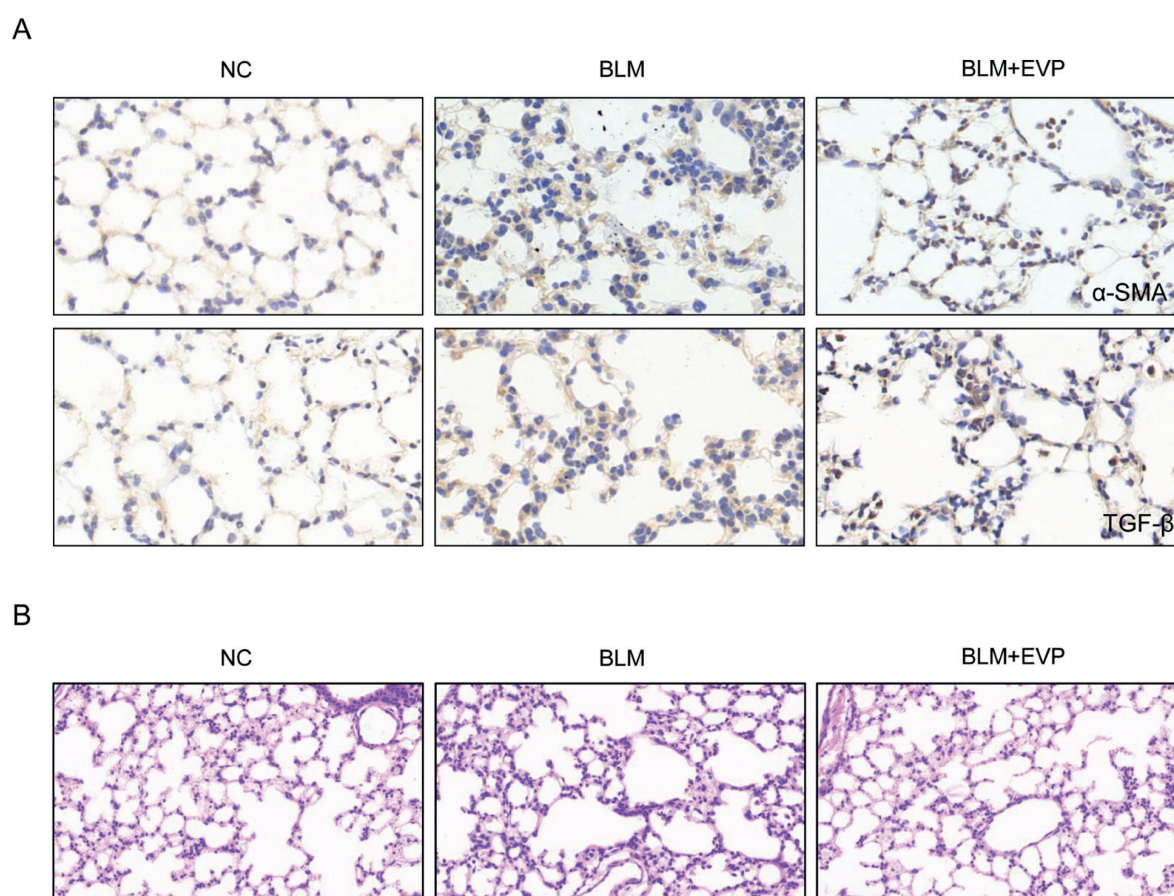
A

B

Figure 3. EVP treatment reduced fibroblast activation level and protected lung tissue integrity. **A,** Representative IHC staining of α-SMA and TGF-β in NC, BLM and BLM+EVP group (magnification: 400×). **B,** Representative HE staining of lung tissue in NC, BLM and BLM+EVP group (magnification: 200×).

that EVP mitigates reduced intrahepatic fibrosis level and alleviated tissue injury after BLM administration.

EVP Alleviated ECM Expression and Mitigated Lung Fibrosis Process

The RNA levels of ECM (COL-1, COL-3 and FB) in lung tissues were measured using qRT-PCR, exhibiting that EVP could negatively modulate the RNA expressions of COL-1, COL-3 and FB after BLM-induced fibrosis (Figure 4A-4C). COL-1 expression was then detected using IHC, exhibiting that EVP treatment reduced COL-1 expression in lung tissues compared with BLM group (Figure 4D). Besides, lung fibrosis degree was examined using Masson staining (Figure 4E). The result exhibited that excessive collagenous fiber was in BLM-induced lung injury, but EVP administration attenuated the fiber deposition in

lung tissue, indicating that EVP alleviates ECM release and lung fibrosis degree.

Discussion

IPF is a complex process that involves lung fibroblast activation in pathogenic process. Excessive fibrosis induces extensive interstitial compensation, dyspnea, and even life-threatening events. The overwhelming fibroblasts in lesion proliferate and differentiate into myofibroblasts secreting a mass of ECM[25]. Long-lasting fibrosis process in lung tissue may replace parenchymal cells by fibroblasts, the deposition of collagenous fiber ultimately causes pulmonary afunction. Effective therapies thereof need to negatively control fibroblasts and ECM secretion, which may reduce the deterioration of IPF. When lung fibro-

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blasts are activated, they rapidly turn to myofibroblasts *via* multiple growth factor induction and secrete varieties of collagens, fibrous proteins[26]. The over-expression of fibers become the adverse factor of the deterioration of IPF. Evogliptin exhibited a distinguished effect against fibrosis process in various diseases. Notably, a study[27] has indicated that evogliptin protects against renal injury by preventing podocyte damage in an animal model of progressive renal injury. Hence, it was we hypothesized that evogliptin may attenuate lung fibrosis *via* regulating lung fibroblasts after TGF-β stimuli. In the current study, it was found that evogliptin down-regulated α-SMA protein expression in the lung fibroblasts with TGF-β treatment, and the RNA level of COL-1,

COL-3 and fibronectin showed that evogliptin employment significantly reduced TGF-β induced ECM expression in the transcription level. To further explore evogliptin effect on inhibiting fibroblast activation, IF co-staining of α-SMA and COL-1 was utilized, showing that evogliptin simultaneously decreased α-SMA and COL-1 expressions in fibroblasts. The results indicate that evogliptin exerts a fibrotic suppressive function in lung fibroblasts. Next, the specific mechanism of evogliptin on the inhibition to fibroblast activation was investigated. Mechanically, TGF-β/Smad2/3 pathway is a critical regulatory signaling loop to modulate fibroblast activation and ECM expression. Hence, p-Smad2/3 level was detected after TGF-β stimulation and evogliptin administration.
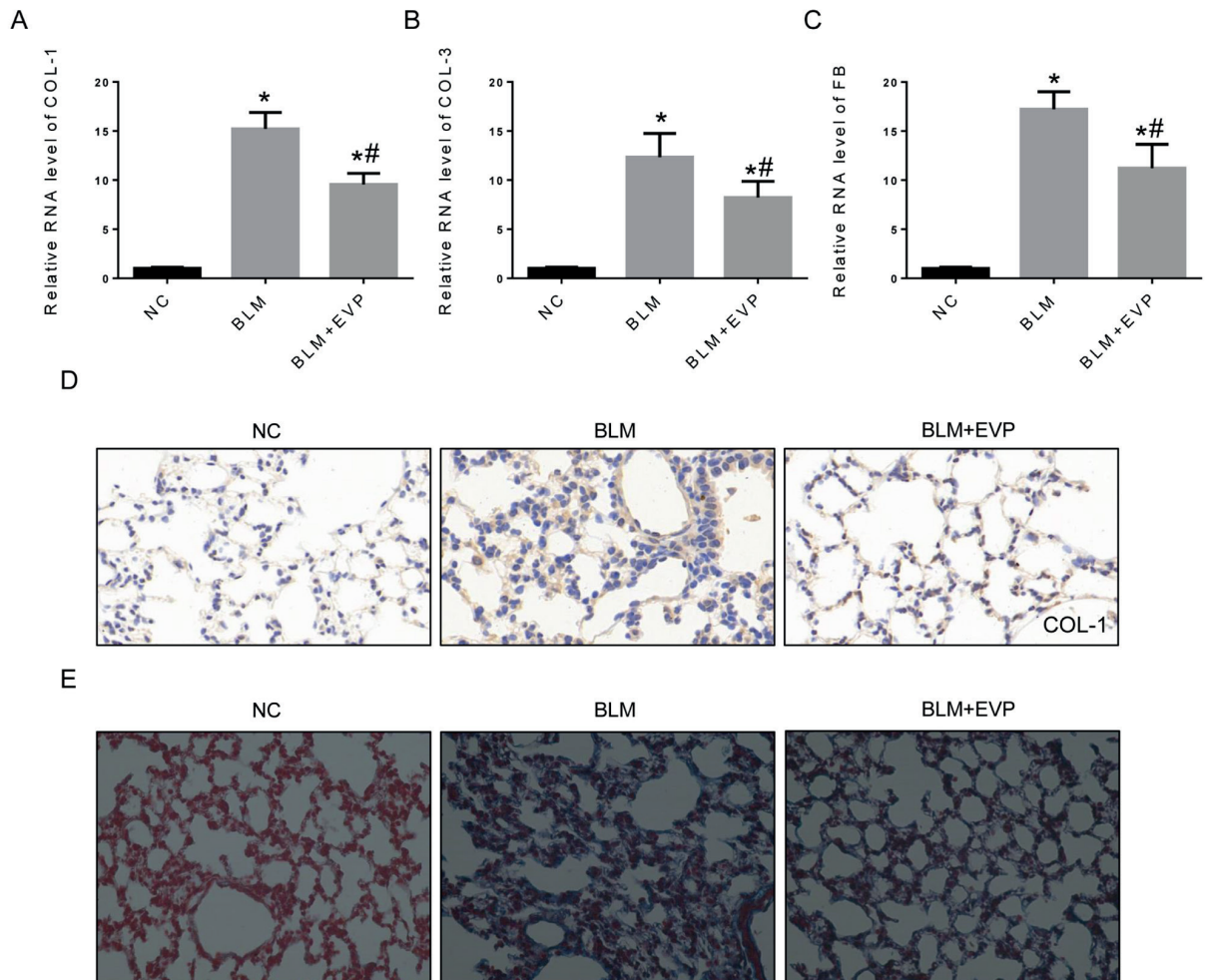

Figure 4. EVP alleviated ECM expression and mitigated lung fibrosis process. **A-C,** Representative RNA level of COL-1, COL-3 and FB in NC, BLM and BLM+EVP group. **D,** Representative IHC staining of COL-1 in NC, BLM and BLM+EVP group (magnification: 400×). **E,** Representative Masson staining of lung tissue in NC, BLM and BLM+EVP group (magnification: 400×). "*" means *vs.* NC group, "#" means *vs.* BLM group with statistical significance.

Western blotting exhibited that p-Smad2/3 protein was increased after TGF-β treatment while evogliptin declined the expression of p-Smad2/3. The result was consistent with the IF staining exhibition, indicating that evogliptin can inhibit TGF-β effect by decreasing the phosphorylation of Smad2/3 pathway. In several fibrosis diseases, effectively inhibiting TGF-β/Smad2/3 activation in fibroblasts could attenuate scarring in repairing tissue, and this study likewise proved the point. Moreover, it was found that evogliptin meanwhile protected the integrity of lung tissues compared with that in BLM group, which may be associated with the anti-fibrotic effect of evogliptin. However, the mechanism of evogliptin in impacting cell proliferation was not demonstrated, so further studies are needed to explore the target of evogliptin to inhibit proliferation in lung fibroblasts. In this study, IHC staining showed apparent increase of α-SMA and TGF-β in the BLM-induced lung fibrosis mice, but evogliptin treatment effectively reduced the expression of α-SMA and TGF-β in lung tissue, indicating that evogliptin may exert anti-fibrotic effect on the BLM-induced lung fibrosis *via* inhibiting fibroblasts activation and TGF-β expression. Furthermore, the function of evogliptin in ECM expression after BLM injection was also assessed. The results of COL-1, COL-3 and FB RNAs in lung tissues showed that evogliptin negatively affected ECM expression in RNA level. Moreover, the IHC exhibited that the expression of COL-1 after evogliptin administration was remarkably decreased compared with the BLM group. On the other hand, the fibrosis degree was measured using Masson staining, exhibiting that BLM could cause serve fiber deposition in large areas, but evogliptin mitigated the degree of fibrosis and decreased the fibrotic area. Therefore, evogliptin played a protective role in lung fibrosis model. Besides, it was proved that evogliptin exerted a promising anti-fibrotic effect in lung fibrosis, but the problems concerning multiple regulation in lung fibroblast or modulation to another cells in lung fibrosis are elusive. Hence, subsequent studies will be concentrated on the above issues.

Conclusions

This study results suggested that evogliptin treatment reduces fibroblasts activation through inhibiting TGF-β/Smad-3 pathway in lung fibrosis. Besides, evogliptin resulted in decreased ECMs and limited proliferation. Thus, evogliptin may be a promising therapeutic agent to inhibit lung fibrosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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