Original Paper

Simvastatin Attenuates TGF- β 1-Induced Epithelial-Mesenchymal Transition in Human Alveolar Epithelial Cells

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Key Words

Fibrosis • EMT • Statin • Simvastatin

Abstract

Background: Transforming growth factor- β 1 (TGF- β 1)-induced epithelial-mesenchymal transition (EMT) of alveolar epithelial cells (AEC) may contribute to idiopathic pulmonary fibrosis (IPF). TGF- β 1-induced EMT in A549 cells (a human AEC cell line) resulted in the adoption of mesenchymal responses that were predominantly mediated via the TGF- β 1-Smad2/3 signaling pathway. Simvastatin (Sim), a 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitor, has been previously reported to inhibit EMT in human proximal tubular epithelial cells and porcine lens epithelial cells and to suppress Smad2/3 phosphorylation in animal models. However, whether Sim can attenuate TGF- β 1-induced EMT in A549 cells and its underlying mechanisms remains unknown. **Methods:** Cells were incubated with TGF- β 1 in the presence or absence of Sim. The epithelial marker E-cadherin (E-Cad) and the mesenchymal markers, α -smooth muscle actin (α -SMA), vimentin (Vi) and fibronectin (FN), were detected using western blotting analyses and immunofluorescence. Phosphorylated Smad2 and Smad3 levels and connective tissue growth factor (CTGF) were analyzed using western blotting. In addition, a cell migration assay was performed. Moreover, the levels of matrix metalloproteinase (MMP)-2 and -9 in the culture medium were examined using ELISA. **Results:** Sim significantly attenuated the TGF- β 1-induced decrease in E-Cad levels and elevated the levels of α -SMA, Vi and FN via the suppression of Smad2 and Smad3 phosphorylation. Furthermore, Sim inhibited the mesenchymal-like responses in A549 cells, including cell migration, CTGF expression and secretion of MMP-2 and -9. However, Sim failed to reverse the cell morphological changes induced by TGF- β 1 in A549 cells. **Conclusion:** Sim attenuated TGF- β 1-induced EMT in A549 cells and might be a promising therapeutic agent for treating IPF.

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Introduction

Idiopathic pulmonary fibrosis (IPF), the most common form of idiopathic interstitial pneumonia, is an age-related fibro-proliferative disorder characterized by interstitial alveolar fibrosis of unknown reasons. IPF exhibits a heterogeneous clinical course with a poor prognosis and a median survival of 2.5–3.5 years after diagnosis [1]. The current treatment for IPF, i.e., glucocorticoids and acetylcysteine, shows poor efficacy and does not prevent disease progression or reduce the high mortality rates [2]. Moreover, there was no significant benefit reported in clinical trials of novel drugs, such as etanercept [3], interferon- γ [4, 5], bosentan [6] and imatinibmesilate [7] in IPF patients with mild to moderate functional impairment, and lung transplantation was the only therapy that prolonged survival [8]. Thus, it is critical to investigate novel therapeutic agents to attenuate IPF progression.

The progressive fibrotic response in IPF is associated with an epithelial-dependent fibroblast-activation process. The presence of fibroblast and myofibroblast foci has been recognized as the central feature of IPF pathogenesis [9]. However, the precise source of fibroblasts and myofibroblasts within the foci has not yet been identified, although there are at least three known sources: proliferation of resident lung interstitial fibroblasts, differentiation of progenitor cells from bone marrow, and epithelial-mesenchymal transition (EMT) [10]. Evidence for EMT in IPF patients includes the immunostaining of lung biopsies from IPF patients, which has revealed fibroblast-like cells expressing the surfactant protein C (SP-C) that is normally synthesized and secreted by type II alveolar epithelial cells [11]. Because EMT has been recognized to play an integral role in the process of fibrosis, the suppression of EMT might alleviate fibrotic progression.

Transforming growth factor- β 1 (TGF- β 1) is a pluripotent growth factor and is produced locally at wound sites and within fibrotic lesions [12]. TGF- β 1 plays a key role in the EMT process and is considered a primary inducer of EMT in lung fibrosis. EMT is characterized by the loss of epithelial markers, including E-Cadherin (E-Cad), and the acquisition of mesenchymal markers, including α -smooth muscle actin (α -SMA), vimentin (Vi) and fibronectin (FN) [13, 14]. These alterations are accompanied by the acquisition of a migratory behavioral response [14, 15], increased secretion of matrix metalloproteinases (MMPs) [16] and increased expression of connective tissue growth factor (CTGF), a fibroblast mitogen and promoter of collagen deposition [17]. EMT in response to TGF- β 1 in fibrosis is mediated predominantly via the Smad-dependent signaling pathway [14]. Smad2 and Smad3 are phosphorylated and subsequently translocated with Smad4 into the nucleus, where they regulate the transcription of EMT-associated genes [18].

Statins, 3-hydroxy-e-methylglutaryl CoA (HMG-CoA) reductase inhibitors, are widely used to treat high cholesterol [19] and are safe and well-tolerated in older adults [20]. Nevertheless, apart from their effects on lowering cholesterol, multiple pleiotropic beneficial effects of statins have been observed [21]. It was demonstrated that Simvastatin prevented Smad2/3 phosphorylation induced by TGF- β 1 in nasal polyp-derived fibroblasts [22] and human intestinal fibroblasts [23]. In addition, other studies have shown that statins inhibited Smad2/3 phosphorylation in animal models [24–27]. Simvastatin also inhibited EMT induced by TGF- β 1 in human proximal tubular epithelial cells [28], and lovastatin inhibited EMT induced by TGF- β 2 in porcine lens epithelial cells [29]. Thus, we hypothesized that Simvastatin could attenuate EMT induced by TGF- β 1 in human alveolar epithelial cells via the inhibition of TGF- β 1-Smad2/3 signaling.

Materials and Methods

Cell Culture and Drug Treatment

Human type II alveolar epithelial cells (A549 cell line) were maintained in DMEM/F12 (HyClone) containing 10% (v/v) fetal bovine serum (FBS, HyClone), 100k U/L penicillin and 100 mg/L streptomycin

at 37°C in a humidified 5% CO₂ atmosphere. The cells were cultured at approximately 70% confluency and starved in serum-free DMEM (SF-DMEM) overnight. Simvastatin (Sim) and resveratrol (dissolved in dimethyl sulfoxide, DMSO (Sigma)) were pretreated at concentrations of 1, 5, 10 and 20 µM for 2 hr prior to incubation with or without TGF-β1 at a concentration of 5 ng/ml for 48 hr. To investigate the effects of TGF-β1 or Sim on cell morphology, a TGF-β1-receptor antagonist LY-364947 was used at 10 µM for 1 hr prior to incubation with or without TGF-β1. The concentration of DMSO in the medium never exceeded 0.1% to avoid toxicity in A549 cells. Each experiment was independently performed at least 3 times.

Western Blotting Analyses

Cellular proteins were extracted using RIPA buffer (SolarBio, 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.1% (w/v) SDS) containing 1% (v/v) PMSF (SolarBio), 0.3% (v/v) protease inhibitor (Sigma Aldrich) and 0.1% (v/v) phosphorylated proteinase inhibitor (Sigma). Lysates were centrifuged at 12000 rpm at 4°C for 15 min and the supernatant was collected for total protein. A BCA protein assay kit (Pierce) was used to determine the protein concentration. Equal amounts of protein (15 µg) was separated on an SDS-PAGE gel (10% (v/v) polyacrylamide) and transferred onto a PVDF membrane. Nonspecific binding was blocked using 8% (w/v) milk in TBS-T for 2 hr at room temperature. The membranes were then incubated with primary antibodies against GAPDH (Abmart), E-Cad (Cell Signaling), α-SMA (Sigma), Fibronectin (FN, BD Bioscience), Vimentin (Vi, Cell Signaling), Smad2 (Cell Signaling), p-Smad2 (Cell Signaling), Smad3 (Epitomics), p-Smad3 (Epitomics) and CTGF (Santa Cruz) overnight at 4°C. After several washes with TBS-T, the membranes were incubated in HRP-conjugated goat anti-rabbit and anti-mouse IgG or HRP-conjugated mouse anti-goat IgG (Abmart, all at a 1:5000 dilution) for 2 hr at room temperature and then washed. The target proteins were visualized using enhanced chemiluminescence (Millipore) according to the manufacturer's recommendations, and quantified using density analysis normalized against GAPDH and expressed as the fold-change compared to control.

Immunofluorescence

Cells grown on chamber slides were washed with PBS for 15 min (total), fixed in 4% paraformaldehyde for 30 min at room temperature (RT) and permeabilized with 0.1% TritonX-100 at RT for 5 min. After several washes with PBS for 15 min (total), nonspecific binding was blocked with 3% bovine serum albumin (BSA) in PBS for 1 hr at RT. Next, the cells were incubated with the following primary antibodies: human E-Cad, α-SMA, Vi, FN and GAPDH, which were all diluted at 1:100 in PBS with 1% BSA. After the cells were incubated with primary antibodies for 2 hr at room temperature, the cells were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG or TRITC-conjugated anti-mouse IgG (Zhongshan Biotechnology) at 1:50 in PBS with 1% BSA for 1 hr at RT. After several washes 15 min (total) with PBS, the cell nuclei were visualized with Hoechst 33258 staining at a concentration of 10 µg/ml for 10 min at RT. The slides were then washed again, dried, mounted, and examined using a fluorescence microscope.

Cell Migration Assay

Cells were grown as a confluent monolayer in six-well plates and were pretreated with DMSO or Simvastatin for 2 hr. To initiate migration, the cell layer was scratched using a pipette tip. Next, the cells were incubated with or without 5 ng/ml TGF-β1. Time-lapse images of cell morphology were captured.

ELISA

After pretreatment with DMSO or Sim and incubation with TGF-β1 for 48 hr, the cell culture medium was centrifuged at 3000 rpm for 20 min, and the supernatant was collected. Quantification of MMP-2 and -9 levels was performed using human MMP-2 and -9 ELISA kits (Beijing Rui'rxinde Technology) according to the manufacturer's instructions.

Statistical Analysis

The data were expressed as the mean ± SEM. The number of independent experiments was represented by "n." Multiple comparisons were performed using one-way ANOVA followed by Tukey's multiple-comparison test, where P<0.05 was considered significant.

Fig. 1. TGF- β 1 induced EMT in A549 cells. A549 cells were exposed to 5 ng/ml TGF- β 1 for 48 hr. Compared to the basal condition, TGF- β 1-treated A549 cells lost their cobblestone shape and adopted a fibroblast-like, spindle-shaped morphology (A). Western blotting revealed that TGF- β 1 significantly downregulated the expression of the epithelial marker E-Cad and upregulated the expression of the mesenchymal markers α -SMA, Vi and FN (B). The data are presented as the means $\pm$ SEM, $n=3$. * $p<0.05$ versus Control.

Fig. 2. Sim attenuated changes in the expression of EMT-markers in a dose-dependent manner. A549 cells were incubated with DMSO (control) or 1, 5, 10 or 20 μ M Sim for 2 hr prior to treatment with TGF- β 1 at 5 ng/ml for 48 hr. Western blotting analyses revealed that expression of E-Cad was increased by Sim in a dose-dependent manner, and expression of the mesenchymal markers α -SMA, Vi and FN were also decreased by Sim in a dose-dependent manner. All of the markers showed significant changes when the Sim concentration reached up to 10 μ M. The data are presented as the means $\pm$ SEM, $n=3$. * $p<0.05$ versus Control.

Results

TGF- β 1 induced EMT in A549 cells

It has previously been shown that TGF- β 1 induces EMT in A549 cells at 5 ng/ml for 48 hr [30, 31]. We thus stimulated A549 cells with TGF- β 1 at 5 ng/ml for 48 hr and examined the morphological characteristics and expression of EMT markers, such as E-Cad, α -SMA, Vi and FN, using western blotting analyses (Fig. 1). A549 cells cultured in basal media showed a classic cobblestone morphology. After stimulation with TGF- β 1 at 5 ng/ml for 48 hr, the cells adopted a more fibroblast-like, elongated and narrower, spindle-shaped morphology (Fig. 1A). Western blotting analyses (Fig. 1B) showed that TGF- β 1 significantly decreased the expression of the epithelial marker E-Cad by approximately 75% and increased the expression of the mesenchymal markers, α -SMA, Vi and FN, by approximately 50%, 120% and 600%, respectively. These results indicated that TGF- β 1 at 5 ng/ml for 48 hr induced EMT in A549 cells.

Sim attenuated changes in the expression of EMT markers induced by TGF- β 1 in a dose-dependent manner

After incubation with SF-DMEM, A549 cells were pretreated with DMSO or Simvastatin (Sim) at 1, 5, 10, and 20 μ M for 2 hr prior to incubation with TGF- β 1. After 48 hr, the

Fig. 3. Sim suppressed TGF- β 1-induced EMT in A549 cells associated with inhibition of the TGF- β 1-Smad2/3 signaling pathway. A549 cells were incubated with DMSO or 10 μ M Sim for 2 hr prior to incubation with 5 ng/ml TGF- β 1 for 48 hr. EMT-markers were detected using western blotting (A) and immunofluorescence (C), and p-Smad2, Smad2, p-Smad3 and Smad3 were detected using western blotting (B). Both western blotting and immunofluorescence revealed that A549 cells exposed to TGF- β 1 significantly decreased the expression of E-Cad and increased the expression α -SMA, Vi and FN, while Sim inhibited these changes of EMT-markers induced by TGF- β 1 (A, C). The effects of Sim on the changes in EMT-markers induced by TGF- β 1 were suppressed by mediating the phosphorylation of Smad2 and Smad3 in A549 cells (B). The data represent the means $\pm$ SEM, n=3. * p<0.05 versus Control, # p<0.05 versus TGF- β 1.

expression of E-Cad, Vi, α -SMA and FN were examined using western blotting analyses (Fig. 2). Downregulation of E-Cad induced by TGF- β 1 was attenuated by Sim at 10 μ M by 90%. In addition, upregulation of the mesenchymal markers, α -SMA, Vi, and FN, were reversed by Sim treatment at 10 μ M by 60%, 45% and 80%, respectively. These results indicated that Sim pretreatment for 2 hr at a dose of 10 μ M could significantly reverse the changes in the expression of both epithelial and mesenchymal markers induced by TGF- β 1.

Consistent with the results obtained above, we used TGF- β 1 at a concentration of 5 ng/ml for 48 hr and Sim pretreatment at a concentration of 10 μ M in subsequent experiments.

Sim suppressed TGF- β 1-induced EMT in A549 cells associated with inhibition of the TGF- β 1-Smad2/3 signaling pathway

In this study, the cells were divided into 4 groups: a control group (DMSO), a Sim group, a TGF- β 1 group (DMSO + TGF- β 1) and a TGF- β 1+Sim group. The EMT-markers, E-Cad, α -SMA, Vi and FN, were detected using western blotting (Fig. 3A) and indirect immunofluorescence (Fig. 3C). In addition, p-Smad2, Smad2, p-Smad3 and Smad3, which are associated with the TGF- β 1-Smad2/3 pathway, were also detected using western blotting (Fig. 3B). A549 cells treated with Sim only showed nonsignificant changes in the expression of E-Cad, α -SMA, Vi and FN. Both western blotting and immunofluorescence demonstrated that treatment of A549 cells with TGF- β 1 significantly decreased the expression of E-Cad and increased the expression of α -SMA, Vi and FN. Compared to the treatment with TGF- β 1 alone, A549 cells treated with both TGF- β 1 and Sim showed a remarkable upregulation of E-Cad and downregulation of α -SMA, Vi and FN (Fig. 3A & 3C). In addition, although Sim rescued TGF- β 1-induced elevation of all three mesenchymal markers back to baseline, it did not result in a TGF- β 1-induced decrease in the expression of E-Cad back to control levels. GAPDH served as an internal control.

Fig. 4. Sim failed to reverse the morphological changes induced by TGF- β 1 in A549 cells. A549 cells were incubated with DMSO or 10 μ M Sim for 2 hr prior to incubation with 5 ng/ml TGF- β 1 for 48 hr and cell morphology was monitored. A549 cells under basal condition displayed a cobblestone-like morphology. Sim alone did not change the A549 cells morphological features. TGF- β 1 induced A549 cells to adopt a fibroblast-like, spindle morphology, while Sim failed to reverse the morphological changes induced by TGF- β 1. However, LY-364947, a TGF- β 1 receptor antagonist, completely prevented the morphological changes induced by TGF- β 1.

We also investigated whether Sim could modulate TGF- β 1-Smad2/3 signaling. The levels of phosphorylated and total Smad2, as well as phosphorylated and total Smad3, were examined using western blotting in the 4 groups (Fig. 3B). Sim alone did not affect the ratio of p-Smad2/Smad2 or p-Smad3/Smad3. Moreover, TGF- β 1 increased these ratios by 120% and 30%, respectively. However, the treatment of A549 cells with both TGF- β 1 and Sim significantly decreased these ratios to baseline.

These results indicated that Sim could partly reverse TGF- β 1-induced EMT in A549 cells associated with inhibition of the TGF- β 1-Smad2/3 signaling pathway.

Sim failed to reverse the morphological changes induced by TGF- β 1 in A549 cells

A549 cells treated with 10 μ M Sim for 48 hr without TGF- β 1 showed non-significant morphological changes. Addition of TGF- β 1 at a concentration of 5 ng/ml for 48 hr altered the morphology of A549 cells from a cobblestone-shaped morphology into a spindle-shaped morphology. However, pretreatment with 10 μ M Sim for 2 hr did not prevent the morphological changes induced by TGF- β 1, but pretreatment with the TGF- β 1-receptor antagonist LY-364947 at a dose of 10 μ M completely prevented the morphological changes induced by TGF- β 1 (Fig. 4). Taken together, these results indicated that Sim could not reverse the morphological changes induced by TGF- β 1 in A549 cells, potentially due to the inability of Sim to completely block TGF- β 1 signaling.

Sim inhibited A549 cell migration, and increased CTGF expression and MMP-2 and -9 secretion induced by TGF- β 1

In addition to the EMT process, A549 cells adopted mesenchymal-like properties, including increased migration activity, and elevation of CTGF expression and MMP-2 and -9 secretion. In this study, we also detected Sim effects on these pathological properties. A cell migration assay was performed by scratching the cell layer prior to drug treatment and imaging of the subsequent cell migration at specific time points (Fig. 5A). TGF- β 1 could

Fig. 5. Sim inhibited the A549 cell migration, CTGF expression and MMP-2 and -9 secretion induced by TGF- β 1. A cell migration assay was performed by scratching the cell layer prior to drug treatment, and time-lapse images were obtained from 0-48 hr. TGF- β 1 increased the migration activity, and Sim decreased TGF- β 1-induced cell migration (Fig. 5A). Western blotting illustrated that Sim could alleviate TGF- β 1-induced CTGF expression (Fig. 5B). ELISA of the cell supernatant showed that Sim significantly suppressed TGF- β 1-induced MMP-2 and -9 secretion (Fig. 5C). N.C.= negative control. The data represent the means $\pm$ SEM, n=3. * $p < 0.05$ versus Control, # $p < 0.05$ versus TGF- β 1.

enhance cell migration activity in a time-dependent manner compared to the control group. Co-incubation of Sim and TGF- β 1 significantly inhibited TGF- β 1-induced cell migration. Western blotting was used to detect CTGF expression in each group and Sim significantly decreased TGF- β 1-induced CTGF expression in A549 cells (Fig. 5B). Furthermore, ELISA was used to measure the secretion of MMP-2 and -9 in the culture medium. Both MMP-2 and -9 secretion were increased by TGF- β 1, which could be alleviated by the addition of Sim (Fig. 5C). This result indicated that the inhibition of EMT resulted in Sim suppressing the ability of A549 cells to adopt a fibroblast-like behavior, including migration, increased expression of CTGF and secretion of MMP-2 and -9.

Discussion

In this study, we used A549 cells as a model of human type II alveolar epithelial cells (AECII) and explored the inhibitory effects of Sim on EMT. A549 cells demonstrated many features of AECII cells [32], and most studies investigating EMT have used A549 cells as a model of AECII [30, 31, 33-35]. In addition, a few studies have used both A549 cells and primary AECIIs to study EMT and showed that A549 cells presented similar features as primary AECIIs during the EMT process [36, 37]. Thus, A549 cells are a suitable human AECII model to study EMT.

EMT is thought to play an important role in cellular trans-differentiation during tissue fibrosis [38]. TGF- β 1-induced EMT in A549 cells is a classic pathological model employed in studies on lung fibrosis and lung cancer. E-Cad is an adherent junction protein that is specifically expressed in epithelial cells [15]. Loss of E-Cad is a universal feature of EMT, and E-Cad can be used as an epithelial marker [14]. However, the acquisition of a mesenchymal phenotype is more difficult to define due to the lack of specificity in many of the available phenotypic markers [39]. In most of the studies on EMT, a combination of 2-3

mesenchymal markers is used. In this study, we chose one epithelial marker (E-Cad) and three mesenchymal markers (α -SMA, Vi and FN) to define EMT. Our results showed that TGF- β 1 induced A549 cells to lose their classic cobblestone-like morphology and to adopt a mesenchymal spindle-like appearance. Moreover, E-Cad expression was decreased and the expression of all the three mesenchymal markers, α -SMA, Vi and FN, was increased. Thus, we succeeded in establishing an EMT model in AECII to study the therapeutic effect of Sim on this pathological process.

The incidence of IPF increases with advancing age, peaking in patients ≥ 75 years old, which indicates that IPF is a geriatric disease [40]. It is generally known that aging causes changes in drug pharmacokinetics, which are dependent on body composition, albumin concentration, liver metabolism and drug elimination, all of which may change with age. This may cause an increase in drug concentration in elderly patients, elevating the risk of side-effects [41]. Although TGF- β is an initiating factor in the development of fibrotic disorders, drugs that block the TGF- β signaling pathway may also be associated with severe side-effects, such as lung tumors [42, 43], and are inappropriate choices for patients, particularly in elderly IPF patients.

Statins are HMG-CoA reductase inhibitors and might attenuate EMT in IPF. More importantly, statins are commonly accepted to be safe and show tolerance in elderly patients [20, 41, 44, 45]. Thus, statins are a potential therapeutic choice for IPF treatment. Among the many species of statins, Simvastatin (Sim) was first identified and is widely described as a potential therapeutic agent of IPF via inhibition of lung fibroblasts expressing CTGF, differentiation of myofibroblasts and secretion of extracellular matrix molecules [17, 46, 47]. Patel et al. [28] explored the effects of statins on EMT in human proximal tubular cells, and discovered that Sim presented similar reversal effects on EMT as Lovastatin and Pravastatin. Thus, Sim might be a potential statin for the treatment of IPF. We chose Sim over the other statins to investigate its therapeutic effect on EMT in AECII.

It has been reported the Sim could suppress TGF- β 1-induced EMT in human proximal tubular epithelial cells [28]. However, whether Sim can inhibit EMT in alveolar cells induced by TGF- β 1 remains unknown. In this study, we used Sim pretreatment prior to TGF- β 1 stimulation in A549 cells and determined that Sim could attenuate changes in EMT-markers at a concentration of up to 10 μ M.

In this study, we used western blotting and immunofluorescence to detect the expression of four EMT-markers, and these two assays showed similar results. TGF- β 1 significantly decreased E-Cad expression and increased the expression of α -SMA, Vi and FN, which was consistent with previous studies [30, 31]. Sim alleviated changes in the expression of these markers induced by TGF- β 1, indicating that Sim could attenuate TGF- β 1-induced EMT in AECII, which supports our hypothesis. Importantly, Sim reversed the effects of elevated expression of mesenchymal markers to baseline, but only partly reversed the decreased expression of the epithelial marker E-Cad. Western blotting analyses revealed that E-Cad levels in the TGF- β 1+Sim group were approximately 50% less than the control group.

TGF- β 1 induced EMT in AEC via 2 specific pathways, a Smad-dependent pathway and a Smad-independent pathway. The former, which is known as the TGF- β 1-Smad2/3 pathway, plays a predominant role in TGF- β 1-induced EMT in A549 cells [13, 14]. The latter, the Smad-independent pathway, includes several pathways, such as ERK, Akt, JNK, Rho, p38 MAPK, and NF- κ B, etc. However, the exact percentage of contribution from each pathway is difficult to estimate because there is complicated cross-talk signaling between the pathways [15]. A transcriptomic analysis of TGF- β -induced EMT in normal mouse and human epithelial cells demonstrated that increased expression of Smad2 or Smad3 with Smad4 induced EMT, whereas expression of dominant-negative versions of Smad2 or Smad3 blocked TGF- β -induced EMT, indicating that Smad signaling was critical in the regulation of all of the examined EMT target genes [48]. However, in most cases, stimulation of Smad-independent pathways induced and specified EMT within a particular tissue. Thus, we concluded that the Smad pathway represented the dominant pathway and that Smad-independent pathways might be necessary, but not sufficient, for the induction of full EMT [39]. The results in

this study showed that Sim attenuated TGF- β 1-induced EMT in A549 cells associated with modulation of the TGF- β 1-Smad2/3 pathway. We did not explore Smad-independent pathways in our study, and thus, the question of whether Sim attenuated EMT via Smad-independent pathways remains unanswered. However, Smad-independent pathways are much less important compared to the Smad-dependent pathway because Smad-independent pathways are not sufficient for EMT, and the Smad-dependent pathway is both sufficient and indispensable for EMT. Blockade of the Smad-dependent pathway, for example, using statins, will be an efficient choice to inhibit EMT.

In this study, we also evaluated whether Sim could reverse TGF- β 1-induced morphological changes. Our results showed that the TGF- β 1 receptor antagonist LY-364947 completely prevented cells from undergoing EMT induced by TGF- β 1, while Sim treatment did not significantly affect TGF- β 1-induced morphological changes. This phenomenon might be due to the ability of Sim to incompletely block TGF- β 1 signaling compared to LY-364947. Another potential explanation may be that Sim could not completely reverse the TGF- β 1-induced decrease in E-Cad. E-Cad is an adherent junction protein that associates with the cytoskeleton. Cell-cell junctions are important for cell maintenance as well as tissue polarity and integrity. Moreover, E-Cad is functionally linked to the generation of a polarized epithelial phenotype [49], which may explain the inability of Sim to prevent TGF- β 1-induced morphological changes. We also discovered that in Fig. 3C, the E-cad staining in control A549 cells is as expected, accentuated in the membrane at epithelial junctions. In the cells treated with both TGF- β 1 and Sim, E-cad expression is restored; however, the distribution of E-cad is primarily cytoplasmic. Interestingly, Sim alone is associated with a redistribution of E-cad staining. This could in part explain the failure of Sim to reverse the morphologic changes induced by TGF- β 1, and perhaps the subtle morphologic change induced by Sim alone depicted in Fig. 4. This is an interesting finding, because it suggests that E-cad redistribution, and acquisition of a spindle-shaped morphology, are not sufficient to indicate EMT. Also, because Sim inhibits the phosphorylation of Smad 2/3, it is likely that the morphologic change is induced via non-Smad pathways. In this study, we discovered that LY-364947 caused A549 cells to exhibit an abnormal morphology. This may be due to the important role of TGF- β 1 in cell growth and maintenance of the cytoskeleton [12]. Complete blockade of TGF- β 1 signaling might cause morphological abnormalities in cells.

By undergoing the EMT process in IPF, AEC also adopted a mesenchymal and fibroblast-like response, which directly promoted the IPF process. In this study, we investigated the effects of Sim on the pathological responses induced by TGF- β 1, including cell migration ability, expression of CTGF, and secretion of MMP-2 and -9. EMT endowed A549 cells with migratory properties, which enabled the cells to transport themselves from their origin to the mesenchyme to aggregate [15]. Our study revealed that Sim could inhibit TGF- β 1-induced cell migration. In addition, CTGF has also been identified to play critical roles in lung fibrosis and is a downstream effector of TGF- β 1 [17, 46, 50]. Sim has been reported to inhibit TGF- β 1-induced CTGF expression in human lung fibroblasts [17] and human intestinal fibroblasts [51]. Thus, we hypothesized that Sim could inhibit TGF- β 1-induced CTGF overexpression in A549 cells. Our results supported this hypothesis. MMP-2 and -9 contributed to the activation of TGF- β 1 and the disruption of basement membranes in IPF [1]. Statins also inhibited MMP expression or secretion in many cells [52, 53]. However, whether Sim can inhibit MMP-2 and -9 secretion induced by TGF- β 1 in A549 cells is still not yet known. This study showed that Sim significantly decreased MMP-2 and -9 secretion induced by TGF- β 1.

Taken together, our study provided the first line of evidence that Simvastatin could attenuate the EMT process induced by TGF- β 1 in human alveolar A549 cells associated with inhibition of the TGF- β 1-Smad2/3 signaling pathway in the absence of regulatory effects on cell morphology. More importantly, Simvastatin also inhibited A549 cells from adopting pathological mesenchymal and fibroblast-like behaviors induced by TGF- β 1, including cell migration, CTGF expression and MMP-2 and -9 secretion. Thus, Simvastatin may be a promising therapeutic agent for the treatment of IPF.

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