Original Research

Rapamycin regulates connective tissue growth factor expression of lung epithelial cells via phosphoinositide 3-kinase

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Abstract

The pathogenesis of idiopathic pulmonary fibrosis (IPF) remains largely unknown. It is believed that IPF is mainly driven by activated alveolar epithelial cells that have a compromised migration capacity, and that also produce substances (such as connective tissue growth factor, CTGF) that contribute to fibroblast activation and matrix protein accumulation. Because the mechanisms regulating these processes are unclear, the aim of this study was to determine the role of rapamycin in regulating epithelial cell migration and CTGF expression. Transformed epithelial cell line A549 and normal human pulmonary alveolar or bronchial epithelial cells were cultured in regular medium or medium containing rapamycin. Real time reverse transcriptase polymerase chain reaction was employed to determine CTGF mRNA expression. Western blotting and an enzyme-linked immunosorbent assay were used for detecting CTGF protein. Wound healing and migration assays were used to determine the cell migration potential. Transforming growth factor (TGF)-β type I receptor (TβRI) inhibitor, SB431542 and phosphoinositide 3-kinase (PI3K) inhibitor, LY294002 were used to determine rapamycin's mechanism of action. It was found that treatment of A549 and normal human alveolar or bronchial epithelial cells with rapamycin significantly promoted basal or TGF-β1 induced CTGF expression. LY294002, not SB431542 attenuated the promotional effect of rapamycin on CTGF expression. Cell mobility was not affected by rapamycin in wound healing and migration assays. These data suggest rapamycin has a profibrotic effect *in vitro* and underscore the potential of combined therapeutic approach with PI3K and mammalian target of rapamycin inhibitors for the treatment of animal or human lung fibrosis.

Keywords: Connective tissue growth factor, epithelial cell, idiopathic pulmonary fibrosis, migration, rapamycin

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Introduction

Idiopathic pulmonary fibrosis (IPF) is the most common form of idiopathic interstitial pneumonia, with a median survival time of 2.5–3.5 y and an increasing incidence.[1–3] IPF is a chronic, progressive, irreversible, and lethal disorder of unknown aetiology and pathogenesis.[4] It was believed that abnormal wound healing and epithelial pathophysiology played a critical role in the initiation and progression of IPF.[4,5]

The profibrotic effects of activated alveolar epithelial cells (AECs) in the lung are diverse. Abnormal AECs are sources of many profibrotic cytokines and growth factors, including connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β). These substances affect the proliferation, differentiation, migration, and matrix protein synthesis of fibroblasts.[4,6,7] Thus, identifying molecules that affect profibrotic factors (such as CTGF) produced by AECs is important for developing novel therapies for IPF.

The profibrotic effects of AECs are also the consequence of their limited capacity to migrate and participate in re-epithelialization. Re-epithelialization requires the migration of neighbouring epithelial cells to cover injured areas.[8] However, epithelial damage associated with lung fibrosis does not heal due to AEC's apoptosis, compromised migration, and failure to proliferate.[9,10] Consequently, therapy for lung fibrosis that targets the processes regulating cell mobility could be effective.

Rapamycin (sirolimus) is a carboxycyclic macrolide that has potent immunosuppressive properties *in vitro* and *in vivo*.[11] Rapamycin inactivates the mammalian target of rapamycin (mTOR). mTOR is critical for cell mRNA translation and modulation of protein transcription[12,13] in response to insulin, growth factors, amino acids, and

drugs. The primary downstream targets of mTOR are p70S6 kinase 1 (p70S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1).[12,13] Independent of anti-inflammatory action, rapamycin could prevent liver, kidney, and pulmonary fibrogenesis in animal models,[14–16] although its mechanism of action is poorly elucidated.

In the parallel study, we showed that rapamycin upregulated CTGF expression in fibrotic human lung fibroblasts (HLFs; unpublished data). CTGF can be produced by various cell types regardless of tissue origin.[17] Injured epithelial cells could also produce CTGF to promote fibrotic process.[4,6,7] So we hypothesized that rapamycin can also modulate CTGF production and the migration of alveolar type II-like epithelial cell line A549 and normal human pulmonary alveolar or bronchial epithelial cells. It was found that rapamycin significantly upregulated basal or TGF-β1 induced CTGF mRNA steady-state level and protein production via its actions on phosphoinositide 3-kinase (PI3K). It was also found that the migration potential of epithelial cells was not affected by rapamycin, suggesting that mTOR signalling is not involved in cell mobility or mTOR may affect cell mobility but the dose of rapamycin or detecting time covers the possible changes.

Materials and methods

Cell stimulation and pharmacological treatments

The human non-small-cell-lung-cancer cell line, A549 (ATCC, Rockefeller, MD, USA), was maintained in RPMI 1640 (Gibco, Grand Island, NYC, USA) containing 10% fetal bovine serum (FBS; Gibco) in a humidified 5% CO_2 atmosphere. Normal human pulmonary alveolar or bronchial epithelial (HPAEpi or HBEpi, ScienCell, San Diego, CA, USA) cells from normal lung tissues were grown as monolayers in humidified 5% CO_2 at 37°C in the special alveolar or bronchial epithelial cell media (ScienCell) and were used on passage between 2 and 4. Subconfluent cultures of cells were maintained in serum-free media for 24 h prior to stimulation with drugs. The modulatory effects of rapamycin on CTGF production of epithelial cells were investigated by incubating for varying periods of time (1, 6, 12, 24 h) at three concentrations of rapamycin (1, 5, 10 ng/mL; Sigma, San Francisco, CA, USA), in the absence or presence of pre-stimulation with TGF-β1 (10 ng/mL; BD, San Diego, CA, USA) for 24 h. The modulatory effects of rapamycin on cell mobility were investigated by treating A549 cells with or without rapamycin (5 ng/mL) for varying durations (0, 24, 48 h). The modulatory mechanisms of rapamycin on epithelial cells were studied using TβRI/Smad inhibitor (SB431542, 1–20 μmol/L, Biotrend, Cologne, Germany) and PI3K specific antagonist (LY294002, 1–20 μmol/L, Biotrend). After the above treatments, cells were assayed for protein and RNA levels, as well as for a cell migration assay.

Characterization of HPAEpiC and HBEpiC and Smad2/3 immunostaining

The identification and purity of the cultured primary HPAEpiC and HBEpiC were confirmed by morphological observation and immunostaining with cytokeratin-18 and prosurfactant protein C (proSP-C). Smad2/3 cellular localization was also analysed by using immunostaining assay. Cells were fixed in 4% paraformaldehyde and permeabilized in phosphate-buffered saline (PBS) containing 0.1% Triton X-100. After washing, non-specific binding was blocked with 4% bovine serum albumin (BSA). Cells were then incubated with mouse anti-human cytokeratin-18 mAb, rabbit anti-human pro-SPC, anti-human Smad2/3 mAb (all from Abcam, Cambridge, MA, USA) according to manufacturer's instruction in a humidified chamber overnight at 4°C. Cells were then incubated with Texas Red-conjugated or FITC-conjugated goat anti-mouse or rabbit secondary antibodies (KPL, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) at 37°C in a humidified chamber in the dark for 60 min. Then, the cover slips were overturned on a microscope slide containing one drop of anti-fade solution with 4′,6-diamidino-2-phenylindole. Images were then taken at room temperature on OLYMPUS inverted microscope (Olympus, Tokyo, Japan).

RNA purification and real time RT PCR

Total RNA was isolated from epithelial cells subsequent to cell stimulation and pharmacological treatments using the RNAeasy Miniprep kit (Tiangen, China), and 2 μg of RNA from each sample was reverse-transcribed at 37°C for 1 h with the Omniscript RT kit (Tiangen, China) using oligo dT primers (1 μmol/L). Real time polymerase chain reaction (PCR) was performed with an ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) using SYBRGreen PCR reagents (Tiangen, China). Reaction mixtures were incubated for 2 min at 94°C, and were amplified for 40 cycles (94°C for 15 s, 55°C for 20 s, and 68°C for 35 s). For each sample, gene expression was corrected against β-actin mRNA levels and the comparative threshold cycle number (CT) method was used to assess the relative quantification of gene expression.[18] The fold changes of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method.[18] The CTGF primers used for PCR reactions were: forward sequence, 5′-CCTGCAGGCTAGAGAA GCAGA-3′; reverse sequence, 5′-TTTGGGAGTACGGAT GCACTT-3′. The β-actin primers used were: forward sequence, 5′-TGCTATCCAGGCTGTGCTAT-3′; reverse sequence, 5′-AGTCCATCACGATGCCAGT-3′.

Protein extraction, SDS-PAGE, and indirect immunoblot analysis

Cells were harvested and lysed using a radioimmunoprecipitation assay (RIPA, Pierce, Rockford, IL, USA) buffer that was supplemented with complete proteinase and phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Protein extracts were obtained by centrifugation at 4°C and concentrations were determined with a bicinchoninic acid assay kit (Pierce). Protein extracts (30 μg) were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were subsequently blocked

with 5% nonfat dry milk or BSA in TBST (10 mmol/L Tris-HCl, pH 7.6; 150 mmol/L NaCl; 0.1% Tween-20) and incubated with the following primary antibodies overnight at 4°C according to the manufacturer's instructions: rabbit anti-phospho-mTOR, mouse anti-phospho-p70S6K, rabbit anti-phospho-4EBP1 (each from Cell Signal Technology, Danvers, MA, USA); and rabbit anti-CTGF, anti-Smad2 + 3, anti-phospho-Smad2, anti-phospho-Smad3, anti-pan-AKT, anti-phospho-AKT(Thr308), anti-phospho-AKT(Ser473), and anti-PI3K (p110α) mAb (each from Abcam). Membranes were incubated for 1 h after washing with the relevant secondary, horseradish peroxidase-labelled antibodies (Proteintech, Chicago, IL, USA). Finally, an enhanced chemiluminescence detection buffer (KPL) with ChemiDoc XRS (Bio-Rad, Hercules, CA, USA) was used for visualization of protein bands. Relative protein levels were normalized with mouse anti-GAPDH mAb (Abcam).

ELISA for CTGF levels in culture supernatant

The levels of CTGF in cell conditioned media were measured with human CTGF enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Carlsbad, CA, USA) using the double antibody sandwich method, as directed by the manufacturer's instructions. In brief, 100 μL of the culture supernatant was added to plate wells that were pre-coated with anti-human CTGF polyclonal antibody. After 2 h incubation at 37°C, the plate was washed five times and 50 μL of anti-human CTGF antibody was added to each well, followed by 1 h incubation at 37°C. The plate was subsequently washed five times and 100 μL of the second antibody was added for 1 h incubation at 37°C. The plate was washed five times again and 100 μL of substrate solution was added to each well for incubation at room temperature in the dark while colour development occurred. After 30 min, 100 μL of stop solution was added to each well. Absorbance was measured at 450 nm using a microplate reader (Tecan, Männedorf, Switzerland). Concentrations of CTGF in the culture supernatant were determined by interpolation from the standard curve.

Wound healing and migration assay

For the wound healing assay,[19] epithelial cells were seeded in six-well plates and were subconfluent at the time scratch wounds were applied to each well. This was accomplished with a 200 μL pipette tip, and cell debris was washed off twice with PBS. Fresh medium (containing 1% FBS), with or without rapamycin, was added to the wells and cells were incubated for up to 48 h. Phase contrast light microscope pictures were taken on an inverted microscope immediately after scratch wounding (0 h), and at 24 h and 48 h. All experiments were conducted in duplicate and repeated three times.

A chamber assay was used to measure migratory potential.[19] Briefly, epithelial cells were seeded in 60 mm dishes and grown to subconfluence. The cells were then incubated in a serum free media for 24 h. Next, the medium was discarded, and rapamycin was added for another 24 h of incubation. The cells were subsequently harvested and seeded at a density of 10,000 cells in 0.1 mL of serum free media in the upper compartment of a two-chamber migratory well (8 μm pore size, Costar, Corning, NY, USA). In the lower compartment, 0.5 mL of media was supplemented with 10% FBS. After incubation for 24 h, non-migrated cells were removed by cotton swabbing and the cells on the lower side were fixed with cold methanol solution, followed by staining with crystal violet solution (Sigma). Images of migrated cells were taken using a digital camera and a fluorescence microscope (Olympus). The cells were dissolved with 33% glacial acetic acid and the absorbance was determined using a multifunctional microplate reader (Tecan, Switzerland).

Statistical analysis

SPSS 13.0 (SPSS, Chicago, IL, USA) software was used for analyses. Data are reported as means ± SEM and analysed using one-way analysis of variance followed by SNK multi-comparison test. The data were presented as relative induction compared with control conditions (*) or compared with rapamycin-stimulated values (#). P values < 0.05 were considered significant.

Results

Phenotype of primary HPAEpiC and HBEpiC cultures

HPAEpiC and HBEpiC had a cobblestone appearance that is unique for epithelial cells as shown in Figure 1a and d. Subcultured lung epithelial cells showed uniform positive immunostaining for cytokeratin or proSP-C (Figure 1b, c and e).

Rapamycin initially upregulates basal CTGF mRNA expression of A549 cells

To determine whether rapamycin modulates basal CTGF mRNA expression of A549 cells, real time reverse transcriptase PCR (RT PCR) was done. It was showed that rapamycin significantly increased basal steady-state CTGF mRNA steady-state levels ($P < 0.001$), with a maximal change of 2.58-fold after 1 h treatment ($P < 0.001$, Figure 2a).

Rapamycin initially upregulates basal CTGF protein expression of A549 followed by induction of CTGF secretion

For determination of rapamycin's effects on CTGF protein synthesis and secretion, Western blot and ELISA were done. Western blot analysis showed that rapamycin (5 ng/mL) significantly reduced mTOR/S6K/4EBP1 activity in a time-dependent manner (Figure 2b). Simultaneously, CTGF protein levels in cell lysates were significantly increased by rapamycin treatments at 1, 6, and 12 h. However, with extended drug stimulation for 24 and 48 h, CTGF expression was decreased. For determination of whether decreased CTGF expression was due to excessive secretion, an ELISA was performed and showed that CTGF protein in culture supernatants was increased in a time-dependent manner (Figure 2c).

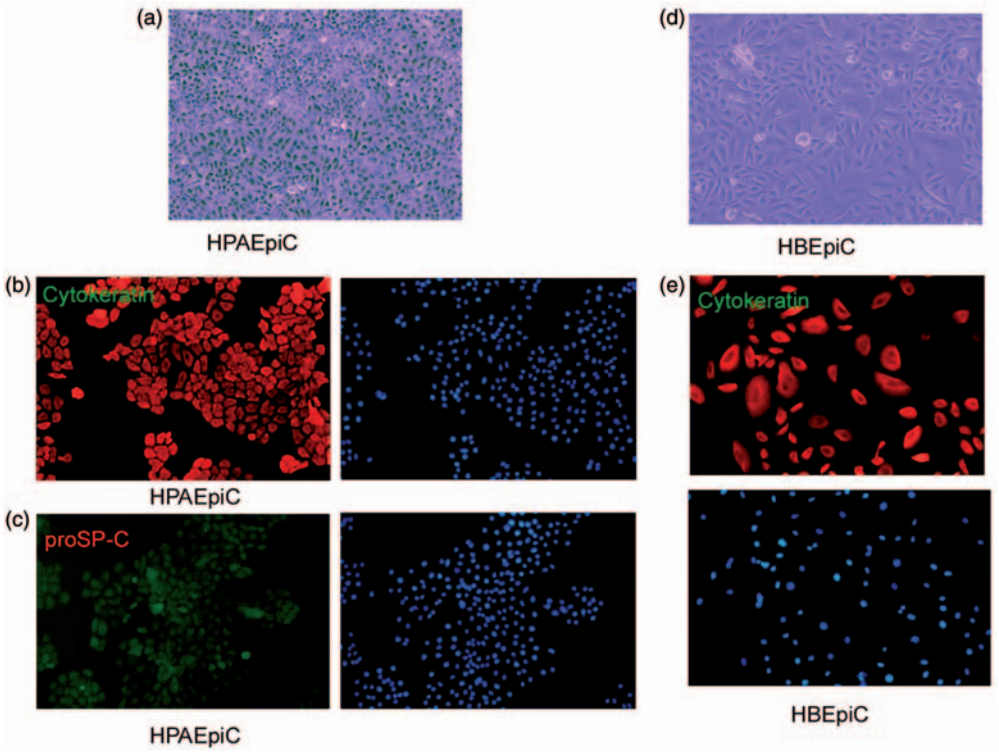

Figure 1 Subculture features of HPAEpiC and HBEpiC. Microphotograph showed that HPAEpiC (a) and HBEpiC (d) exhibited the typical cobblestone morphology of epithelial cells. Magnification 100×. Cells stained for epithelium-specific markers. HPAEpiC showed positive for cytokeratin-18 (b) and proSP-C (c). HBEpiC was also positive for cytokeratin-18 (e). Magnification 100 ×

Rapamycin augments CTGF expression and secretion from A549 cells incubated with TGF-β1 during later stages

The role of rapamycin in the expression of CTGF mRNA in A549 cells induced by TGF-β1, a major profibrogenic factor, was investigated. Real time RT PCR analysis showed that rapamycin significantly induced CTGF steady-state mRNA expression in A549 cells that were potentiated by incubation with TGF-β1 for 24 h ($P < 0.001$, vs. control group; $P < 0.001$ vs. TGF-β1 treated group; Figure 3a). Western blot analyses demonstrated that CTGF in cell lysates significantly decreased after co-stimulation with TGF-β1 and rapamycin for 24 h ($P = 0.01$, Figure 3b). However, CTGF levels in cell culture media were significantly increased after incubation for 24 h in TGF-β1 and rapamycin ($P < 0.001$, Figure 3c).

Rapamycin upregulates CTGF expression in HPAEpiC and HBEpiC

Because A549 cells are a transformed human lung cancer cell line that may not reflect the regulatory properties of normal bronchial or AECs, we performed experiments using or pulmonary alveolar or bronchial epithelial cells isolated from normal human lungs (HPAEpiC and HBEpiC). We assessed whether basal or cytokine induced CTGF expression of HPAEpiC and HBEpiC was upregulated by rapamycin (Figures 4 and 5). When exposed to rapamycin, HPAEpiC also exhibited an increase in CTGF mRNA (Figure 4a, $P < 0.05$), with a maximal change of 1.51-fold after 6 h treatment ($P < 0.01$). Western blot analysis showed CTGF protein levels in cell lysates were also significantly increased by rapamycin treatments at 1, 6, 12, and 24 h (Figure 4b, $P < 0.001$). ELISA was also performed and showed that CTGF protein in culture media was simultaneously increased in a time-dependent manner (Figure 4e, left column, $P < 0.001$). Rapamycin also potentiated TGF-β1 induced CTGF mRNA and protein expression in HPAEpiC (Figure 4c, d and e, right column). The parallel studies were also performed on HBEpiC. Data showed that rapamycin also potentiated basal or TGF-β1 induced CTGF mRNA and protein expression (mRNA, Figure 5a and c; protein, Figure 5b, d and e). These studies suggest a similar and universal effect of rapamycin on CTGF in different kinds of epithelial cell types.

Rapamycin has no modulatory effect on epithelial cell migration

To study the role of rapamycin in epithelial cell migration, wound healing scratch assay was used to measure cell migration towards injuries. It was found that cell mobility was not affected by rapamycin (5 ng/mL; Figure 6a and b). The chamber migration assay also showed that rapamycin (5 ng/mL) had no effect on cell mobility (Figure 6c and d). The same study was done with HPAEpiC. It was also shown that rapamycin (5 ng/mL) also failed to affect cell migration of HPAEpiC (Figure 7a and b, scratching assay; c and d, migration assay).

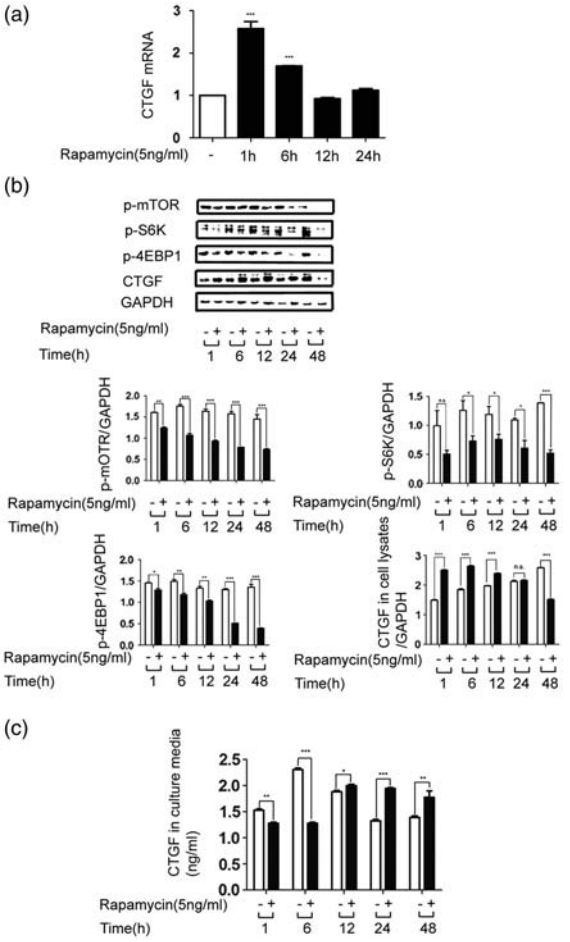

Figure 2 Modulatory effect of rapamycin on A549 cell's basal CTGF mRNA and protein levels. Quiescent A549 was incubated with rapamycin (5 ng/mL) for multiple time points. a: Whole cell mRNA was extracted carefully according to the RNAeasy Miniprep Kit manual. Changes in CTGF steady-state mRNA levels were determined by real time RT PCR. The results are representative of three independent experiments. Values shown are means ± SEM. ***$P < 0.001$ vs. vehicle control group. b: Whole cell lysates were harvested in RIPA buffer, 30 μg of cell extracts were subjected to SDS-PAGE. Phospho-mTOR/S6K/4EBP1, CTGF (in whole cell lysates), and GAPDH expression was analysed by Western blot. Values shown are means ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. vehicle control group. c: Cell supernatants were harvested and secretory CTGF protein was determined with an ELISA. Values shown are means ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. vehicle control group

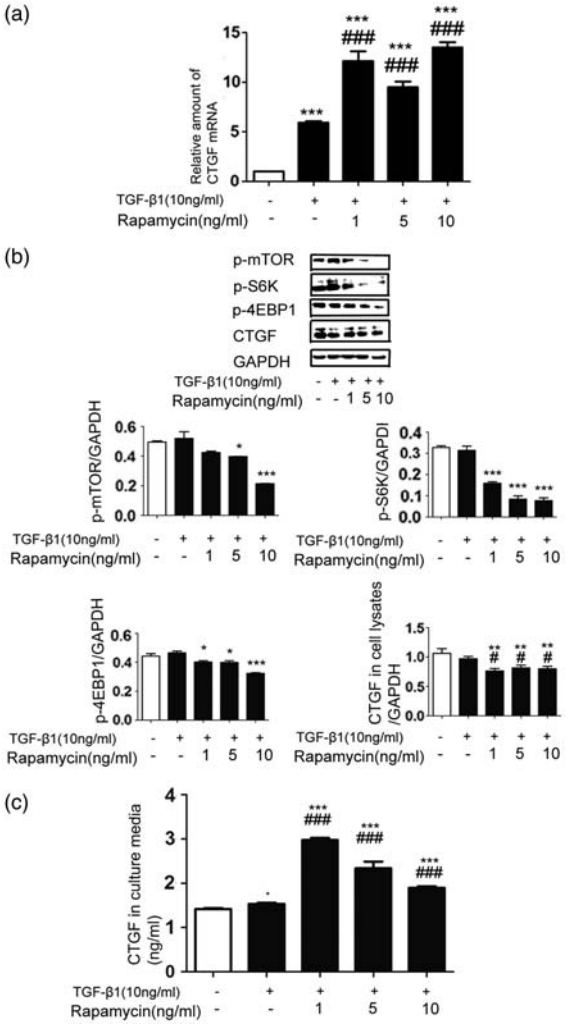

Figure 3 Modulatory effects of rapamycin on CTGF mRNA and protein levels in A549 cells incubated with TGF-β1. A549 cells were treated with TGF-β1 (10 ng/mL) for 24 h and then treated with varying doses of rapamycin (1–10 ng/mL) for an additional 24 h. a: CTGF mRNA levels were determined by real time RT PCR. ***Significantly different from vehicle-treated cells, $P < 0.001$. ###Significantly different from cells treated with TGF-β1 (10 ng/mL), $P < 0.001$. Data are expressed as mean ± SEM relative to vehicle-treated cells from three independent experiments. b: Whole cell lysates were harvested in RIPA buffer and subjected to SDS-PAGE. Phospho-mTOR/S6K/4EBP1, CTGF (in whole cell lysates), and GAPDH expression were analysed by Western blot. Values shown are means ± SEM, $n = 3$ experiments per point. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. vehicle control group. #, ### significantly different from cells treated with TGF-β1 (10 ng/mL), $P < 0.05$, $P < 0.001$, respectively. c: Cell supernatants were harvested and secretory CTGF protein was determined with an ELISA. Values shown are means ± SEM of three independent experiments. *$P < 0.05$, ***$P < 0.001$ vs. vehicle control group. ###Significantly different from cells treated with TGF-β1 (10 ng/mL), $P < 0.001$

PI3K, not Smad 2/3 is involved in rapamycin's effects on CTGF expression in lung epithelial cell

The underlying mechanism of rapamycin's effects on lung epithelial cells was investigated. Previous study[20] showed that rapamycin induces the TGFβ1/Smad signalling cascade in renal mesangial cells, and concomitantly causes an increase of CTGF and PAI-1. So we want to examine whether TβR1/Smad is also involved in rapamycin's positive effect on CTGF expression in lung epithelial cells. Firstly, we tested whether rapamycin modulates the Smad signal by phosphorylation and nuclear translocation of the Smad2/3. As shown in Figure 8b–e, simulation of A549 and HPAEpiC with rapamycin (5 ng/mL) failed to induce a rapid phosphorylation of Smad2 and Smad3. Rapamycin treatment also failed to induce a significant nuclear translocation of Smad2/3 in A549 and HPAEpiC as detected by indirect immunofluorescence microscopy (Figure 9). Next, we used SB431542 to determine whether it could block rapamycin's positive action. We found SB431542 (from 1 to 20 μmol/L) did not affect profibrotic effects of rapamycin on A549 (Figure 8a, left column). Larger dose of SB431542 (10∼20 μmol/L) could even potentiate rapamycin's positive action on CTGF expression of HPAEpiC (Figure 8a, right column).

Recently, it was also demonstrated that negative feedback loops exist in the mTOR/S6K1/4EBP1 signal

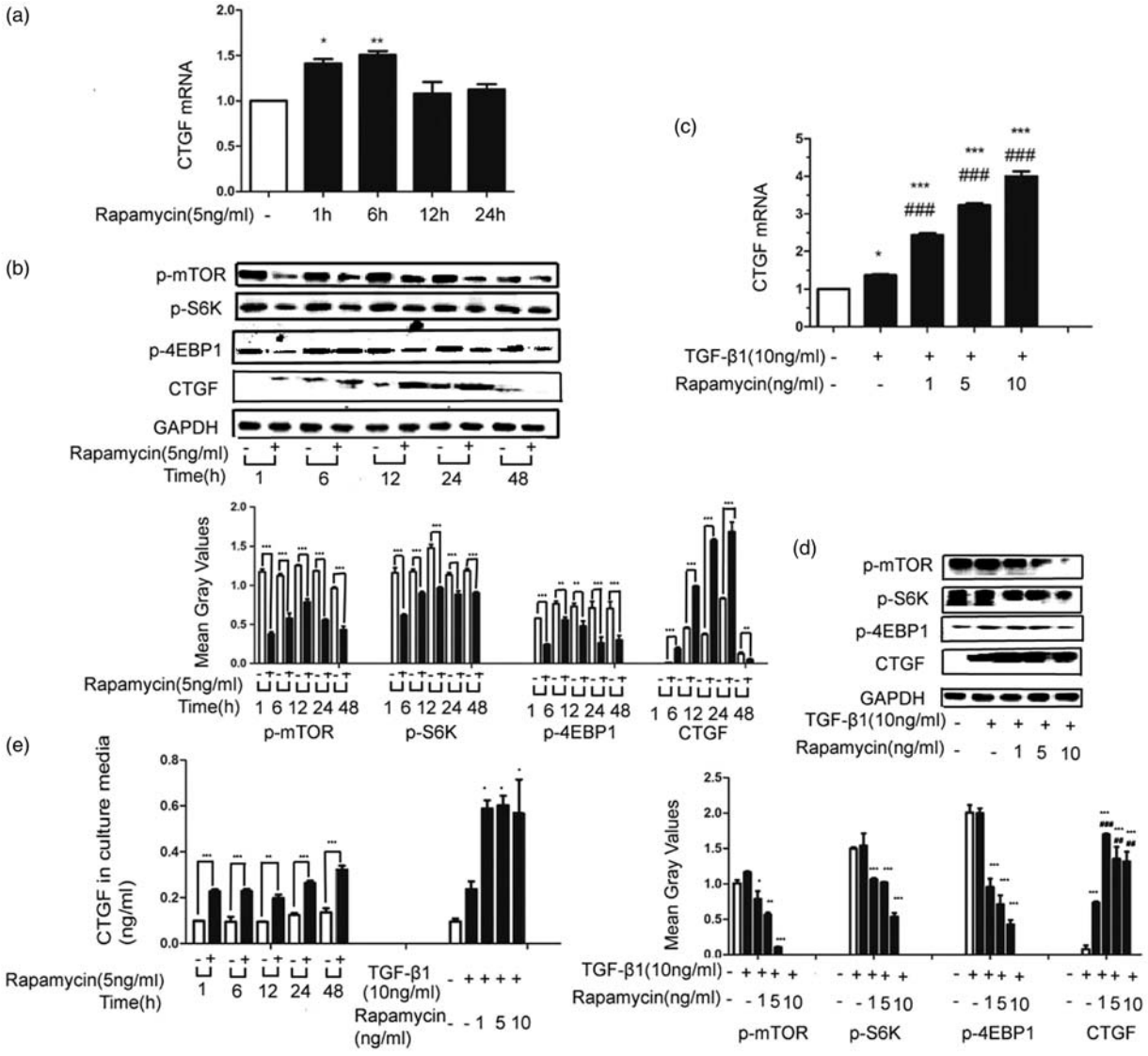

Figure 4 Rapamycin upregulates basal or induced CTGF expression of HPAEpiC. HPAEpiC were incubated with rapamycin (5 ng/mL) for multiple time points in regular medium or with different doses of rapamycin for 24 h in medium containing TGFβ1 (10 ng/mL) in triplicate. a and c: CTGF mRNA levels were determined by real time RT PCR. b and d: CTGF expressions in whole cell lysates were analysed by Western blot. E: CTGF protein in culture media was determined with an ELISA. Values shown are means ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. vehicle control group. ###Significantly different from cells treated with TGF-β1 (10 ng/mL), $P < 0.001$

system.[13,21] Inhibition of mTOR activity with rapamycin may result in a hyperactive receptor tyrosine kinase (RTK)/(PI3K) pathway. So whether the PI3K pathway regulates rapamycin's positive effects on CTGF expression in A549 and HPAEpiC was examined. We first showed that rapamycin (5 ng/mL) treatment for 30 min and 1 h significantly promoted PI3K (p110α), p-AKT (T308), and p-AKT (S473) expression in A549 and HPAEpiC (Figure 10b–f, $P < 0.05$). We then showed that LY294002 (from 1 to 20 μmol/L) largely blocked basal CTGF mRNA upregulation that was induced by rapamycin ($P < 0.001$, 20 μmol/L LY294002 vs. rapamycin treated group, Figure 10a).

Discussion

Recently, rapamycin was mainly used for immunosuppressive therapy because of its anti-inflammatory actions.[22,23] However, considering its extensive functions on different cell types in addition to T, B immunocytes, rapamycin was gradually used in anti-cancer and anti-fibrotic studies.[13–16,24] This was based on the hypothesis that rapamycin, after direct binding and inhibiting mTOR activity of cancer cells or fibrosis response cells, could attenuate cell growth and tumour- or fibrosis-associated protein expression.[12]

However, mTOR signal network is very different and complicated.[25] So inhibition of mTOR with rapamycin would induce even more complex changes on mTOR targeted proteins. In the parallel studies, we explored rapamycin's effects on primary cultured fibrotic HLFs and found that it significantly potentiated basal or cytokine induced CTGF mRNA and protein expression (unpublished data). Because epithelium is also the most important targeted effector of IPF, we, in this present study, wanted to

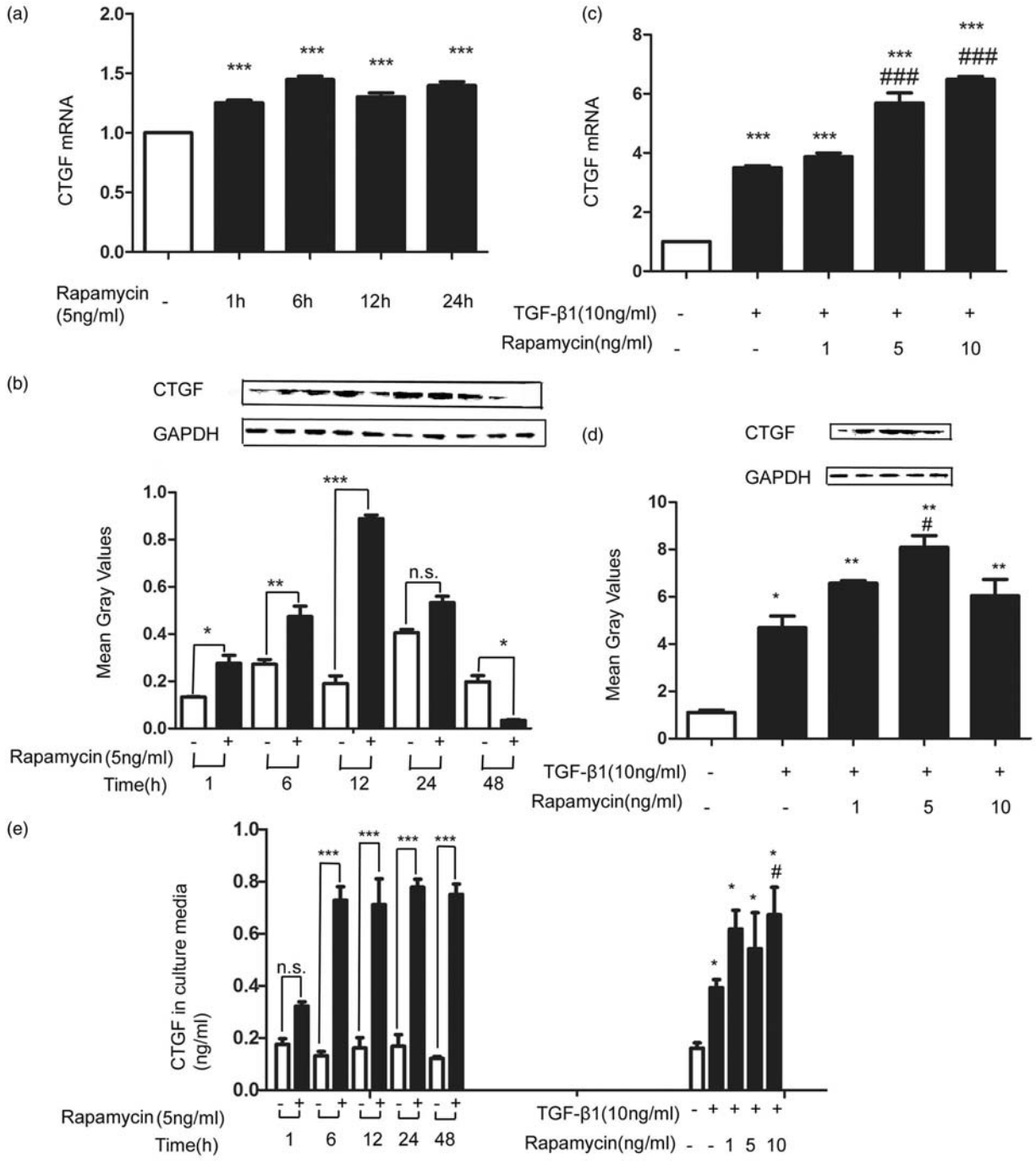

Figure 5 Rapamycin upregulates basal or induced CTGF expression of HBEpiC. HBEpiC were incubated with rapamycin (5 ng/mL) for multiple time points in regular medium or with different doses of rapamycin for 24 h in medium containing TGFβ1 (10 ng/mL) in triplicate. a and c: CTGF mRNA levels were determined by real time RT PCR. b and d: CTGF expressions in whole cell lysates were analysed by Western blot. e: CTGF protein in culture media was determined with an ELISA. Values shown are means ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. vehicle control group. #, ### significantly different from cells treated with TGF-β1 (10 ng/mL), $P < 0.05$ and $P < 0.001$

investigate whether rapamycin had similar effects on epithelial cells. Our results using A549 cell line showed that rapamycin upregulates CTGF mRNA steady-state level via the PI3K pathway. This occurred at baseline levels and when A549 cells were incubated with TGF-β1. It was also found that rapamycin promotes CTGF protein synthesis and secretion of A549 cells. Importantly, because A549 cells are transformed, immortalized cancer cell line, we also done the parallel experiments with primary human normal pulmonary alveolar and bronchial epithelial cells (HPAEpiC and HBEpiC). We observed an almost identical pattern of CTGF expression following stimulation with rapamycin in HPAEpiC and HBEpiC. These results establish that rapamycin's positive effect on CTGF expression is not limited to transformed type II AECs but can also be induced in normal human pulmonary alveolar and bronchial epithelial cells *in vitro*. Because CTGF is the result and also the cause of fibrosis establishment,[26–28] this

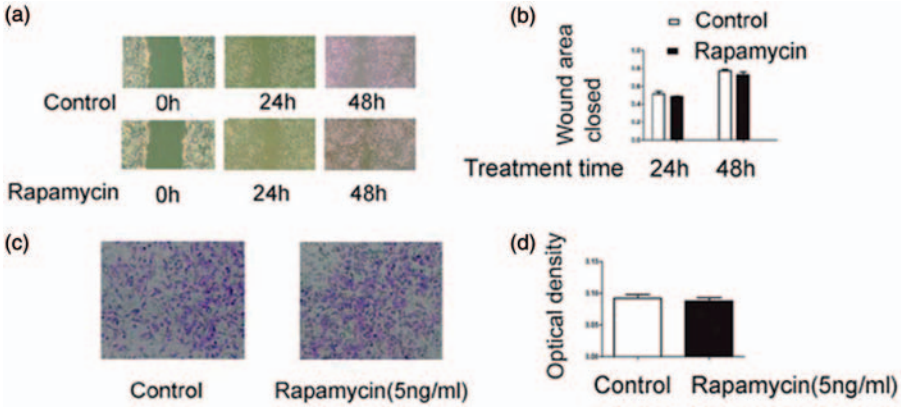

Figure 6 Wound healing and migration assays show rapamycin without a modulatory effect on A549 cell's mobility. For the wound healing assay, serum starved A549 cells were incubated for 24 h and 48 h in the absence or presence of rapamycin (5 ng/mL) in DMEM containing 1% FBS. a: A representative example of the wound healing assay (40×). b: Summary of wound assay results. Values are average healed distance (measured at three different points) compared to the healing time; bars show SEM. For the migration assay, A549 cells were incubated with serum free medium for 24 h, and then rapamycin (5 ng/mL) was added for an additional 24 h of incubation. Cells were harvested and seeded on the upper side of millipore chambers for the migration analysis. c: Representative example of the transwell migration assay (100×). d: Summary of the wound assay results. Cells were dissolved with 33% glacial acetic acid. Values are the absorbance determined by multifunctional microplate reader; bars show the SEM of three independent experiments

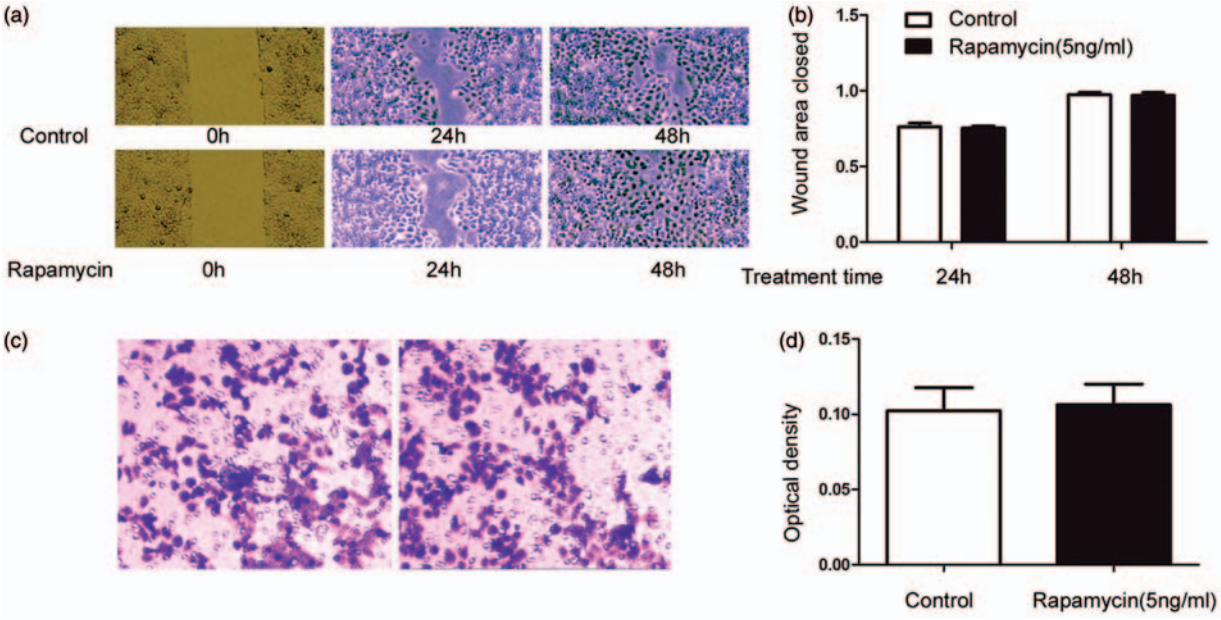

Figure 7 Rapamycin failed to affect cell migration of normal HPAEpiC. a: A representative example of the wound healing assay (40×). b: Summary of wound assay results. c: Representative example of the transwell migration assay (100×). d: Summary of the wound assay results. Bars show the SEM of three independent experiments

suggests that rapamycin has a profibrotic effect *in vitro*. However, migration of A549 and HPAEpiC was not affected by rapamycin, suggesting that a rapamycin/mTOR signal is not involved in epithelial cell mobility or that it may be involved in cell mobility but current inhibition is incomplete under the conditions used for this study.

CTGF is a member of the CCN family of proteins, and is a multifunctional, matricellular protein which plays a central role in the progression of various fibrotic disorders and is associated with disease severity parameters and outcome and may have diagnostic and prognostic value.[26–30] Studies showed that CTGF affects a variety of cell types that are involved in fibrotic disorders, regardless of tissue origin, including pulmonary type II alveolar cells, tubular epithelial cells, interstitial fibroblasts, mesangial cells, vascular smooth muscle cells, mesenchymal stem cells, mesothelial cells, cardiomyocytes, pericytes and so on.[17] Our study showed that rapamycin (5 ng/mL) significantly upregulated basal CTGF mRNA steady-state level by transformed and normal human lung epithelial cells (Figures 2a, 4a, 5a). Because TGF-β1 is a potent inducer of CTGF,[17] we also explored whether rapamycin has a synergistic effect on CTGF expression

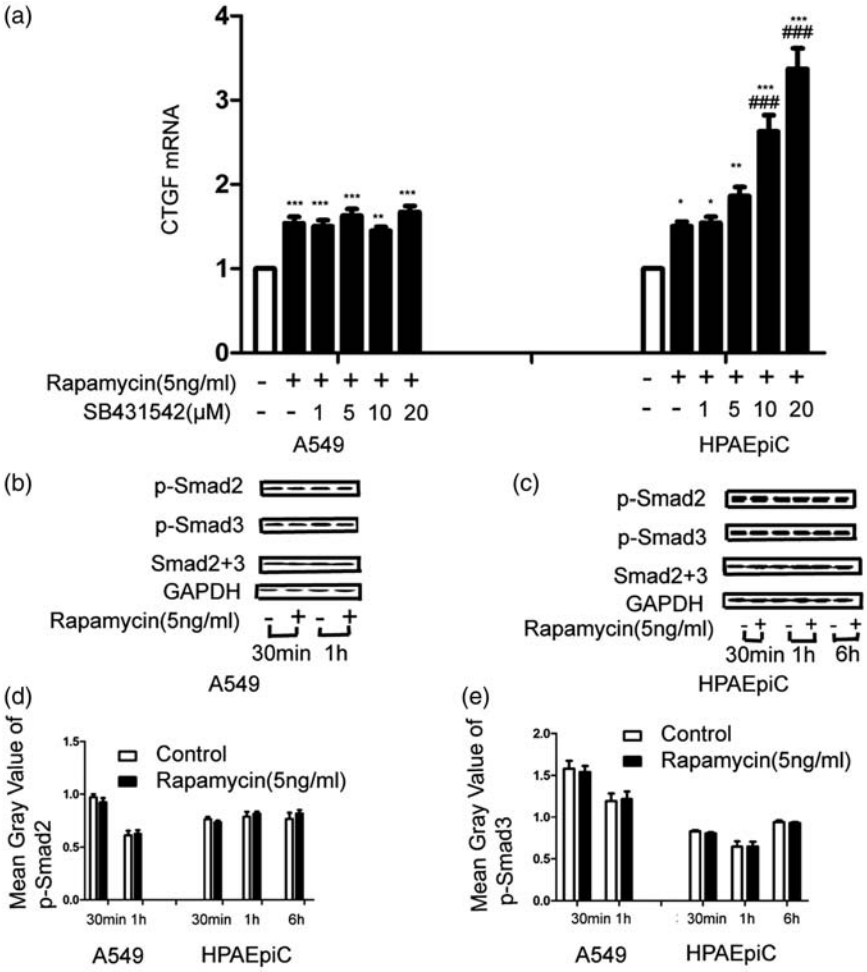

Figure 8 TβR1/Smad is not involved in the positive role of rapamycin on CTGF expression of A549 and HPAEpiC. a: Serum-starved A549 and HPAEpiC were treated with rapamycin (5 ng/mL) for 1 h in the presence or absence of SB431542 (1–20 μmol/L). After incubations, CTGF mRNA steady-state levels were determined by quantitative RT-PCR. Bars indicate mean ± SEM of at least three independent experiments performed, each with duplicates. *, **, *** Significant difference, $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively, compared with vehicle group; ###, Significant difference, $P < 0.001$, compared with rapamycin group. b and c: Representative example of immunoblotting analysis for p-Smad2/3. Quiescent A549 and HPAEpiC were stimulated with either vehicle (–) or with rapamycin (5 ng/mL) for 30 min, 1 h, or 6 h before cells were harvested for immunoblotting analysis. GAPDH staining was used for loading control. d and e: Densitometry results from three independent experiments

when treated with TGF-β1. We found rapamycin (1, 5, 10 ng/mL) induced CTGF mRNA expression in lung epithelial cells that were concurrently incubated with TGF-β1 for 24 h (Figures 3a, 4c, 5c). Although CTGF is a down stream activator of TGF-β1, there are also interdependent rather than only sequential relationship between TGF-β1 and CTGF. They both have respective profibrotic activities. So we believe that rapamycin exerts a profibrotic effect *in vitro* by upregulating basal or TGF-β1 induced CTGF expression. Findings that used other cell models are consistent with our results. One *in vitro* study[31] showed that rapamycin and cyclosporine A synergistically upregulated human mesangial cells' CTGF expression and may also exert profibrotic effects. Another *in vitro* study showed that rapamycin activated CTGF promoter and promoted CTGF mRNA and protein expression in rat mesangial cells (RMCs).[20] This indicated that rapamycin upregulated CTGF expression in transcriptional way. An *in vivo* study[32] used a rat model of chronic nephrotoxicity to show that rapamycin further augmented CTGF expression in kidney tissue and promoted kidney fibrosis. However, other data[33] showed that rapamycin attenuated human chronic allograft nephropathy by reducing CTGF expression. Collectively, various doses of rapamycin and different animal models may serve to reconcile discrepancies regarding the modulatory effects of rapamycin on CTGF expression.

But how does rapamycin inhibition of the mTOR signal cascade promote CTGF mRNA transcription, protein synthesis, and protein secretion in A549 cells at baseline and when incubated with TGF-β1? It was shown that rapamycin activated Smad signal to upregulate CTGF expression in RMCs.[20] However, in this study, we found that rapamycin failed to induce smad2/3 phosphorylation and nucleus localization in A549 and HPAEpiC (Figures 8b–e and 9). Also, TβRI/Smad inhibitor, SB431542, failed to attenuate rapamycin's positive effect on CTGF expression in these cells (Figure 8a). So Smad activation may not be served for the positive effect of rapamycin on CTGF expression in epithelial cells.

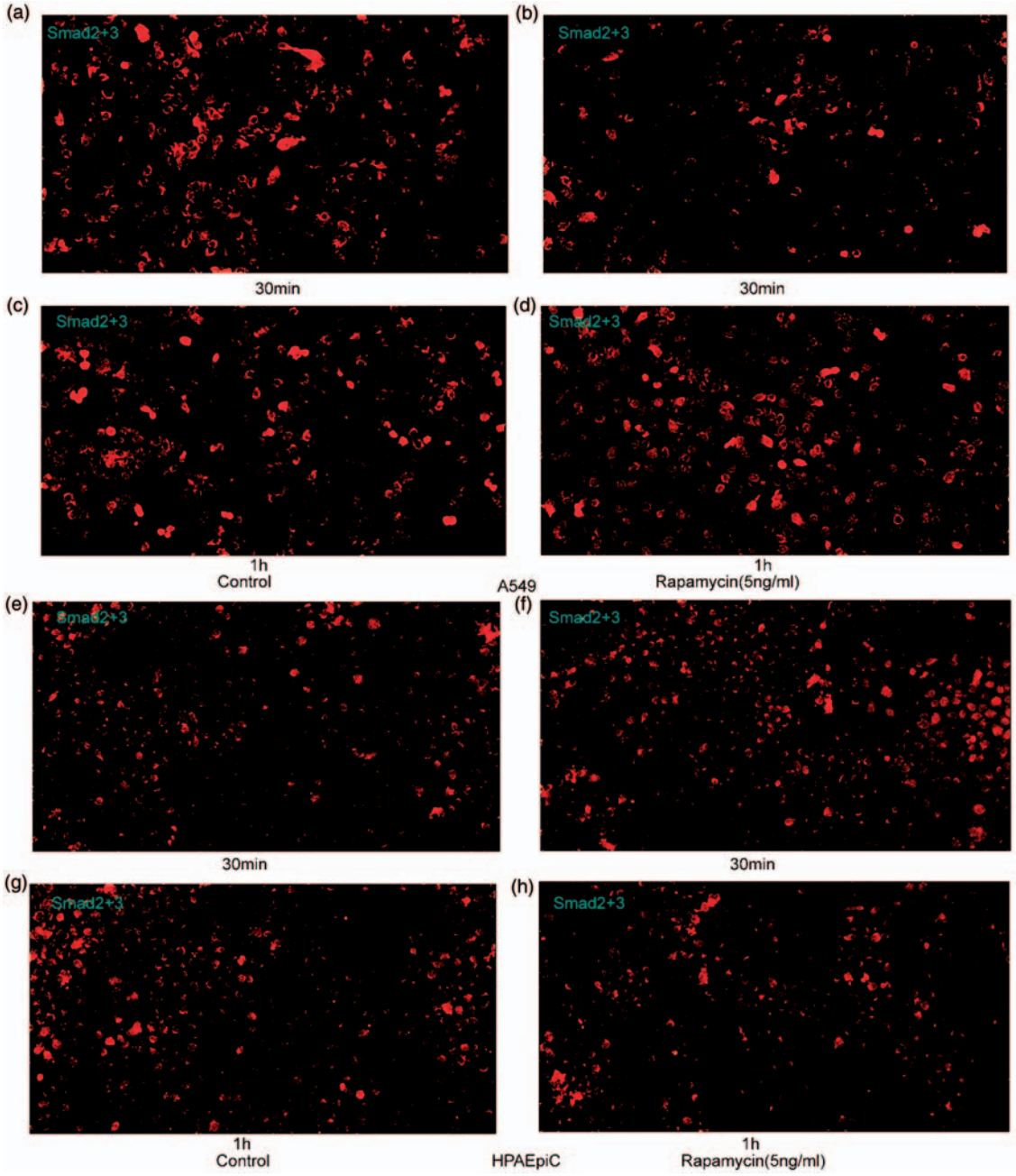

Figure 9 Immunofluorescent staining for Smad2/3. Quiescent A549 and HPAEpiC were first simulated with vehicle (control, a, c and e, g) or with rapamycin (5 ng/mL, b, d and f, h) for 30 min and 1 h. Then cells were fixed and stained with anti-Smad2/3 antibody. Texas Red-conjugated goat anti-rabbit secondary antibody was used for colour making. It showed that rapamycin failed to induce a significant nuclear entry of Smad2/3 in A549 and HPAEpiC. Magnification 100×

Studies also found that there is a negative feedback loop in the mTOR/S6K1/4EBP1 signal system.[13,21] Activation of mTOR leads to PI3K and mitogen-activated protein kinase (MAPK) inhibition via a negative feedback loop originating from S6K1. Inhibition of the mTOR activity with rapamycin results in a hyperactive RTK/PI3K pathway.[13,21] So we also explored whether PI3K-AKT was involved in rapamycin's action on CTGF expression. Firstly, whether PI3K-AKT pathway was activated after mTOR inhibition with rapamycin was studied. PI3 kinases are lipid kinases that can be divided into three classes according to their structure, regulation, and substrate specificity.[34,35] Among these enzymes, class 1A PI3 kinase (a heterodimer consisting of a p85 regulatory and a p110 catalytic subunit, p110α, p110β, or p110δ) has been identified as the most critical for cell growth and survival.[34] We found that rapamycin would promote PI3K (p110α) expression in A549 and HPAEpiC (Figure 10b, c and d), suggesting that PI3K may be activated by mTOR inhibition with rapamycin. After PI3K activation, Akt will be transferred to the plasma membrane from the cytosol and phosphorylated at Thr308 by phosphoinositide-dependent kinase to gain its active form.[36] In this study, we

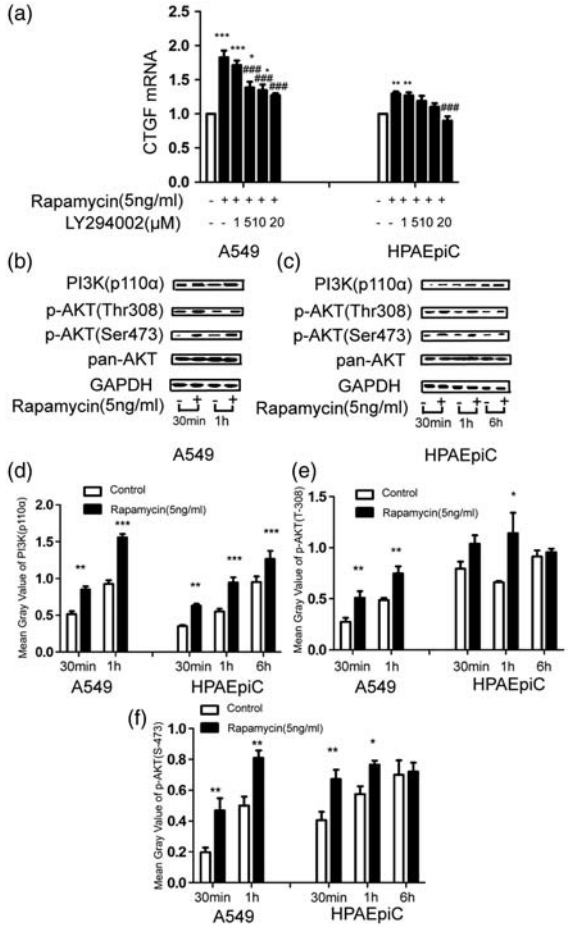

Figure 10 Rapamycin induces expressions of CTGF by an involvement of the PI3K-AKT. a: A549 and HPAEpiC were treated with either the vehicle or rapamycin (5 ng/mL) in the absence or presence of LY294002 (1–20 μmol/L) for 1 h. CTGF mRNA levels were determined by real time RT PCR. *, **, *** Significant difference, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared with vehicle group; ###Significant difference, $P < 0.001$, compared with rapamycin treated group. Data are expressed as mean ± SEM relative to vehicle-treated cells from three independent experiments. b and c: Representative example of immunoblotting analysis for PI3K (p110α), p-AKT (Thr308), and p-AKT (Ser 473). Quiescent A549 and HPAEpiC were stimulated with either vehicle (–) or with rapamycin (5 ng/mL) for 30 min, 1 h or 6 h before cells were harvested for immunoblotting analysis. GAPDH staining was used for loading control. d, e and f: Densitometry from Western blots normalized for GAPDH. *, **, *** Significant difference, $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively, compared with vehicle group. Data are expressed as mean ± SEM relative to vehicle-treated cells from three independent experiments

also found that rapamycin treatment significantly induced p-AKT (Thr308) expression in both A549 and HPAEpiC at 30 min and 1 h (Figure 10b, c and e). For full activation of Akt, phosphorylation of Ser473 in a C-terminal regulatory domain by mTOR complex 2 (mTORC2) is also required.[37] So we also examined whether rapamycin also modulated p-AKT (Ser473) expression. Not surprisingly, we found that after rapamycin treatment, p-AKT (Ser473) expression in A549 and HPAEpiC was also remarkably elevated at 30 min and 1 h. Taken together, the data showed that PI3K-AKT pathway in both normal and transformed lung epithelial cells may be activated after mTOR inhibition with rapamycin. Some other tumour studies were in line with us, which showed that rapamycin significantly upregulated AKT (Ser473) activity through PI3K activation.[38,39]

In order to further determine whether activated PI3K-AKT was involved in rapamycin's effect on CTGF expression, a specific PI3K inhibitor (LY294002) was used. It was found that LY294002 significantly attenuated rapamycin's promotion of CTGF mRNA expression in A549 and HPAEpiC (Figure 10a). This indicates that the PI3K pathway activated after mTOR inhibition and contributes to rapamycin's promotion of CTGF. In our parallel studies, it was also found that rapamycin upregulated CTGF in HLFs via the PI3K pathway (unpublished data). Collectively, we draw the conclusion that rapamycin monotherapy may activate PI3K-AKT signal pathway, probably due to inhibition of an mTOR-dependent retrograde signal.[13,21] Secondary activated PI3K-AKT pathway may be account for the profibrotic effect of rapamycin, such as promoting CTGF expression. This observation suggests that rapamycin blocks one output of PI3K-Akt signalling (mTORC1) at the expense of activating other outputs. So, we, together with other groups,[20,38,39] suggest that via direct inhibition of mTOR, rapamycin may secondarily activate other pathways. The pathway that is alternatively activated should be cell specific. Mono-treatment with rapamycin for lung fibrosis may not be a proper method because it has positive action on CTGF and other profibrotic genes.

IPF is often due to insufficient epithelial regeneration. Migration, proliferation, and differentiation of epithelial cells are required for normal wound healing. Promotion of AEC migration offers a potential target for therapies that attenuate fibrosis. In this study, it was demonstrated that rapamycin had no effect on the wound healing process and cell mobility as indicated by scratching and transwell assays (Figures 6 and 7). These results offer at least two possibilities. One is that mTOR signal pathway does not affect the motor ability of fibroblasts and epithelial cells. Another is that mTOR may affect cell mobility but the dose of rapamycin or detecting time covers the possible changes.

Our study has limitations. Although we used transformed and normal human lung epithelial cells to show rapamycin's positive effect on CTGF expression, nuanced modulatory roles of rapamycin on it should also be further studied. We should confirm that whether rapamycin directly upregulates CTGF expression in transcriptional or translational way or both by CTGF promoter activity and/or protein turnover assay. Furthermore, these *in vitro* studies should be further explored by using animal trials. In spite of these shortages, the data still have the power to show rapamycin may exert a profibrotic effect by accumulating CTGF mRNA steady-state level, protein synthesis, and secretion in different lung epithelial cell types by activating PI3K pathway. It was also shown that mTORC1 inhibition could activate PI3K or MAPK signal in cancer cells. This had important implications for cancer therapy because it provided a further explanation for the limited benefit observed in clinical trials utilizing rapamycin analogues as single agent.[21,40] Combined treatment with mTOR inhibitors and P3IK/MAPK inhibitors would show more antitumour activity in a wide range of human malignancies.[41] The dual feedback loop further unravels the complex pathways involved in the resistance to mTORC1-blocking

drugs of fibrotic models or clinical trials[42,43] and provides a rationale for using combinatorial therapy. In line with this idea, combined inhibition of PI3K and mTOR pathways may have additive anti-fibrotic effects.

Author contributions: XX designed and made the experiments, analysed the data, draft, and revised the manuscript. XW participated in the experiments and analysis of the data, revision of the manuscript. JG, FL, and TY participated in the experiments and analysis of the data. HD contributed to the conception and design of the study, the analysis and interpretation of the data, revision of the article and final approval of the version to be published. All authors read and approved the final manuscript.

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