Original Paper

TGFβ1-Smad Signaling Pathway Participates in Interleukin-33 Induced Epithelial-to-Mesenchymal Transition of A549 Cells

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Key Words

Epithelial-to-mesenchymal transition • Pulmonary fibrosis • Transforming growth factor β • TGFβ1-smad signaling pathway • Interleukin-33

Abstract

Backgrounds/Aims: Epithelial-to-mesenchymal transition (EMT) has been proven to be involved in development and progression of pulmonary fibrosis. This study aims to investigate the role of transforming growth factor β1 (TGFβ1)-smad signaling pathway in the interleukin-33 (IL-33) induced EMT. **Methods:** The human type II alveolar epithelial cell line, A549, and small airway epithelial cells (SAEC) were cultured and divided into 4 groups including Control, LY-2109761 (TGFβ receptor inhibitor), IL-33 and IL-33+LY-2109761 group. Expression of TGFβ1, E-cadherin (E-cad) and α-smooth muscle actin (α-SMA) were examined by using real-time PCR (RT-PCR) and western blot assay, respectively. The smad3 signaling pathway factors, including smad3 and phosphorylated smad3 (p-smad3), were also detected by using western blot assay. **Results:** IL-33 significantly activated T1/ST2 expression in A549 cells ($P<0.05$). TGFβ1 receptor inhibitor significantly suppressed the IL-33 caused down-expression of E-cad compared to IL-33 alone ($P<0.05$). IL-33 significantly increased the α-SMA levels compared to Control group ($P<0.05$) and TGFβ1 receptor inhibitor inhibited the other effects of IL-33. IL-33 significantly enhanced the levels of TGFβ1 compared to Control group ($P<0.05$). TGFβ1 receptor inhibitor suppressed the IL-33 induced up-expression of p-smad3. **Conclusion:** The TGFβ1-smad signaling pathway participates in the IL-33 induced epithelial-to-mesenchymal transition of A549 cells.

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Introduction

Interstitial lung disease (ILD) is caused by many factors, such as infection and exposure to tobacco smoke [1]. The ILD inflicts pulmonary interstitium, alveolar and/or bronchial [2]. Idiopathic pulmonary fibrosis (IPF) is a common form of ILD, with a prevalence of 50 patients in 100000 individuals [3, 4]. IPF is also correlated with the aging and is characterized by the histopathological pattern of usual interstitial pneumonia [5]. IPF is also characterized by extra-cellular matrix (ECM) accumulation in lung tissues, thereby replacing normal lung architecture [6, 7]. Although the mechanism of IPF is still elusive, several pathogenic investigations have been explored in its development and progression. The previous investigations mainly focus on the epithelial-to-mesenchymal transition (EMT) [8], fibroblast activation, collagen deposition [9], aberrant repair of injured epithelium [10] and the immune cell dysfunction [11].

EMT was firstly discovered by Greenberg and Hay in 1982 [12]. In EMT processes, following with the transition of cellular morphology, the epithelium biomarkers, including E-cadherin (E-cad), Keratin, Laminin-1 (LN-1), Claudin, are down-regulated. Meanwhile, the mesenchymal cell biomarkers, including α -smooth muscle actin (α -SMA), Vimentin, Laminin-5 (LN-5), fibronectin (FN) and N-cadherin (N-cad) are up-regulated [13-15]. EMT participates in the processes of fibrosis formation, development and wound-healing [14, 16-18].

Interleukin-33 (IL-33) is a cytokine of IL-1 family, and plays an important role in regulating inflammatory processes [19]. IL-33 is critical for the progression and development of the pulmonary fibrosis by promoting the differentiation of myofibroblasts and production of extra-cellular matrix proteins [20]. Luzina *et al.* [21] reported that the IL-33 mRNA expression is significantly enhanced in the IPF lung tissues. A previous study [22] also discovered that transforming growth factor β 1 (TGFβ1) is a critical factor in the pathogenesis for pulmonary fibrosis. TGFβ1 could significantly promote myofibroblast survival by triggering the canonical TGFβ1 signaling pathway (via phosphorylation of smad2/3) [23]. Willis *et al.* [24] found that TGFβ1 could cause alveolar epithelial cells to undergo EMT both *in vitro* and *in vivo* by activating smad2. Ming *et al.* [25] discovered that the serum TGFβ1 expression was positively correlated with IL-33 levels.

In this study, we speculated that IL-33 may trigger smad2/3 phosphorylation by up-regulating TGFβ1 expression, and thereby contribute to pulmonary fibrosis. Therefore, we employed the specific inhibitor of TGFβ receptor, LY2109761 [26], to inhibit the phosphorylation and activation of smad2/3, and block the smad2/3 signaling pathway. Meanwhile, we investigated the role of TGFβ1-smad2/3 signaling in the processes of EMT in alveolar epithelial cell line, A549 cell.

Materials and Methods

Cell culture

The human type II alveolar epithelial cell line (A549) and normal small airway epithelial cells (SAEC) were purchased from the Scientific Research Department of Wuhan University, Wuhan, China. A549 cells and SAEC cells were cultured in RPMI-1640 (Hyclone, Logan, UT, USA) medium supplementing with the 10% (w/v) fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 mg/l streptomycin in a humidified 5% CO₂ atmosphere at 37°C.

Cell treatment and trial grouping

The cells were adjusted to the density of 10⁷ cells/ml in medium. A549 cells or SAEC cells were seeded into 6-well plates (100 μ l per well). Then, the cells were divided into 4 groups, including Control group (adding RPMI-1640 medium), LY2109761 group (5 μ g/ml TGF- β 1 receptor inhibitor), IL-33 group (10 ng/ml IL-33) and LY2109761 + IL-33 group (5 μ g/ml LY210976 and 10 ng/ml IL-33). Recombinant human

IL-33 was purchased from Peprotech (Rocky Hill, NJ, USA) and LY2109761 was purchased from Selleckchem (Houston, TX, USA).

RNA isolation, cDNA synthesis and RT-PCR assay

The total RNA of A549 cells or SAEC cells was extracted by utilizing TRIzol solution (Invitrogen/Life Technologies, Carlsbad, CA, USA) according to the instruction of manufacturer. cDNAs were synthesized with a

first chain synthesis kit (Permentas, Waltham, MA, USA) according to the instruction of the manufacturer. E-cad, TGF-β1 and α-SMA mRNA expressions were examined by using the SYBR green/Flourescein qPCR Master (Permentas, Waltham, MA, USA). The PCR amplification conditions were listed as the followings: 95 °C for 10 min, 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The primer sequences utilized for the RT-PCR are listed in Table 1. Amplified DNAs were detected on 1.5% agarose gels, and the DNA images were digitally captured by using a CCD camera. A DL2000 DNA marker (Takara, Japan) was used to evaluate the length of PCR products of the target genes.

Western blot analysis

A549 cells or SAEC cells were harvested and lysed in the lysis buffer. The lysate of the A549 cells or SAEC cells were centrifuged at speed of 12,000 × g, for 5 min at 4°C. Protein concentration was examined by utilizing the BCA assay kit (Biyotime Biotech, Shanghai, China). The same amounts of proteins were loaded and separated by using the SDS-PAGE and standard protocols. Then, the proteins were transferred onto a PVDF membrane (Millipore, Bedford, MA, USA) for 2 h at 4°C. Membranes were blocked by using 5% non-fat milk in PBST and washed with PBST for 3 times (5 min per time). Then, the membranes were treated with antibodies at 4°C overnight. The following antibodies were used, including rabbit anti-human TGFβ1 polyclonal antibody (Catalogue No. ab9758; 1:2000; Abcam Biotech, Cambridge, Massachusetts, USA), mouse anti-α-SMA monoclonal antibody (Catalogue No. sc-71626; 1:4000; Santa Cruz, CA, USA), rabbit anti-human smad2/3 antibody (Catalogue No. ab202445; 1:2000; Abcam), mouse anti-human E-cad monoclonal antibody (Catalogue No. ab40772; 1:4000, Abcam), rabbit anti-human T1/ST2 polyclonal antibody (Catalogue No. ab25877, 1:2000, Abcam) and mouse anti-human β-actin monoclonal antibody (Catalogue No. ab8226; 1:4000; Abcam). Subsequently, the VEGF membranes were incubated with HRP-conjugated goat anti-rabbit (Catalogue No. BA1054; 1:2000, Boster, Inc., Wuhan, China) or goat anti-mouse (Catalogue No. BA1051; 1:2000, Boster, Inc., Wuhan, China) at room temperature for 2 h. Then, the membranes were detected by using an electrochemiluminescence (ECL) kit (Thermo Electron Corp, Waltham, MA, USA). The band intensities were quantified by utilizing a AlphaEaseFC image analyzer system (Alpha Innotech, Inc., CA, USA). The relative protein expression was normalized to that of β-actin (ratio of OD values of proteins and β-actin).

Statistical analysis

All of the data were analyzed by using SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA). The pair-wise comparisons between two groups were analyzed by using the Student's *t*-test. Multiple comparisons were analyzed by using ANOVA. All of the data are represented as the means ± standard deviation (SD). All of the experiments or tests were conducted repeated at least for six times. A *P* value < 0.05 is considered as the statistically significant.

Table 1. Primer sequences for the RT-PCR

Genes	Primer sequences	Length (bp)
ACTIN	Forward: CACCCAGCACAATGAAGATCAAGAT	317
	Reverse: CCAGTTTTAAATCCTGAGTCAAGC	
TGFβ1	Forward: CAGCAACAATTCCTGGCGATA	139
	Reverse: GCTAAGGCGAAAGCCCTCAAT	
E-cad	Forward: GAGAACGCATTGCCACATACAC	162
	Reverse: GAGCACCTTCCATGACAGACCC	
α-SMA	Forward: ATGCTCCAGGGCTGTTTTTC	112
	Reverse: CTTTTGCTCTGTGCTTCGTC	

Results

IL-33 activated T1/ST2 expression in A549 cells

In order to investigate the effects of IL-33 on EMT, the IL-33 specific receptor, T1/ST2, was examined in both A549 and SAEC cells. The results indicated that the endogenous T1/ST2 levels in A549 cells were higher significantly compared to SAEC cells (Fig. 1A, $P < 0.05$). In the SAEC cells, there were no significant differences for the T1/ST2 levels between IL-33 treated SAEC cells and untreated SAEC cells (Fig. 1B, $P > 0.05$). However, the T1/ST2 levels in IL-33 treated A549 cells were significantly higher compared to the untreated A549 cells (Fig. 1C, $P < 0.05$).

TGFβ1 receptor inhibitor suppressed

IL-33 caused down-expression of E-cad

IL-33 significantly decreased E-cad mRNA levels compared to Control group (Fig. 2A, $P < 0.05$). The TGFβ1 receptor inhibitor, LY-2109761, blocked this effect of IL-33 (Fig. 2A, $P < 0.05$). Western blots also indicated that the E-cad levels were significantly decreased with IL-33 exposure (Fig. 2B, $P < 0.05$). LY-2109761 significantly increased the E-cad expression in LY-2109761 + IL-33 group compared to the IL-33 group (Fig. 2B, $P < 0.05$).

However, these were not the effects of the IL-33 and LY-2109761 on the E-cad expression in SAEC cells according to the PCR and western blot analysis (Fig. 3A, B).

IL-33 increased α-SMA levels and TGFβ1 receptor inhibitor inhibited effects of IL-33

There were no significant differences for the α-SMA mRNA expression between LY-2109761 and Control groups (Fig. 4A, $P > 0.05$). The α-SMA mRNA expression in IL-33 group was significantly increased compared to the Control group (Fig. 4B, $P < 0.05$). When treating cells with LY-2109761 and IL-33, the α-SMA mRNA expression was significantly decreased compared to IL-33 group (Fig. 4A, $P < 0.05$).

Moreover, western blots were also used to detect α-SMA protein expression. The results also showed that the α-SMA levels were significantly increased in IL-33 group compared to the Control group (Fig. 4B, $P < 0.05$). LY-2109761 significantly blunted the effects of IL-33 on α-SMA expression (Fig. 3B, $P < 0.05$).

Fig. 1. T1/ST2 expression in IL-33 treated or untreated SAEC and A549 cells. A. T1/ST2 expression in SAEC and A549 cells was examined by using western blot assay. B. T1/ST2 expression in IL-33 treated or untreated SAEC cells. C. T1/ST2 expression in IL-33 treated or untreated A549 cells. * $P < 0.05$ and * $P > 0.05$ represent the differences of between the illustrated groups.

Fig. 2. Examination for the E-cad expression in A549 cells in every group. A. E-cad mRNA expression examined by using RT-PCR and statistical analysis. B. E-cad protein expression examined by using western blot assay and statistical analysis. * $P < 0.05$ represents the differences of E-cad expression between the illustrated groups. E-cad: E-cadherin, RT-PCR: real-time PCR.

Fig. 3. Examination for the E-cad and α-SMA expression in SAEC cells in every group. A. Statistical analysis for E-cad mRNA expression due to RT-PCR assay. B. Statistical analysis for E-cad protein expression due to western blot assay. C. Statistical analysis for α-SMA mRNA expression due to RT-PCR assay. D. Statistical analysis for α-SMA protein expression due to western blot assay. * $P > 0.05$ represents the differences of E-cad expression between the illustrated groups. E-cad: E-cadherin, α-SMA: α-smooth muscle actin, RT-PCR: real-time PCR.

Fig. 4. Observation for α -SMA expression in A549 cells in every group. A. α -SMA mRNA expression examined by using RT-PCR and statistical analysis. B. α -SMA protein expression examined by using western blot assay and statistical analysis. * $P < 0.05$ represents the differences of α -SMA expression between the illustrated groups. α -SMA: α -smooth muscle actin, RT-PCR: real-time PCR.

However, these were not the effects of the IL-33 and LY-2109761 on the α -SMA expression in SAEC cells according to the PCR and western blot analysis (Fig. 3C, D).

IL-33 enhanced levels of TGF β 1

In order to investigate the effects of IL-33 on the TGF β 1 expression, the TGF β 1 levels were examined by using RT-PCR and western blot assays, respectively. The results indicated that the TGF β 1 receptor inhibitor, LY-2109761, did not significantly affect TGF β 1 mRNA (Fig. 5A) and protein (Fig. 5B) levels compared to the Control group (both $P > 0.05$). Both TGF β 1 mRNA and protein levels were significantly increased in IL-33 group compared to the Control group (Fig. 5, both $P < 0.05$). However, LY-2109761 also presented this effect of IL-33 on TGF β 1 compared to IL-33 group (Fig. 5, $P > 0.05$).

However, these were not the effects of the IL-33 on the levels of the TGF β 1 in SAEC cells according to the PCR and western blot analysis (Fig. 6A, B).

TGF β 1 receptor inhibitor suppressed the IL-33 induced up-expression of p-smad3

The smad2/3 signaling pathway was also examined by using western blot assays. The results showed that there were no significant differences for smad3 or p-smad3 between LY-2109761 and Control groups (Fig. 7, $P > 0.05$). Also, there was no significant difference for smad3 levels in the IL-33 group compared to the Control group (Fig. 7, $P > 0.05$). However, the p-smad3 levels were significantly enhanced by IL-33 (Fig. 7, $P < 0.05$). Moreover, the TGF β 1 receptor inhibitor, LY-2109761, significantly inhibited the IL-33 induced up-expression of p-smad3 compared to IL-33 group (Fig. 7, $P < 0.05$).

However, the TGF β 1 receptor inhibitor, LY-2109761, did not have any effects on the expression of smad3 and the p-smad3 in SAEC cells (Fig. 8).

Fig. 5. Detection for TGF β 1 expression in A549 cells in every group. A. TGF β 1 mRNA expression examined by using RT-PCR and statistical analysis. B. TGF β 1 protein expression examined by using western blot assay and statistical analysis. * $P < 0.05$ represents the differences of TGF β 1 expression between the illustrated groups. TGF β 1: transforming growth factor β 1, RT-PCR: real-time PCR.

Fig. 6. Examination of the TGF β 1 expression in SAEC cells in every group. A. Statistical analysis for TGF β 1 mRNA expression due to RT-PCR assay. B. Statistical analysis for TGF β 1 protein expression due to western blot assay. $P > 0.05$ represents the differences of TGF β 1 expression between the illustrated groups. TGF β 1: transforming growth factor β 1, RT-PCR: real-time PCR.

Fig. 7. Observed Smad3 and the p-Smad3 protein expression in A549 cells. A. Western blot assay for Smad3 and p-Smad3 expression. B. Statistical analysis for Smad3 and p-Smad3 expression. *P < 0.05 represents the differences of Smad3 or p-Smad3 expression between the illustrated groups. p-Smad3: phosphorylated Smad3.

Fig. 8. The statistical analysis for Smad3 and p-Smad3 expression in SAEC cells due to western blot assay. *P > 0.05 represents the differences of Smad3 or p-Smad3 expression between the illustrated groups. p-Smad3: phosphorylated Smad3.

Discussion

The alveolar epithelial cells (AECs) include type I alveolar epithelial cell (AEC I) and type II alveolar epithelial cell (AEC II) [27]. The AEC I mainly plays the biological roles of gas exchange, and AEC II mainly plays the biological role of secreting cytokines [including TGFβ1, tumor necrosis factor alpha (TNFα)] and the alveolar surfactants [28]. However, when the normal biological function of AEC I is damaged, pulmonary fibrosis progression may be initiated. Epithelial-mesenchymal transition is considered as an important mechanism in pulmonary fibrosis pathogenesis [29].

The IL-33 pathway is also involved in the Th2 type immune response, which is involved in other fibrotic diseases [30]. One previous study [31] also reported that the TGFβ1-smad signaling pathway is an important up-stream mechanism of EMT. Meanwhile, the TGFβ1-smad is positively correlated with the IL-33 expression. Therefore, we investigated the effects of IL-33 on the pulmonary fibrosis formation.

In this study, we firstly examined the expression of IL-33 specific receptor, T1/ST2, in both of the A549 cells and SAEC cells. The results showed that the endogenous T1/ST2 levels in A549 cells were higher significantly compared to SAEC cells. Also, the IL-33 treatment significantly triggered the T1/ST2 expression in A549 cells, but not in SAEC cells. These results suggest that the A549 cells are better cell line to investigate the effects of IL-33 due to the higher levels of IL-33 receptor, T1/ST2. Therefore, in the following experiments, we would mainly study the effects of IL-33 on A549 cells.

Our results indicated that the IL-33 treatment significantly decreased E-cad expression and significantly increased α-SMA expression compared to the Control group, which suggest that IL-33 could induce the occurrence of the EMT. Meanwhile, the levels of TGFβ1 were also increased in IL-33 treatment cells, which suggests that TGFβ1 may participate in the IL-33 induced EMT of A549 cells. TGFβ1 via TGFβ receptors activates the phosphorylation of smad2/sm3, and triggers the biological effects of TGFβ1 [32, 33]. Therefore, this study applied the TGFβ1/Smad signaling pathway inhibitor, LY-2109761, to inhibit the phosphorylation and activity of the smad2/sm3. The previous studies [34, 35] illustrated that 5 μl LY-2109761 treatment could completely inhibit the TGFβ1 induced phosphorylation of smad2/3. The LY-2109761 could further inhibit the TGFβ1 induced cell proliferation, migration and differentiation. Our results show that LY-2109761 by itself does not change the expression of smad3 and p-smad3. However, LY-2109761 significantly blunted IL-33 induced p-smad3 expression. E-cad levels were significantly decreased and α-SMA levels were significantly increased by IL-33, which suggests that the IL-33 promotes the processes of EMT. The biological activity of TGFβ1 *in vivo* mainly conducts through phosphorylated-smad3, but not smad3 [36]. The present study also discovered that when p-smad3 expression was decreased, the IL-33 induced EMT process was obviously weakened. This result suggests that the TGFβ1-smad signaling pathway participates in the IL-33 induced changes. Therefore, we speculated that IL-33 secretion is significantly enhanced when the AECs undergoing various stimuli. Therefore, IL-33 induces the TGFβ1 up-regulation and smad2/3 phosphorylation, triggering EMT and contributing to pulmonary fibrosis. However, there were no effects of the IL-33 on the SAEC cells, and IL-33 could not induce the EMT in the SAEC cells.

Although this study received some interesting results, there were also a few limitations. Firstly, the present study only employed two cell lines, A549 cells and SAEC cells, to investigate the effects of IL-33. Other cell lines important in fibrosis, especially primary fibroblasts, will need to be studied to further understand IL-33 effects relative to fibrosis. Moreover, this study only investigated the effects of *in vitro* IL-33 levels, but not *in vivo* levels. In future studies we will use other cell lines and animal models to investigate the effects of IL-33. That phospho-smad bands became more dense while unphosphorylated smad bands were unchanged seems unusual; this will also require further study.

Conclusion

IL-33 triggers the down-expression of E-cad and up-expression of α-SMA, TGFβ1 and p-smad3. The TGFβ1-smad signaling pathway participates in the IL-33 induced epithelial-to-mesenchymal transition of A549 cells.

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Disclosure Statement

The authors declare no conflict of interest.

References

- 1 Yin X, Liang Z, Yun Y, Pei L: Intravenous transplantation of BMP2-transduced endothelial progenitor cells attenuates lipopolysaccharide-induced lung injury in rats. *Cell Physiol Biochem* 2015;35:2149-2158.
- 2 Maitra M, Wang Y, Gerard RD, Mendelson CR: Surfactant protein A2 mutations associated with pulmonary fibrosis lead to protein instability and endoplasmic reticulum stress. *J Biol Chem* 2010;285:22103-22113.
- 3 Raghu G, Weycker D, Edelsberg J, Bradford WZ, Oster G: Incidence and prevalence of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2006;174:810-816.
- 4 Wang L, Wang Y, Yang T, Guo Y, Sun T: Angiotensin-converting enzyme 2 attenuates bleomycin-induced lung fibrosis in mice. *Cell Physiol Biochem* 2015;36:679-711.
- 5 Craig VJ, Zhang L, Hagood JS, Owen CA: Matrix metalloproteinases as therapeutic targets for idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2015;53:585-600.
- 6 Yan Z, Kui Z, Ping Z: Reviews and perspectives of signaling pathway analysis in idiopathic pulmonary fibrosis. *Autoimmun Rev* 2014;13:1020-1025.
- 7 Kim HJ, Perlman D, Tomic R: Natural history of idiopathic pulmonary fibrosis. *Respir Med* 2015;109:661-670.
- 8 Liang H, Gu Y, Li T, Zhang Y, Huangfu L, Hu M, Zhao D, Chen Y, Liu S, Dong Y, Li X, Lu Y, Yang B, Shan H: Integrated analyses identify the involvement of microRNA-26a in epithelial-mesenchymal transition during idiopathic pulmonary fibrosis. *Cell Death Dis* 2014;5:e1238.
- 9 King TE, Pardo A, Selman M: Idiopathic pulmonary fibrosis. *Lancet* 2011;378:1949-1961.
- 10 Camelo A, Dunmore R, Sleeman MA, Clarke DL: The epithelium in idiopathic pulmonary fibrosis: breaking the barrier. *Front Pharmacol* 2014;4:173.
- 11 Glasgow CG, El-Chemaly S, Moss J: Lymphatics in lymphangioleiomyomatosis and idiopathic pulmonary fibrosis. *Eur Respir Rev* 2012;21:196-206.
- 12 Greenburg G, Hay ED: Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. *J Cell Biol* 1982;95:333-339.
- 13 Lamouille S, Xu J, Derynck R: Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* 2014;15:178-196.
- 14 Guarino M, Tosoni A, Nebuloni M: Direct contribution of epithelium to organ fibrosis: epithelial-mesenchymal transition. *Hum Pathol* 2009;40:1365-1776.
- 15 Zeisberg M, Neilson EG: Biomarkers for epithelial-mesenchymal transitions. *J Clin Invest* 2009;119:1429-1437.
- 16 Carew RM, Wang B, Kantharidis P: The role of EMT in renal fibrosis. *Cell Tissue Res* 2012;347:103-116.
- 17 Xi Y, Tan K, Brumwell AN: Inhibition of epithelial-to-mesenchymal transition and pulmonary fibrosis by methacycline. *Am J Respir Cell Mol Biol* 2014;50:51-60.

- 18 Ohbayashi M, Kubota S, Kawase A, Kohyama N, Kobayashi Y, Yamamoto T: Involvement of epithelial-mesenchymal transition in methotrexate-induced pulmonary fibrosis. *J Toxicol Sci* 2014;39:319-330.
- 19 Mirchandani AS, Salmond RJ, Liew FY: Interleukin-33 and the function of innate lymphoid cells. *Trends Immunol* 2012;33:389-396.
- 20 Wynn TA, Ramalingam TR: Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med* 2012;18:1028-1040.
- 21 Luzina IG, Kopach P, Lockatell Y, Kang PH, Nagarsekar A, Burke AP: Interleukin-33 protentiates bleomycin-induced lung injury. *Am J Respir Cell Mol Biol* 2013;49:999-1008.
- 22 Zhao J, Shi W, Wang YL, Chen H, Bringas P, Datto MB, Frederick JP, Wang XF, Warburton D: Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L585-L593.
- 23 Biernacka A, Dobaczewski M, Frangogiannis NG: TGFβ signaling in fibrosis. *Growth Factors* 2011;29:196-202.
- 24 Willis BC, Borok Z: TGF-beta-induced EMT: mechanism2 and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L525-L534.
- 25 Ming DI, Yu X, Guo R, Deng Y, Li J, Lin C, Su M, Lin Z, Su Z: Elevated TGFβ1/IL-33 pathway is associated with the disease severity of Hepatitis B virus-related liver cirrhosis. *Viral Immunol* 2015;28:209-216.
- 26 Melisi D, Ishiyama S, Sclabas GM, Fleming JB, Xia Q, Tortora G, Abbruzzese JL, Chiao PJ: LY2109761, a novel transforming growth factor beta receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis. *Mol Cancer* 2008;7:829-840.
- 27 Tan WJ, Tan QY, Wang T, Lian M, Zhang L, Cheng ZS: Calpain 1 regulates TGF-beta induced epithelial-mesenchymal transition in human lung epithelial cells via PI3K/Akt signaling pathway. *Am J Transl Res* 2017;9:1402-1409.
- 28 Maloney JP, Gao L: Proinflammatory cytokines increase vascular endothelial growth factor expression in alveolar epithelial cells. *Mediators Inflamm* 2015;2015:387842.
- 29 Ward C, Forrest IA, Murphy DM, Johnson GE, Robertson H, Cawston TE, Fisher AJ, Dark JH, Lordan JL, Kirby JA, Corris PA: Phenotype of airway epithelial cells suggests epithelial to mesenchymal cell transition in clinically stable lung transplant recipients. *Thorax* 2005;60:865-871.
- 30 Marvie P, Lisbonne M, L'helgoualc'h A, Rauch M, Turlin B, Preisser L, Bourd-Boittin K, Theret N, Gascan H, Piguet-Pellorce C, Samson M: Interleukin-33 over-expression is associated with liver fibrosis in mice and humans. *J Cell Mol Med* 2010;14:1726-1739.
- 31 Xiang Y, Zhang Y, Tang Y, Li Q: MALAT1 modulates TGF-β1-induced endothelial-to-mesenchymal transition through downregulation of miR-145. *Cell Physiol Biochem* 2017;42:1309-1310.
- 32 Shi Y, Massague J: Mechanisms of TGFβ1 signaling from cell membrane to the nucleus. *Cell* 2013;113:685-700.
- 33 Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K: Two major Smad pathways in TGFβ1 superfamily signaling. *Genes Cells* 2002;7:1191-1204.
- 34 Melisi D, Ishiyama S, Sclabas GM, Fleming JB, Xia Q, Tortora G, Abbruzzese JL, Chiao PJ: LY2019761, a novel transforming growth factor B receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis. *Mol Cancer Ther* 2008;7:829-840.
- 35 Zhang B, Halder SK, Zhang S, Datta PK: Targeting transforming growth factor beta signaling in liver metastasis of colon cancer. *Cancer Lett* 2009;277:114-120.
- 36 Warburton D, Shi W, Xu B: TGFβ-Smad2 signaling in emphysema and pulmonary fibrosis: an epigenetic aberration of normal development? *Am J Physiol Lung Cell Mol Physiol* 2013;304:L83-85.