p38 MAPK is Crucial for Wnt1- and LiCl-Induced Epithelial Mesenchymal Transition

Chun-xiao FANG[1†], Chun-mei MA[2†], Ling JIANG[3], Xi-ming WANG[1], Na ZHANG[1], Ji-na MA[1], Tai-hua WU[1], Zhong-he ZHANG[1], Guang-dong ZHAO[1], Ya-dong ZHAO[1#]
[1]Department of Pulmonary Medicine, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, China
[2]Department of Radiology, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, China
[3]Department of Pulmonary Medicine, Dalian Central Hospital Affiliated to Dalian Medical University, Dalian 116033, China

Summary: Idiopathic pulmonary fibrosis (IPF) is characterized by myofibroblast foci in lung parenchyma. Myofibroblasts are thought to originate from epithelial-to-mesenchymal transition (EMT). Wnt1 and lithium chloride (LiCl) induce EMT in alveolar epithelial cells (AECs), but the mechanisms are unclear. AECs were treated with Wnt1 and LiCl, respectively; morphological change and molecular changes of EMT, including E-cadherin, fibronectin, and vimentin, were observed. SB203580 was administrated to test the role of p38 MAPK signaling in EMT. Then AECs were treated with siRNAs targeting p38 MAPK to further test the effects of p38 MAPK, and the role was further confirmed by re-expression of p38 MAPK. At last β-catenin siRNA was used to test the role of β-catenin in the EMT process and relationship of β-catenin and p38 MAPK was concluded. Exposure of AECs to Wnt1 and LiCl resulted in upregulation of vimentin and fibronectin with subsequent downregulation of E-cadherin. Wnt1 and LiCl stimulated the p38 MAPK signaling pathways. Perturbing the p38 MAPK pathway either by SB203580 or through p38 MAPK siRNA blocked EMT and inhibited fibronetin synthesis, which were reversed by transfection of p38 MAPK expression plasmid. β-catenin siRNA attenuated the EMT process and decreased p38 MAPK phosphorylation, indicating that β-catenin is involved in the EMT-related changes through regulation of p38 MAPK phosphorylation. These findings suggest that p38 MAPK participates in the pathogenesis of EMT through Wnt pathway and that p38 MAPK may be a novel target for IPF therapy.
Key words: Wnt; p38 MAPK; epithelial-to-mesenchymal transition

Pulmonary fibrosis may be caused by lung injury, chemotherapy, systemic autoimmune diseases, and environmental particles and toxin inhalation; it can also be considered as an idiopathic disease, termed as idiopathic interstitial pneumonia (IIP)[1]. Idiopathic pulmonary fibrosis (IPF) represents a fatal and highly progressive lung disease that destroys the lung architecture. IPF is an idiopathic disease that is poorly responsive to any of the currently available treatments[2]. The lung architecture in IPF is distorted showing temporal and spatial histological heterogeneity in areas with relatively normal parenchyma, interstitial inflammation, septal fibrosis with fibroblast foci, and end stage honeycombing. It is well established that fibroblast foci formation is the hallmark of IPF. The foci usually consist of aggregates of fibroblasts that deposit extracellular matrix (ECM) within the alveolar space[3]. The lethal fibrotic reaction in IPF is associated with an epithelial-dependent fibroblast-activated process, namely epithelial-mesenchymal transition (EMT), which is an origin of functional myofibroblast/fibroblast[4, 5]. Several studies have demonstrated that EMT and fibroblast activation through functional pathways are driven by the dysfunctional bronchiolar and alveolar epithelial cells (AECs)[6–8]. The EMT of pulmonary epithelial cells is proposed as an orchestrated and highly regulated process. Among these signaling pathways, the Wnt pathway and p38 MAPK have been

Chun-xiao FANG, E-mail: Candy-fun@163.com
Chun-mei MA, E-mail: Machunmei1979@163.com
†These authors contributed equally to this study.
#Corresponding author, E-mail: zhaoyadongdmu@163.com

suggested to play an important role in this process[9].

The Wnt family regulates various cellular processes such as proliferation, differentiation, and apoptosis[10]. The canonical Wnt pathway is β-catenin-dependent and is known to induce mesenchymal transdifferentiation in epithelial cells[10]. In fact, Wnt ligands are known to regulate β-catenin stability and degradation through activation of upstream mediators of β-catenin dephosphorylation, and subsequently reduce β-catenin degradation[11]. The accumulated β-catenin typically localizes within the nucleus where it interacts with downstream transcription factors such as the T-cell-specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF), regulating gene transcription[10]. Interestingly, a recent report showed increased nuclear β-catenin localization in IPF tissue sections, indicating increased Wnt signaling activity[12, 13]. The Wnt pathway is involved in EMT observed in various developmental and disease settings[10–12, 14]. Recently, several *in vivo* and *in vitro* studies have shown that pulmonary epithelial cells undergo EMT[15, 16]. Overexpression of transforming growth factor β (TGF-β) in transgenic mice model, in which β-galactosidase was specifically expressed in alveolar cells, caused pulmonary fibrosis. The research found an accumulation of mesenchymal markers in β-galactosidase positive cells, which was evidence of pulmonary epithelial cells undergoing EMT. In lung tissues from patients and animals with pulmonary fibrosis, myofibroblast and epithelial cell markers are co-localized with alveolar epithelial cells overlying fibroblastic foci, a key feature of the disease, further supporting an active EMT process in pulmonary fibrosis[6–8].

Many researchers have demonstrated the involvement of several signaling pathways, such as TGF-β[17, 18], leptin[19], IL-8, and VEGF[20] during EMT using the alveolar epithelial A549 cell line. The Wnt pathway-induced EMT process is thought to act through the canonical Wnt/βcatenin pathway[10]. The Wnt pathway can activate p38 MAPK as a reinforcement of Wnt pathway itself[21]. The Wnt pathway and p38 MAPK signaling pathways are involved in the EMT process separately, but the underlying mechanisms and the relationship between Wnt pathway and p38 MAPK have not been fully elucidated. In the present study, we attempted to demonstrate the role of p38 MAPK signaling in Wnt pathway-induced EMT in alveolar epithelial cells (A549 cell line). Our results may help validate p38 MAPK as a novel target for therapeutic intervention of lung fibrosis.

1 MATERIALS AND METHODS

1.1 Cells, siRNAs, and Reagents

A549 cell line was obtained from Center of Cell Resources of Shanghai Institutes for Life Sciences, China. p38 MAPK siRNA and non-specific siRNA controls were from Shanghai R&S Biotechnology (China). Lipofectamine 2000 was purchased from Invitrogen (USA). All antibodies were obtained from Sigma (USA). The p38 MAPK inhibitor SB203580 was obtained from Sigma. The ELISA assay kit for fibronectin was from Amersham (China) and the assay for dispositive fibronectin in a 96-well plate was performed according to the manufacture's guide. All PCR primers were synthesized by Takara (China).

1.2 Cell Culture and Treatment with Wnt and LiCl

The cell culture method was similar to that of Zheng's with slight modifications[22]. Briefly, A549 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 1% of Pen/Strep, and 10 mmol/L of HEPES, at 37°C with 5% CO_2. Wnt1 protein and LiCl were used to activate the Wnt pathway. LiCl (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) and then diluted with the phosphate buffered saline (PBS) to make a final concentrations of 20 mmol/L[23]. Wnt1 protein (Abcam, USA) was kept at –20°C and then diluted with PBS. According to a previously published method, Wnt1 protein was added to culture medium at 100 ng/mL final concentration[24].

The cells were plated at 3×10^3 cells per well in a 96-well plate with 200 μL of culture medium. LiCl and Wnt1 solutions were added to the wells with desired final concentrations. After 2 h, medium was replaced with drug-free medium and then further incubated for 48 h. For controls, culture medium containing the same concentration of DMO was used. The cell morphology was monitored under an inverted microscope. The fibronectin level was analyzed by the aforementioned ELISA kit, following the manufacturer's instructions.

1.3 Gene Constructs and Transfection

The experimental method was similar to that of Wei with slight modifications[25]. Total RNA was isolated from A549 cells by Trizol reagent and then cDNA was synthesized from the isolated RNA by reverse transcription, and the full-length cDNA was amplified with the Takara RNA PCR Kit. Recombinant pEGFP-N1-p38 MAPK plasmid was constructed as previously reported[26]. The coding region of p38 MAPK was subcloned into the pcDNA3.1 vector after removing the pEGFP-N1 vector. pcDNA3.1-p38 MAPK was trasnsfected into cells using Lipofectamine 2000 (Invitrogen, USA), following the manufacturer's instructions.

1.4 Transfection of siRNA

The cell transfection was conducted following the instruction of the manufacturer. Briefly, p38 MAPK siRNA and non-specific siRNA control, in RNase-free water, was prepared at stock concentration of 20 μmol/L. Cells were transfected with 40 nmol/L of siRNA using lipofectamine 2000.

1.5 Western Blot Analysis

For preparing the whole-cell extracts, cells (90%

confluence) were washed with PBS and incubated on ice for 10 min with lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, pH 8.0, 0.2 mmol/L Na_3VO_4, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). The cell lysates were centrifuged at 9000 g for 10 min, and the supernatants were transferred to new tubes. The Coomassie Protein Assay Reagent (Sigma, USA) was used to measure total protein concentrations. The samples (50 μg protein) were run on 10% SDS-polyacrylamide gel electrophoresis and then transferred into nitrocellulose membranes. The membranes were blocked for 2 h with 5% fat-free dry milk in TBST (Tris-Buffered saline, 0.1% Tween 20) and then incubated with primary antibodies against GAPDH, p38 MAPK, phospho-p38 MAPK, fibronectin for 3 h at room temperature. After incubation with the primary antibody, membranes were washed with TBST and then incubated with HRP-conjugated anti-rabbit secondary antibody for 1 h. Proteins were visualized using a chemiluminescent ECL detection system. The density of protein bands was quantified by densitometry analysis using Labworks 4.6 (UVP Products, USA). The protein band intensity was normalized to that of the corresponding β-actin in each sample and the average protein levels were determined from three separate experiments. Data below Western blots represent relative expression levels of proteins.

1.6 Statistical Analysis

The data derived from experiments were shown as $\bar{x}\pm s$. Difference in mean values between groups were analyzed using student's *t*-test, and a *P*-value of <0.05 was considered statistically significant. The statistical analyses were performed with the SPSS 13.0 statistical package (USA).

2 RESULTS

2.1 Wnt Pathway Induces EMT in A549 Cells

The morphological changes of LiCl (20 mmol/L)- and Wnt1 (100 nmoL/L)-treated cells and controls were observed by an inverted microscope. The phase-contrast imaging of A549 cells showed a transition from the typical epithelial appearance to elongated spindle shapes. Under the culture conditions, confluent A549 cells appeared as monolayer adherent squamous-like cells (fig. 1A). After 48-h exposure to LiCl (fig. 1B) or Wnt1 (fig. 1C) conditioned medium, the cells showed a spindle-shaped, disseminated, and pseudopodia stretched morphology. These morphological changes suggested that both LiCl and Wnt1 could transform A549 cells into mesenchymal-like cells. To further elucidate the EMT in A549 cells, Western blotting for E-cadherin, fibronectin, and vimentin was performed. There was a marked increase in vimentin and fibronectin and a decrease in E-cadherin expression after Wnt1 or LiCl treatment (fig. 2). In the subsequent experiments, we used fibronectin as a marker for LiCl- and Wnt1-induced EMT.

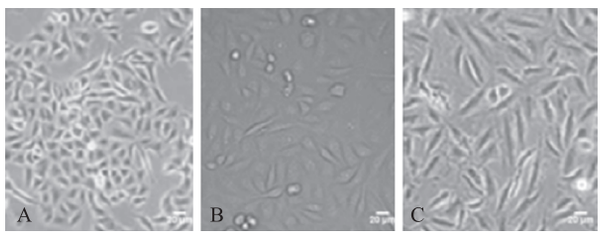

Fig. 1 Morphological changes induced by LiCl or Wnt1
A: control; A549 cells appeared as monolayer adherent squamous-like cells. B: A549 cells cultured with LiCl; C: A549 cells in Wnt1 conditioned medium. Morphological changes of cells treated with LiCl (20 mmol/L) or Wnt1 (100 nmol/L) were observed by inverted microscopy. After 48 h of culture with LiCl (B) or Wnt1 (C) conditioned medium, both cell types became spindle-shaped, disseminated, and pseudopodia stretched.

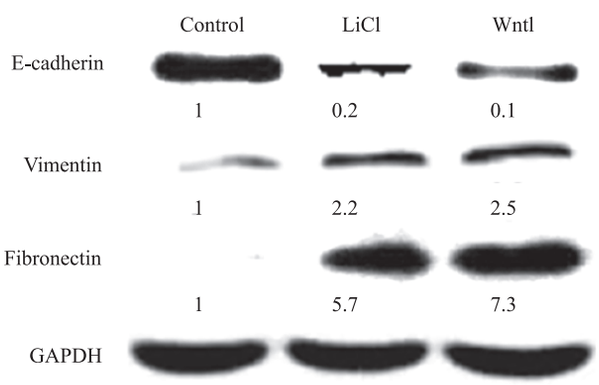

Fig. 2 A549 cells cultured in LiCl- and Wnt1-conditioned medium showed a significant increase in vimentin and fibronectin, and decrease in E-cadherin expression ($P<0.05$).

2.2 Elevated p38 MAPK in EMT Cells

As shown in fig. 3, phospho-p38 MAPK was significantly elevated after treatment with LiCl and Wnt1 for 30 min, suggesting that p38 MAPK was involved in LiCl- and Wnt1-induced EMT in A549 cells.

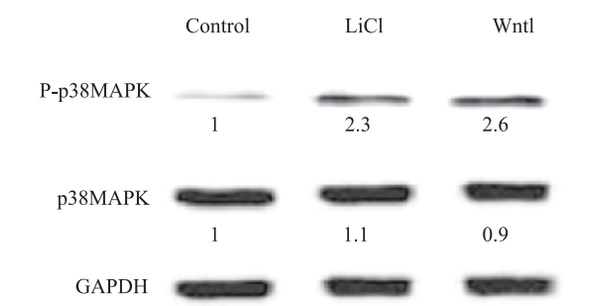

Fig. 3 Phospho-p38 MAPK expression after treatment with LiCl and Wnt1 for 30 min
Western blotting analysis shows that phospho-p38 MAPK was significantly elevated in LiCl- and Wnt1-treated A549 cells ($P<0.05$).

2.3 Crucial Role of p38 MAPK Signaling in LiCl- and Wnt1-induced EMT

We next determined the role of p38 MAPK in EMT. First, the cells were treated with SB203580, a specific inhibitor of the p38 MAPK pathway, for 30 min before LiCl or Wnt1 was added. As shown in table 1, fig. 4A and fig. 4B, SB203580 treatment resulted in a decrease in the phospho-p38 MAPK level and in expression of fibronectin, indicating that the EMT process was blocked. Cells treated with siRNA targeting p38 MAPK showed decreased levels of fibronectin, compared to those transduced with scrambled siRNA controls (table 2, fig. 5A and 5B), suggesting that p38 MAPK is important for LiCl- and Wnt1-induced EMT. Furthermore, re-transfection of p38 MAPK restored the LiCl- and Wnt1-induced EMT (table 3, fig. 6A and 6B), further confirming that p38 MAPK plays a critical role in LiCl- and Wnt1-induced EMT.

Table 1 Effects of SB203580 on LiCl- and Wnt1-induced fibronectin expression

Groups	Fibronectin (ng/mL)
Control	7.4±0.4
LiCl	20.7±2.1
Wnt1	22.9±3.6
LiCl+SB203580	6.7±0.3
Wnt1+SB203580	6.9±0.3

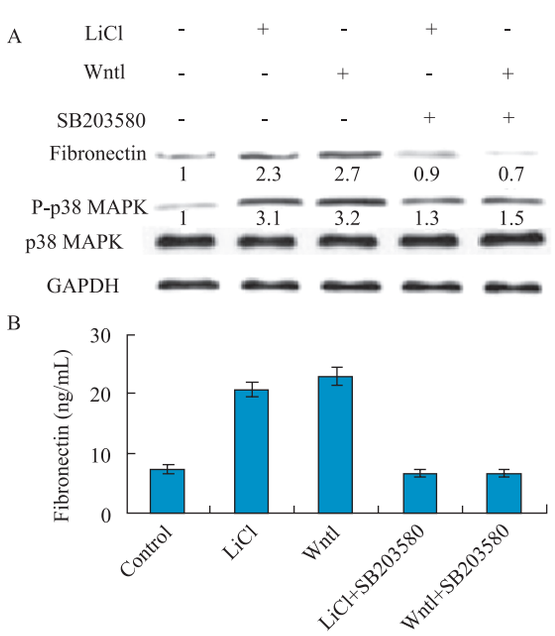

Fig. 4 Fibronectin expression in A549 cells treated with p38 MAKP inhibitor SB203580
A: Reduced expression of fibronectin (P<0.05) and p38 MAPK in the presence of SB203580 shown by Western blot analysis; B: Decreased expression of fibronectin in A549 cells after treatment of SB203580 assayed using ELISA (P<0.05).

Table 2 Effect of RNAi targeting p38 MAPK on LiCl- and Wnt1-induced fibronectin expression

Groups		Fibronectin (ng/mL)
Control		8.3±0.6
LiCl	Scrambled siRNA	40.7±3.8
	p38 MAPK-RNAi	7.4±0.6
Wnt1	Scrambled siRNA	51.9±5.6
	p38 MAPK-RNAi	8.1±0.6

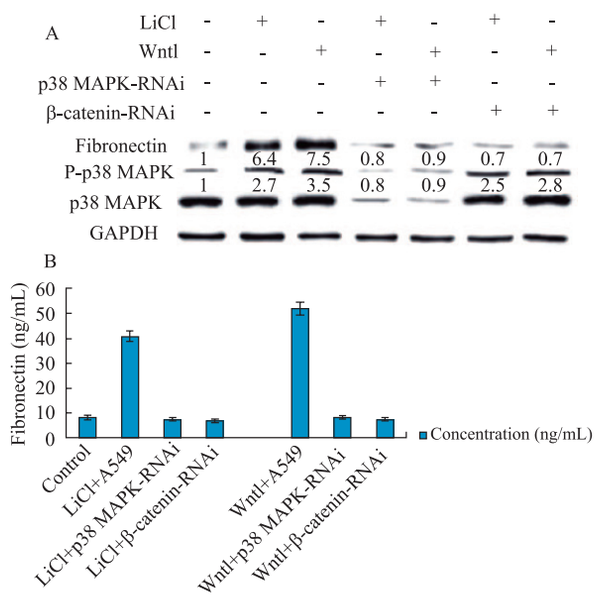

Fig. 5 A: Fibronectin was blotted in scrambled- and p38 MAPK-siRNA-transduced cells. p38 MAPK-siRNA downregulated expression of fibronectin (P<0.05). β-catenin siRNA significantly decreased the fibronectin expression and phosphorylation of p38 MAPK (P<0.05). B: ELISA in scrambled- and p38 MAPK-siRNA-transduced cells. p38 MAPK-silenced A549 cells showed downregulated expression of fibronectin. β-catenin-siRNA-transduced cells showed decreased fibronectin expression (P<0.05).

Table 3 ELISA of re-transfection of p38 MAPK on restoration of LiCl- and Wnt1-induced fibronectin expression

Groups		Fibronectin (ng/mL)
LiCl (p38 MAPK-RNAi)	A549	8.1±0.5
	p38 MAPK-transfect	25.7±2.4
	p38 MAPK-mock	7.9±0.7
Wnt1 (p38 MAPK-RNAi)	A549	7.8±0.6
	p38 MAPK-transfect	23.1±2.6
	p38 MAPK-mock	7.5±0.6

2.4 β-catenin Is Critical for Phosphorylating p38 MAPK in A549 Cells

The cells transfected with β-catenin siRNA expression plasmid showed a low level of fibronectin

expression, compared to that of Wnt1- or LiCl-treated cells (fig. 5A). Considering that the Wnt pathway can activate p38 MAPK as a reinforcement of the Wnt pathway itself[21], we examined the levels of phosphorylated p38 MAPK. Our results showed that inhibiting β-catenin blocked LiCl- and Wnt1-induced EMT (table 4), and it really decreased p38 MAPK phosphorylation (fig. 5A). These results indicate that p38 MAPK is the downstream signal and β-catenin was the upstream molecule. Therefore, β-catenin is essential for phosphorylated p38 MAPK in LiCl- and Wnt1-induced A549 cells EMT.

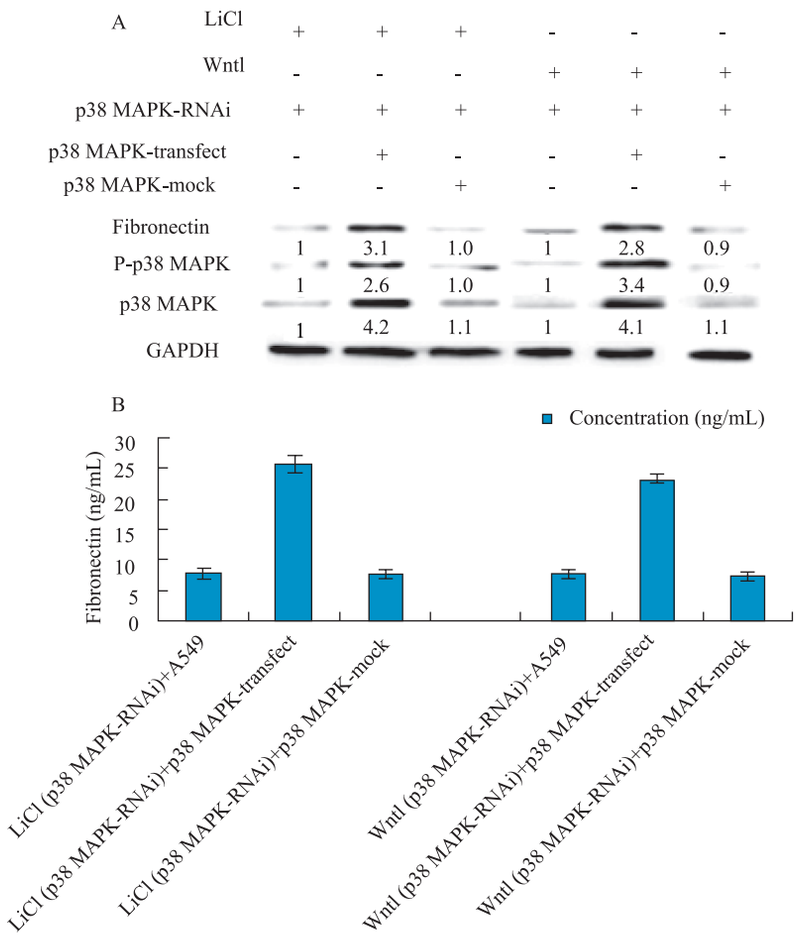

Fig. 6 A: p38 MAPK expression plasmid transfection restored the LiCl- and Wnt1-induced EMT in A549 cells; the control plasmid did not. (P<0.05); B: ELISA of fibronectin expression in p38 MAPK expression plasmid transfected cells. p38 MAPK expression plasmid transfection restored the LiCl- and Wnt1- induced fibronectin expression in A549 cells (P<0.05).

Table 4 ELISA of RNAi targeting β-catenin on LiCl- and Wnt1-induced fibronectin expression

Groups		Concentration (ng/mL)
Control		8.3±0.6
LiCl	Scrambled siRNA	40.7±3.8
	β-catenin-RNAi	6.9±0.5
Wnt1	Scrambled siRNA	51.9±5.6
	β-catenin-RNAi	7.3±0.5

3 DISCUSSION

IPF is a common form of idiopathic interstitial lung diseases (ILDs), which are non-neoplastic pulmonary disorders that are thought to result from inflammatory or aberrant repair mechanisms in the lungs[27]. IPF is usually associated with poor prognosis (median survival at 3–5 years after diagnosis), and there is no effective therapy currently available[28]. IPF is histologically characterized by usual interstitial pneumonia (UIP) and existence of fibroblastic foci[28]. Pathogenesis of IPF is still unclear; however, recent advances show that IPF development is associated with deregulated fibroblasts and abnormal epithelial cells. Epithelial cell dysfunction is related to EMT, where epithelial cells express a mesenchymal phenotype and contribute to pathogenesis of pulmonary fibrosis[29].

EMT was first described in the 1980s as a cellular

process in the primitive streak of chick embryos, and modulates many processes in development such as palate and lip fusion, gastrulation, neural crest development and somite dissociation[30]. EMT is an essential mechanism in tissue repair and embryonic development, but also is implicated in diseases such as organ fibrosis and cancer. EMT results from the induction of transcription factors that alter gene expression resulting in a loss of cell-cell adhesion, leading to a change in cytoskeletal dynamics and a change in cell morphology and physiology to the mesenchymal phenotype. This process permits the cells to leave the parenchyma and enter the systemic circulations during cancer metastasis. Vimentin, fibronectin, fibroblast specific protein 1 (FSP-1), α-smooth muscle actin (α-SMA), and N-cadherin increase during EMT. Characteristics of EMT include the abundance of mesenchymal markers, and decrease in expression of E-cadherin, zona occludens 1 (ZO-1), occludin and cytokeratins[31]. Spindle-shaped cells penetrate the basal ECM and move into connective tissues along a secreted matrix of fibronectin. In the present study, we demonstrated that the stimulation of Wnt signaling in A549 cells was associated with typical signs of EMT: loss of the adherence junction, cell elongation, and increase in fibronectin.

Transcription switching in EMT is primarily induced by the following signaling pathways and molecules, such as TGF-β pathway and Wnt-β-catenin pathway, Notch pathway, Hedgehog pathway, bone morphogenetic protein (BMP) pathway and some receptor tyrosine kinases. These pathways are activated by various dynamic stimuli from the local environment, including hypoxia, growth factors and cytokines and contact with the surrounding ECM. Stressful microenvironments, such as hypoxia or free radicals[32], trigger EMT. TGF-β superfamily[33], hedgehog (HH) family, Wnt family, and interleukin (IL) family[34], have been shown to initiate EMT. Several transcription factors induce EMT, such as the zinc-finger binding transcription factors Snail1 and Snail2 (also known as Slug) and several other basic helix-loop-helix (bHLH) factors such as zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2[35], and Twist[36] A T cell factor (TCF) transcription factor family member called lymphoid enhancer binding factor-1 (LEF-1), which is a downstream transcription factor of Wnt pathway[33]. These proteins interact with the transcription machinery to repress transcription of genes associated with cell adhesion, which is the key initiating step of EMT.

In humans, there are three isoforms of TGF-β protein (TGF-β1, TGF-β2 and TGF-β3), and two types of TGF-β receptors (TGFBR). Generally, first the type II TGFBR (TGFBR-II) receptors recognize and bind to TGF-β. Next, the complex recruits other TGFBRs and forms a complex with two copies of TGFBR-I, two copies of TGFBR-II, and one copy of active TGF-β. The complex activates the phosphorylation of the intracellular complex of TGFBRs to transduce the TGF-β signals downstream of the pathway. The canonical TGF-β pathway involves phosphorylation and activation of SMADs. Meanwhile, TGFBRs can also phosphorylate other pathway proteins or transmit signals via crosstalk with other pathways. Like the TGF-β pathway, the sonic hedgehog (SHH) pathway starts from secretive glycoproteins termed hedgehog proteins. The precursors of SHH proteins undergo several steps of post-translational modification before maturation, which are then secreted as oligomers or soluble multimers which can act in a paracrine manner. Three mammalian HH proteins have been identified in the HH family: SHH, Indian hedgehog (IHH) and desert hedgehog (DHH). Notably, SHH is crucial in cancer progression, embryo development and body patterning[37]. The Wnt pathway appears to be playing a certain role in regulating IPF[38].

The Wnt pathway has been postulated to be a key factor in the induction of EMT in lungs and alveolar type II cells[39]. In patients with IPF[40], the gene expression of the Wnt pathway is increased in fibroblasts and the Wnt proteins levels are elevated in bronchoalveolar lavage fluid, as compared with non-IPF patients[41]. Therefore, the Wnt pathway appears to be an important target for understanding the underlying signaling pathways contributing to EMT. Previous studies have shown that the canonical Wnt pathway is responsible for Wnt pathway-induced EMT[42]. The canonical Wnt pathway starts from reception of signaling molecules on the cell membrane complex. GSK3β is a major downstream regulator of the receptors. Without Wnt signals, GSK3β remains in an active form, which can phosphorylate target proteins, such as β-catenin, for further degradation[43]. When the Wnt pathway is activated, GSK3β is phosphorylated into an inactive form. Thus, β-catenin is accumulated in the cell and further transported into the nucleus. In the nucleus, in combination with TCF/LEF, β-catenin binds to the promoter region of target gene, such as SNAIL1, and activates its transcription. Furthermore, SNAIL1 expression results in a positive feedback loop with β-catenin by interacting with the β-catenin physically, or increasing the amount of free β-catenin indirectly through the EMT process[44, 45].

The Wnt pathway can interact with several signaling pathways, including the SHH and TGF-β pathways. GSK3β affects GLI proteins positively or negatively. First, GSK3β phosphorylates GLI proteins for degradation. Second, GSK3β phosphorylates SUFU and releases free GLI proteins[46]. GSK3β also stabilizes GLI mRNA indirectly, which leads to an increase of the amount of GLI proteins[47]. When the

TGF-β pathway is activated, the Wnt pathway can be activated via an SMAD-independent pathway. For example, in human lung fibroblast cells, TGF-β1 can activate the mitogen-activated protein kinase (MAPK) signaling and phosphorylate ERKs that inactivate GSK3β[48]. In some cell lines TGF-β1 can activate the ARK pathway, which can inhibit GSK3β. In addition, GSK3β negatively modulates the TGF-β pathway by phosphorylating SMAD3 and triggering its degradation when TGF-β is absent[49]. It has been documented that several extracellular stimuli, such as cytokines, growth factors and environmental stress are capable of activating p38 MAPK[50], leading to biological changes related to EMT[51, 52]. We have shown that LiCl and Wnt1 activate p38 MAPK. Also, we demonstrated that the pharmacological inhibition of p38 MAPK pathway reduced the expression of fibronectin as well as the EMT process in Wnt-stimulated A549 cells, which support the p38 MAPK signaling is capable of inducing EMT. p38 MAPK activation is a downstream event within the Wnt pathway in the development of EMT[25]. However, little is known about the significance of Wnt pathway-mediated p38 MAPK signaling in EMT.

Recent studies have reported a link between the p38 MAPK and Wnt/β-catenin pathways. Activated p38 MAPK appears to regulate the canonical Wnt/β-catenin signaling through regulating GSK3β inactivation[21, 53]. P38 MAPK was reported as a non-canonical downstream effector that mediates the biological effect of the Wnt pathway[54]. β-catenin is normally phosphorylated by GSK3β, then β-catenin is ubiquitinated and degraded. Wnt stimulation suppresses GSK3β activity, leading to accumulation of cytosolic and nuclear β-catenin, which in turn activates the genes necessary for embryonic development, patterning and cellular proliferation. Phosphorylation on Ser9 by Akt is the characterized mechanism for GSK3β inhibition. However, p38α also inactivates GSK3β by direct phosphorylation of Ser389, leading to β-catenin accumulation and Wnt pathway activation. This p38 MAPK dependent phosphorylation of GSK3β seems to provide a potential mechanism for p38 MAPK-mediated survival in specific tissues[55]. Also, GSK3β has been reported to inhibit p38 MAPK activation by phosphorylating the MAP3K MEKK4[56]. In the current experiment, we used siRNA to knockdown β-catenin expression. After the knockdown of β-catenin, EMT, induced by LiCl and Wnt1 was blocked and phosphorylation of p38 MAPK decreased, indicating that both β-catenin and p38 MAPK are involved in Wnt1- or LiCl-induced A549 cell EMT. β-catenin is essential for LiCl and Wnt1-induced EMT of A549 cells and β-catenin is the upstream molecule for phosphorylating p38 MAPK.

In summary, we have demonstrated that p38 MAPK plays a crucial role in LiCl- and Wnt1-indued EMT in A549 cells. β-catenin is necessary for p38 MAPK signaling and plays a role in phosphorylation of p38 MAPK. The results may be helpful in better understanding of the pathogenesis of IPF.

Conflict of Interest Statement

The authors declare no competing interests.

REFERENCES

1 Travis WD, Costabel U, Hansell DM, *et al*. An official American Thoracic Society/European Respiratory Society statement: Update of the international multidisciplinary classification of the idiopathic interstitial pneumonias. Am J Respir Crit Care Med, 2013,188(6):733-748

2 Olson AL, Swigris JJ. Idiopathic pulmonary fibrosis: diagnosis and epidemiology. Clin Chest Med, 2012,33(1):41-50

3 King TE, Jr., Pardo A, Selman M. Idiopathic pulmonary fibrosis. Lancet, 2011,378(9807):1949-1961

4 Kim KK, Kugler MC, Wolters PJ, *et al*. Alveolar epithelial cell mesenchymal transition develops *in vivo* during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci USA, 2006,103(35):13180-13185

5 Betensley A, Sharif R, Karamichos D. A Systematic Review of the Role of Dysfunctional Wound Healing in the Pathogenesis and Treatment of Idiopathic Pulmonary Fibrosis. J Clin Med, 2016,6(1):2

6 Hinz B. The myofibroblast: paradigm for a mechanically active cell. J Biomech, 2010,43(1):146-155

7 Rock JR, Barkauskas CE, Cronce MJ, *et al*. Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. Proc Natl Acad Sci USA, 2011,108(52):E1475-1483

8 Tanjore H, Xu XC, Polosukhin VV, *et al*. Contribution of epithelial-derived fibroblasts to bleomycin-induced lung fibrosis. Am J Respir Crit Care Med, 2009,180(7):657-665

9 Li LF, Lee CS, Lin CW, *et al*. Trichostatin A attenuates ventilation-augmented epithelial-mesenchymal transition in mice with bleomycin-induced acute lung injury by suppressing the Akt pathway. PLoS one, 2017,12(2):e0172571

10 Moon RT, Kohn AD, De Ferrari GV, *et al*. Wnt and beta-catenin signalling: diseases and therapies. Nat Rev Genet, 2004,5(9):691-701

11 Gordon MD, Nusse R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. J Biol Chem, 2006,281(32):22429-22433

12 Wang C, Zhu H, Sun Z, *et al*. Inhibition of Wnt/beta-catenin signaling promotes epithelial differentiation of mesenchymal stem cells and repairs bleomycin-induced lung injury. Am J Physiol-Cell Ph, 2014,307(3):C234-244

13 Konigshoff M, Balsara N, Pfaff EM, *et al*. Functional Wnt signaling is increased in idiopathic pulmonary

fibrosis. PLoS One, 2008,3(5):e2142
14 Wang Y, Li YP, Paulson C, *et al*. Wnt and the Wnt signaling pathway in bone development and disease. Front Biosci (Landmark edition), 2014,19:379-407
15 Kawami M, Harabayashi R, Miyamoto M, *et al*. Methotrexate-Induced Epithelial-Mesenchymal Transition in the Alveolar Epithelial Cell Line A549. Lung, 2016,194(6):923-930
16 Mikami Y, Yamauchi Y, Horie M, *et al*. Tumor necrosis factor superfamily member LIGHT induces epithelial-mesenchymal transition in A549 human alveolar epithelial cells. Biochem Biophys Res Commun, 2012,428(4):451-457
17 Choi JH, Hwang YP, Kim HG, *et al*. Saponins from the roots of Platycodon grandiflorum suppresses TGFbeta1-induced epithelial-mesenchymal transition via repression of PI3K/Akt, ERK1/2 and Smad2/3 pathway in human lung carcinoma A549 cells. Nutr Cancer, 2014,66(1):140-151
18 Kawata M, Koinuma D, Ogami T, *et al*. TGF-beta-induced epithelial-mesenchymal transition of A549 lung adenocarcinoma cells is enhanced by pro-inflammatory cytokines derived from RAW 264.7 macrophage cells. J Biochem, 2012,151(2):205-216
19 Feng H, Liu Q, Zhang N, *et al*. Leptin promotes metastasis by inducing an epithelial-mesenchymal transition in A549 lung cancer cells. Oncol Res, 2014,21(3):165-171
20 Desai S, Laskar S, Pandey BN. Autocrine IL-8 and VEGF mediate epithelial-mesenchymal transition and invasiveness via p38/JNK-ATF-2 signalling in A549 lung cancer cells. Cell Signal, 2013,25(9):1780-1791
21 Bikkavilli RK, Feigin ME, Malbon CC. G alpha o mediates Wnt-JNK signaling through dishevelled 1 and 3, RhoA family members, and MEKK 1 and 4 in mammalian cells. J Cell Sci, 2008,121(Pt 2):234-245
22 Zheng X, Wang Y, Liu B, *et al*. Bmi-1-shRNA inhibits the proliferation of lung adenocarcinoma cells by blocking the G1/S phase through decreasing cyclin D1 and increasing p21/p27 levels. Nucleic Acid Ther, 2014,24(3):210-216
23 Huang J, Xiao D, Li G, *et al*. EphA2 promotes epithelial-mesenchymal transition through the Wnt/beta-catenin pathway in gastric cancer cells. Oncogene, 2014,33(21):2737-2747
24 Song P, Zheng JX, Liu JZ, *et al*. Effect of the Wnt1/beta-catenin signalling pathway on human embryonic pulmonary fibroblasts. Mol Med Report, 2014,10(2):1030-1036
25 Wei J, Li Z, Chen W, *et al*. AEG-1 participates in TGF-beta1-induced EMT through p38 MAPK activation. Cell Biol Int, 2013,37(9):1016-1021
26 Jing YM, Luo J, Zhang YL, *et al*. Construction of a recombinant lentiviral vector of p38 MAPK and establishment of a human prostatic carcinoma cell line stably expressing p38 MAPK. Nan Fang Yi Ke Da Xue Xue Bao (Chinese), 2012,32(3):317-321
27 Daccord C, Maher TM. Recent advances in understanding idiopathic pulmonary fibrosis. F1000Res, 2016,5:F1000 Faculty Rev-1046
28 Raghu G, Rochwerg B, Zhang Y, *et al*. An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline: Treatment of Idiopathic Pulmonary Fibrosis. An Update of the 2011 Clinical Practice Guideline. Am J Respir Crit Care Med, 2015,192(2):e3-e19
29 Selman M, King TE, Pardo A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Ann Intern Med, 2001,134(2):136-151
30 Gonzalez DM, Medici D. Signaling mechanisms of the epithelial-mesenchymal transition. Sci Signal, 2014,7(344):re8
31 Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest, 2009,119(6):1420-1428
32 Min AL, Choi JY, Woo HY, *et al*. High expression of Snail mRNA in blood from hepatocellular carcinoma patients with extra-hepatic metastasis. Clin Exp Metastasis,2009,26(7):759-767
33 Nawshad A, Hay ED. TGFbeta3 signaling activates transcription of the LEF1 gene to induce epithelial mesenchymal transformation during mouse palate development. J Cell Biol, 2003,163(6):1291-1301
34 Cano A, Perez-Moreno MA, Rodrigo I, *et al*. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol, 2000,2(2):76-83
35 Peinado H, Portillo F, Cano A. Transcriptional regulation of cadherins during development and carcinogenesis. Int J Dev Biol, 2004,48(5-6):365-375
36 Yang J, Mani SA, Donaher JL, *et al*. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell, 2004,117(7):927-939
37 Zhang J, Tian XJ, Xing J. Signal Transduction Pathways of EMT Induced by TGF-beta, SHH, and Wnt and Their Crosstalks. J Clin Med, 2016,5(4):E41
38 Bruno NE, Kelly KA, Hawkins R, *et al*. Creb coactivators direct anabolic responses and enhance performance of skeletal muscle. EMBO J, 2014,33(9):1027-1043
39 Song P, Zheng JX, Xu J, *et al*. Beta-catenin induces A549 alveolar epithelial cell mesenchymal transition during pulmonary fibrosis. Mol Med Red, 2015,11(4):2703-2710
40 Brandenburg J, Reiling N. The Wnt Blows: On the Functional Role of Wnt Signaling in Mycobacterium tuberculosis Infection and Beyond. Front Immunol, 2016,7:635
41 Ramos C, Montano M, Garcia-Alvarez J, *et al*. Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. Am J Respir Cell Mol Biol, 2001,24(5):591-598
42 Vincan E, Barker N. The upstream components of the Wnt signalling pathway in the dynamic EMT and MET associated with colorectal cancer progression. Clin Exp Metastasis, 2008,25(6):657-663
43 Fodde R, Brabletz T. Wnt/beta-catenin signaling in

cancer stemness and malignant behavior. Curr Opin Cell Biol, 2007,19(2):150-158
44 Stemmer V, de Craene B, Berx G, *et al*. Snail promotes Wnt target gene expression and interacts with beta-catenin. Oncogene, 2008,27(37):5075-5080
45 Yook JI, Li XY, Ota I, *et al*. A Wnt-Axin2-GSK3beta cascade regulates Snail1 activity in breast cancer cells. Nat Cell Biol, 2006,8(12):1398-1406
46 Takenaka K, Kise Y, Miki H. GSK3beta positively regulates Hedgehog signaling through Sufu in mammalian cells. Biochem Biophys Res Commun, 2007,353(2):501-508
47 Noubissi FK, Goswami S, Sanek NA, *et al*. Wnt signaling stimulates transcriptional outcome of the Hedgehog pathway by stabilizing GLI1 mRNA. Cancer Res, 2009,69(22):8572-8578
48 Caraci F, Gili E, Calafiore M, *et al*. TGF-beta1 targets the GSK-3beta/beta-catenin pathway via ERK activation in the transition of human lung fibroblasts into myofibroblasts. Pharmacol Res, 2008,57(4):274-282
49 Guo X, Ramirez A, Waddell DS, *et al*. Axin and GSK3- control Smad3 protein stability and modulate TGF- signaling. Genes Dev, 2008,22(1):106-120
50 Zhang Y, Dong C. Regulatory mechanisms of mitogen-activated kinase signaling. Cell Mol Life Sci, 2007,64(21):2771-2789
51 Xia JM, Zhang J, Zhou WX, *et al*. Downregulation of p38 MAPK involved in inhibition of LDL-induced proliferation of mesangial cells and matrix by curcumin. J Huazhong Univ Sci Technolog Med Sci, 2013,33(5):666-671
52 Xiao Y, Zou P, Wang J, *et al*. Lower phosphorylation of p38 MAPK blocks the oxidative stress-induced senescence in myeloid leukemic CD34(+)CD38(-) cells. J Huazhong Univ Sci Technolog Med Sci, 2012, 32(3):328-333
53 Cervenka I, Wolf J, Masek J, *et al*. Mitogen-activated protein kinases promote Wnt/beta-catenin signaling via phosphorylation of LRP6. Mol Cell Biol, 2011,31(1):179-189
54 Jin EJ, Lee SY, Choi YA, *et al*. BMP-2-enhanced chondrogenesis involves p38 MAPK-mediated down-regulation of Wnt-7a pathway. Mol Cells, 2006,22(3):353-359
55 Thornton TM, Pedraza-Alva G, Deng B, *et al*. Phosphorylation by p38 MAPK as an alternative pathway for GSK3beta inactivation. Science, 2008,320(5876):667-670
56 Abell AN, Granger DA, Johnson GL. MEKK4 stimulation of p38 and JNK activity is negatively regulated by GSK3beta. J Biol Chem, 2007,282(42):30476-30484

(Received May 20, 2017; revised Jan. 25, 2018)