

## Neferine Attenuates Epithelial-Mesenchymal Transition of Alveolar Epithelial Cells

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Received date: July 22, 2017; Accepted date: August 11, 2017; Published date: August 16, 2017

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### Abstract

**Background:** Idiopathic pulmonary fibrosis (IPF) is an incurable, progressive, and fatal interstitial lung disease with the characteristics of lung tissue damage and an enhancement in extracellular matrix (ECM). Alveolar epithelial cells (AMs) are major target cells that can directly promote to occurring of pulmonary fibrosis by acquisition of a mesenchymal phenotype through epithelial- mesenchymal transition (EMT). Neferine, a component of Chinese herbs, has been thought to be involved in anti-fibrotic activity in experimental lung fibrosis. However, its mechanism is not clear. In this study, we explore the regulation of neferine in TGF- $\beta$ -induced EMT in lung fibrosis model and illustrate its mechanism of action.

**Methods:** The alveolar epithelial cell line A549 was stimulated with TGF- $\beta$ 1 with or without Neferine pretreatment in advance. Morphologic variations and expression of EMT-related markers, including E-cadherin,  $\beta$ -catenina-SMA and Vimentin were detected. Expressions of Smad2, p- Smad 2, Smad3 and p-Smad3 were measured.

**Results:** TGF- $\beta$ 1-treated A549 cells were transformed into the mesenchymal morphology with less E-cadherin,  $\beta$ -catenin and more  $\alpha$ -SMA, Vimentin expression. The addition of Neferine inhibited the TGF- $\beta$ 1-induced change of the mesenchymal phenotype. Furthermore, Neferine inhibited the TGF- $\beta$ 1-induced increase in the expression of p-Smad2 and p-Smad3.

**Conclusions:** Our study illustrate that Neferine inhibits TGF- $\beta$ 1-induced EMT in lung fibrosis model *via* TGF- $\beta$  signaling pathway.

**Keywords:** Neferine; Transforming growth factor beta1; Epithelial-mesenchymal transition; Alveolar epithelial cells; Signalling pathway

### Introduction

IPF is a fatal and aggressive disease, characterized by the abnormal accumulation of extracellular matrix and alveolar epithelial cell injury [1]. Currently, there are no pharmaceuticals or therapies that prevent IPF progression, and life expectancy is 2.5 to 3.5 years after being diagnosed [2]. Although several anti-fibrotic agents have been shown to block progression to fibrosis, no clinically available agent has been developed to reduce fibrotic nodules and reverse established fibrosis [3].

Neferine, a major bisbenzylisoquinoline alkaloid derived from seed embryo of the traditional Chinese medicine herbs *Nelumbo nucifera* Gaertn's [4]. Previous studies have demonstrated that Neferine has a number of biological and pharmacological activities, such as anti-inflammation, antioxidation [5], anti-apoptosis [6], neuroprotective [7], nephroprotection [8] and anticancer effects [9-11]. In addition, Neferine exerts antifibrotic effects. A recent study revealed that Neferine had antifibrogenic effects in diabetes-related myocardial fibrosis, which may have been partly due to inhibiting cardiac fibroblast multiplication, migration, and differentiation into

myofibroblasts [12]. Chen et al. demonstrated that Neferine had an antifibrotic effect on CCl<sub>4</sub>-induced hepatic fibrosis in mice because of the reduced expression of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) in the liver [13]. In addition, it was reported that Neferine has anti-fibrotic and anti-inflammatory effects on the amiodarone and bleomycin-induced lung fibrosis model [4,14]. Although, the previous data represented anti-fibrotic activities of Neferine in pulmonary fibrosis, the protective mechanism of Neferine in pulmonary fibrosis is not known.

Emerging evidence has demonstrated that EMT is a key event playing an important role in the development of IPF [15]. EMT is genetically characterized by a decreased expression of epithelial cell-related gene (E-cadherin/ $\beta$ -catenin) and increased expression of mesenchymal cell and fibrotic related genes, such as  $\alpha$ -SMA Vimentin, and also Collagen I [16]. TGF- $\beta$ 1 plays a pivotal role in the EMT process and is considered to be the major inducer of EMT in lung fibrosis. Alveolar epithelial cells (AECs) are major target cells that can directly promote pulmonary fibrosis by acquiring mesenchymal phenotype through EMT. The TGF- $\beta$ /Smad signalling pathway is necessary in the process of EMT and fibrosis in a variety of organs [17].

At present, the role of Neferine in IPF is not clearly defined. Deng et al. found that Neferine might be involved in hepatocellular carcinoma pathogenesis by regulating EMT [11]. Therefore, we hypothesized that Neferine may participate in EMT in lung epithelial cells. In this research, We found that treatment A549 cells with Neferine inhibited EMT-related changes and alleviated the effects of TGF- $\beta$ 1 *via* restraining of TGF- $\beta$ /Smad signal pathway.

## Methods

### Cell culture

The human type II alveolar epithelial cells, A549, were obtained from the Scientific Research Department of Central South University, Changsha, China. A549 cells were cultured in RPMI-1640 medium and supplemented with 10% fetal bovine serum, 100 mg/l streptomycin and 100 U/mL penicillin in a humidified 5% CO<sub>2</sub> atmosphere at 37°C.

### Cell treatment

Cell cultures were seeded into 6-well plates (100  $\mu$ L per well). The cells were allocated into three groups: control group (only RPMI-1640 medium was added), TGF- $\beta$ 1 treatment group (10 ng/mL TGF- $\beta$ 1) and TGF- $\beta$ 1+Neferine treatment group (10 ng/mL TGF- $\beta$ 1 and 10  $\mu$ M Neferine). TGF- $\beta$ 1 and Neferine were purchased from Sigma-Aldrich.

### Western blot analysis

Total protein was extracted with RIPA lysis buffer (Beyotime, China), which contains PMSF and cocktail, and then the protein concentrations were measured by a BCA protein assay kit (Beyotime, China). Thirty-five micrograms of denatured proteins were used in the experiment. SDS-PAGE gels (10%) were used to separate the proteins, followed by transfer to PVDF membranes (Millipore, IPVH00010). The membranes were blocked in 5% non-fat milk for 2 h and were incubated overnight with one of the following antibodies in PBST: rabbit anti-E-cadherin, anti- $\beta$ -catenin, anti- $\alpha$ -SMA and anti-Vimentin (CST, Beverly, MA, USA), rabbit anti-Smad2, anti-pSmad2, anti-Smad3 and anti-P-Smad3 obtained from Abcam. Next, the membranes were incubated for 2 h at room temperature with HRP-conjugated secondary antibody. The blots were scanned using a ChemiDoc™ MP Imaging System, and other steps were performed as previously described [18]. Each experiment was repeated for three times.

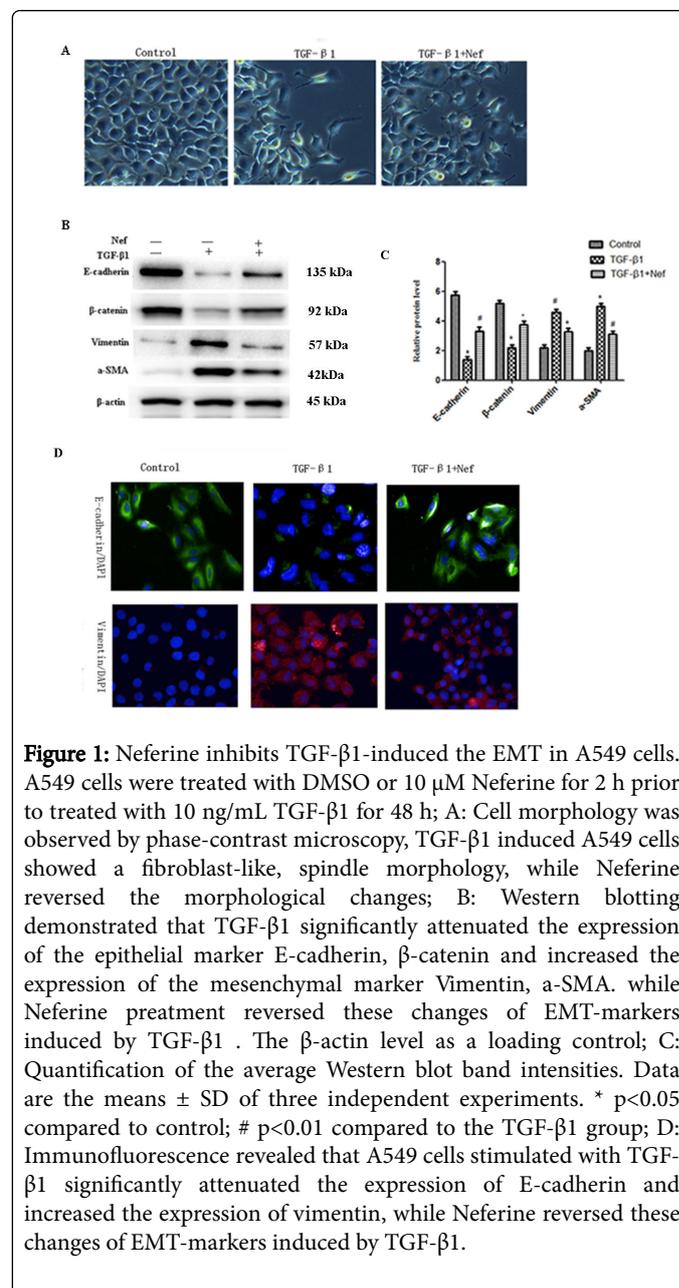
### Immunofluorescence microscopy

A549 cells were seeded in 96-well plate with a concentration of 5  $\times$  10<sup>4</sup>/mL, after incubated overnight, Neferine along or with 10 ng/mL TGF- $\beta$ 1 were added in, after incubated for 48 h, washed with PBS for two times, then fixed for 20 min with 4% paraformaldehyde in PBS at room temperature, blocked with goat serum for 1 h. The cells were first stained with E-cadherin and Vimentin rabbit antibody (1:500) at 4°C overnight, and then with Cy3-conjugated goat anti-rabbit IgG (1:4000) for 1 h at room temperature. DAPI was used for nuclear staining. Immunofluorescence images were taken with PerkinElmer. All the experiments were repeated for three times.

### Statistical analysis

The results were expressed by the mean  $\pm$  standard deviation (SD). Student's t test and one-way analysis of variance (ANOVA) were used

for determining the significant differences between the control and experimental groups by SPSS18.0. A p value of less than 0.05 was considered significant difference.



**Figure 1:** Neferine inhibits TGF- $\beta$ 1-induced the EMT in A549 cells. A549 cells were treated with DMSO or 10  $\mu$ M Neferine for 2 h prior to treated with 10 ng/mL TGF- $\beta$ 1 for 48 h; A: Cell morphology was observed by phase-contrast microscopy, TGF- $\beta$ 1 induced A549 cells showed a fibroblast-like, spindle morphology, while Neferine reversed the morphological changes; B: Western blotting demonstrated that TGF- $\beta$ 1 significantly attenuated the expression of the epithelial marker E-cadherin,  $\beta$ -catenin and increased the expression of the mesenchymal marker Vimentin,  $\alpha$ -SMA. while Neferine pretreatment reversed these changes of EMT-markers induced by TGF- $\beta$ 1. The  $\beta$ -actin level as a loading control; C: Quantification of the average Western blot band intensities. Data are the means  $\pm$  SD of three independent experiments. \*  $p$ <0.05 compared to control; #  $p$ <0.01 compared to the TGF- $\beta$ 1 group; D: Immunofluorescence revealed that A549 cells stimulated with TGF- $\beta$ 1 significantly attenuated the expression of E-cadherin and increased the expression of vimentin, while Neferine reversed these changes of EMT-markers induced by TGF- $\beta$ 1.

## Results

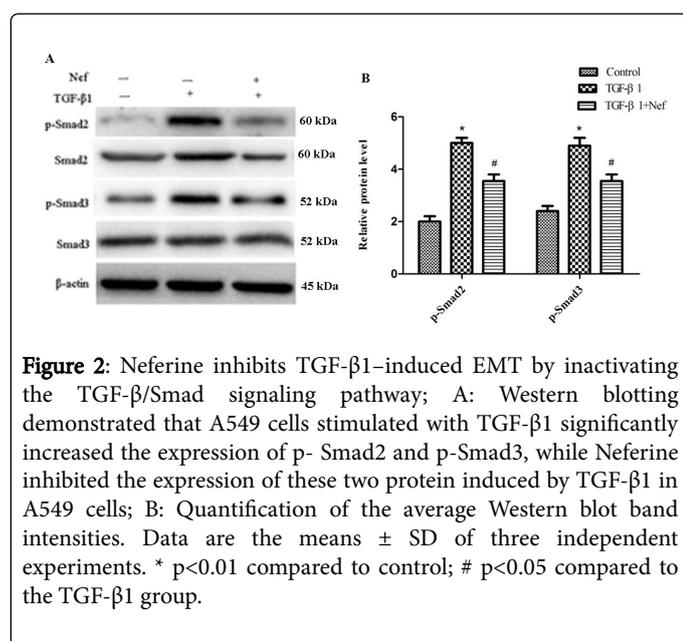
### Neferine inhibits TGF- $\beta$ 1-induced EMT in A549 cells

As reported previously, treated with TGF- $\beta$ 1, A549 cells transformed from an epithelial phenotype to a mesenchymal phenotype, and treatment with Neferine partly restored the cells to the traditional epithelial shape (Figure 1A). To confirm the effect of Neferine in A549 cells, the protein levels of E-cadherin,  $\beta$ -catenin,  $\alpha$ -SMA and Vimentin were evaluated using Western blot. We found that TGF- $\beta$ 1 induced Vimentin and  $\alpha$ -SMA expression whereas decrease E-

cadherin and  $\beta$ -catenin expression. The effect of TGF- $\beta$ 1 could be partly restored by the presence of Neferine (Figure 1B and C). And all these were further proved by the immunofluorescence assay (Figure 1D), which suggested that Neferine can effectively inhibit TGF- $\beta$ 1 induced EMT.

### Neferine inhibits TGF- $\beta$ 1-induced EMT by inactivating the TGF- $\beta$ /Smad signaling pathway

To further investigate the mechanism of neferine on EMT, the expression of the phosphorylated Smad2 and Smad3 was determined using Western blot, which have been shown to be the major signal pathways for TGF- $\beta$ 1-induced EMT. P-Smad2 and p-Smad3 in A549 cells markedly increased after TGF- $\beta$ 1 treatment. When the cells were pretreated with Neferine, the expression of p-Smad2 and p-Smad3 was significantly reduced (Figure 2A and 2B). This finding suggests that Neferine inhibits TGF- $\beta$ 1-induced EMT by inactivating the Smad-dependent signaling pathway.



**Figure 2:** Neferine inhibits TGF- $\beta$ 1-induced EMT by inactivating the TGF- $\beta$ /Smad signaling pathway; A: Western blotting demonstrated that A549 cells stimulated with TGF- $\beta$ 1 significantly increased the expression of p-Smad2 and p-Smad3, while Neferine inhibited the expression of these two protein induced by TGF- $\beta$ 1 in A549 cells; B: Quantification of the average Western blot band intensities. Data are the means  $\pm$  SD of three independent experiments. \*  $p < 0.01$  compared to control; #  $p < 0.05$  compared to the TGF- $\beta$ 1 group.

### Discussions

In this research, we used A549 cells as a model of AECs and explored the effect and mechanism of Neferine on EMT. Some studies have used A549 cells to research EMT and demonstrated that A549 cells showed similar characteristics as AECs in the EMT process [19]. Thus, A549 cells are a suitable cell model for the study of EMT

Many studies have identified the important role of EMT in the development of IPF [2,20,21]. E-cadherin is an adherent junction protein that is specifically expressed in epithelial cells. Loss of E-cadherin is a universal feature of EMT, and E-cadherin can be used as an epithelial marker. However, the acquisition of a mesenchymal phenotype is more difficult to define due to the lack of specificity in many of the available phenotypic markers. In our study, we chose two epithelial marker (E-cadherin,  $\beta$ -catenin) and two mesenchymal markers ( $\alpha$ -SMA, Vimentin) to define EMT. EMT in lung epithelial cells is associated with decreases in E-cadherin levels and increases in Vimentin levels [22]. During the induction of EMT *in vitro* and *in vivo*, TGF- $\beta$ 1 is considered to be a typical cytokine [23]. Our results

demonstrated that treatment with TGF- $\beta$ 1 (10 ng/mL) could promote EMT marked by the increase of Vimentin,  $\alpha$ -SMA the decrease of E-cadherin,  $\beta$ -catenin. Thus, we successfully established the EMT model in A549 cells to research the therapeutic effect of Neferine on this pathological process.

In previous research, it was demonstrated that Neferine had anti-inflammation, antioxidation and anti-pulmonary fibrosis activities [4,14]. However, the mechanism is not clear. It has been reported that Neferine could suppress EMT induced by TGF- $\beta$ 1 in Hepatoma cells [11]. However, whether Neferine can inhibit EMT in AECs remains unknown. In this study, we found that Neferine pretreatment before TGF- $\beta$ 1 stimulation in A549 cells could attenuate the expression of Vimentin,  $\alpha$ -SMA and increased the expression of E-cadherin,  $\beta$ -catenin.

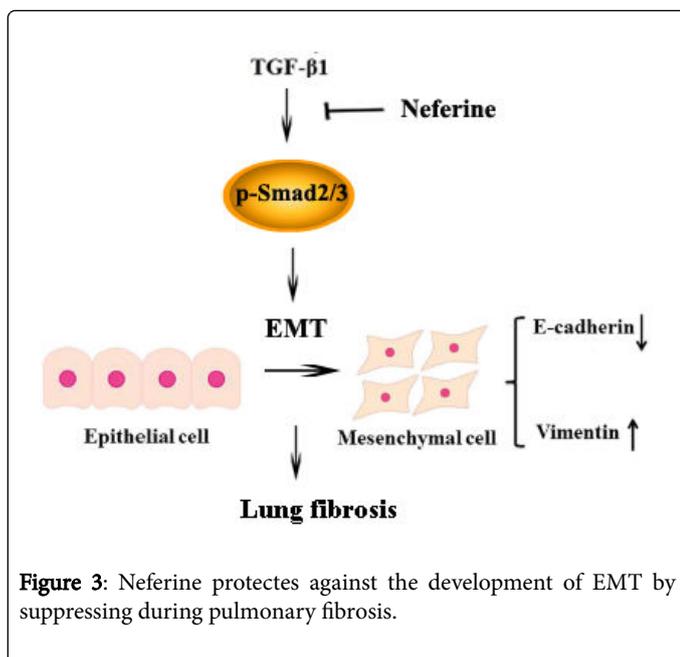
In this study, we detected the expression of the EMT markers by western blotting and immunofluorescence. These two assays showed the same results. TGF- $\beta$ 1 significantly attenuated E-cadherin expression and increased the expression of Vimentin. Neferine alleviated EMT changes induced by TGF- $\beta$ 1 and the expression of Vimentin, indicating that Neferine could inhibit TGF- $\beta$ 1-induced EMT in A549 cells, which is consistent with our hypothesis.

The Smad dependent signal, which is acknowledged as the TGF- $\beta$ 1-Smad2/3 Signaling pathway, plays a major role in TGF- $\beta$ 1-induced EMT in A549 cells. Transcriptome analysis of EMT induced by TGF- $\beta$  in normal epithelial cells demonstrated that increased expression of p-Smad2 or p-Smad3 induced EMT, whereas dominant-negative versions expression of p-Smad2 or p-Smad3 inhibited TGF- $\beta$ -induced EMT [24]. When the TGF- $\beta$  ligand binds to the TGF- $\beta$  receptor in the cell membrane, the receptor activated Smads (RSMADS) and both smad2 and 3 are phosphorylated by the intracellular T $\beta$ RI kinase domain, and then bind to the common Smad mediator (Co-SMAD), Smad4. These Smad proteins transfer into the nucleus, combined with Smad-binding elements to form a transcription factor complex, and then activate TGF- $\beta$  target genes (e.g., Vimentin), as well as inhibiting epithelial-related genes (e.g., E-cadherin) through the combination of various transcription factors [25]. In this study, it was shown that TGF- $\beta$ 1 promoted the expression levels of p-Smad2 and p-Smad3. Similarly, Neferine decreased EMT induced by TGF- $\beta$ 1 in A549 cells related to modulation of the TGF- $\beta$ 1-Smad2/3 pathway. Inhibition of the Smad-dependent pathway by Neferine will be an efficient strategy to reverse EMT.

Our study shows the protective mechanism of neferine against IPF for the first time, however, it should be mentioned that our study possesses certain defects, such as the dose-response effects of Neferine and the different treatment time course after TGF- $\beta$  stimulated were not presented. Currently, related studies employing the lung tissue of human IPF patients are underway.

### Conclusions

Our research offered the first line of proof that Neferine could attenuate the EMT induced by TGF- $\beta$ 1 in A549 cells related to inhibition of the TGF- $\beta$ 1-Smad signaling pathway (Figure 3). Therefore, Neferine may be a good prospect agent for the treatment of IPF.



**Figure 3:** Neferine protects against the development of EMT by suppressing during pulmonary fibrosis.

## Acknowledgment

This study was financially supported by the Human Medical Research Plan Foundation (B2014-145).

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