Melatonin attenuates TGFβ1-induced epithelial-mesenchymal transition in lung alveolar epithelial cells

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Abstract. Idiopathic pulmonary fibrosis (IPF) is the most common interstitial lung disease. However, the pathogenesis remains to be fully elucidated. Melatonin is secreted by the pineal gland, it has a strong antioxidant effect, and exerts an anti-fibrosis effect. Whether melatonin attenuates pulm -onary fibrosis by inhibiting epithelial-mesenchymal transition (EMT) requires further research. The present study aimed to investigate whether melatonin prevents transforming growth factor-β1 (TGF-β1)-induced EMT and underlying signaling pathways using reverse transcription-quantitative polymerase chain reaction, western blot analysis and immunofluorescence. The results demonstrated that melatonin inhibits EMT in A549 cells, and the Wnt/β-catenin and Smad2/3 signaling pathways are involved in the EMT of the A549 cell line as they were suppressed by melatonin. The present study indicates that melatonin inhibited TGFβ1-induced epithelial-mesenchymal transition in the A549 cell line and may potentially be useful in the treatment of IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, irreversible and progressive lung disease of unknown origin, marked by progressive dyspnoea, and ultimately, respiratory failure and mortality (1). Idiopathic pulmonary fibrosis occurs more commonly in older individuals and men, cases are predominantly observed in male ex-smokers, it has a mean survival of 3-5 years from the time of diagnosis (2,3). Research over the past decade aiming to determine an effective treatment to slow disease progression and improve survival, has lead to unsatisfactory results.

Epithelial-mesenchymal transition (EMT) is an essential mechanism and process in embryonic development and tissue repair by which differentiated epithelial cells undergo a phenotypic conversion and acquire a mesenchymal phenotype, including a change from epithelial morphology and physiology, the loss of cell-cell adhesion, and enhanced migratory and invasion capacity (4,5). However, EMT also contributes to the progression of disease, including organ fibrosis and cancer (6-8). A previous study determined that EMT has been observed in pulmonary epithelial cells and the lung *in vivo* (9). A number of myofibroblasts are derived from lung epithelial cells by epithelial-mesenchymal transition (10). However, the molecular mechanisms underlying IPF are largely unknown, the epithelial-mesenchymal transition is likely responsible for the enhanced synthesis of abnormal matrix observed in pulmonary fibrosis. The present study aimed to investigate transforming growth factor-β1 (TGF-β1)-induced EMT and whether melatonin prevents EMT in the A549 cell line.

Melatonin is secreted from the pineal gland, it regulates circadian rhythms, sleep and immune system activity. In addition, melatonin is a powerful and effective free radical scavenger in oxidative stress and inflammation (11). As oxidative stress is important in epithelial-mesenchymal transition (EMT) and fibrosis (12,13), melatonin may protect cells, tissues, and organs against oxidative damage and partially reverse EMT by reducing the oxidative stress. Previous studies have also indicated that melatonin exerts an anti-fibrotic effect at tissue level in liver, lung and heart disease (14-16). However, the exact underlying mechanism requires further elucidation.

From the guidelines on IPF treatment by the National Clinical Guideline Centre (UK) in 2013, the limitations of current pharmacological therapies for IPF demonstrate the importance of other forms of treatment, including lung transplantation and best supportive care, such as oxygen therapy, pulmonary rehabilitation and palliation of symptoms (17). Research into treatment of IPF has produced limited results. Pirfenidone and nintedanib have been demonstrated to reduce functional decline and disease progression with equivalent efficacy and an acceptable safety profile, and have also resulted in improved survival (18,19). However, the treatment outcomes

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do not match expectations. The present study hypothesizes that melatonin may be a potential therapeutic candidate for IPF and aimed to investigate underlying mechanisms.

Materials and methods

Reagents. RPMI 1640 and fetal bovine serum (FBS) were obtained from (Abcam, Cambridge, UK), penicillin-streptomycin (5,000 U/ml penicillin; 5,000 U/ml streptomycin), were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Dimethyl sulfoxide (DMSO), paraformaldehyde, Triton X-100, Hoechst 33342 and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Melatonin was purchased from J&K Scientific Ltd. (Beijing, China). Anti-β-actin, anti-E-cadherin, anti-vimentin, anti-N-cadherin, anti-phosphorylated (p)-β-catenin, anti-p-Smad2 and anti-p-Smad3 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). ECL kit was purchased from Thermo Fisher Scientific, Inc. All reagents used were trace element analysis grade and all water used was glass distilled.

Cell culture. The A549 human alveolar epithelial cell line was obtained from the American Type Cell Collection (Manassas, VA, USA). Cells were cultured in RPMI 1640 with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were maintained at 37˚C and 5% CO_2 in a humidified atmosphere.

Cell viability assay. Cellular viability was assessed using the MTT assay. Cells were plated in 96-well plates containing 200 μl culture medium at a concentration of 5,000 cells/well with ≥90% viability. Cells were cultured with various concentrations of melatonin (0, 0.25, 0.5, 1, 1.25, 1.5, 1.75 and 2 mM) for 24 h. Following incubation, MTT dye (20 μl; 5 mg/ml) was added to the cells for 4 h followed by incubation with DMSO for 10 min. Absorbance was measured at a wavelength of 570 nm using a microplate reader (UVM340; Asys-Hitech GmbH, Eugendorf, Austria). The cell viability was determined as the ratio of signal between the treated and control cultures. Melatonin alone at 1 to 2 mM suppressed cell viability in a dose-dependent manner, however, above this concentration the cell inhibition rate was almost unchanged. Treatment of cells with melatonin (from 0.25 to 1.0 mM) did not markedly enhance the inhibition of cell viability, compared with the control cells. Thus, 0.5 mM was used as the treated concentration in all subsequent experiments.

Western blotting. The A549 cell lysates were homogenized in a radioimmunoprecipitation buffer (Thermo Fisher Scientific, Inc.) comprised of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid and 0.02% sodium azide. Protein concentrations were determined using the Pierce BCA Protein assay kit (Thermo Fisher Scientific, Inc.) according to manufacturer's protocols. Equal quantities of protein from each sample were separated electrophoretically using 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% non-fat milk overnight at 4˚C The primary antibodies used in the present study were as follows: Mouse monoclonal anti-β-actin (1:400; cat. no. sc-47778), mouse monoclonal anti-N-cadherin (1:400; cat. no. sc-121905), mouse monoclonal anti-vimentin (1:400; cat. no. sc-373717), mouse monoclonal anti-E-cadherin (1:400; cat. no. sc-52327), mouse monoclonal anti-p-β-catenin (1:400; cat. no. sc-101651), mouse monoclonal anti-p-Smad2 (1:400; cat. no. sc-101801 2), and mouse monoclonal anti-p-Smad3 (1:400; cat. no.sc-130218). The membranes were then washed with TBST on a shaker twice at room temperature for 10 min each timefollowed by TBS for 10 min. The membranes were then incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibodies (1:400; Santa Cruz Biotechnology, Inc.; cat. no. sc-358920) overnight. Detection was performed using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) and the images were captured by X-ray film. The relative quantities of various proteins were analyzed and the results were quantified by Quantity One software V4.62 (Bio-Rad, Hercules, CA, USA).

Immunofluorescence experiments. The protein expression levels of E-cadherin in A549 cells was examined using immunofluorescence. The cells were seeded in 6-well plates, washed with ice-cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min at 4˚C. Following washing with PBS three times, the cells were incubated with 1% Triton X-100 for 10 min. The cells were subsequently incubated mouse monoclonal anti-E-cadherin (1:400) overnight. Tetramethylrhodamine-conjugated goat anti-mouse IgG (1:100; Santa Cruz Biotechnology, Inc.; cat. no. sc-2086) was incubated with the cells for 30 min at room temperature. Finally, Hoechst 33342 was added to the cells for 15 min and following three times with PBS, cells were visualized under fluorescence microscopy.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from A549 cells was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RNA concentration was measured using absorbance at a wavelength 260 nm. Total RNA (2 μg) was reverse transcribed to cDNA using a PrimeScript RT reagent kit (Takara Biotechnology, Co., Ltd., Dalian, China). The reverse transcription program was as follows: 15 min at 37˚C and 5 sec at 85˚C. The sequences of WNT1 gene was obtained from the GenBank database, and specific primers were designed over an exon-exon junction with Primer Premier 5.0 (Jiran Biotechnology, Co., Ltd., Shanghai, China). The primers used for amplification were as follows: Forward, 5'-CGGAGTCAA CGGATTTGGTCGTAT-3' and reverse, 5'-AGCCTTCTCCAT GGTGGTGAAGAC-3' for GAPDH; and forward, 5'-TACCTC CAGTCACACTCCCC-3' and reverse, 5'-CCATGGCAGGAG AATAGGAA-3' for WNT1. For PCR, the cDNA templates were initially heat-denatured at 94˚C for 3 min; followed by 35 cycles of 94˚C for 1 min, annealing at 60˚C for 1 min, and extension at 72˚C for 1 min; with a final extension cycle at 72˚C for 10 min. The appropriate negative control reactions were performed to demonstrate the absence of DNA contamination. Products were analyzed on ethidium bromide-stained 2% agarose gels and photographed under ultraviolet light.

Statistical analysis. Statistical analysis was performed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA). The data are expressed as the mean ± standard error.

The statistical significance of the differences was calculated using Student's t-test and one-way analysis of variance and Student-Neuman-Keuls test. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

TGF-β1 induces EMT in A549 cells. A549 cells were treated with 5 ng/ml TGF-β1 for 0, 24, 48 and 72 h (20). To assess the TGF-β1-induced EMT, A549 cells served as an *in vitro* model system for investigating the expression of E-cadherin, vimentin and N-cadherin. Exposure of A549 cells to 5 ng/ml TGF-β1 for 12-72 h significantly decreased protein expression levels of the epithelial cell marker E-cadherin ($P<0.01$ at 72 h) and significantly increased the expression levels of the mesenchymal markers vimentin and N-cadherin compared with non-treated cells (Fig. 1A and B; $P<0.01$ at 72 h). Similarly, treatment with TGF-β1 resulted in decreased expression of E-cadherin by immunofluorescence (Fig. 1C). Thus, these results indicated that TGF-β1-treated A549 cells lost their epithelial characteristics and gained a mesenchymal phenotype. Therefore, TGFβ1 positively induced the EMT of A549 cells.

Melatonin inhibits TGF-β1-induced EMT in A549 cells. The MTT assay was used to test the effects of melatonin on cell viability in A549 cells. Melatonin alone (1-2 mM) suppressed cell viability in a dose-dependent manner (Fig. 2A). However, the treatment of the cells with doses of melatonin between 0.25 and 1.0 mM did not significantly enhance the inhibition of cell viability compared with the control cells. Above 2 mM, the cell inhibition rate was almost unchanged. To detect whether melatonin inhibited TGF-β1 mediated EMT, A549 cells were cultured with 5 ng/ml TGF-β1 with or without 0.5 mM melatonin for 48 h and the protein expression levels of E-cadherin, vimentin and N-cadherin were detected by western blotting. As demonstrated in the results of Fig. 2, the TGF-β1-induced EMT was attenuated by co-treating the A549 cells with 0.5 mM melatonin. This resulted in significant reduction of N-cadherin and vimentin, and increased expression of E-cadherin. Treatment with melatonin (0.5 mM) alone did not inhibit the cell viability as shown in Fig. 2A; however, it markedly inhibited the TGF-β1-induced EMT in A549 cells. (Fig. 2B and C). These results indicated that melatonin supplementation reversed TGF-β1-induced EMT in A549 cells.

Melatonin inhibits TGF-β1-induced EMT via suppression of Wnt/β-catenin signaling. The Wnt signaling pathway has previously been associated with the development and progression of cancer due to EMT-like transition (21). Thus, the present study aimed to investigate the effect of melatonin on the Wnt/β-catenin signaling pathway. A549 cells were cultured with 5 ng/ml TGF-β1 with or without 0.5 mM melatonin for 48 h, and the expression levels of WNT1 and p-β-catenin were detected by RT-PCR and western blotting. As demonstrated in Fig. 3, TGF-β1 alone resulted in significantly increased mRNA and protein expression levels of WNT1 and p-β-catenin compared with the control. Notably, it was observed that melatonin markedly inhibited TGF-β1-induced upregulation of WNT1 and p-β-catenin compared with the treatment with TGF-β1 alone ($P<0.01$). These results indicate that the anti-EMT effect of melatonin treatment may partially be mediated via the inactivation of the Wnt/β-catenin signaling pathway.

Melatonin inhibits TGF-β1-induced EMT via suppression of Smad signaling. TGF-β1 has been regarded as a 'master switch' in the regulation of EMT and demonstrated to signal primarily via the Smad2/3 pathway (22). Thus, the present study aimed to investigate whether melatonin treatment of A549 cells inhibited the expression of Smad2/3 in blocking EMT. Cells were cultured with 5 ng/ml TGF-β1 with or without 0.5 mM melatonin for 48 h, and the protein expression levels of p-Smad2/3 were detected by western blotting. As presented in Fig. 4, TGF-β1 alone resulted in significantly increased expression levels of p-Smad2/3 compared with the control ($P<0.01$). Melatonin markedly inhibited the upregulation of p-Smad2/3 when used with TGF-β1 compared with treatment with TGF-β1 alone ($P<0.01$). These results indicate that the anti-EMT effect of melatonin may partially be mediated via inactivation of the Smad signaling pathway.

Discussion

Idiopathic pulmonary fibrosis (IPF) has a low incidence (4.6-16.3/100,000), and a high mortality rate. It is characterised by an interstitial fibrotic process (23). Over 10 years, the mortality of pulmonary fibrosis has increased year by year (24). IPF patients require pharmacological and non-pharmacological management strategies, however, numerous attempts to elucidate therapeutic agents have not been successful. Understanding of the pathobiology of IPF has increased, however, the causative factors require further elucidation and the disease pathogenesis is incompletely understood. Current concepts suggest that alveolar epithelial cell injury characterized by proliferation, migration, and activation of fibroblasts, and secretion of excessive quantities of extracellular matrix components, result in scarring of the lung, architectural distortion, and irreversible loss of function (25). A previous study demonstrated that EMT is essential in the pathogenesis of lung fibrosis (26). The present study aimed to investigate the therapeutic potential and possible mechanisms of action of melatonin in lung fibrosis.

Melatonin is predominantly produced by the pineal gland, however it is also produced by other organs, including the cerebellum and ovaries. It has numerous physiological functions, such as sleep improvement, antioxidative effects, and endothelial function, and does not have toxic or mutagenic effects (27,28). A previous study demonstrated that melatonin is closely associated with expression of TLR4 and TLR4-mediated inflammation, which is key in the development of EMT (29). Melatonin may inhibit TLR4-mediated inflammation (30). Reactive oxygen species (ROS) have also been demonstrated to be important in early EMT, and increase EMT in developmental and pathological EMT (31). The present study demonstrated that melatonin inhibited TGFβ1-induced EMT in the A549 cell line. Inflammation and oxidative stress are important in the process of epithelial-mesenchymal transition and lung fibrosis. As a potent antioxidant, melatonin may prevent the development of atherosclerosis, hyperlipidemia

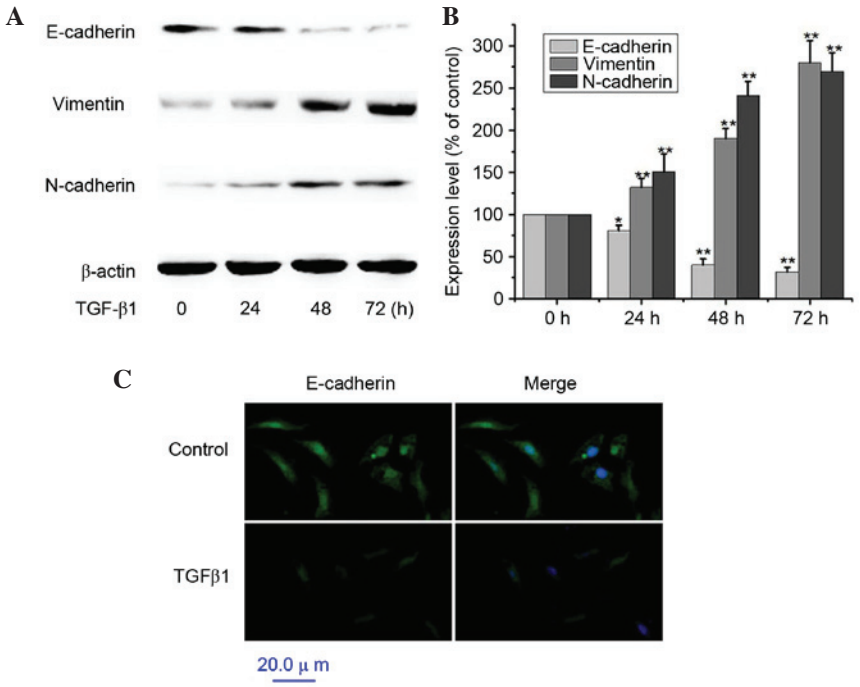

Figure 1. TGF-β1 induced epithelial-mesenchymal transition in A549 cells. (A) Western blots demonstrating the expression of E-cadherin, vimentin, and N-cadherin in total lysates of A549 cells treated with 5 ng/ml TGF-β1 for 24, 48 and 72 h. β-actin served as a loading control. (B) All experiments were repeated three times and results are presented as the mean ± standard error of the mean. (C) Immunofluorescence demonstrating the expression of E-cadherin in A549 cells treated with or without 5 ng/ml TGF-β1 for 48 h. Magnification, x100. *P<0.05 and **P<0.01 vs. the control group. TGF-β1, transforming growth factor-β1.

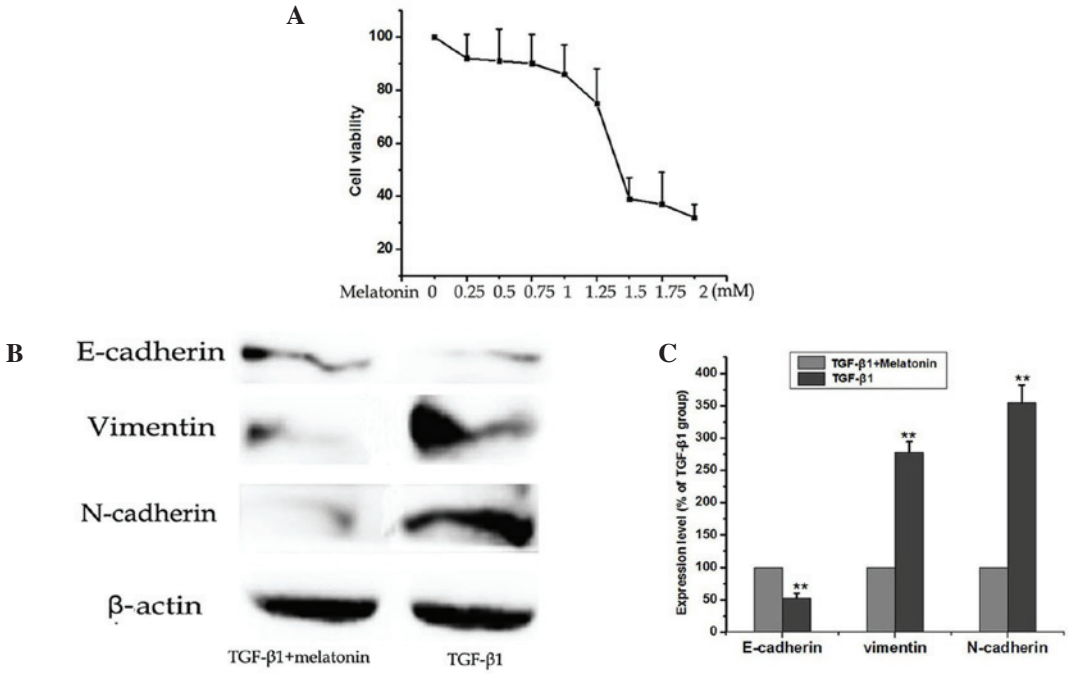

Figure 2. Effects of melatonin on epithelial-mesenchymal transition in A549 cells. (A) The A549 cells were treated with different dose (0-2 mM) of melatonin for 48 h, and the cell viability was detected using the MTT assay. All experiments were repeated three times and results are presented as the mean ± standard error of the mean. (B) Western blots demonstrating the expression of E-cadherin, vimentin, and N-cadherin in total lysates of A549 cells treated with 5 ng/ml TGF-β1 with or without melatonin (0.5 mM) for 48 h. β-actin served as a loading control. (C) All experiments were repeated three times and results are presented as the mean ± standard error of the mean. **P<0.01 vs. the TGF-β1 group. TGF-β1, transforming growth factor-β1.

and other consequences of aging (32). Melatonin may have a protective effect against ROS and EMT. The current study also indicated that the Wnt/β-catenin and Smad2/3 signaling pathways are involved in EMT in the A549 cell lines. Melatonin inhibits the Wnt/β-catenin and Smad signaling pathways and ameliorates the TGF-β1-induced EMT in A549 cells.

Melatonin markedly inhibits TGF-β1-induced activation of EMT and has a protective effect in lung fibrosis, this indicates

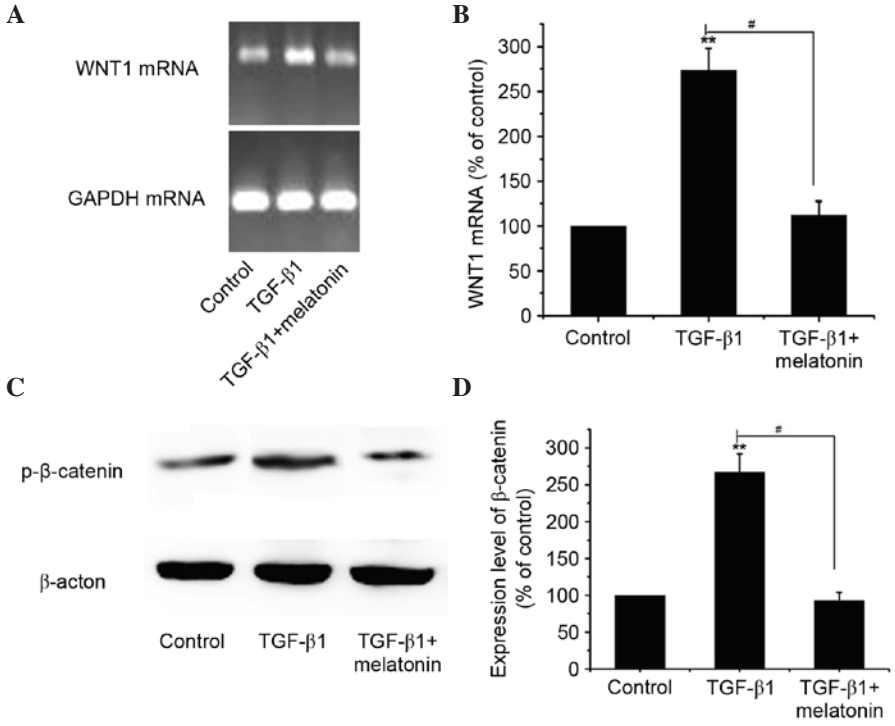

Figure 3. Effects of melatonin on the Wnt/β-catenin signaling pathway in A549 cells. (A) A549 cells were cultured with 5 ng/ml TGF-β1 with or without 0.5 mM melatonin for 48 h and the level of WNT1 mRNA was detected by reverse transcription-polymerase chain reaction. (B) All experiments were repeated three times and results are presented as the mean ± standard error of the mean. GAPDH served as a loading control. (C) Cells were treated as described above, and the expression levels of p-β-catenin were detected by western blotting. (D) All experiments were repeated three times and results are presented as the mean ± standard error of the mean. β-actin served as a loading control. **P<0.01 vs. the control group; $^{\#}$P<0.01 vs. the TGF-β1 group. TGF-β1, transforming growth factor-β1; p, phosphorylated; WNT1, Wnt family member 1.

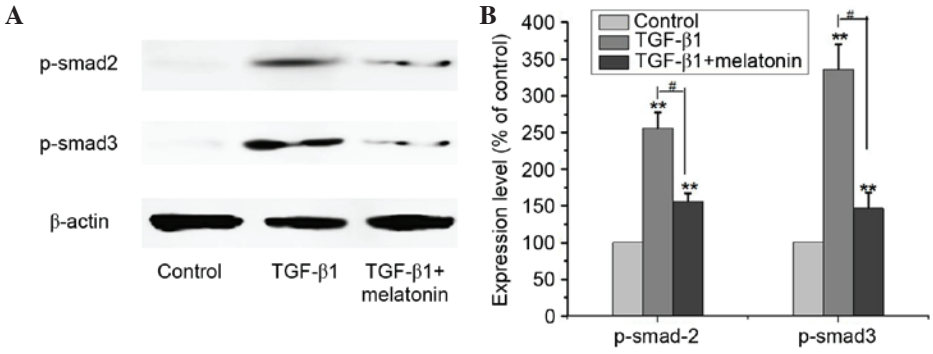

Figure 4. Effects of melatonin on Smad signaling in A549 cells. (A) A549 cells were cultured with 5 ng/ml TGF-β1 with or without 0.5 mM melatonin for 48 h and the level of p-Smad2 and p-Smad3 were detected by western blotting. (B) All experiments were repeated three times and results are presented as the mean ± standard error of the mean. β-actin served as a loading control. **P<0.01 vs. the control group; $^{\#}$P<0.01 vs. the TGF-β1 group. TGF-β1, transforming growth factor-β1; p, phosphorylated; Smad, SMAD family member.

that melatonin may be a promising therapeutic agent for fibrosis of the lung. However, further studies on suppression of pulmonary fibrosis by melatonin are required, including animal experiments, research into side effects and assessment of the effect of melatonin on experimentally-induced lung fibrosis. Furthermore, there are potential limitations in the present study. Animal experiments were not conducted and the direct impact of melatonin on pulmonary fibrosis was not assessed, however, they may be investigated in the future. IPF is closely associated with TGF-β1-induced EMT, while melatonin could inhibit TGF-β1-induced epithelial-mesenchymal transition in the A549 cell line. These studies have opened up a new road for the treatment of pulmonary fibrosis and provide a theoretical basis for clinical research.

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