Silencing of heart and neural crest derivatives expressed transcript 2 attenuates transforming growth factor-β1-enhanced apoptosis of human bronchial epithelial cells

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Abstract. Human bronchial epithelial (HBE) cells form the first protective barrier of the airway to protect patients from pulmonary diseases. The present study was performed to illustrate the mechanism underlying the effect of silencing heart and neural crest derivatives expressed transcript 2 (HAND2) on attenuating the transforming growth factor (TGF)-β1-enhanced apoptosis of HBE cells. TGF-β1 (10 μg/ml) was applied to HBE cells, and the HBE cells were transfected with small interfering RNA targeting HAND2 or were transfected with non-specific sequence. Subsequently, cell proliferation was measured using a Cell Counting kit-8 assay, whereas cell cycle and apoptosis status were measured using a flow cytometer. Reverse transcription-quantitative polymerase chain reaction and western blot analyses were performed to detect the expression levels of cell cycle- and apoptosis-related factors. Western blot analysis was also used to detect the phosphorylation levels of extracellular signal-regulated kinase (ERK), P38 and c-Jun-N-terminal kinase (JNK) of mitogen-activated protein kinase (MAPK) pathways. The results showed that TGF-β1 decreased HBE cell proliferation ability, arrested cell cycle at the G_2 phase and promoted cell apoptosis with statistical significance. The expression levels of P21 and Cyclin D1 were inhibited, and those of caspase-3, caspase-8 and caspase-9 were promoted by TGF-β1. The phosphorylation levels of ERK, P38 and JNK were increased by TGF-β1. HAND2-silencing significantly alleviated the above functions of TGF-β1 on the HBE cells. In conclusion, the silencing of HAND2 attenuated the TGF-β1-stimulated apoptosis of HBE cells through regulating cell cycle, apoptosis-related factors and ERK/P38/JNK MAPK pathways. This may provide a novel treatment strategy for pulmonary disease, with HAND2 as the novel gene target.

Introduction

Chronic obstructive pulmonary disease (COPD), asthma, pulmonary fibrosis and acute lung injury are common pulmonary diseases, which are acknowledged as public health problems. COPD is reported to rank as the fifth leading contributor to morbidity and mortality rates of chronic diseases, as a worldwide burden, according to the World Band/World Health Organization (1). The median survival rate of patients with idiopathic pulmonary fibrosis is 4-5 years (2). Airway hyper-responsiveness is considered to be a main inducing factor of pulmonary diseases. Epithelial cell injury is considered to initiate pulmonary fibrosis. Human bronchial epithelial (HBE) cells form the first protective barrier of the airway, secreting protective cytokines. The balance of pro- and anti-apoptotic mechanisms is involved in the occurrence and development of several pulmonary diseases, including COPD, asthma, pulmonary fibrosis and acute lung injury.

Previous studies have demonstrated that transforming growth factor (TGF)-β1 is important in promoting the fibrotic processes of pulmonary fibrosis (3). It is an extracellular matrix inducer and a chemotactic factor of fibroblasts (4). TGF-β1 is a multifunctional cytokine, which is involved in cell growth, differentiation, cell cycle and apoptosis. It is reported to stimulate the apoptosis of pulmonary epithelial cells (5), gastric carcinoma cells (6) and hepatocytes (7). TGF-β1 can induce apoptosis through different pathways, including the small mothers against decapentaplegic (Smad)-dependent pathway and non-Smad dependent pathway involving pathways which include the mitogen-activated protein kinase (MAPK), Fas (8,9), or extracellular signal-regulated kinase (ERK)/P38/c-Jun terminal kinase (JNK) pathway (10-12).

Although corticosteroids remain the main treatment option for pulmonary diseases, the cure rate is <30% (13). It is important to identify novel treatments for pulmonary diseases. Heart and neural crest derivatives expressed transcript 2 (HAND2), belonging to the basic helix-loop-helix family, is a transcription

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factor which is required for organ growth and development, including the heart, limb buds and branchial arches, adjusting stem cell differentiation (14). It has been found that a missense mutation of HAND2 inpatients with congenital heart disease significantly decreases the interactions of HAND2 with other key developmental genes (15). Another study showed that HAND2 was expressed in developing gut tissue and was associated with enteric neuron formation in avian species (16). Uterine tissue-specific HAND2-knockout was found to maintain epithelial proliferation by inducing paracrine mitogenic mediator function in the uterine tissues of mice. However, whether and how HAND2 possess any function on pulmonary disease remain to be elucidated.

The present study hypothesized that HAND2 is associated with HBE cell proliferation, therefore, HAND2 interference was applied to investigate whether HAND2 was able to repair TGF-β1-enhanced apoptosis in HBE cells. Variations in cell proliferation, cell cycle, apoptosis and the ERK/P38/JNK pathway were all examined in the present study. The results may provide a novel gene target and treatment strategy for pulmonary diseases.

Materials and methods

Cell culture. 16HBE cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C, in a humidified 5% CO_2-containing atmosphere. Cells of the logarithm phase were used in the following experiments.

To examine the cell morphology of the TGF-β1-treated HBE cells, 10 ng/ml TGF-β1 (R&D systems, Inc., Minneapolis, MN, USA) was added to the culture media during cell growth. The cells were observed under a light microscope following culture for 48 h.

Small interfering (si)RNA transfection. siRNA transfection was used to validate the effect of HAND2 on the TGF-β1-treated HBE cells. siRNA-HAND2 (5'-AAGATCAAGACACTGCGC CTG-3') was designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). To perform siRNA transfection, the cells were initially incubated in 6-well cell culture plates at a density of $6x10^4$ cells/well and transfected with siRNA-HAND2 (siHAND2 group) and non-specific siRNA (mock group) respectively for 2 days at 37°C, when the cells were 70% confluent, using Lipofectamine RNAi transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). HBE cells without any treatment were included as a control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses were performed to detect the levels of HAND2 in the different groups to measure the interference efficiency of siHAND2 at 48 h-post transfection.

Cell treatment. For the following experiments, the cells were divided into five groups: HBE cells transfected with siHAND2 and treated with 10 μg/ml TGF-β1 (siHAND2 + TGF-β1 group),

HBE cells transfected with non-specific sequence and treated with 10 μg/ml TGF-β1 (mock + TGF-β1 group), HBE cells only treated with 10 μg/ml TGF-β1 (TGF-β1 group), HBE cells only transfected with non-specific sequence (mock group), HBE cells without any treatment (control group).

Cell viability assay. The effect of siHAND2 on the reduced HBE cell viability induced by TGF-β1 was measured using a Cell Counting kit-8 (CCK8) assay (Beyotime Institute of Biotechnology, Haimen, China). In brief, the different groups of HBE cells were seeded in 96-well plates at a density of $5x10^3$ cells/well and incubated in a 5% CO_2-containing atmosphere at 37°C for determined times (12, 24, 48 and 72 h). Subsequently, 10 μl CCK8 was added to each well and the cells were incubated for another 4 h. The CCK8 assay is optimized from the traditional MTT assay, for the production to be detected is dissolved formazan. Therefore, the addition of organic solution to dissolve formazan is not required, which can reduce errors. In addition, the toxicity of CCK8 is lower than that of MTT. Therefore, CCK8 was selected in favor of MTT in the present study. The optical density (OD) values were read at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Cell cycle analysis. The cell cycle progression of the different cell groups was measured using a propidium iodide (PI) staining assay and detected by flow cytometry. Following washing twice with phosphate buffered saline (PBS), the cells were fixed by 70% ice-cold ethanol at 4°C and washed with PBS again. The cells were then incubated in 400 μl PI (50 μg/ml) for 30 min at room temperature, and analyzed immediately using a FACS flow cytometer (BD Biosciences, San Diego, CA, USA). The cell proportions in the G_0/G_1, S and G_2/M phases were detected.

Cell apoptosis analysis. The apoptotic rates of the different cell groups were measured using an Annexin V/PI double-stain assay (Roche Diagnostics, Inc., Indianapolis, IN, USA), according to the manufacturer's protocols. Briefly, the cells ($1x10^6$/ml) were collected and reacted with 5 μl Annexin-V fluorescein isothiocyanate and 10 μl PI. Following incubation in the dark for 15 min at room temperature, the cells were detected using a flow cytometer (BD Biosciences) and analyzed using Cell Quest Pro software version 5.1 (BD Biosciences).

RT-qPCR analysis. The cells were washed and collected, total RNA was extracted respectively from the different groups using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and reverse transcribed into cDNA using a cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The reaction system involved: RNase Free ddH2O 9.5 μl, Master Mix 12.5 μl, 10 μmol/l up primer 1 μl, 10 μmol/l down primer 1 μl and cDNA template 1 μl. The PCR amplification was performed at 95°C for 30 sec, followed by 40 cycles, including denaturation at 95°C for 5 sec, and annealing/extension at 60°C for 30 sec using Fast SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), in an ABI 7300 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Table I. Primers used in the present study.

Name	Type	Sequence (5'-3')	Size (bp)
GAPDH	Forward	CCATCTTCCAGGAGCGAGAT	222
	Reverse	TGCTGATGATCTTGAGGCTG	
HAND2	Forward	GAACCCCTACTTCCATGGCT	236
	Reverse	CGCTGTTGATGCTCTGAGTC	
Caspase-3	Forward	TGAGCCATGGTGAAGAAGGA	220
	Reverse	TCGGCCTCCACTGGTATTTT	
Caspase-8	Forward	GGAGGAGTTGTGTGGGGTAA	207
	Reverse	CCTGCATCCAAGTGTGTTCC	
Caspase-9	Forward	AAAGTTGTCGAAGCCAACCC	237
	Reverse	GACTCACGGCAGAAGTTCAC	
P21	Forward	GGATGTCCGTCAGAACCCAT	222
	Reverse	GTGGGAAGGTAGAGCTTGGG	
Cyclin D1	Forward	CCCTCGGTGTCCTACTTCAA	219
	Reverse	CTTAGAGGCCACGAACATGC	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAND2, heart and neural crest derivatives expressed transcript 2.

The quantification was conducted according to the $2^{-\Delta\Delta Cq}$ method (17). The primer sequences are listed in Table I.

Western blot analysis. The cells were lysed using the protein lysis reagent P0013 (Beyotime Institute of Biotechnology) and cell lysis was centrifuged at 10,000 x g for 5 min at 4˚C, and supernatants with proteins were collected for western blot analysis. The concentration of proteins was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Identical quantities of proteins (20 µg/well) were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (GE Healthcare Life Sciences, Chalfont, UK). Following being blocked with 5% nonfat dried milk in PBS for 1.5 h, the blotting membranes were incubated overnight respectively at 4˚C in the presence of each of the following primary antibodies: Rabbit anti-HAND2 (1:500; cat no. ab10131), anti-Cleaved-caspase-3 (1:200; cat no. ab2302), anti-caspase-8 (1:1,000; cat no. ab25901), anti-caspase-9 (1:500; cat no. ab69514), anti-P21 (1:1,000; ab227443), anti-Cyclin D1 (1:2,000; cat no. ab226977) and anti-GAPDH (1:2,000; cat no. ab9485; all Abcam, Cambridge, UK). The membranes were then washed with 0.1 mol/l TBS with 0.2% Tween-20 (Wuhan Boster Biological Technology, Ltd., Wuhan, China) 5 times, 10 min each time, and incubated with appropriate secondary antibodies conjugated with HRP (1:5,000; cat no. ab6721; Abcam) for 2 h at room temperature. The PVDF membrane was exposed to X-ray and enhanced chemiluminescence detection system reagents (GE Healthcare Life Sciences), which were used to assist in visualizing bands. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Lab Works Image Acquisition and VisionWorks®LS Analysis software version 7.0 (UVP, Inc., Upland, CA, USA) were used to quantify band intensities.

Detection of phosphorylation. The phosphorylation levels of ERK, P38 and JNK of the MAPK pathway were measured using the above western blot analysis protocol. The antibodies used were: Anti-JNK (1:1,000; cat no. ab179461), anti-phospho-JNK (1:1,000; cat no. ab124956), anti-ERK (1:1,000; cat no. ab17942), anti-phospho-ERK (1:1,000; cat no. ab201015), anti-p38 (1:2,000; cat no. 170099) and anti-phospho-p38 (1:1,000; cat no. ab47363; all Abcam).

Statistical analysis. Data are expressed as the mean ± standard deviation of at least three independent experiments. Statistical analysis was performed by SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA) and data were analyzed by one-way analysis of variance and Dunnett's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Function of TGF-β1 on HBE cell morphologies and siHAND2-interfering efficiency. The effects of TGF-β1 on HBE cell morphologies were observed under a light microscope, which showed that 10 µg/ml TGF-β1 led to fewer cells and cell shrinkage (Fig. 1A). Interference of the mRNA expression of HAND2 was induced in the HBE cells, as described above. The interference efficiency was identified by RT-qPCR and western blot analyses. Transfection with siRNA-HAND2 resulted in a significant decline in the mRNA and protein levels of HAND2 in the siHAND2 group, compared with those in the control and mock group, which suggested high interference efficiency (P<0.01; Fig. 1B-D).

siHAND2 interference recovers HBE cell viabilities inhibited by TGF-β1. The cell viabilities in the siHAND2 + TGF-β1 group, mock + TGF-β1 group, TGF-β1 group, mock group, and control group were evaluated using a CCK8 assay. It was
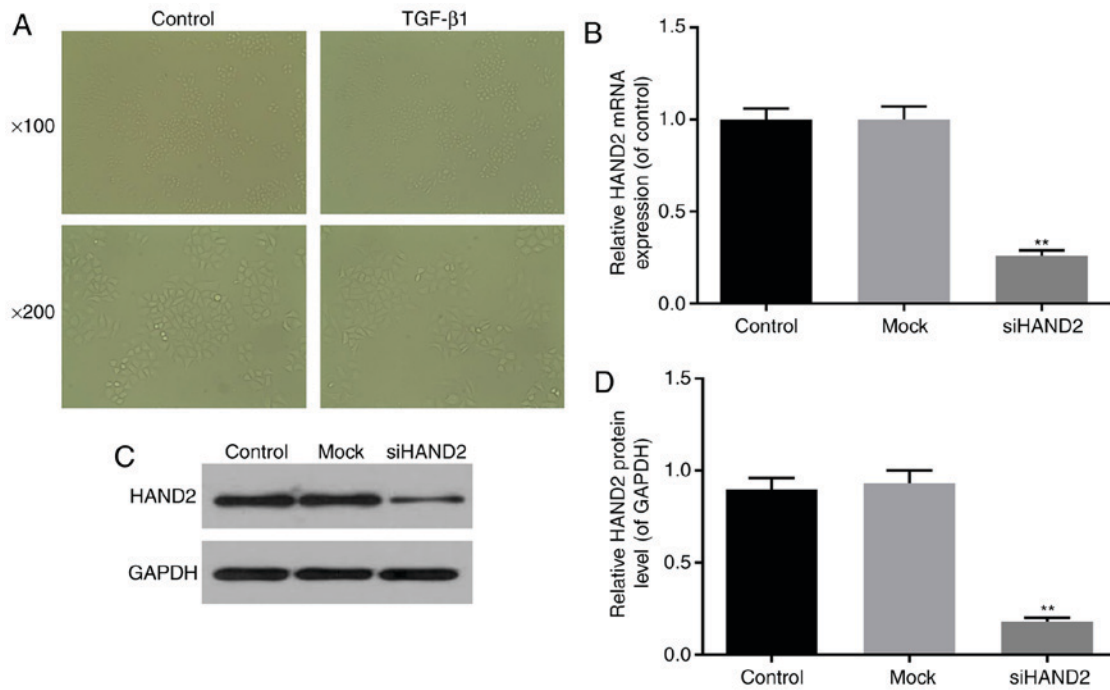
Figure 1. Function of TGF-β1 on HBE cell morphologies and determination of siHAND2 interference efficiency. (A) HBE cell morphologies were observed under a light microscope. TGF-β1 (10 μg/ml) treatment induced cell shrinkage and a reduction in cell numbers. (B) HAND2 interference efficiency on HBE cells was identified using reverse transcription-quantitative polymerase chain reaction analysis. The mRNA expression of HAND2 decreased significantly, compared with that in the control group (similar to that in the mock group; P<0.01). (C and D) HAND2 interference efficiency on HBE cells was identified using western blot analysis. The protein of HAND2 decreased significantly, compared with that in the control group (similar to that in the mock group; P<0.01). Data are presented as the mean ± standard deviation (n=3). **P<0.01 vs. control group. HBE, human bronchial epithelial; TGF-β1, transforming growth factor-β1; HAND2, heart and neural crest derivatives expressed transcript 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; si, small interfering RNA.

demonstrated that TGF-β1 significantly inhibited HBE cell viability in a time-dependent manner (12, 24, 48 and 72 h), compared with that in the control group, which was similar to that in the mock group (P<0.01). siHAND2 significantly recovered the TGF-β1-inhibited viabilities of HBE cells in the siHAND2 + TGF-β1 group, compared with that in the TGF-β1 group, which was similar to that in the mock + TGF-β1 group (P<0.05; Fig. 2).

siHAND2 interference repairs the promotion of cell cycle arrest and apoptosis induced by TGF-β1 in HBE cells. As shown in Fig. 3A and B, cell cycle status of the different groups was determined using a PI assay and apoptotic status was detected using an Annexin-V/PI assay, with the results of each analyzed using a flow cytometer. The results showed that, in the TGF-β1-treated HBE cells (TGF-β1 group), cell cycle was arrested at the G_2 phase, with the percentage of cells increased significantly in the G_2 phase and decreased significantly in the G_1 phase, compared with those in the control group (P<0.01). The transfection of cells with siHAND2 in the siHAND2 + TGF-β1 group significantly alleviated the cell cycle-inhibitory effect of TGF-β1 (P<0.01). In addition, as shown in Fig. 3C and D, the apoptotic rate was significantly promoted in the TGF-β1 group, compared with that in the control group, and siHAND2 significantly inhibited the apoptotic rate in the siHAND2 + TGF-β1 group (P<0.01).

siHAND2 restores the expression levels of cell cycle- and apoptosis-related factors in HBE cells treated with TGF-β1.
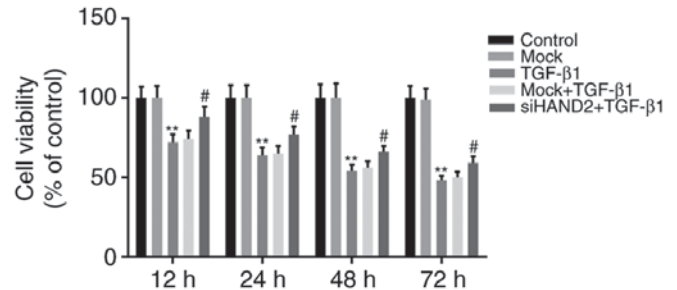

Figure 2. siHAND2 interference recovers human bronchial epithelial cell viabilities inhibited by TGF-β1. Cell viabilities of cells in the siHAND2 + TGF-β1 group, mock + TGF-β1 group, TGF-β1 group, mock group and control group were evaluated using a Cell Counting kit-8 assay. The viability of cells in the TGF-β1 group decreased significantly in a time-dependent manner (12, 24, 48 and 72 h), compared with that in the control group (P<0.01). The viability of cells in the siHAND2 + TGFβ1 group recovered significantly in a time-dependent manner, compared with that in the TGF-β1 group (similar to that in the TGF-β1 + mock group; P<0.01). Data are presented as the mean ± standard deviation (n=3). **P<0.01 vs. control group; #P<0.05 vs. TGF-β1 group. TGF-β1, transforming growth factor-β1; HAND2, heart and neural crest derivatives expressed transcript 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; si, small interfering RNA.

RT-qPCR and western blot analyses were performed to detect the expression levels of cell cycle- and apoptosis-related factors in the different groups. As shown in Fig. 4A-C, the mRNA and protein levels of cell cycle-related factors (Cyclin D1 and P21) were decreased significantly in the TGF-β1 group, compared with those in the control group. siHAND2 interference
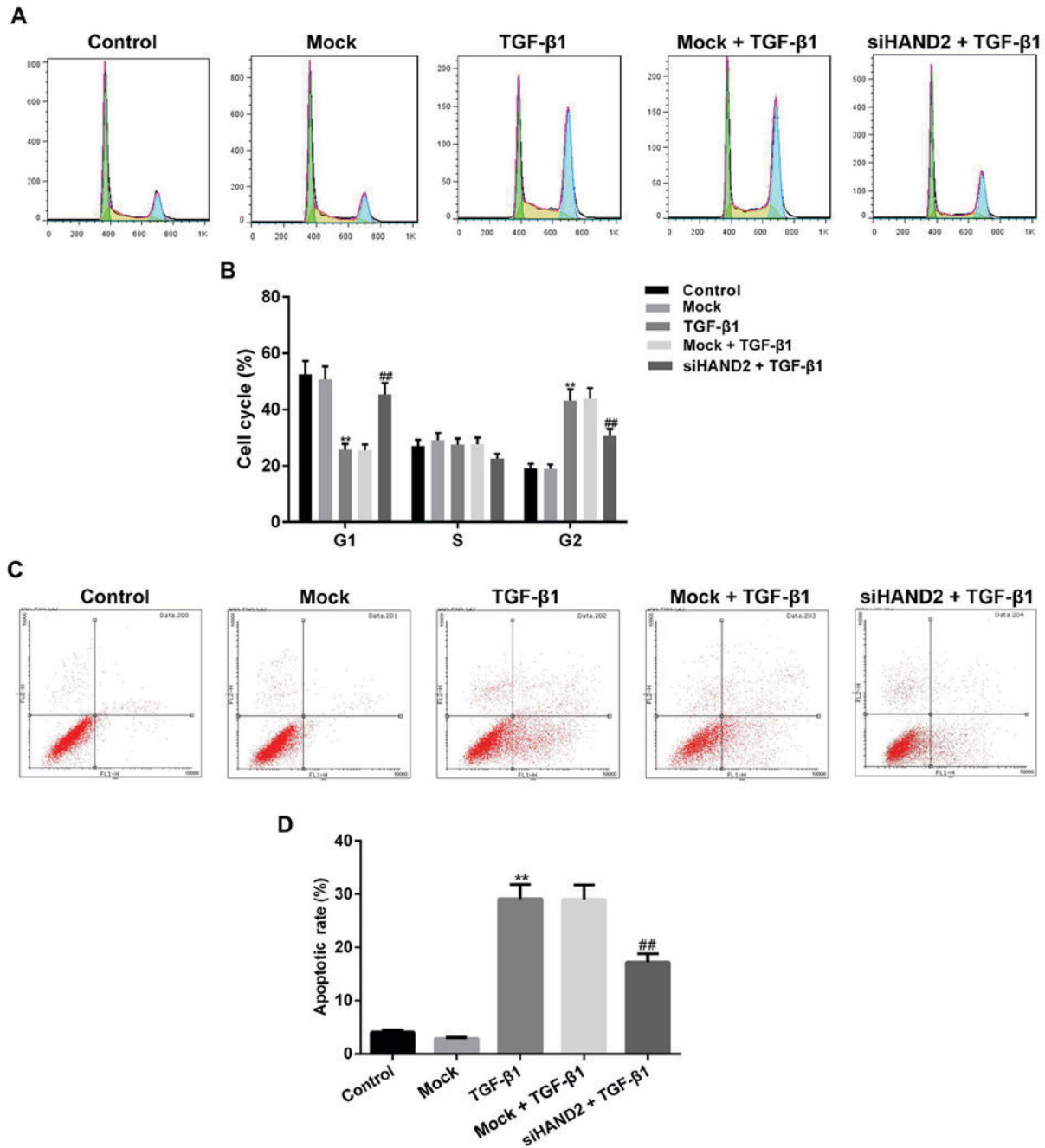
Figure 3. siHAND2 interference reverses cell cycle arrests and promotion of apoptosis induced by TGF-β1 in human bronchial epithelial cells. (A) Cell cycle status of the siHAND2 + TGF-β1 group, mock + TGF-β1 group, TGF-β1 group, mock group and control group were determined using a PI assay and analyzed by flow cytometry. (B) Cell cycle of TGF-β1 group was arrested at the G_2 phase, compared with that in the control group, and siHAND2 interference in the siHAND2 + TGF-β1 group significantly alleviated the inhibitory effect of TGF-β1 (P<0.01). (C) Cell apoptosis status was detected using an Annexin-V/PI assay and analyzed by flow cytometry. (D) Apoptotic rate was promoted in the TGF-β1 group, compared with that in the control group, and siHAND2 interference significantly inhibited the apoptotic rate in the siHAND2 + TGF-β1 group (P<0.01). Data are presented as the mean ± standard deviation (n=3). **P<0.01 vs. control group; ##P<0.01 vs. TGF-β1 group. TGF-β1, transforming growth factor-β1; HAND2, heart and neural crest derivatives expressed transcript 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; si, small interfering RNA; PI, propidium iodide.

promoted the expression levels of Cyclin D1 and P21 in the siHAND2 + TGF-β1 group, compared with those in the TGF-β1 group (P<0.01). The mRNA and protein expression levels of apoptosis-related factors (caspase-3, caspase-8 and caspase-9) were increased markedly in the TGF-β1 group, compared with those in the control group. siHAND2 interference inhibited the expression levels of caspase-3, caspase-8 and caspase-9 in the siHAND2 + TGF-β1 group, compared with those in the TGF-β1 group (P<0.01).

siHAND2 inhibited the phosphorylation of ERK/P38/JNK in the HBE cells treated with TGF-β1. Western blot analysis was performed to detect the phosphorylation levels of ERK, P38 and JNK of the MAPK pathway. As shown in Fig. 5A-C, the phosphorylation levels of ERK, P38 and JNK were increased significantly in the TGF-β1 group, compared with levels in the control groups, and were decreased in the siHAND2 + TGF-β1 groups, compared with levels in the TGF-β1 groups (P<0.01).
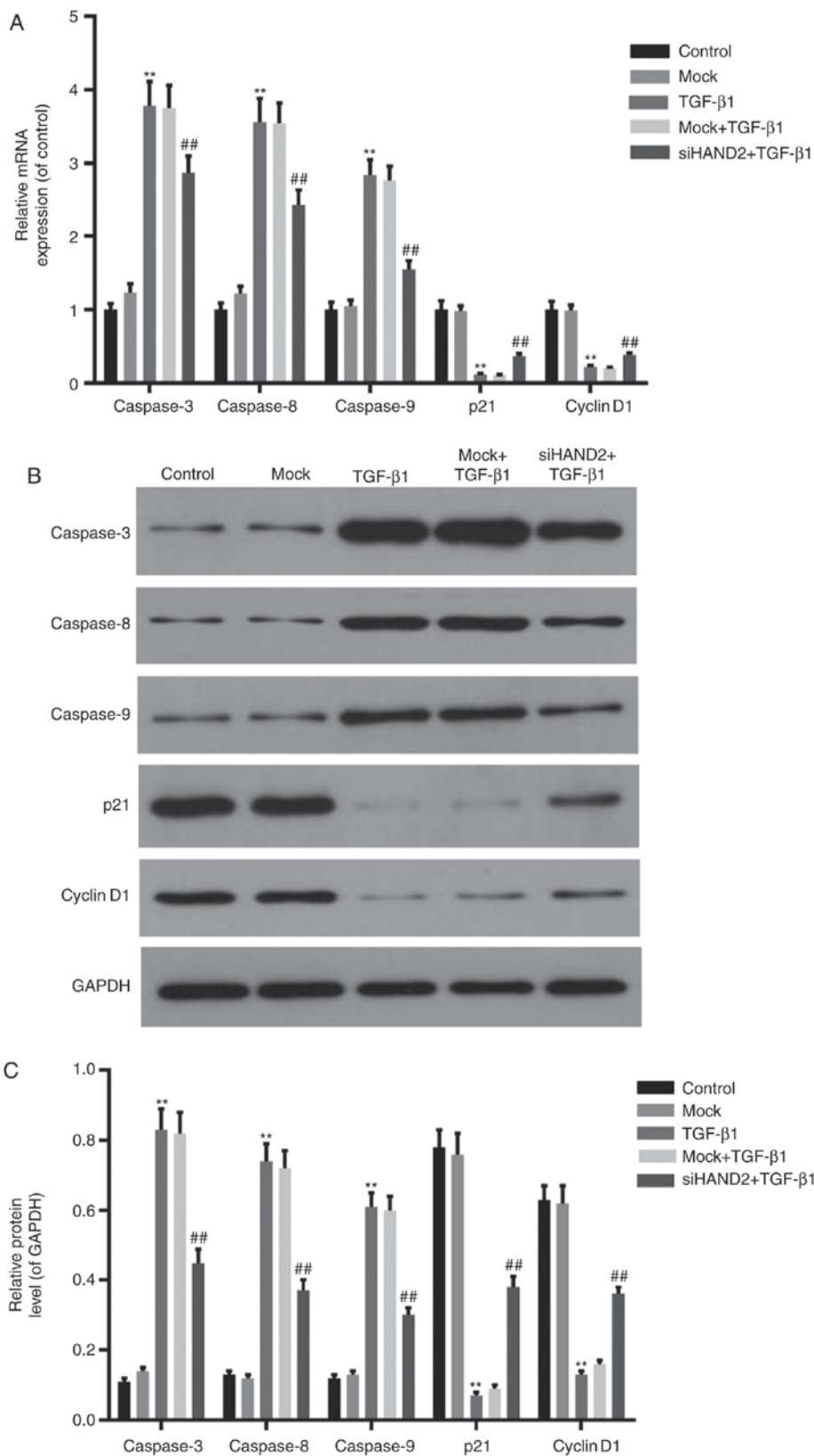
Figure 4. siHAND2 restores the expression levels of cell cycle- and apoptosis-related factors in HBE cells treated with TGF-β1 (A) Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect mRNA expression of cell cycle- and apoptosis-related factors in HBE cells of different groups. The mRNA expression of cell cycle-related factors (Cyclin D1 and P21) decreased, and apoptosis-related factors (caspase-3, caspase-8 and caspase-9) increased significantly in the TGF-β1 group, compared with those in the control group. All expression levels were recovered in the siHAND2 + TGF-β1 group, compared with those in the TGF-β1 group (P<0.01). (B and C) Western blot analysis was performed to detect protein levels of cell cycle- and apoptosis-related factors in HBE cells of different groups. The protein levels of Cyclin D1 and P21 decreased, and those of caspase-3, caspase-8 and caspase-9 increased significantly in the TGF-β1 group, compared with those in the control group. All levels were recovered in the siHAND2 + TGF-β1 group, compared with those in the TGF-β1 group (P<0.01). Data are presented as the mean ± standard deviation (n=3). **P<0.01 vs. control group; ##P<0.01 vs. TGF-β1 group. HBE, human bronchial epithelial; TGF-β1, transforming growth factor-β1; HAND2, heart and neural crest derivatives expressed transcript 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; si, small interfering RNA.
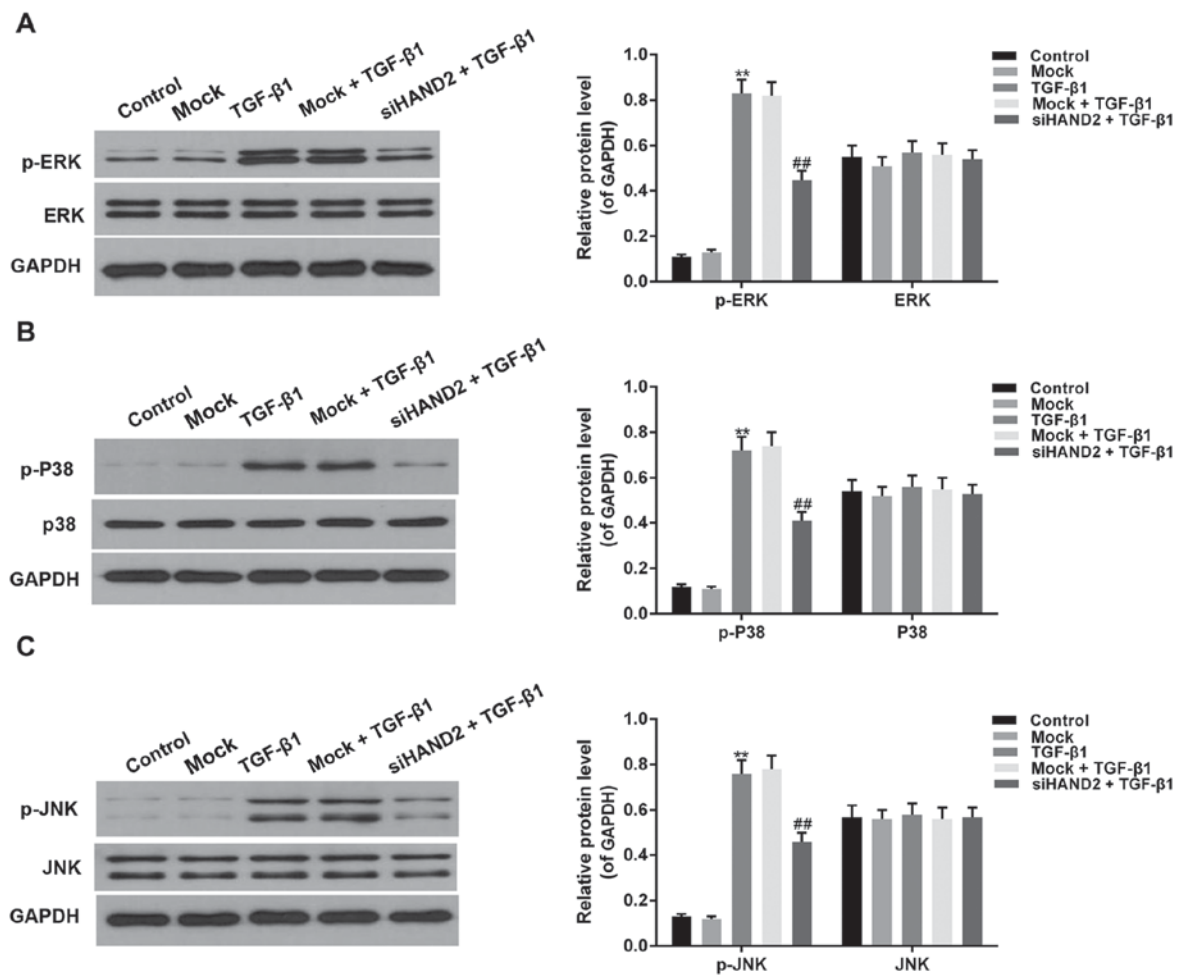
Figure 5. siHAND2 inhibits ERK/P38/JNK phosphorylation in human bronchial epithelial cells treated with TGF-β1. Western blot analysis was performed to detect the phosphorylation levels of ERK, P38 and JNK. The phosphorylation levels of (A) ERK, (B) P38 and (C) JNK increased significantly in the TGF-β1 group, compared with those in the control group, and were decreased in the siHAND2 + TGF-β1 group, compared with those in the TGF-β1 group (P<0.01). Data are presented as the mean ± standard deviation (n=3). **P<0.01 vs. control group; ##P<0.01 vs. TGF-β1 group. TGF-β1, transforming growth factor-β1; HAND2, heart and neural crest derivatives expressed transcript 2; ERK, extracellular signal-regulated kinase; JNK, c-Jun terminal kinase; p-, phosphorylated GAPDH, glyceraldehyde-3-phosphate dehydrogenase; si, small interfering RNA.

Discussion

Pulmonary diseases, including COPD and pulmonary fibrosis, are common public health problems. HBE cells represent the first protective barrier of the airway, and epithelial cell injury is considered to initiate pulmonary fibrosis. Apoptosis, also termed programmed cell death, is involved in the development of several diseases, including pulmonary diseases. In the present study, TGF-β1 was used to enhance apoptosis in HBE cells, on which the function of HAND2 interference was detected.

TGF-β1 is considered to be critical in promoting pulmonary fibrosis development (18), and to stimulate the apoptosis of pulmonary epithelial cells through different signaling pathways, including the ERK/P38/JNK pathway. The present study verified that treatment of HBE cells with TGF-β1 (10 µg/ml) resulted in a decreased number of cells and cell shrinkage, indicating that TGF-β1 caused damage to the HBE cells. HAND2 is a transcription factor, which is associated with the growth and development of organs, including the heart, limb buds and branchial arches. HAND2-knockout has been previously been reported to induce epithelial proliferation. In

the present study, thesiRNA-HAND2 sequence was used to establish a HAND2-silencing model. RT-qPCR and western blot analyses were performed to detect the efficiency of HAND2 interference, revealing high interference efficiency.

TGF-β1 (10 µg/ml) was used to treat HBE cells, mock cells and siHAND-treated HBE cells, following which cell proliferation, cell cycle, apoptosis variations were measured. The results showed that cell proliferation was decreased significantly in the TGF-β1-treated HBE cells in a time-dependent manner, compared with that in the control group. When HAND2 was silenced in the HBE cells prior to TGF-β1 treatment, cell proliferation increased significantly in a time-dependent manner, compared with that in cells treated with TGF-β1 only. Therefore, HAND2 interference promoted HBE cell proliferation when treated with TGF-β1. To identify the mechanism underlying the effect of HAND2 interference on the promotion of HBE cell proliferation, flow cytometry was used to detect cell cycle progression and apoptotic status. When treated with TGF-β1, the HBE cell cycle was arrested at the G_2 phase, with cells in the G_2 phase increased significantly, compared with those in the control group. However, HAND2 interference almost recovered the condition. Higher cell

apoptotic rates were detected in the TGF-β1-treated HBE cells, compared with the HBE cells without any treatment; whereas HAND2 interference markedly decreased the apoptotic rate. These results indicated that TGF-β1 inhibited HBE cell proliferation, accompanied by cell cycle arrest at the G_2 phase and promotion of cell apoptosis, whereas HAND2 interference in the HBE cells repaired all these effects induced by TGF-β1.

To examine the molecular mechanisms underlying the effect of HAND2 interference on the repair of TGF-β1-induced inhibition of HBE cell proliferation and promotion of apoptosis, the present study attempted to identify the expression levels of cell cycle- and apoptosis-related factors. Cyclin D1 is the most representative factor in promoting cells transferring from the G_1 phase to the S phase. Cyclin-dependent kinase (CDK) inhibitor 1A (p21) is an inhibitor of cell cycle, known for its extensive kinase inhibitory activity (19-22). It has been reported that TGF-β1 can induce lung epithelial cell apoptosis, through downregulating the expression of p21. The decrease of p21 promoted TGF-β1-stimulated apoptosis (23,24), It is possible that, in the presence of TGF-β1, p21 protects HBE cells from apoptosis (25,26). Previous studies have found that the activation of caspase-3 is regulated by p21, and the complex formation of procaspase-3-p21 is an essential mechanism for cell survival (27,28). Therefore, the downregulation of p21 by TGF-β1 may activate caspase-3 to enhance apoptosis. The results of the present study verified that TGF-β1 treatment inhibited the expression of P21 and Cyclin D1 in HBE cells, whereas siHAND2 interference recovered the expression of P21 and Cyclin D1 in HBE cells treated with TGF-β1, to inhibit cell apoptosis, recover cell cycle progression and promote cell proliferation. Caspases (cysteinyl aspartate-specific proteinases), are a family of protease enzymes which are closely associated with cell apoptosis, and are involved in cell development and differentiation (29). The activation of caspase-3 is a key element in the TGF-β1-induced apoptotic signaling pathway. Caspase-8 and caspase-9 are important initiator caspases, the activation of which cleaves and stimulates caspase-3, functioning on downstream factors. The results of the present study demonstrated that TGF-β1 treatment promoted the expression of apoptosis-activating factors, including caspase-3, caspase-8 and caspase-9, to promote HBE cell apoptosis. In addition, siHAND2 effectively decreased the promotion of apoptosis induced by TGF-β1 in HBE cells. As Hagimoto *et al* reported, B-cell lymphoma-2-related proteins were not affected by the addition of TGF-β1 in small airway epithelial cells (23). In addition, there are several other biomarkers involved in cell cycle and apoptosis, including CDKs (CDK4 and CDK6). It was a potential limitation that these were not investigated in the present study.

Subsequently, the present study performed western blot analysis to determine whether the effect of HAND2 interference on TGF-β1-enhanced apoptosis was mediated by the ERK/P38/JNK signaling pathway. ERK, a critical factor of the MAPK family, transmits signals from the cytoplasm to the nucleus, when it is activated as a phosphorylated form, and functions in cell development and differentiation (30,31). The P38 and JNK pathways are activated to induce the release of cytochrome *c* from mitochondria, and to activate

caspase-9 and promote cell apoptosis. The phosphorylation levels of ERK, P38 and JNK were markedly increased in the TGF-β1-treated HBE cells, and decreased significantly with siHAND2 interference in the HBE cells. This indicated that siHAND2 interference promoted HBE cell proliferation and inhibited apoptosis through the ERK/P38/JNK signaling pathway in HBE cells treated with TGF-β1.

In conclusion, the novel function of HAND2 interference on repairing TGF-β1-inhibited cell proliferation, arrested cell cycle and promoted apoptosis, through regulating cell cycle- and apoptosis-related factors and the ERK/P38/JNK pathway, maybe considered as a novel strategy in the treatment of pulmonary diseases. To address the limitation of using only one cell line in the present study, future investigations using other pulmonary cell lines or *in vivo* may be performed to support these conclusions.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XJ conducted all the experiments in the present study.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

All patients involved provided consent for publication.

Competing interests

The authors declare that they have no competing interests.

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