Exogenous BMP9 promotes lung fibroblast HFL-1 cell activation via ALK1/Smad1/5 signaling *in vitro*

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Abstract. Bone morphogenetic protein 9 (BMP9) has recently been described as a crucial regulator in modulating fibroblast-type cell activation. Activin receptor-like kinase 1 (ALK1) is a high affinity receptor for BMP9 that exerts its role via Smad1/5. However, the functional roles of BMP9 in activating lung fibroblasts and the underlying signaling pathway are not completely understood. The present study aimed to explore the effect of exogenous BMP9 on human lung fibroblast HFL-1 cell proliferation and differentiation, as well as the potential role of the ALK1/Smad1/5 signaling pathway. In the present study, fibroblast proliferation was assessed using Cell Counting Kit-8 and colony formation assays, and the mRNA and protein expression of target genes was examined using reverse transcription-quantitative PCR and western blot assays, respectively. Compared with the control group, BMP9 treatment increased HFL-1 cell proliferation, mRNA and protein expression of differentiated markers, including α-smooth muscle actin, type I collagen and type III collagen, and the expression of ALK1 and phosphorylated Smad1/5 expression. Furthermore, the effects of BMP9 were partially rescued by dorsomorphin-1, an inhibitor of ALK1. The results indicated that BMP9 may serve as a key inducer of lung fibroblast activation and ALK1/Smad1/5 signaling might be associated with BMP9-mediated effects in HFL-1 cells. Therefore, the present study highlighted that the potential role of the BMP9/ALK1/Smad1/5 signaling pathway in the development of pulmonary fibrosis requires further investigation.

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Introduction

Pulmonary fibrosis is a chronic interstitial lung disease that can destroy the normal architecture of lung tissues and lead to progressive respiratory dysfunction (1). Fibroblasts are the major effector cells during pulmonary fibrosis due to their ability to differentiate into myofibroblasts, which can synthesize excess extracellular matrix (ECM) proteins, resulting in excessive ECM deposition and pulmonary fibrosis (2). The key difference between quiescent fibroblasts and activated fibroblasts (myofibroblasts) manifests as aberrant proliferative capacity and increased production of α-smooth muscle actin (α-SMA) and ECM proteins, such as type I collagen (collagen I), type III collagen (collagen III), connective tissue growth factor (CTGF) and fibronectin, when fibroblasts are activated (3). Therefore, targeting fibroblast activation may serve as a therapeutic strategy for pulmonary fibrosis.

The transforming growth factor-β (TGF-β) family, including TGF-β, bone morphogenetic proteins (BMPs) and activins, is involved in the development of fibrotic diseases (4-6). TGF-β1 is widely considered as the primary cytokine responsible for the induction of fibroblast activation (7); however, the precise functions of other BMPs, such as BMP9, in activating fibroblasts and fibrosis development are not completely understood.

BMP9 was originally identified in liver tissues and considered as an important participant of several biological functions, including hepatocyte proliferation, chondrogenesis, angiogenesis, glucose metabolism and neuronal differentiation (8-12). BMP9 primarily binds to its high affinity receptor activin receptor-like kinase 1 (ALK1) to phosphorylate Smad1/5 and regulate gene transcription (13,14). In addition, non-Smad signaling proteins, such as mitogen-activated protein kinases, PI3K and c-met, can also be activated by BMP9 (15,16). The BMP9/ALK1 signaling pathway is involved in the angiogenesis and metastasis of tumors (17-19). For instance, exogenous BMP9 can significantly inhibit breast cancer MDA-MB-231 cell invasion and migration *in vitro* and *in vivo* (19). However, BMP9 is overexpressed in hepatocellular carcinoma tissues, and can induce epithelial-mesenchymal transition and promote hepatocellular carcinoma metastasis (20). BMP9 also induces proliferation in multiple types of endothelial cells *in vitro* and promotes tumor angiogenesis and metastasis in a xenograft model of pancreatic cancer (21).

It has been reported that BMP9 is a vital cytokine that regulates the activation of fibroblasts and development of fibrosis (9,22-26). Increasing studies have indicated that BMP9 has a bidirectional response to fibroblast activation and fibrotic diseases (9,22-26). Breitkopf-Heinlein *et al* (9) indicated that BMP9 serves as a profibrogenic cytokine in hepatic tissues. Selective inhibition or deletion of BMP9 in mice results in reduced liver fibrosis compared with the control group, and BMP9 overexpression promotes hepatic stellate cell proliferation and migration, which are the major fibroblast-type cells involved in hepatic fibrosis (9). Moreover, Li *et al* (22) demonstrated a positive correlation between serum levels of BMP9 and the stage of fibrosis in 52 patients, suggesting that BMP9 might serve as an early diagnostic marker for hepatic fibrosis (22). BMP9 also stimulates mouse embryo fibroblast activation and increases the expression of fibronectin and collagen I by triggering Smad1/5 signaling (23). By contrast, other studies have identified an inhibitory role of BMP9 and its receptor ALK1 during fibrogenesis. For instance, Morine *et al* (24) observed that BMP9 is expressed by cardiac fibroblasts, but its expression is upregulated in the circulation and left ventricle of human patients with heart failure (24). Furthermore, BMP9 decreases the synthesis of collagen I in cardiac fibroblasts, and loss of BMP9 activity increases cardiac fibrosis by inhibiting Smad1 phosphorylation (24). Meanwhile, ALK1 has been reported to suppress cardiac fibrosis by maintaining Smad1 activity in a mouse model of heart failure (25). Moreover, reduced levels of ALK1 have also been reported to accelerate heart failure without increasing markers of cardiac fibrosis, such as collagen I, CTGF or plasminogen activator inhibitor 1 (26). Therefore, the contribution of BMP9 to fibroblast activation and fibrogenesis requires further investigation. Certain strategies targeting BMP9 have been applied in the preclinical treatment of fibrotic diseases and have displayed positive effects (9,16,22,27). For example, mice treated with BMP9-short hairpin RNA and monoclonal antibodies display limited liver fibrosis (27).

At present, there is no direct evidence that BMP9 regulates pulmonary fibrosis. However, several studies have indicated that BMP9 serves a pivotal role in several chronic lung diseases related to fibrosis, such as bronchopulmonary dysplasia and pulmonary arterial hypertension (28,29). In the present study, the role of BMP9 in regulating human lung fibroblast proliferation and differentiation, as well as underlying signaling pathway, were investigated.

Materials and methods

Cell culture. Human fetal lung fibroblasts HFL-1 (The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were cultured in DMEM (HyClone; Cytiva) supplemented with 6% FBS (HyClone; Cytiva) and penicillin/streptomycin solution (Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C in a humidified incubator with 5% CO_2. Cells in the exponential growth phase were used for subsequent experiments. For the experiment of rBMP9 treatment, DMEM without rBMP9 is used as control. For the experiment of rBMP9 combined with dorsomorphin-1 (Dor), DMEM with 0.1% dimethyl sulfoxide is used as control.

Table I. Sequences of the primers used for reverse transcription-quantitative PCR.

Gene	Sequence (5'-3')
α-SMA	F: AGCGTGGCTATTCCTTCGT
	R: CTCATTTTCAAAGTCCAGAGCTACA
Collagen I	F: AACCAAGGCTGCAACCTGGA
	R: GGCTGAGTAGGGTACACGCAGG
Collagen III	F: CTCCTGGGATTAATGGTAGT
	R: CCAGGAGCTCCAGGAAT
GAPDH	F: AGAAGGCTGGGGCTCATTTG
	R: AGGGGCCATCCACAGTCTTC

α-SMA, α-smooth muscle actin; F, forward; R, reverse.

Cell viability assay. A Cell Counting Kit-8 (CCK-8) assay (Beijing Transgen Biotech Co., Ltd.) was used to detect cell viability. Briefly, HFL-1 cells ($1x10^3$) were cultured in a 96-well plate for 24 h and starved in DMEM with 0.1% FBS at 37˚Covernight. Subsequently, cells were treated with fresh DMEM containing: i) Different concentrations of recombinant BMP9 (rBMP9; 0, 2, 10 or 50 ng/ml; BioLegend, Inc.) at 37˚C for 24, 48 or 72 h; or ii) 50 ng/ml rBMP9 with different concentration of Dor (0, 0.1, 0.5 or 1 μM; MedChemExpress LLC), a BMP type I receptor ALK1 inhibitor, at 37˚C for 48 h. Subsequently, 10 μl CCK-8 solution was added to each well. After incubation at 37˚C for 30 min, the absorbance of each well was measured at a wavelength of 450 nm using a multi-mode microplate reader (Molecular Devices, LLC). The absorbance value recorded was directly proportional to the number of viable cells.

Colony formation assay. Briefly, HFL-1 cells ($1x10^3$) were subcultured in 6-cm dishes at 37˚C for 24 h. Subsequently, cells were treated with fresh DMEM containing different concentrations of rBMP9 (0, 2, 10 or 50 ng/ml) in the presence or absence of 0.1 μM Dor at 37˚C. The culture medium was replaced every 3 days. Following incubation for 10 days, cells were fixed with 4% paraformaldehyde at room temperature for 20 min and stained with 1% crystal violet (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 15 min. The images of 6-cm dish were obtained by Scanner. The number of colonies (the cell clusters >0.5 mm is defined as a colony) in each 6-cm dish was calculated using ImageJ software (version 1.80; National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). Following incubation with 50 ng/ml rBMP9 and/or 0.1 μM Dor for 48 h, HFL-1 cells ($5x10^6$) in a 6-cm dish were washed twice with ice-cold PBS. Total RNA was extracted from cells using TRIzol® reagent (Beijing Transgen Biotech Co., Ltd.) according to the manufacturer's protocol. Total RNA was reverse transcribed to cDNA using the Reverse Transcription System (cat. no. A3500; Promega Corporation). The temperature protocol for reverse transcription was 42˚C for 15 min, 95˚C for 5 min and 4˚C for 5 min. Subsequently, qPCR was
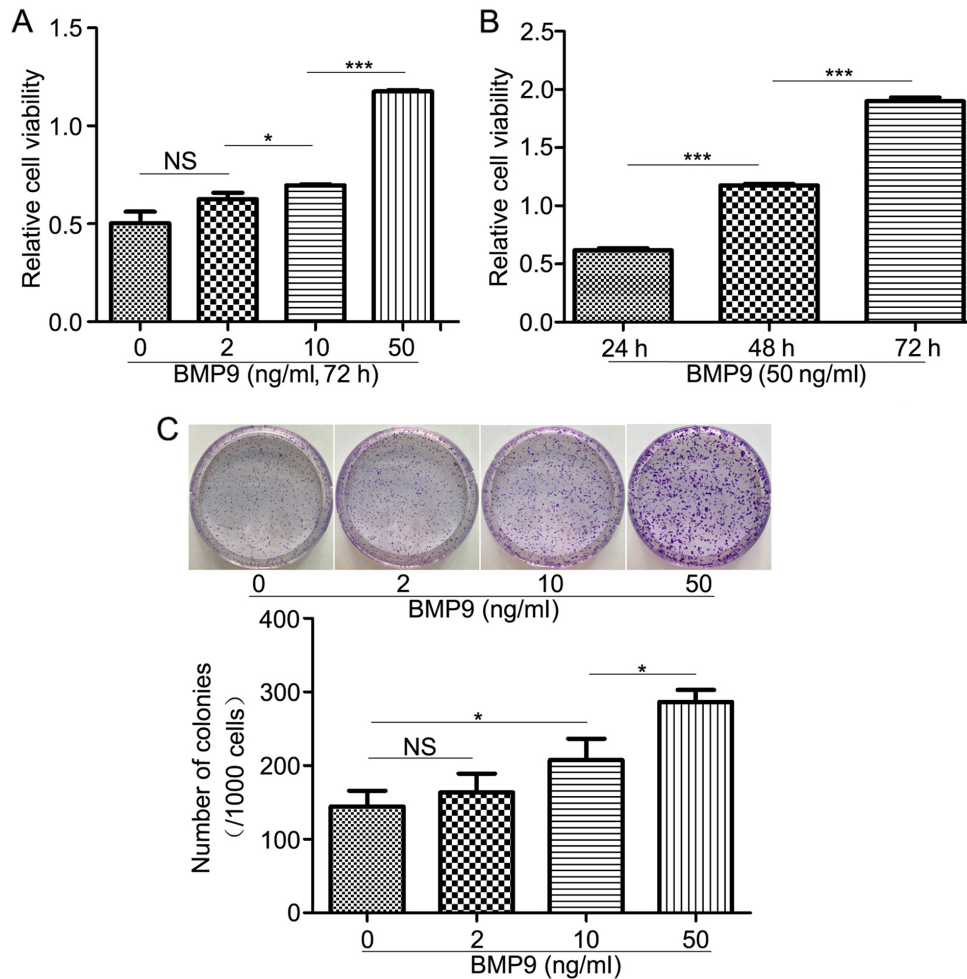
Figure 1. BMP9 promotes HFL-1 cell proliferation. HFL-1 cells were incubated with (A) different concentrations of BMP9 or (B) 50 ng/ml BMP9 for different incubation periods. (C) Effect of BMP9 on HFL-1 cell colony formation. *P<0.05 and ***P<0.001. BMP9, bone morphogenetic protein 9; NS, not significant.

performed using the SYBR Green kit (Beijing Transgen Biotech Co., Ltd.) and an ABI Prism 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation for 10 min at 95°C; followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 10 sec. The sequences of the primers used for qPCR are listed in Table I. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (30) and normalized to the internal reference gene GAPDH.

Western blotting. Briefly, HFL-1 cells ($5x10^6$) were cultured in 6-cm dish with different concentrations of rBMP9 (0, 2, 10 or 50 ng/ml) or 0.5 μM Dor at 37°C for 24 or 48 h. Cells were then washed twice with ice-cold PBS. Total protein was extracted from cells using RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) on ice. Cell debris was discarded by centrifugation at 12,000 x g at 4°C for 5 min. Total protein was quantified using a Bicinchoninic Acid Protein Assay kit (cat. no. P0010; Beyotime Institute of Biotechnology). Proteins (40 μg/lane) were separated via 8-10% SDS-PAGE and transferred to nitrocellulose membranes (EMD Millipore). The membranes were blocked with 5% non-fat milk for 1 h at room temperature. Subsequently, the membranes were

incubated overnight at 4°C with primary antibodies targeted against: ALK1 (1:500; cat. no. DF3170; Affinity Biosciences), collagen I (1:500; cat. no. AF7001; Affinity Biosciences), collagen III (1:500; cat. no. AF5457; Affinity Biosciences), α-SMA (1:1,000; cat. no. ab124964; Abcam), phosphorylated (p)-Smad1/5 (1:1,000; cat. no. CST9516; Cell Signaling Technology, Inc.), total-Smad1/5 (1:1,000; cat. no. CST6944; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. T0004; Affinity Biosciences). Following primary incubation, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. nos. S0001 and S0002; Affinity Biosciences) for 2 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence kit (EMD Millipore). Protein expression was quantified using ImageJ software (version 1.80; National Institutes of Health) with GAPDH as the loading control (31).

Statistical analysis. Data are presented as the mean ± standard deviation from at least three independent experiments. Statistical analyses were performed using GraphPad Prism software (version 5; GraphPad Software, Inc.). Comparisons between two groups were analyzed using the unpaired Student's t-test. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc
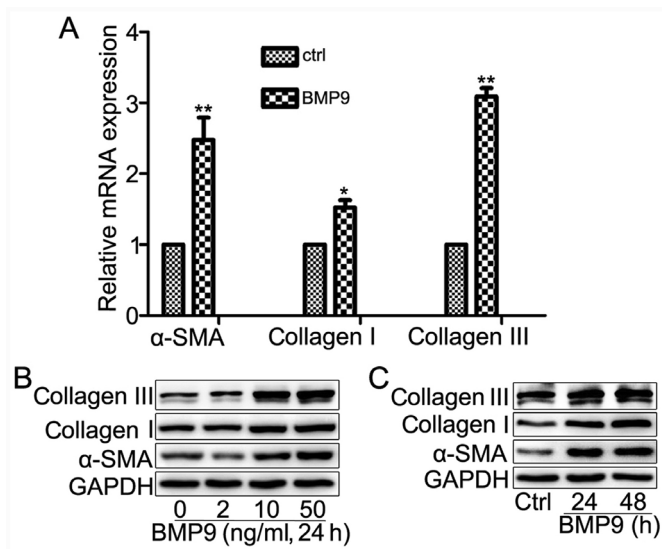
Figure 2. BMP9 increases HFL-1 cell transdifferentiation. (A) mRNA expression levels of α-SMA, collagen I and collagen III. Effect of (B) different concentrations of BMP9 and (C) incubation with BMP9 for different time periods on the protein expression levels of α-SMA, collagen I and collagen III. *P<0.05 and **P<0.01 vs. ctrl. BMP9, bone morphogenetic protein 9; α-SMA, α-smooth muscle actin; ctrl, control.
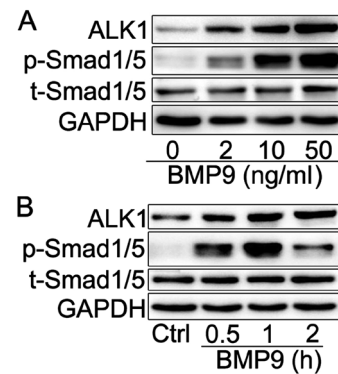

Figure 3. BMP9 activates ALK1/Smad1/5 signaling in HFL-1 cells. Effect of (A) different concentrations of BMP9 and (B) incubation with BMP9 for different time periods on the protein expression levels of ALK1, p-Smad1/5 and t-Smad1/5. BMP9, bone morphogenetic protein 9; ALK1, activin receptor-like kinase 1; p, phosphorylated; t, total; ctrl, control.

test. P<0.05 was considered to indicate a statistically significant difference.

Results

BMP9 increases HFL-1 cell proliferation. Abnormal proliferation is a characteristic of lung fibroblast activation (32). Therefore, to determine whether BMP9 altered the proliferation of HFL-1 cells, the CCK-8 assay was performed to assess HFL-1 cell proliferation following treatment with BMP9. BMP9 enhanced HFL-1 cell proliferation in a concentration- and time-dependent manner (Fig. 1A and B). Similarly, the colony formation assay suggested that BMP9 increased colony formation in a dose-dependent manner (Fig. 1C). The results indicated that BMP9 stimulation promoted HFL-1 cell proliferation.

BMP9 induces HFL-1 cell differentiation. To investigate whether BMP9 served an important role in lung fibroblast activation, the effect of BMP9 on HFL-1 cell transdifferentiation was investigated. Firstly, the mRNA expression levels of myofibroblast marker α-SMA, and ECM markers collagen I and collagen III were assessed. The results indicated that BMP9 significantly increased the expression levels of α-SMA, collagen I and collagen III in HFL-1 cells compared with the control group (Fig. 2A). Furthermore, the protein expression levels of collagen I, collagen III and α-SMA were increased in a dose- and time-dependent manner by BMP9 treatment (Fig. 2B and C). The results indicated that BMP9 induced HFL-1 cell transdifferentiation and increased HFL-1 cell ECM protein synthesis.

BMP9 activates Smad1/5 signaling in HFL-1 cells. In the majority of cell types, BMP9 activates Smad1/5 to regulate its biological function via ALK1, a receptor with high affinity to BMP9 (13,14). To assess whether BMP9 promoted HFL-1 cell

proliferation and differentiation via ALK1/Smad1/5 signaling, the expression levels of ALK1 expression and p-Smad1/5 were assessed via western blotting. BMP9 increased the expression levels of ALK1 and p-Smad1/5 in a dose-dependent manner. At 10 ng/ml, BMP9 increased the expression levels of ALK1 and p-Smad1/5 in a time-dependent manner (Fig. 3B), reaching a maximal level at the 1 h time point. In addition, the expression level of p-Smad1/5 decreased when HFL-1 cells were exposed to BMP9 for 2 h compared with 1 h, which may be associated with the rapid turnover of p-Smad1/5 in HFL-1 cells. Lo *et al* (33) suggested that once Smad proteins enter the nucleus, they might be degraded by ubiquitination. Therefore, the results suggested that BMP9 might trigger Smad1/5 activation via ALK1 in HFL-1 cells, which may serve as a mechanism underlying BMP9-induced HFL-1 cell activation.

ALK1 inhibitor suppresses the effects of BMP9 in HFL-1 cells. To investigate the effect of BMP9 on HFL-1 cells via ALK1/Smad1/5 signaling, the inhibitory role of Dor, an ALK1 inhibitor, on BMP9-mediated HFL-1 cell responses was assessed. Dor notably reduced BMP9-induced ALK1 and p-Smad1/5 expression levels (Fig. 4A). Dor also significantly inhibited BMP9-induced cell viability and colony formation (Fig. 4B and C). Similarly, Dor decreased BMP9-mediated upregulation of α-SMA, collagen I and collagen III expression (Fig. 4D and E). The results further suggested that BMP9 increased HFL-1 cell proliferation and differentiation via activating ALK1/Smad1/5 signaling.

Discussion

Fibroblast activation is a major mechanism underlying the fibrotic process that occurs during pulmonary fibrosis, in which a variety of cytokines have been reported to be associated with, such as TGF-β1 (34), BMP4 and BMP7 (35). TGF-β1 is a profibrotic cytokine (34), whereas BMP4 displays antifibrotic properties (35). However, the effect of BMP9 on lung fibroblast activation is not completely understood. The present study suggested that BMP9 was a profibrotic regulator that displayed a key role in inducing HFL-1 cell proliferation and differentiation via Smad1/5 activation. Therefore, the
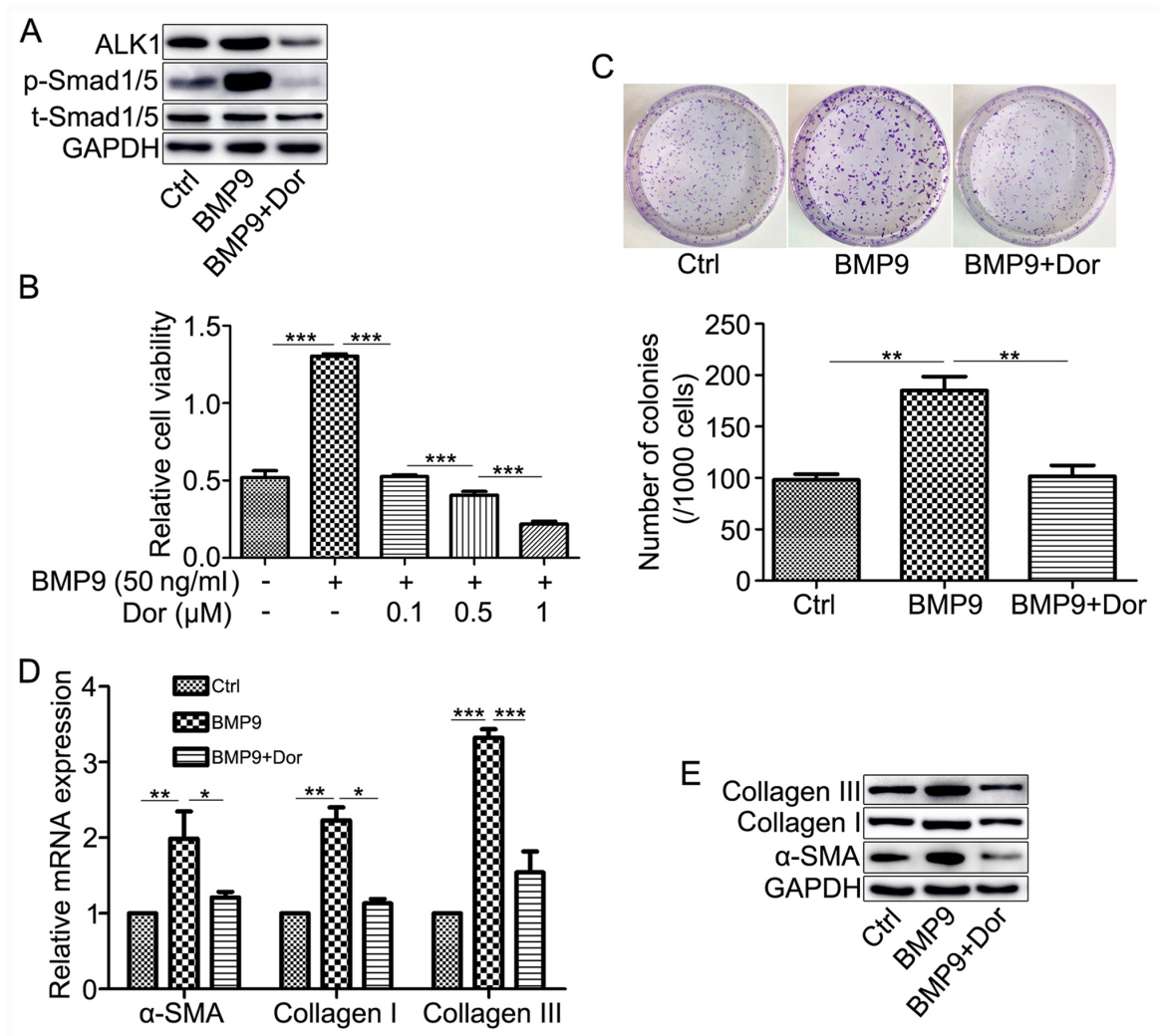
Figure 4. ALK1 inhibitor Dor suppresses the effects of BMP9 on HFL-1 cells. (A) Protein expression levels of α-SMA, collagen I and collagen III. (B) Cell viability was detected by performing the Cell Counting Kit-8 assay. (C) Colony formation was measured by performing colony formation assays. (D) mRNA and (E) protein expression levels of α-SMA, collagen I and collagen III. *P<0.05, **P<0.01 and ***P<0.001. ALK1, activin receptor-like kinase 1; Dor, dorsomorphin-1; BMP9, bone morphogenetic protein 9; α-SMA, α-smooth muscle actin; ctrl, control.

present study suggested a potential mechanism underlying pulmonary fibrosis and indicated that BMP9 may serve as a potential therapeutic target for fibroblast activation-associated lung diseases, such as lung fibrosis and cancer.

BMP9 is primarily synthesized in hepatic tissues, but actively circulates in the bloodstream (36), performing physiological and pathophysiological functions, such as angiogenesis (11), neuronal differentiation (12), osteogenic development (37) and carcinogenesis (38). Previous studies have indicated that BMP9 is a key regulator of fibrogenesis (22-26). For example, BMP9 promotes liver fibrosis as evidenced by rBMP9 increasing human hepatic stellate cell proliferation and migration, whereas BMP9 inhibition or knockout reduces liver fibrosis (22). By contrast, BMP9 decreases the synthesis of collagen I in cardiac fibroblasts, and loss of BMP9 activity increases cardiac fibrosis (24). Similarly to previous studies, the present study indicated that rBMP9 significantly promoted cell proliferation and induced differentiation into myofibro-blasts, as evidenced by increased α-SMA and ECM protein (collagen I and collagen III) expression levels, which were suppressed by BMP type I receptor inhibitor. Therefore, the

present study supported the hypothesis that BMP9 may induce lung fibroblast activation and serve as a profibrotic cytokine during lung fibrosis. The role of BMP9 in lung fibrosis requires further investigation in an animal model of pulmonary fibrosis or in patients with pulmonary fibrosis.

Additionally, accumulating studies have demonstrated that fibroblast-type cells are a key host cell type in tumor metastasis via microenvironmental modulation, and fibroblast activation is a critical event in tumor progression and metastasis (39-42). A number of ECM proteins have been identified, including an elevated deposition of fibroblast-derived fibronectin, in the tumor microenvironment (43-45). Moreover, previous studies have indicated that the transformation of normal lung fibroblasts to a myofibroblast phenotype and the formation of cancer-associated fibroblasts promoted tumor progression in non-small cell lung cancer (46-48). Therefore, targeting cancer-associated fibroblasts is an important alternative treatment strategy for lung cancer. The present study further suggested that exogenous BMP9 activated lung fibroblasts, which indi-cated that BMP9 might be involved in the progression and metastasis of lung cancer via regulating cancer-associated

fibroblasts, a process that is similar to lung fibroblast activation during pulmonary fibrosis. Meanwhile, several studies have reported that BMP signaling, including BMP receptor type II, endoglin, growth differentiation factor 15 and Smad4, is involved in tumor progression and metastasis via regulating cancer-associated fibroblasts (49-51). For example, Paauwe *et al* (49) demonstrated that endoglin, a coreceptor for TGF-β and BMP9, is expressed in cancer-associated fibroblasts, and endoglin-expressing cancer-associated fibroblasts contribute to colorectal cancer progression and metastasis. Therefore, whether BMP9 is implicated in cancer-associated fibroblast metastasis requires further investigation.

As a member of the TGF-β superfamily, BMP9 binds to type I and type II transmembrane receptors, including ALK1, ALK2, BMP receptor type II and activin receptor type IIB, to exert its effects (17,18,27). Type I BMP receptor ALK1 has been identified as displaying the highest affinity with BMP9 in the majority of cell types (52). Typically, when BMP9 binds to the ALK1 receptor, it primarily activates the expression of downstream p-Smad1/5 (17,18). Numerous studies have reported that Smad1/5 is activated in fibrotic diseases (20,53-55). During hepatic fibrosis, BMP9 induced the expression of inhibitor of differentiation 1 via the Smad1 signaling pathway, thereby triggering hepatic stellate cells to differentiate into myofibroblasts (52,53). In addition, Li *et al* (20) demonstrated that the BMP9/ALK1/Smad1 signaling pathway is involved in the epithelial-mesenchymal transition of liver cancer cells, which is an important mechanism underlying hepatic fibrosis. In the cellular context of scleroderma fibroblasts, ALK1/Smad1/5 signaling promotes ECM proteins, such as collagen I and CTGF (54). Consistent with the aforementioned studies, the present study indicated that rBMP9 increased ALK1 expression and Smad1/5 phosphorylation, and promoted cell viability, colony formation and the expression of α-SMA, collagen I and collagen III in HFL-1 cells. Moreover, the present study suggested that Dor partly rescued BMP9-induced effects. Therefore, the results supported the hypothesis that the ALK1/Smad1/5 signaling pathway might be implicated in BMP9-mediated HFL-1 cell proliferation and differentiation.

Collectively, the present study indicated that BMP9 directly induced HFL-1 cell proliferation. In addition, the results suggested that the activated ALK1/Smad1/5 signaling pathway may mediate BMP9-induced HFL-1 cell proliferation and differentiation. Moreover, the results indicated that BMP9 may serve as an activator of lung fibroblast proliferation and differentiation and may also serve as a profibrotic cytokine in pulmonary fibrosis, supporting its potential role in the treatment of lung fibrosis. Furthermore, BMP9-mediated lung fibroblast activation indicated that the potential role of BMP9-regulated cancer-associated fibroblasts during the progression and metastasis of lung cancer requires further investigation. However, the present study had a number of limitations. For example, the study focused on *in vitro* experiments and only HFL-1 cells were used in the study. Therefore, future studies should explore the role of BMP9 in other cell lines, *in vivo* or by using clinical tissue samples to identify the exact role of BMP9 in pulmonary fibrosis and evaluate the possible effect of BMP9-regulating cancer-associated fibroblasts on lung cancer.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YW designed and performed the main experiments and analyzed the data. XS analyzed the data. YY performed the experiments and analyzed the data. YH contributed to designing the study. YW and YH can authenticate the raw data in this study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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