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ORIGINAL RESEARCH

High BMP7 Expression May Worsen Airway Disease in COPD by Altering Epithelial Cell Behavior

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Purpose: Airway disease is the main pathological basis of chronic obstructive pulmonary disease (COPD), but the underlying mechanisms are unknown. Bone morphogenetic protein-7 (BMP7) is a multi-functional growth factor that belongs to the transforming growth factor superfamily, which affects the regulation of proliferation, differentiation, and apoptosis. Previous research has shown that BMP7 is highly expressed in the airway epithelia of patients with COPD, but its role in airway disease has not been fully elucidated.

Methods: A lung tissue cohort and a sputum cohort were included in the study. BMP7 expression in the airway epithelium and the BMP7 level in sputum supernatants were detected. Human primary bronchial epithelial cells (HPBECs) were isolated by bronchoscopy from healthy individuals. The functional consequences of adding recombinant human BMP7 or BMP7 overexpression to HPBECs were explored.

Results: BMP7 expression in bronchial epithelial cells of patients with COPD was significantly higher than that in smoking and nonsmoking controls. The expression of BMP7 in the bronchial epithelia of patients with COPD was negatively correlated with the airway counts measured by quantitative computed tomography, positively correlated with airway wall thickness, and negatively correlated with FEV1. The BMP7 level in the induced sputum of patients with COPD was higher than that in controls, and was related to the levels of interleukin-6 (IL-6), IL-8, and IL-1β. The addition of rhBMP7 (100 ng/mL) inhibited the proliferation of HPBECs and promoted squamous metaplasia and inhibit ciliated cell differentiation in human bronchial epithelial cells. BMP7 overexpression promotes apoptosis in human bronchial epithelial cells, through regulating MKK7/JNK2 signaling pathway and activating the caspase-3 pathway.

Conclusion: High expression of BMP7 in the bronchial epithelia may play a crucial role in airway disease of COPD through inhibiting proliferation and promoting abnormal differentiation and excessive apoptosis of human bronchial epithelial cells.

Keywords: COPD, BMP7, airway disease, remodeling, apoptosis

Introduction

Chronic obstructive pulmonary disease (COPD) is a common disease that seriously threatens human health.[1] In 2019, COPD was the third leading cause of death worldwide, with over 3.3 million deaths.[2] Airway disease involves both the large and small airways in COPD and is not only the cause of persistent symptoms, but also the main pathological basis

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of airflow limitation.[3] In patients with COPD, repetitive injury and aberrant repair of the airway leads to structural remodeling, resulting in airway narrowing, loss of airway counts and airflow obstruction.[2] The mechanisms of continuous inflammation, mucus hypersecretion, squamous epithelial metaplasia, and structural remodeling of the airway in COPD are poorly understood.[4]

As the first barrier, the airway epithelium is inevitably exposed to harmful stimuli, leading to production of cytokines, chemokines, activating stimulating factors, and growth factors, all of which are involved in tissue damage and repair. Due to the abnormally high inflammatory response to stimuli, these factors may be abnormally expressed in COPD[5] and lead to series of pathological changes.[6] Investigating the influence of abnormally expressed cytokines may help to elucidate the pathogenesis and seek new prevention strategies for COPD.

In the preliminary work, we found that the level of bone morphogenetic protein-7 (BMP7) in induced sputum was higher in patients with COPD compared to controls. Further immunohistochemistry results showed that BMP7 expression in the bronchial epithelia was higher in patients with COPD than controls.

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor beta (TGF-β) superfamily.[7] Previous studies have shown that the BMP family may play a significant role in COPD airway pathology. The aberrant overexpression of BMP4 in COPD smokers promoted abnormal airway epithelial differentiation, leading to squamous metaplasia and impaired mucociliary differentiation.[8] However, given the diversity of BMP molecules, the specific roles of individual BMPs in the development of COPD airway lesions remain to be elucidated. Among more than 20 members of BMPs, BMP7 has attracted much attention in recent years.[9] BMP7 is expressed in multiple tissues, such as the thymus, bone marrow, heart, kidney, lung, and prostate, and plays a key role in the development of bone and kidney. Moreover, BMP7 can regulate the proliferation, differentiation, and apoptosis of epithelial cells, and participates in various pathological processes, such as osteoarthritis, tumor bone metastasis, renal fibrosis, and obesity.[10,11] Previous studies have shown that the expression of BMP7 in the airway epithelia of smokers was increased,[12] and suggested that BMP7 might participate in the repair of the airway epithelium in mice.[13] However, the function and mechanism of BMP7 in chronic pulmonary disease have not yet been fully elucidated.

In this study, we investigated the following aspects: 1) the correlation between BMP7 expression in the bronchial epithelia and clinical characteristics in patients with COPD; 2) the role of BMP7 in the proliferation and differentiation of bronchial epithelial cells; and 3) the role and mechanisms of BMP7 in apoptosis of bronchial epithelial cells.

Materials and Methods

Subjects

Two subject cohorts were recruited, including a tissue specimen cohort and a sputum cohort. All the participants were from mainland China, aged between 40 and 85 years, and presented to the Qilu Hospital between July 2017 and November 2020. This study was conducted according to the principles of the Declaration of Helsinki and was approved by Ethics Committee of Qilu Hospital, Shandong University (No. 2015091). Written informed consent was obtained from each subject before recruitment.

All subjects underwent pulmonary function tests and chest computed tomography (CT) scan before recruitment. Patients were diagnosed with COPD based on more than 10 pack-years smoking history or confirmed noxious gas or particle exposure, and persistent airflow limitation (postbronchodilator FEV1/FVC < 0.70), according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD).[14]

The tissue cohort consisted of nonsmoking controls (nonsmokers, n = 25), smoking controls (smokers, n = 27), and patients with COPD (n = 35). Lung tissue specimens were collected from patients who underwent lobectomy for lung lesions. Lung tissue used for analysis was resected more than 5 cm away from the lesion margin. Smoking controls had more than 10 pack-years smoking history and no airflow limitation (postbronchodilator FEV1/FVC > 0.70). Nonsmoking controls were subjects with no smoking history or airflow limitation. The exclusion criteria were as follows: 1) presence of asthma, bronchiectasis, interstitial lung disease, tuberculosis, central lung mass; and 2) systematic disease, such as congestive heart failure, autoimmune disease, or infection.

The sputum cohort consisted of 42 stable patients with COPD and 38 non-COPD controls. Non-COPD controls were defined as subjects with no respiratory symptoms, chronic respiratory diseases, and no airflow limitation. Patients with COPD with exacerbation in the past 6 weeks and a history of long-term antibiotics/systemic steroid use were excluded. Other exclusion criteria were the same as those for the tissue cohort.

Quantitative CT Analysis of Emphysema and Airway Remodeling

Quantitative CT analysis was conducted using the Chest Imaging Platform (CIP) in 3D Slicer.[15] Emphysema was evaluated by the percentage of voxels below −950 hounsfield units (Hu) to total lung voxels (%LAA-950). The airway-remodeling parameters, including the square root of the wall area of a 10-mm internal perimeter (Pi10), represent the thickness of the airway wall, and the total airway counts of the 4th to 9th airway in the right upper lobe, represent the loss of airways.

The wall thickness was measured in the following target airways: right apical (RB1), right basal posterior (RB10), left apical (LB1), and left basal posterior bronchus (LB10). The upper middle 1/3 from the bronchial origin to the bifurcation was chosen as the measurement site. The wall area (WA) and lumen perimeter of the 3rd to 5th airways of each target airway were collected. Pi10 was calculated with a linear regression of the square root of WA versus the internal perimeter.[16]

The airway counts in the right upper lobe were quantified according to the methods described by Diaz of Harvard Medical School.[17] Slice-by-slice examination was performed under the conditions of a window width of 1000 hu and a level of −500 hu to identify the bifurcation of airways. Airway counts from the 4th to 9th airways were summed to evaluate the loss of airway counts.

Detection of BMP7 and Other Cytokines in Sputum

The sputum induced by hypertonic saline was collected following a standardized process.[18] Sputum samples were collected and processed within 2 h once harvested. Dithiothreitol was added in a volume four times the weight of the sputum samples. The samples were then dissolved gently, filtered through sterile nylon mesh, and centrifuged at 800 × g at 4°C for 10 min. The supernatants were obtained and stored at −80°C. The levels of BMP-7, BMP-4, IL-6, IL-8/CXCL8, IL-1β, and MMP-12 in sputum were detected using Luminex liquid phase microarray technology.[19]

Isolation, Culture, and Identification of Human Primary Bronchial Epithelial Cells (HPBECs)

HPBECs were obtained by gently brushing the airway epithelium from the third- to fourth-order bronchi of healthy nonsmoking volunteers using flexible bronchoscopy as previously described.[20] Cells were collected and cultured in PneumaCult-Ex Medium (STEMCELL, Canada) in 5% CO_2 in a humidified chamber at 37°C. The medium was changed after the first 12 h in culture to remove unattached cells, and every 2–3 days thereafter. Experiments were performed on HPBECs at passage 2 or 3.

After the first passage, HPBECs were seeded onto glass cover slips in a 24-well plate and incubated for 24 h. Cells were fixed in 4% paraformaldehyde for 15 min and blocked with bovine serum albumin (BSA) for 30 min at 37°C. Cells were incubated with primary antibody (Cytokeratin 5 [1:100] and p63 [1:100]) overnight at 4°C. Slides were rinsed, incubated with Tetramethylrhodamine (TRITC) conjugated anti-rabbit IgG (1:500) and Fluorescein (FITC)-conjugated anti-mouse IgG at room temperature for 30 min, counterstained with DAPI (4′,6-diamidino-2-phenylindole), and visualized with fluorescence microscopy.

Generation of BMP7 Overexpressing (OE) Cells

Puromycin-resistant lentiviruses containing the human BMP7 gene (BMP7-OE-LV) and scramble negative control (NC-LV) were provided by GeneChem Company (Shanghai, China). HPBECs were cultured at 1×10^5 cells/well in 6-well tissue culture plates overnight. The lentiviral supernatant was added to cells, and the multiplicity of infection (MOI) was 100. After 24 h, puromycin (1 μg/mL; Sigma-Aldrich) in new culture medium was added to select for transduced cells. Western blotting was performed to confirm the overexpression of BMP7.

Antibodies

The anti-BMP7 and anti-involucrin antibodies were purchased from Proteintech (Tokyo, Japan). The anti-GAPDH antibody was purchased from Cell Signaling Technologies (Danvers, MA, USA). The anti-p63 and anti-Cytokeratin 5 antibodies were purchased from Abcam (Cambridge, UK). The anti-FOXJ1 antibody was purchased from Affinity (USA).

Immunohistochemistry

Tissue sections with a 4-μm thickness were made from paraffin-embedded lung specimens. Staining was performed with an ultrasensitive streptavidin-peroxidase kit (PV-9001; ZSGB-Bio, Beijing, China) according to the manufacturer's instructions. The mean-staining density of BMP7 in the airway epithelium was quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

Cell Proliferation Assay

HPBECs were cultured into 96-well plates with at a density of 5000 cells/well. A 5-ethynyl-20-deoxyuridine (EdU) incorporation assay kit (Beyotime, China) was used to detect HPBEC proliferation according to the manufacturer's instructions.

Spheroid-Formation Assay

A spheroid-formation assay was conducted using ultra-low attachment surface spheroid microplates (Corning, USA). Cell suspension (100 μL) with 1000 hPBECs was dispensed to each well. After 72 h, the 3D multicellular spheroids in each wall were observed using a light microscope.

Air-Liquid Interface (ALI) Culture

The HPBECs from nonsmokers were seeded into 0.4-μm pore size transwell polyester membrane inserts (Corning) at a density of 3–5 × 10^5 cells/cm^2. The cells were cultured in PneumaCult-Ex Medium (STEMCELL, Canada). When the HPBECs had reached 100% confluence, the upper chamber medium was removed, the PneumaCult-ALI medium (STEMCELL, Canada) was added to the lower chamber, and the apical surface was exposed to air to establish the ALI. The cells were cultured at 37°C, 5% CO_2 for 28–35 days until the ciliary metaplasia was observed.

Western Blot

Proteins were extracted from HPBECs and quantitated with a BCA protein assay kit. A total of 10 μg protein of each sample was separated on 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was then blocked with 5% skim milk at 37°C for 1 h. Target proteins on the membrane were recognized by incubating with appropriate primary antibodies at 4°C overnight. After rinsing with TBST three times for 5 min each time, the protein-antibody complexes were recognized by the antibody by incubating at room temperature for 1 h. After rinsing with TBST three times for 10 min each, the gray scale images were scanned and protein expression was analyzed using GAPDH as the internal control. Each experiment was repeated three times.

Flow Cytometry

HPBECs were washed twice using precooling PBS, and then digested in 75% alcohol at 4°C overnight. Propidium iodide (PI) was added to the cell suspensions and the cell cycle distribution was detected by flow cytometry (Beckman, USA). The results were analyzed following the manufacturer's protocol using the Kaluza software (Beckman, USA).

TMT-Based Proteomics

TMT-based proteomics were performed to screen the differentially expressed proteins (DEPs) between the BMP7-OE and control groups of HPBECs. Using a cluster of orthologous group (COG) analysis, DEPs were classified into 24 categories, including signal transduction mechanisms, defense mechanisms, and transcription, etc.

Statistical Analysis

Descriptive data are presented as the mean ± standard deviation (SD). SPSS 21.0 (IBM, Armonk, NY, USA) was used for statistical analysis. Comparison between continuous variables with a normal distribution was performed via Student's *t*-tests and one way analysis of variance. We conducted a power analysis using G*Power (version 3.1) with an alpha level (α) of 0.05 and power (1-β) of 0.80 to detect significant differences in BMP7 levels of induced sputum between COPD patients and Non-COPD controls. Based on preliminary data indicating an effect size of 0.632, we calculated a required sample size of approximately 32 per group. Categorical variables were analyzed by chi-square tests. A correlation analysis for continuous variables with a normal distribution was performed by Pearson's correlation analysis. $P < 0.05$ was considered statistically significant.

Results

Participants' Characteristics

The characteristics of the subjects from the tissue cohort and sputum cohort are shown in Tables 1 and 2.

In the tissue cohort, no significant difference was found in the age of the patients among the three groups ($P = 0.279$). The ratio of males to females was significantly lower in nonsmokers than in smokers and patients with COPD ($P < 0.05$), but no significant difference was shown between smokers and patients with COPD ($P > 0.05$). The quantitative CT parameters,

Table 1 Clinical and Quantitative CT Characteristics of the Lung Tissue Cohort

	Nonsmokers (n = 25)	Smokers (n = 27)	COPD (n = 35)	P-value
Age, years	62.57 ± 9.26	60.37 ± 8.06*	63.54 ± 6.33*	0.279[a]
Sex, female/male	20/5	2/25	3/32	<0.01[b]
BMI, kg/m^2	25.16 ± 4.63	25.4 ± 3.35	23.77 ± 2.21	0.162[a]
Smoking, pack-years	0.18 ± 0.65	31.56 ± 29.32	36.10 ± 28.64	<0.01[a]
FEV1, % predicted	107.83 ± 13.56	105.97 ± 15.50	72.89 ± 20.29*#	<0.01[a]
FEV1/FVC, %	78.29 ± 3.36	77.10 ± 4.39	59.53 ± 10.12*#	<0.01[a]
%LAA-950, %	0.69 ± 0.40	1.60±1.94*	4.80±4.05*#	<0.01[a]
Pi-10	5.48 ± 1.25	5.99 ± 0.31*	6.38 ± 0.49*#	<0.01[a]
Airway counts (n = 27)				
4th to 9th airway	96.27±13.27	93.11±23.50	76.57±14.57*#	0.015[a]

Note: *P < 0.05 vs nonsmokers, #P < 0.05 vs smokers, [a]one-way ANOVA analysis, [b]chi-square test.
Abbreviations: BMI, Body mass index; FEV1, Forced expiratory volume in 1 second; FVC, Forced vital capacity; %LAA-950, Percentage of low attenuation area with voxels below −950 hu.

Table 2 Clinical Characteristics of Subjects in the Sputum Cohort

	Non-COPD Controls (n = 38)	COPD (n = 42)	P-value
Age, years	61.03 ± 6.68	64.21 ± 8.74	0.075[a]
Sex, female/male	3/35	5/37	0.414[b]
BMI, kg/m^2	24.43 ± 3.64	24.55 ± 3.19	0.909[a]
Smoking, pack-years	33.81 ± 24.60	37.63 ± 26.42	<0.01[a]
FEV1, % predicted	110.42 ± 16.02	51.44 ± 18.36	<0.01[a]
FEV1/FVC, %	79.84 ± 5.21	47.29 ± 15.23	<0.01[a]

Note: [a]t-test, [b]chi-square test.
Abbreviations: BMI, Body mass index; FEV1, Forced expiratory volume in 1 second; FVC, Forced vital capacity.

including Pi10 and %LAA-950, were significantly higher in the COPD group compared to smokers and nonsmokers ($P < 0.05$). The airway counts (4th to 9th) of patients with COPD were significantly lower than those of nonsmokers and smokers ($P < 0.05$), but no significant difference was observed between smokers and nonsmokers ($P = 0.682$).

In the sputum cohort, there was no significant difference in age (61. 03 ± 6.68 vs 64.21 ± 8.74, P= 0.075), sex ratio (P= 0.414), and BMI (24 ± 3.64 vs 24.55 ± 3.19 kg/m^2, P = 0.909) between patients with COPD and non-COPD controls. The FEV1% and FEV1/FVC of patients with COPD were significantly lower than non-COPD controls ($P < 0.05$).

BMP7 Expression in the Airway Epithelia of Patients with COPD

As shown by immunohistochemistry (Figure 1), BMP7 was expressed in both the large and small airways. In the large airway, immunostaining of BMP7 was mainly located in the basal side, whereas in the small airway, the difference in distribution between the lumen side and basal side was not obvious (Figure 1). BMP7 was also expressed in epithelial cells of the large airway mucus glands.

Semi-quantitative immunohistochemistry showed significantly higher expression of BMP7 in the airway epithelia of patients with COPD than in smokers and nonsmokers ($P < 0.05$), and the expression of BMP7 was higher in smokers than nonsmokers ($P < 0.05$). BMP7 was also expressed in the epithelial cells of the large airway mucus glands.

Correlation Between BMP7 Expression with Clinical Characteristics and Quantitative CT Parameters

Because BMP7 expression in the airway epithelia of patients with COPD was increased, the correlation between BMP7 expression with clinical characteristics and quantitative CT parameters was analyzed (Figure 2). In terms of airway-

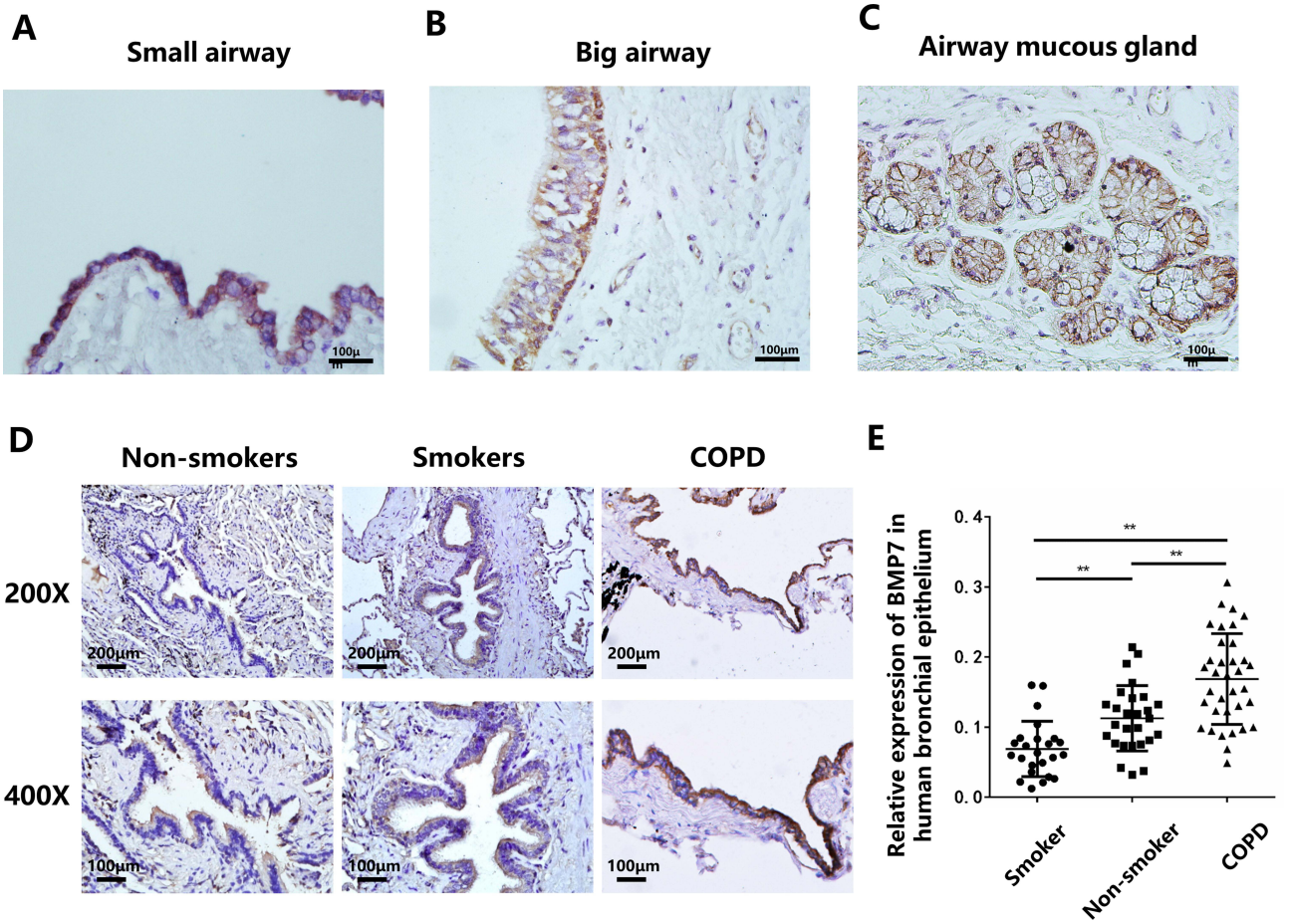

Figure 1 Immunohistochemistry of BMP7 in the airway epithelia. ** P<0.01. (**A**–**C**) Distribution of BMP7 in big and small airways. (**D**–**E**) Comparison of BMP7 expression in patients with COPD, nonsmokers, and smokers.

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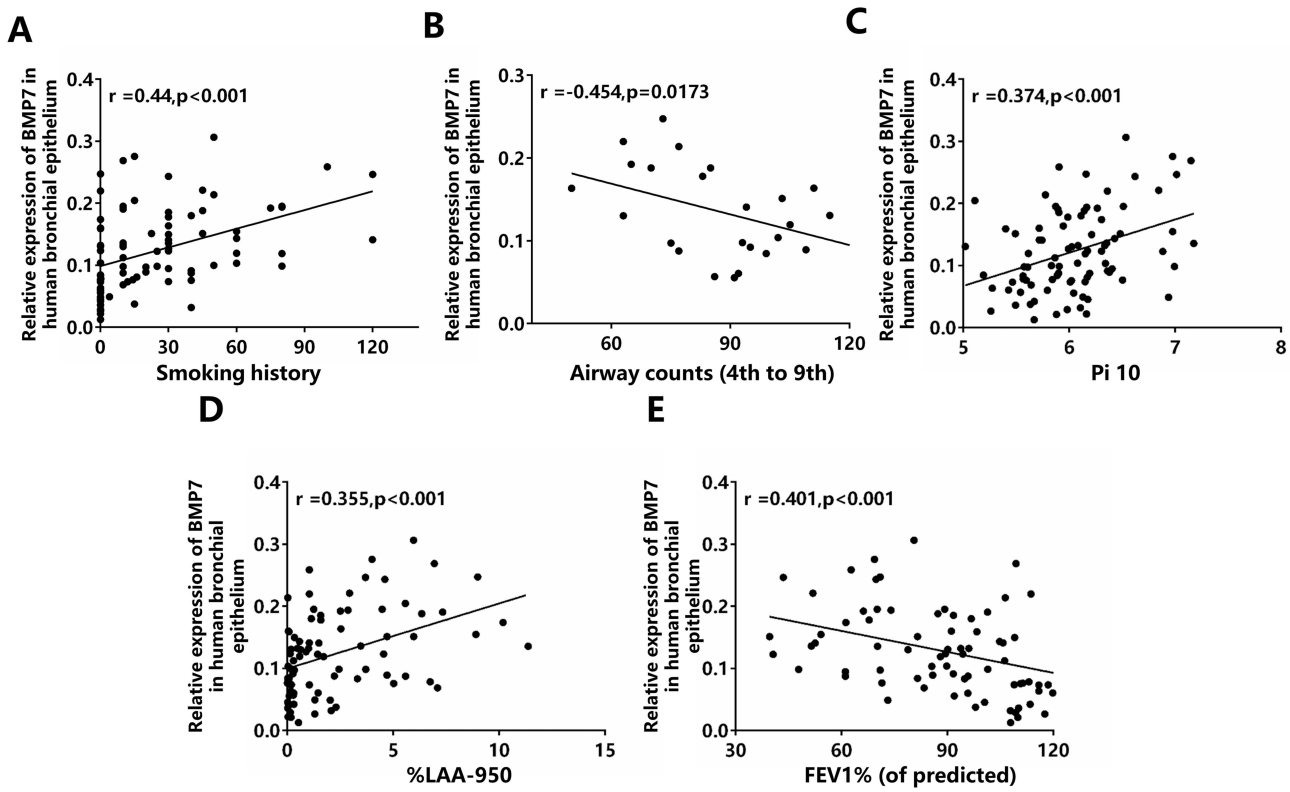

Figure 2 Correlation between BMP7 expression with clinical characteristics and quantitative CT parameters. (**A**) Smoking history. (**B**)Airway counts; (**C**) Pi-10; (**D**) %LAA-950; (**E**) FEV1% (of predicted).

remodeling parameters, bronchial epithelial BMP7 expression was significantly correlated with airway counts (r = –0.454, P = 0.0173) and airway wall thickness parameter Pi10 (r = 0.374, P < 0.001). In addition, BMP7 expression in the bronchial epithelia was also correlated with the emphysema index %LAA-950 (r = 0.355, P < 0.001) and FEV1.0% (r = –0.401, P < 0.001).

Sputum BMP7 Level in Patients with COPD and the Relationship with Inflammatory Factors

As shown in Table 3, the level of BMP7 in the induced sputum of patients with COPD was significantly higher than that in non-COPD controls (355.38 ± 287.64 vs 213.56 ± 85.73 pg/mL, P = 0.013). The levels of IL-6, IL-8, and IL-1β in the induced sputum of patients with COPD were significantly higher than those in the control group (P < 0.05). However, the level of BMP4 and MMP-12 showed no significant difference between the two groups (P > 0.05).

Table 3 Levels of Cytokines of Induced Sputum

	Non-COPD Controls (n = 38)	COPD (n = 42)	P-value
BMP7, pg/mL	213.56 ± 85.73	355.38 ± 287.64	0.004[a]
BMP4, pg/mL	48.60 ± 46.69	44.95 ± 44.93	0.724[a]
IL-6, pg/mL	19.06 ± 22.31	167.96 ± 299.77	0.003[a]
IL-8/CXCL8, pg/mL	111.72 ± 165.83	551.62 ± 903.64	0.005[a]
IL-1β, pg/mL	124.90 ± 148.86	353.92 ± 692.20	0.038[a]
MMP-12, pg/mL	656.43 ± 502.70	667.70 ± 567.81	0.926[a]

Note: [a]*t*-test.

Abbreviations: BMP7, bone morphogenetic protein 7; BMP4, bone morphogenetic protein 4; IL-6, interleukin-6; IL-8, interleukin-8; IL-β, interleukin-1β; MMP-12, matrix metalloproteinase-12.

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The level of BMP7 in induced sputum was significantly related to the levels of IL-6 (r = 0.461, P < 0.001), IL-8 (r = 0.306, P = 0.005), and IL-1β (r = 0.246, P = 0.027) (Table 4). The BMP7 level in the induced sputum was also negatively correlated with FEV1% (r = –0.242, P = 0.029), but was not associated with smoking history.

Role of BMP7 in HPBEC Proliferation

Abnormally high BMP7 expression in the airway of patients with COPD was related to airway-remodeling parameters, and BMP7 played a key role in regulating proliferation, differentiation, and apoptosis; thus, BMP7 may affect abnormal proliferation and differentiation of bronchial epithelial cells and be involved in airway disease in COPD. To elucidate the functional consequences of increased BMP7 expression, in vitro experiments were conducted using HPBECs.

HPBECs were identified by immunofluorescence staining before functional experiments. The results showed that more than 95% of cells expressed both KRT5 and p63 (basal cell biomarkers), with KRT5 expressed in the cytoplasm and cell membrane, and P63 expressed in the nucleus (Figure 3A), indicating that most of the HPBECs are basal cells.

An EdU assay was performed to assess the possible function of BMP7 in the proliferation of HPBECs. The results showed that the proportion of EdU-positive cells was significantly reduced in the rhBMP7 (100 ng/mL) group compared to the control group (Figure 3B and C), while the proportion of EdU-positive cells was significantly increased in rhBMP7 + LDN193189 (inhibition of BMP signaling) group compared to the rhBMP7 group (P < 0.05). The results of the spheroid formation assay (Figure 3D and E) showed that the diameter of the spheroids of the rhBMP7 group were significantly lower than controls, and those of the rhBMP7 + LDN193189 group had a significantly longer diameter than those of the rhBMP7 group (P < 0.05).

Effects of BMP7 on HPBEC Differentiation

To investigate the effects of BMP7 on HPBEC differentiation, monolayer-cultured HPBECs were treated with different concentrations of rhBMP7 (0, 10, 20, 40, and 80 ng/mL). As shown in Figure 4, no significant difference in involucrin (marker of squamous cell) and FOXJ1 (marker of ciliated cell) expression was observed between the 10 ng/mL group and the control group (P > 0.05). However, involucrin expression was significantly higher and expression of FOXJ1 was significantly lower in the 20, 40, and 80 ng/mL groups compared to the control group (P < 0.05). Compared to the rhBMP7 (40 ng/mL) treated group, the expression of involucrin was significantly reduced, and the expression of FOXJ1 was significantly increased in the group treated with both rhBMP7 (40 ng/mL) and the BMP pathway inhibitor LDN193189 (50 nM) (P < 0.05).

The effect of BMP7 on HPBEC differentiation was further investigated using air-liquid interface models. Immunofluorescence and WB (Figure 5) showed that involucrin expression was higher in the rhBMP7 (40 ng/mL) treated group than that in the control group, and that FOXJ1 expression was lower than that in the control group (P < 0.05).

Role of BMP7 in HPBEC Apoptosis

High expression of BMP7 showed a negative effect on proliferation and differentiation of HPBECS, and BMP7 plays a key role in the regulation of apoptosis; thus, we hypothesize that BMP7 may be further involved in the regulation of

Table 4 Relationship of Sputum BMP7 Level with Clinical Characteristics and Other Cytokines

	Correlation with Sputum BMP7 level	**P-value**
Smoking history	0.136	0.222
FEV1	–0.242	0.029
IL-6	0.461	< 0.001
IL-8/CXCL8	0.306	0.005
IL-1β	0.246	0.027

Abbreviations: IL-6, interleukin-6; IL-8, interleukin-8; IL-1β, interleukin-1β.

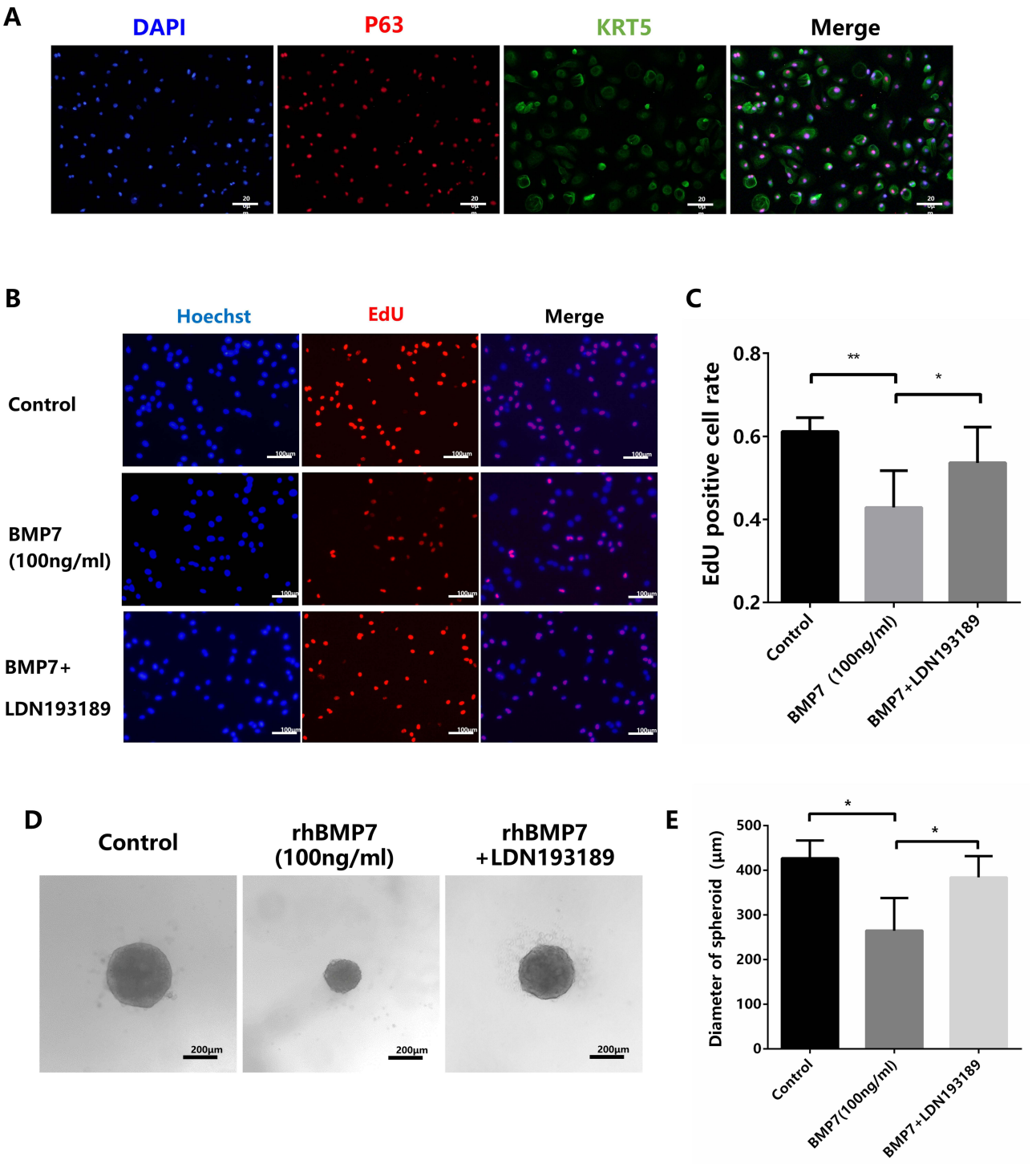

Figure 3 Immunofluorescence of HPBECs and the effect of BMP7 on cell proliferation and spheroid-formation ability. * P<0.05, ** P<0.01. (**A**) Immunofluorescence staining of KRT5 and p63 of HPBECs. (**B** and **C**) EdU assay. (**D** and **E**) Spheroid formation assay.

bronchial epithelial cell apoptosis, and that highly expressed BMP7 may generate an imbalanced state of cell proliferation/death.

To investigate the consequences of BMP7 overexpression, lentivirus (OE-BMP7-LV) and negative control lentivirus (NC-LV) were used to transfect human bronchial epithelial cells, respectively. The results of WB (Figure 6) showed that

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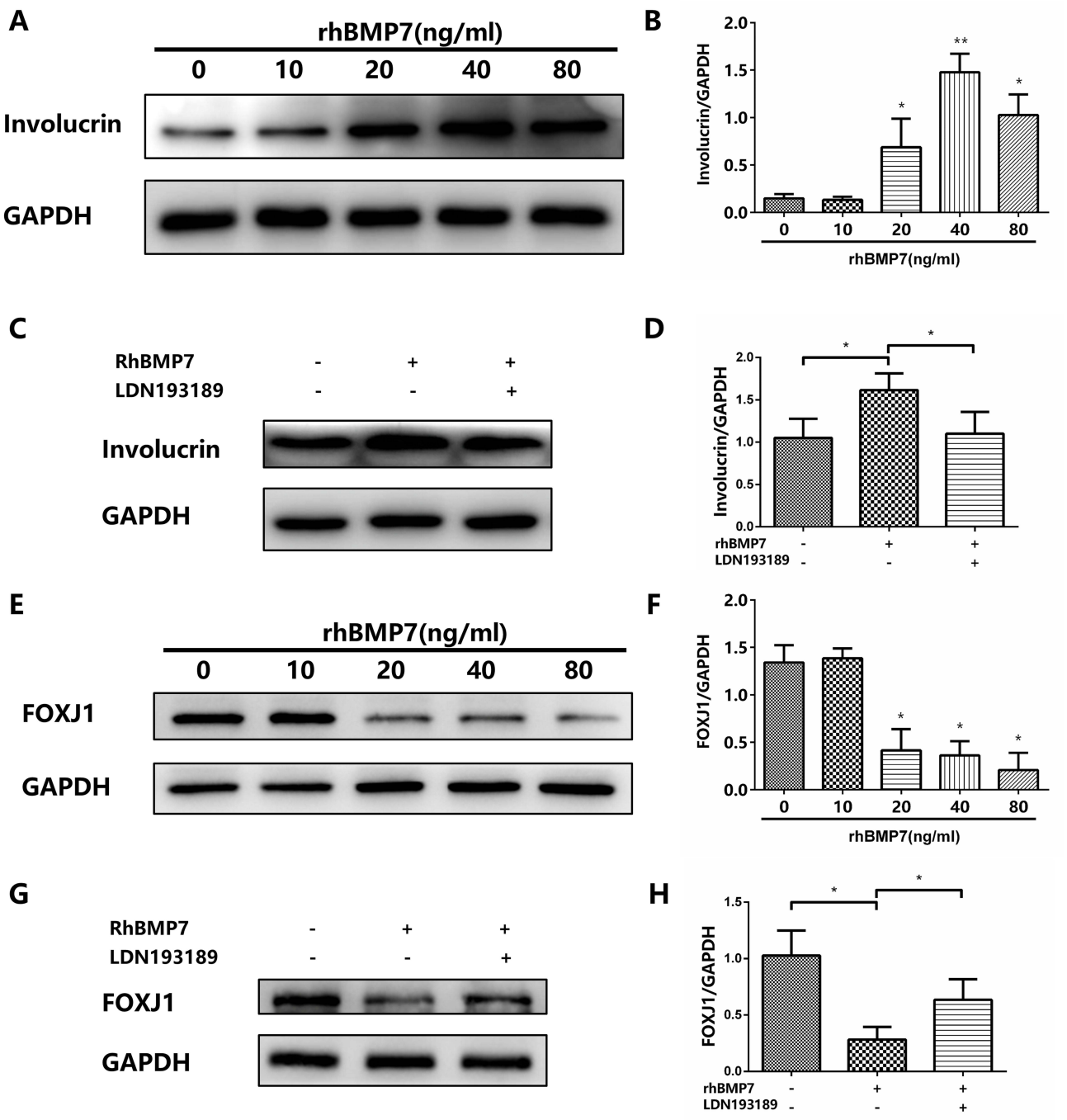

Figure 4 Effect of BMP7 on differentiation of monolayer-cultured HPBECs. * P<0.05, ** P<0.01. (**A**–**D**) WB of involucrin. (**E**–**H**) WB of FOXJ1.

the expression of BMP7 in the OE-BMP7-LV transfection group was significantly higher than that in the NC-LV transfection group (P < 0.001), indicating successful construction of BMP7-OE HPBECs (Figure 6A).

The results of flow cytometry (Figure 6) showed that the percentage of early apoptotic cells of the BMP7-OE group was significantly higher than that of the control group (12.1% ± 2.2% vs 5.26% ± 1.16%, P = 0.015). Moreover, the percentage of early apoptotic cells was significantly lower in the BMP7-OE HPBECs + LDN193189 (50 nM) group than that in the BMP7-OE group (5.24% ± 0.92% vs 9.29% ± 1.55%, P = 0.0042). In terms of apoptosis-related proteins, compared to the control group, the BMP7-OE group showed significant decreases in pro-caspase-3 and BCL-2 expression (Figure 7), and significant

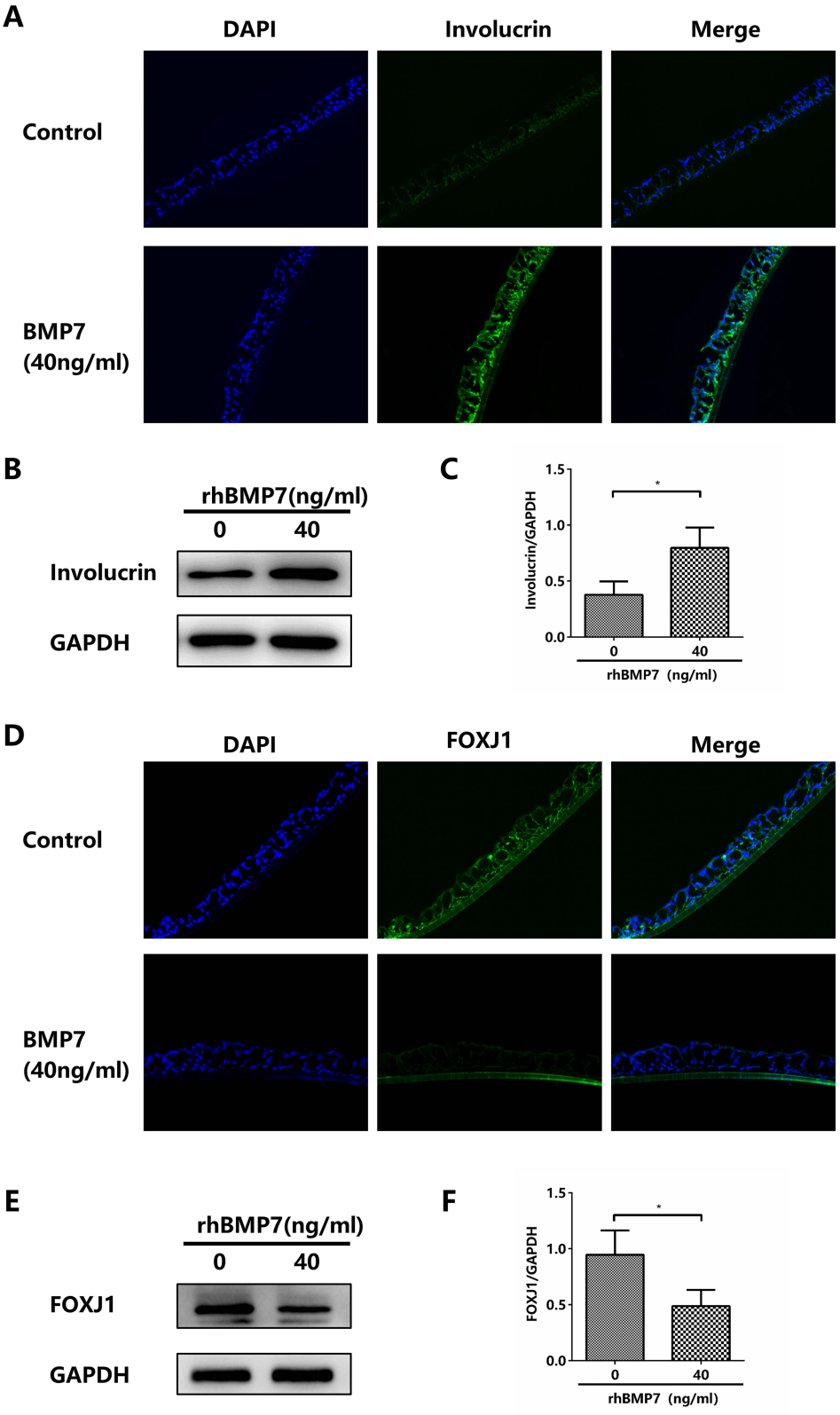

Figure 5 Immunofluorescence and WB of air-liquid interface (ALI) culture. * P<0.05. (**A**–**C**) immunofluorescence and WB of involucrin. (**D**–**F**) Immunofluorescence and WB of FOXJ1.

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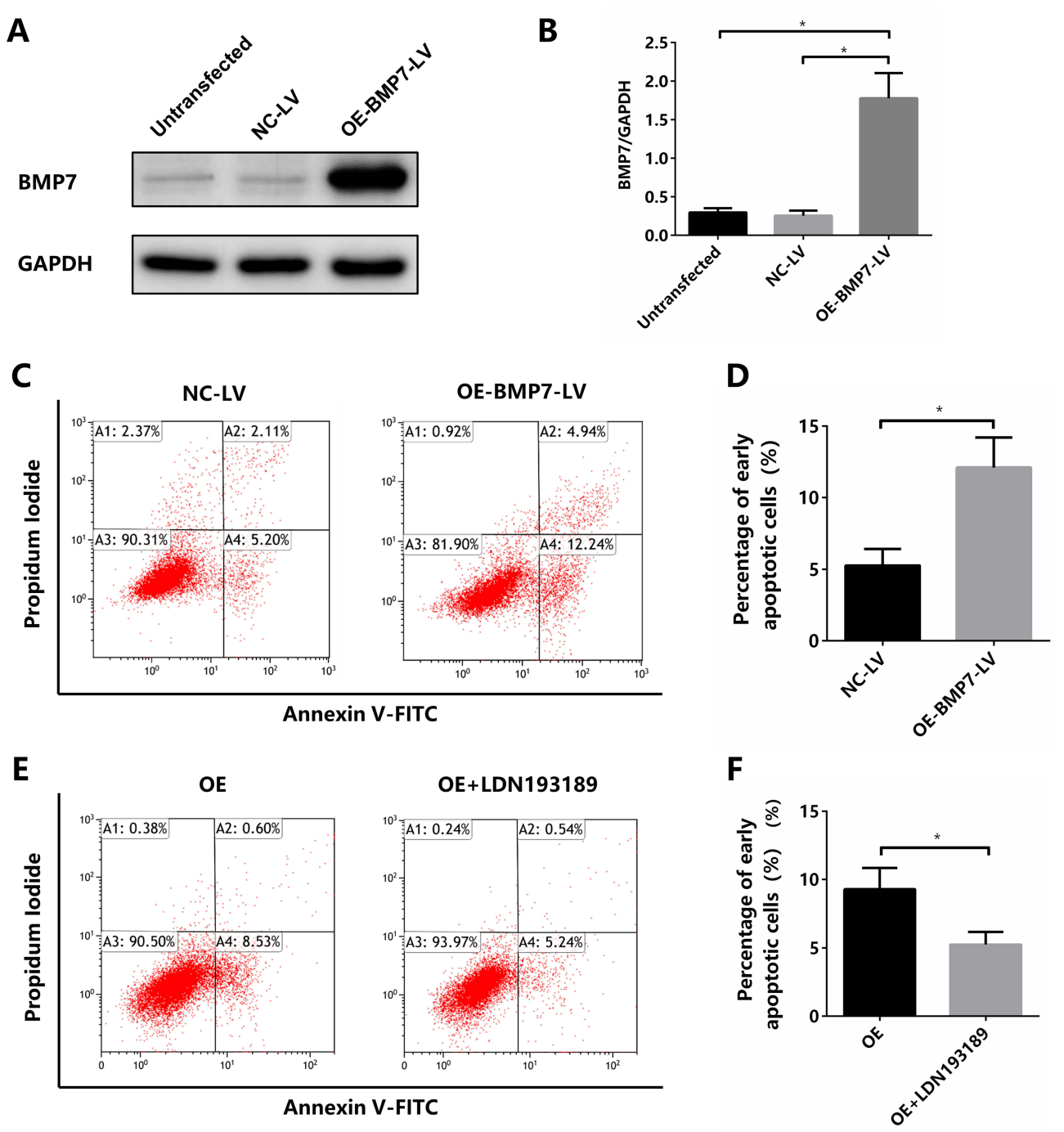

Figure 6 Effect of BMP7 on HPBECs apoptosis. * P<0.05. (**A** and **B**) Verification of BMP7-OE HPBECs using WB. (**C**–**F**) Comparison of the percentage of apoptotic cells in different groups of HPBECs.

increases in cleaved-caspase-3 and BAX expression ($P < 0.05$). In the BMP7-OE group, treatment with LDN193189 significantly reduced cleaved-caspase-3 and BAX expression and promoted BCL-2 expression ($P < 0.05$).

Mechanism of BMP7 in Promoting HPBEC Apoptosis

To explore the signaling pathway of apoptosis induced by BMP7 overexpression, proteomics was used to detect the differentially expressed proteins between the BMP7-OE group and the control group. The results showed that 189 proteins were upregulated and 169 proteins were downregulated in the BMP7-OE group compared to the control group;

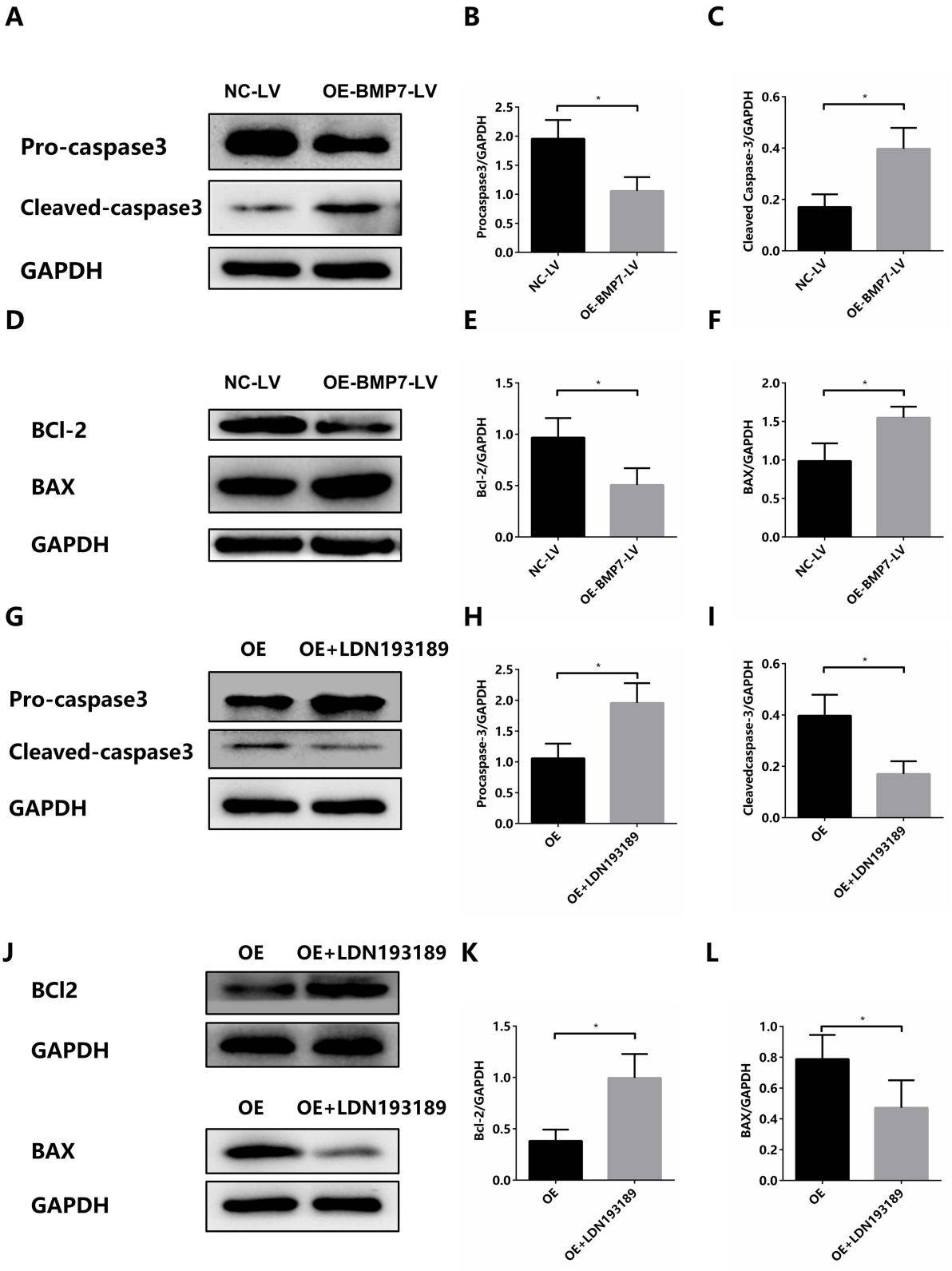

Figure 7 Effect of BMP7 on the expression of apoptosis-related proteins. (**A**–**F**) Comparison between BMP7-OE and control groups. (**G**–**L**) Comparison between BMP7-OE and BMP7-OE + LDN193189. * P<0.05.

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Table 5 Top 10 Differentially Expressed Proteins Related to Signal Transduction Between the BMP7-OE Group and Control Group

Proteins (Genes)	Fold Change	P-value
MAP2K7	2.698	0.0013098
CLK2	2.674	0.0032831
SASH1	2.405	0.0026652
FOLR1	2.176	0.0001542
HCAR3	1.911	0.0002789
ARHGDIB	1.859	0.0339526
INPP4B	1.848	0.0031395
CDH2	1.818	0.0205078
HCAR2	1.779	0.0242734
INHA	1.751	0.0019072

Abbreviations: MAP2K7, mitogen-activated protein kinase kinase 7; CLK2, CDC Like Kinase 2; SASH1, SAM And SH3 Domain Containing 1; FOLR1, Folate Receptor Alpha; HCAR3, hydroxycarboxylic acid receptor 3; ARHGDIB, Rho GDP Dissociation Inhibitor Beta; INPP4B, Inositol Polyphosphate-4-Phosphatase Type II B; CDH2, Cadherin 2; HCAR2, hydroxycarboxylic acid receptor 2; INHA, Inhibin Subunit Alpha.

of which, 44 proteins were associated with signal transduction. Sorted by differential expression fold, MAP2K7 (also known as MKK7 [mitogen-activated protein kinase 7], which specifically activates the JNK signaling pathway) was the most differentially expressed in the two groups (2.698-fold change) (Table 5).

The results of proteomics were verified using WB. As shown in Figure 8, MKK7, and p-JNK2 expression was significantly higher in the BMP7-OE group than the control group ($P < 0.05$). Using the JNK pathway inhibitor SP600125 significantly reduced p-JNK2 expression in the BMP7-OE group ($P < 0.05$). Flow cytometry results showed that the percentage of early apoptotic cells was significantly lower in the BMP7-OE + SP600125 group than the BMP7-OE group (5.28% ± 0.86% vs 9.74% ± 0.60%, $P = 0.018$). WB showed that cleaved-caspase-3 expression in the BMP7-OE + SP600125 group was lower than the BMP7-OE group ($P < 0.05$).

Discussion

In this study, we investigated the role of BMP7 in airway disease of COPD. We demonstrated that the expression of BMP7 in the bronchial epithelia of COPD was elevated and correlated with quantitative CT airway-remodeling parameters. BMP7 can inhibit the proliferation, promote squamous metaplasia, and inhibit ciliated cell metaplasia in human bronchial epithelial cells. BMP7 overexpression may promote apoptosis in human bronchial epithelial cells through regulating the MKK7/JNK2 signaling pathway and activating caspase-3.

Airway disease is one of the main causes of airflow limitation in COPD. Its connotation includes not only airway wall thickening, characterized by squamous metaplasia, inflammatory cell infiltration, and subepithelial interstitial fibrosis, but also the reduction of distal airway number, characterized by abnormal death of airway epithelial cells and destruction of the airway structure.[21] Airway disease also plays a key role in the pathogenesis of emphysema.[22] Previous studies have shown that the loss of alveolar attachment points caused by small airway inflammation represents the early stage of lung parenchyma destruction.[23] The pathogenesis of airway disease in COPD has not yet been fully elucidated.

As a member of the TGF-β superfamily, BMP7 has attracted much attention for its essential role in organ development and the pathogenesis of diseases such as osteoarthritis, tumor bone metastasis, renal fibrosis, and obesity.[10,11] However, whether BMP7 is involved in the pathogenesis of COPD has not yet been elucidated. We examined the expression of BMP7 in the airway of COPD, and the results showed that BMP7 was highly expressed in COPD compared to controls, which was consistent with the results of a previous study.[8] The correlation study showed that the expression of BMP7 in airway was associated with airway-remodeling

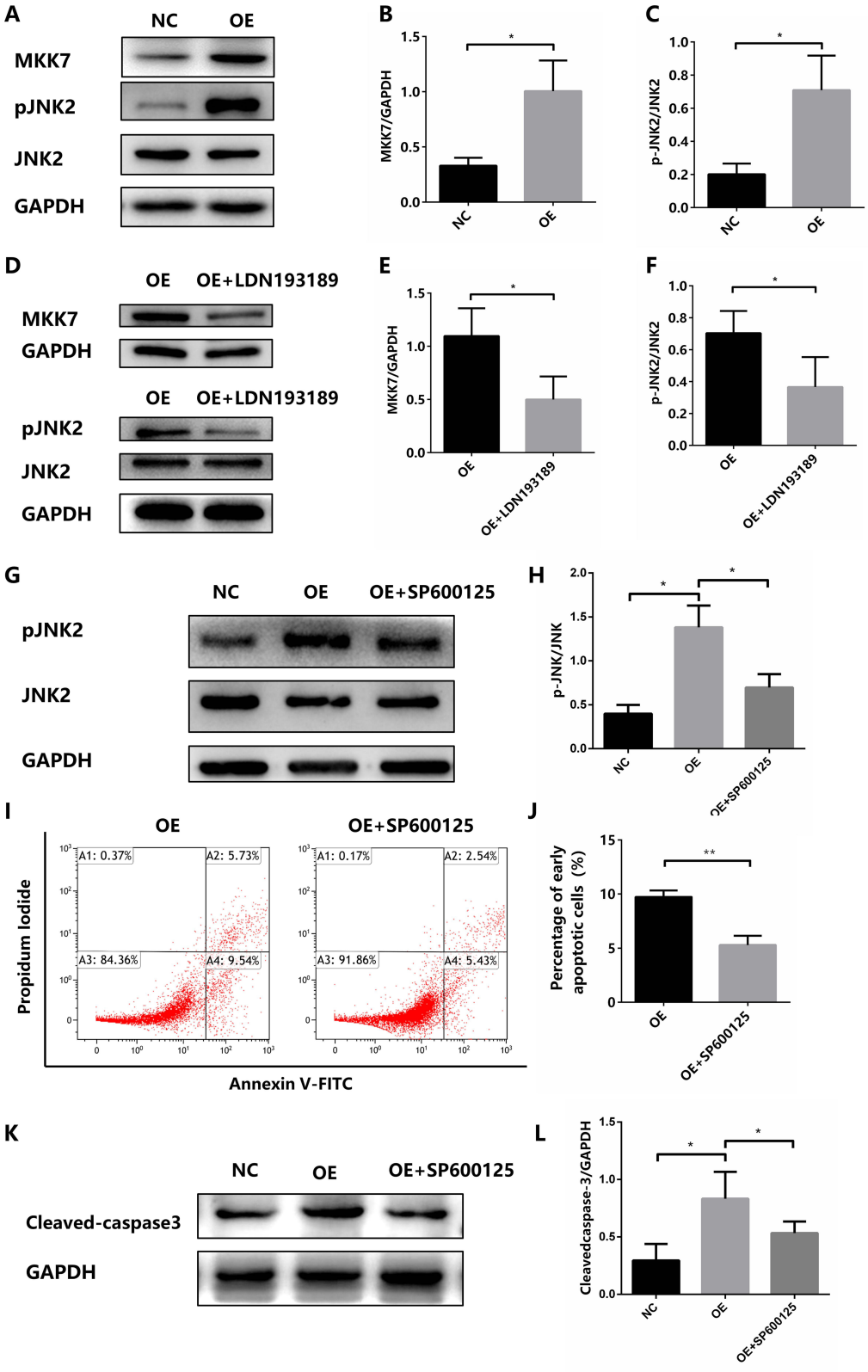

Figure 8 Role of the MKK7/JNK2 signaling pathway in BMP7-induced HPBEC apoptosis. * P<0.05, ** P<0.01. (**A**–**F**) Expression of MKK7 and p-JNK2 in different groups. (**G** and (**H**) The effect of SP600125 on the phosphorylation of JNK2. (**I** and **J**) Effect of SP600125 on the HPBEC apoptosis. (**K** and **L**) Effect of SP600125 on the expression of cleaved caspase-3.

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parameters of quantitative CT. BMP7 also showed a high level in the sputum of COPD and was correlated with inflammatory cytokines, including IL-6, IL-8, and IL-1β. Owing to constraints in research conditions, objective limitations, and challenges in sample collection, the present study was limited in its ability to conduct comprehensive investigations into the role of BMP7 in COPD inflammation. Current research indicates that BMP7's role in inflammation varies across different diseases and tissues. Studies have shown that BMP7 could enhance inflammatory responses under certain conditions. For instance, BMP7 was abnormally expressed in psoriatic lesions, leading to increased production of pro-inflammatory cytokines such as TNF-α, IL-6, and GM-CSF in Langerhans cells.[24] Similarly, in a bleomycin-induced mouse model of lung fibrosis, rhBMP7 administration resulted in elevated IL-6 levels.[25] Conversely, BMP7 has also been shown to exert anti-inflammatory effects. In a rat model of uremic peritoneal fibrosis, BMP7 reduced macrophage infiltration and decreased IL-1β release from fibroblasts in vitro.[26] Whether BMP7 acts as a pro-inflammatory or anti-inflammatory factor and influences airway disease progression in COPD through its effects on inflammation remains to be elucidated and warrants further investigation.

Abnormal proliferation and differentiation of airway epithelial cells is core in airway disease in COPD.[27] The airway epithelium is the first line of defense of the respiratory system against harmful stimulation.[28] Under normal conditions, after airway injury caused by exogenous harmful stimulation, basal cells proliferate and migrate rapidly, differentiate into ciliated and secretory cells, and maintain the integrity of the epithelial structure.[29] However, in patients with COPD, after long-term exposure to harmful gases and particles, basal cells show decreased self-renewal ability and abnormal differentiation, resulting in decreased airway repair ability, increased cell death, squamous metaplasia, and decreased ciliary cell differentiation, all of which constitute the pathological basis of COPD airway disease.[8,30]

To elucidate the role of BMP7 in airway disease, the effect of BMP7 in the proliferation and differentiation of human bronchial epithelial cells was investigated. The results showed that a high dose of rhBMP7 inhibits the proliferation of HPBECs. A previous study found that the effect of BMP7 in cell proliferation was concentration dependent; low-dose BMP7 increased cell proliferation and high-dose BMP7 inhibited cell proliferation and stimulated apoptosis in embryonic kidney explants.[31] Further research showed that BMP7 promoted squamous metaplasia and inhibit ciliated cell metaplasia in human bronchial epithelial cells. This result is similar to the role of BMP7 in squamous metaplasia of psoriasis, which demonstrates that BMP7 can promote the differentiation of skin epithelial progenitor cells into keratinocytes and is involved in the squamous metaplasia of psoriasis.[24] These results indicate that high expression of BMP7 in the bronchial epithelia of COPD contributes to airway remodeling through abnormal regulation of cell proliferation and differentiation.

The imbalance of apoptosis and anti-apoptosis is an important mechanism of COPD. The increase in apoptosis can lead to an imbalance in the destruction and reconstruction of airway epithelial tissue and a decrease in tight junction and secondary necrosis, resulting in remodeling of the airway structure and a decrease in the airway number.[32] As BMP7 can also regulate epithelial cell apoptosis, we further investigated the effect of BMP7 in HPBECs. The results showed that high expression of BMP7 increased HPBEC apoptosis. Proteomics were conducted to clarify the downstream signaling pathway responsible for the BMP7-induced apoptosis of HPBECs. The results suggested that the activation of the MKK7 signaling pathway may be involved in this process. MKK7 is illa member of the mitogen activated protein kinase (MAPK) family and one of the specific kinases of the JNK signaling pathway. Moreover, MKK7 participates in the signal transduction-mediated cell response under various cytokines and environmental stresses, and plays an important role in cell proliferation, apoptosis and tumorigenesis.[33,34] We verified the activation of the MKK7/JNK2 pathway in BMP7 overexpressing HPBECs. Our results demonstrated that the inhibition of the JNK pathway significantly reduced the level of apoptosis induced by BMP7 overexpression. These results indicated that BMP7 promoted apoptosis of human bronchial epithelial cells by regulating the MKK7/JNK2 signaling pathway.

Previous research has indicated that members of the BMP family, including BMP4, might contribute to the pathological processes in COPD airways. But, the function of BMP7 in the pathogenesis of COPD has not been fully explored. This study demonstrates that BMP7 promotes airway lesions via the MKK7/JNK2 pathway, inhibiting proliferation, promoting squamous metaplasia, and inducing apoptosis in human bronchial epithelial cells (HPBECs). In contrast, BMP4 activates the Smad1/5/8 pathway, leading to aberrant epithelial differentiation toward a smoking-associated phenotype, notably squamous metaplasia.[21] Given the diversity of BMPs and the complexity of downstream signaling pathways, our study focused on the role of MKK7/JNK2 in BMP7-mediated airway remodeling. Further research is needed to elucidate the involvement of other BMP members and pathways like SMAD in COPD airway lesions.

The correlation between BMP7 and airway remodeling in COPD suggests potential clinical applications, definitely further investigation is needed. Considering the relation among BMP7 and key clinical parameters such as FEV1 and airway wall thickness, BMP7 can be a biomarker for disease severity or progression. However, the utility of BMP7 as a biomarker need validation through larger, more diverse cohorts. BMP7's involvement in promoting apoptosis and abnormal differentiation presents intriguing possibilities. Manipulating BMP7 levels or inhibiting its downstream pathways, such as MKK7/JNK2, might alleviate airway epithelial damage. Nevertheless, due to the complexity of BMP7 and its associated biological functions—BMP7 may exert pro-inflammatory and anti-inflammatory effects depending on the context—its potential role as a therapeutic target in COPD interventions requires more in-depth researches.

The limitations of this study are as followed. Firstly, most lung tissue specimens were from patients with COPD with mild to moderate airflow limitation, and there was a lack of specimens from patients with COPD with severe airflow limitation. Secondly, we were unable to fully incorporate CAT scores and dyspnea index data due to incomplete recordings, which may have affected the clinical significance of our findings. Thirdly, the specific signal transduction mechanism of BMP7 that regulates bronchial epithelial cell proliferation and differentiation has not yet been elucidated, and further research is needed.

Conclusions

The expression of BMP7 is increased in the airway epithelium of COPD. BMP7 can inhibit the proliferation of human bronchial epithelial cells, promote squamous metaplasia, and inhibit ciliary metaplasia. BMP7 promotes apoptosis of human bronchial epithelial cells through the MKK7/JNK2 signaling pathway, which may play a role in the pathogenesis of airway disease in COPD.

Abbreviations

BMP7, bone morphogenetic protein-7; COPD, Chronic obstructive pulmonary disease; FEV1, Forced expiratory volume in first second; FVC, Forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease; JNK, c-Jun N-terminal kinase; MAP2K7/MKK7, mitogen-activated protein kinase kinase 7 / mitogen-activated protein kinase 7.

Ethics Approval and Consent to Participate

This study was approved by the Research Ethics Committee of Qilu Hospital of Shandong University (No 2015091). All participants in the study provided informed consent.

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Disclosure

The authors declare that there are no conflicts of interest in this article.

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