

# Effects of ozone stimulation of bronchial epithelial cells on proliferation and collagen synthesis of co-cultured lung fibroblasts

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**Abstract.** Ozone (O<sub>3</sub>) as a major air pollutant is widely recognized for causing pathological changes of the airway system. However, it is not clear whether O<sub>3</sub> exposure of bronchial epithelial cells (BECs) influences the proliferation and collagen synthesis of submucosal fibroblasts and contributes to the pathogenesis of airway remodeling in diseases, including asthma. In the present study, a co-culture method was applied to culture human lung fibroblasts (HLFs) with human bronchial epithelial cells (HBECs) that were pre-stimulated with O<sub>3</sub>. Following co-culture for up to 24 h, the proliferation of HLFs was measured using MTT colorimetry. Furthermore, the collagen synthesis capacity of HLFs was determined by the level of hydroxyproline. In addition, the protein expression levels of cytokines, including transforming growth factor (TGF)-β1, tumor necrosis factor (TNF)-α and prostaglandin E2 (PGE2) were assessed. Results indicated that the proliferation of HLFs co-cultured with HBECs was significantly inhibited when compared with HLFs cultured alone (P<0.05). By contrast, co-culture with O<sub>3</sub>-stimulated HBECs significantly promoted the proliferation of HLFs compared with the HLFs cultured alone or those cultured with HBECs but no O<sub>3</sub> stimulation, respectively (P<0.05 and P<0.01). Furthermore, similar effects were observed regarding the collagen synthesis capacity of HLFs co-cultured with HBECs for 24 h. In the supernatant, TGF-β1 concentration was continuously increased over 24 h, whereas the concentration of PGE2 increased and plateaued between 12 to 24 h and TNF-α concentration was not significantly altered during the assessed time period. To conclude, the present results suggest that O<sub>3</sub> pre-exposure of HBECs may promote the transformation of

HLFs from the typical inhibitory state into a promoting state with respect to proliferation and collagen synthesis, which may likely occur through a mechanism that influences the balance between pro- and anti-inflammatory factors, including TGF-β1 and PGE2. The present findings may improve the understanding of the mechanism involved in O<sub>3</sub>-induced airway remodeling from a novel perspective of maintenance/loss of steady-state function of the airway epithelium.

## Introduction

Bronchial asthma is a chronic airway inflammatory disease mediated by a variety of cell types and factors (1). A key hallmark associated with bronchial asthma is marked airway remodeling, which at tissue and cellular levels primarily includes airway wall thickening, airway epithelial fibrosis, airway smooth muscle cell hyperplasia and hypertrophy and myofibroblast proliferation and activation (2). At a molecular level, the pathological changes of airway remodeling are known to be associated with increased deposition of extracellular matrix (ECM) components, including type I, III and IV collagen fibers and fibronectin (3-5). Although the underlying mechanism of airway remodeling is not fully understood, it is generally acknowledged that airway remodeling is predominantly a result of the imbalance of airway injury and repair following repeated airway injury from chronic exposure to hazardous environmental factors and abnormal repair due to chronic airway inflammation (2). This association between airway remodeling and environmental hazards may be a contributing factor to the rising incidence of bronchial asthma, particularly in industrial countries worldwide during recent decades; the highest prevalence of clinical asthma worldwide were reported in Australia, Sweden, UK, Netherlands and Brazil were 21.5, 20.2, 18.2, 15.3 and 13.0%, respectively (6).

Among the environmental hazardous factors known to cause adverse effects on health, ozone (O<sub>3</sub>) is a major factor in air pollution, which is widely recognized as a particularly harmful risk to airway health (7,8). In human and animal studies, it has been demonstrated that early exposure at post-natal stage or long-term exposure at later stages to O<sub>3</sub> may result in pathological changes in the respiratory system, including modifications of time and mode of alveolar growth

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and development (9,10). It has been reported that O<sub>3</sub> exposure may also cause inflammation in the airways and the lungs and structural reorganization of smooth muscle cells (11,12). Previous studies indicated that O<sub>3</sub> induced oxidative stress to airway epithelial cells cultured *in vitro*, resulting in the release of nitric oxide and epithelial cell injury (13), and that exposure to O<sub>3</sub> of the bronchial epithelium resulted in airway inflammation and airway hyperresponsiveness (14,15).

Notably, there is little evidence to support whether O<sub>3</sub> exposure may result in airway remodeling. The limited reports regarding this may partly be due to the fact that O<sub>3</sub> directly targets bronchial epithelial cells (BECs) but not the fibroblasts that reside beneath these cells, which serve a key role in airway remodeling (16).

In order to explore the association between O<sub>3</sub> exposure and airway remodeling, it was hypothesized that BECs exposed to O<sub>3</sub> will release inflammatory growth factors, including transforming growth factor (TGF)- $\beta$ 1, which may subsequently enhance the secretion and activity of collagen from adjacent fibroblasts in the airway wall and ultimately result in airway remodeling. To investigate this hypothesis in the present study, a co-culture model of human lung fibroblasts (HLFs) and human bronchial epithelial cells (HBECs) was constructed. O<sub>3</sub> stress was applied to the HBECs and cell proliferation and secretion of cytokines in the HLFs were assessed. The present study aimed to elucidate the mechanism of O<sub>3</sub>-induced airway remodeling from a novel perspective of maintenance/loss of steady-state function of the airway epithelium.

## Materials and methods

**HBE and HLF culture.** HBECs from a cell line were provided by Professor Gruenent of University of California, San Francisco (San Francisco, USA) (17). Embryonic diploid HLF-02 HLFs were purchased from the Cell Center of Central South University (Changsha, China). HBECs were seeded in Dulbecco's modified Eagle medium (DMEM)/F12 (1:1 ratio; Hyclone, Logan, UT, USA) supplemented with 15% newborn calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 U/ml streptomycin in a 100-ml culture flask. Cells grew adherently at 37°C until 80% confluence was reached. Subsequently, the culture medium was displaced and the cells were rinsed with phosphate-buffered saline one to two times. Following this, HBECs were trypsinized with 1 ml 0.25% trypsin with 0.05% EDTA and scattered gently through a pipette to create the cell suspension. HBECs were divided in 1:2 or 1:3 proportions for subculture. When cells in subculture grew to 80% confluence with a healthy, cuboid morphology, they were seeded on culture plates for subsequent experiments. HLFs were cultured and prepared for experiments as described above.

**Co-culture of HLFs and O<sub>3</sub>-stimulated HBECs.** Prior to co-culture, confluent HBECs in subculture flasks were harvested by trypsinization and then counted manually using trypan blue (0.4%) staining to detect the live cells. HBECs were seeded onto coverslips that had been placed in 24-well culture plates (Thermo Fisher Scientific, Inc.) at 1x10<sup>5</sup> cells/ml (1 ml per well). Subsequently, the cells were cultured at 37°C and 5% CO<sub>2</sub> for 48 h in DMEM medium supplemented with 15% newborn calf serum to allow the cells to adhere to the coverslip. After

48 h, the medium was replaced with DMEM supplemented with 1% newborn calf serum (Gibco; Thermo Fisher Scientific, Inc.).

To establish the O<sub>3</sub> stimulated HBECs group, HBECs were prepared as above and were incubated in DMEM supplemented with 1% newborn calf serum for 8 h. Subsequently, HBECs were stimulated with O<sub>3</sub> (2 ppm) for 30 min and incubated for 1 h prior to co-culture with HLFs. For the control (HBECs) group, the HBECs followed the same time course and procedure, with the exception that the HBECs were not exposed to O<sub>3</sub> for 30 min.

HLFs were also harvested and counted as described above for HBECs. HLFs were seeded into the 24-well plates at 2x10<sup>4</sup> cells/ml (1 ml per well). HLFs were cultured in DMEM supplemented with 15% newborn calf serum for 12 h to allow the cells to adhere to the culture dish. The medium was replaced with DMEM supplemented with 1% newborn calf serum and cells were incubated for a further 12 h. Subsequently, a sterile stainless steel screen mesh was inserted into each well containing the HLFs and a coverslip with HBECs that had been stimulated or not simulated with O<sub>3</sub> was placed on top of the stainless steel screen mesh that provided separation between the HBECs and HLFs in the same well. HBECs and HLFs were co-cultured in the same well for 24 h. following this, the coverslip and the mesh were removed from the well, respectively, and the proliferation of HLFs was assessed by MTT colorimetric method.

**MTT colorimetry for evaluation of cell proliferation.** The proliferation of HLFs was evaluated using the MTT colorimetric method. HLFs were divided into the following three groups: Fibro group, which contained HLFs that had been cultured alone; co-culture group, which contained HLFs co-cultured with HBECs without O<sub>3</sub> stimulation; and O<sub>3</sub> co-culture group, which contained HLFs co-cultured with O<sub>3</sub>-stimulated HBECs. Cells were seeded at 2x10<sup>4</sup> cells/ml (1 ml/well) in 24-well plates and treated with 100  $\mu$ l of 0.5% MTT solution. Cells were incubated at 37°C for 4 h and the supernatant was removed. A total of 800  $\mu$ l dimethyl sulfoxide was added to each well followed by 10 min steady oscillation of the 24-well plates. The cell suspension was aliquoted into 96-well plates and was analyzed using an automatic microplate reader (Elx800, Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 570 nm. Culture medium without cells served as a blank control. Each experiment was repeated six times.

The proliferation of HBECs in the O<sub>3</sub> stimulated and control groups was assessed as described above. The proliferation of HBECs was measured at 1, 12 and 24 h. Each measurement was repeated four times.

**Assessment of HLF collagen synthesis.** Collagen synthesis in HLFs was determined by assessing the hydroxyproline levels in HLF culture medium supernatant using a hydroxyproline kit (cat. no. A030-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Hydroxyproline is a major component of collagen (18). Therefore, measurement of hydroxyproline levels in cell culture medium indicates the ability of the cell to produce collagen (18-21). To Pleasure the hydroxyproline levels in the culture medium supernatant, 0.5 ml of culture medium was combined with 1 ml hydrolysate and hydrolyzed for 20 min in a boiling water bath (pH 6.0-6.8). A blank tube with distilled water, a standard tube with 5  $\mu$ g/ml standard solution and a measurement tube with

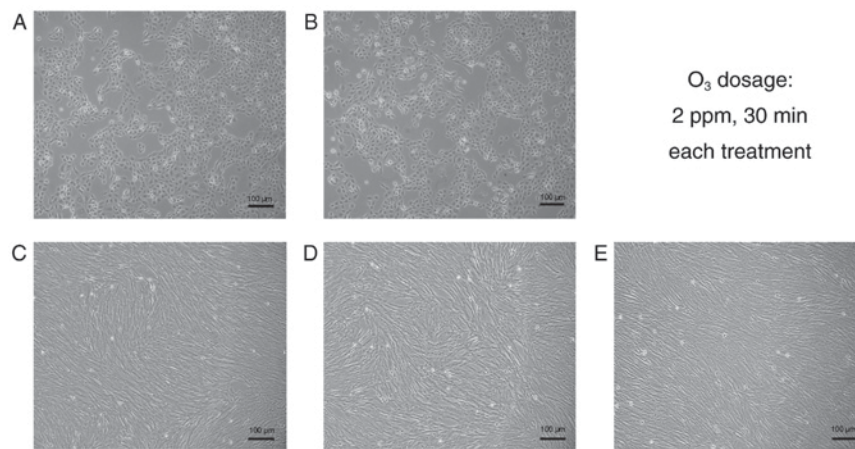


Figure 1. Representative microscopic images of HBECs and HLFs cultured alone or co-cultured with or without O<sub>3</sub> stimulation. (A) HBECs cultured alone without O<sub>3</sub> stimulation, (B) HBECs cultured alone with O<sub>3</sub> stimulation, (C) HLFs cultured alone, (D) HLFs co-cultured with HBECs without O<sub>3</sub> stimulation and (E) HLFs co-cultured with O<sub>3</sub>-stimulated HBECs (magnification, x50). O<sub>3</sub> stimulation was given at a dosage of 2 ppm, 30 min for each treatment. Scale bar=100 μm. HBECs, human bronchial epithelial cells; HLFs, human lung fibroblasts; O<sub>3</sub>, ozone.

the hydrolyzed supernatant of culture medium were prepared, respectively. Subsequently, a 1-ml sample was taken from each tube and mixed with reagent One. Following 10 min standing at room temperature, each sample was mixed with reagent Two and left to stand for 5 min at room temperature. Following this, the sample was mixed with reagent Three, incubated at 60°C for 15 min, cooled and centrifuged at 4°C, 2,054 x g for 10 min. The absorbance of the supernatant was measured at 550 nm. Each tube was repeatedly measured four times. Total protein was assayed using the Lowry protein assay (22). Hydroxypyrrolone levels were calculated as follows: Hydroxypyrrolone (μg/mg protein)=[optical density (OD) of test tube/OD of standard sample tube]x(concentration of standard sample/total protein).

**Detection of TGF-β1, TNF-α and PGE2 concentration in the supernatant of the co-cultured system and HBECs cultured alone.** The concentration of inflammatory mediators TGF-β1 and TNF-α in the supernatant of O<sub>3</sub>-stimulated HBECs co-cultured with HLFs was assessed at 6, 12, 18 and 24 h following co-culture using ELISA kits (cat. no. MM-0090H1 and MM-0122H1, respectively; Jingmei Bioengineering Co., Ltd., Yancheng, China) according to the manufacturer's protocol. The OD value measured at 450 nm was directly proportional to the concentration of the indicator. By constructing a standard curve, the concentration of TGF-β1 and TNF-α in the specimen could be determined accordingly. The concentration of the inflammatory mediator PGE2 in the supernatant of the co-cultured system was measured using a PGE2 radioimmunoassay kit (cat. no. ADI-905-025-1; Enzo Life Sciences, Inc., Farmingdale, NY, USA) according to the manufacturer's protocol. The concentration of PGE2 in the sample was directly calculated using an automatic γ immuno-counter. The TGF-β1, TNF-α and PGE2 concentration was detected in the supernatant of O<sub>3</sub>-stimulated HBECs and HBECs cultured alone at 24 h as described above.

**Detection of TGF-β1 protein expression levels in HBECs alone or co-cultured with HLFs.** The protein expression levels of TGF-β1 in HBECs alone or co-cultured with HLFs were detected using an immunocytochemistry method. Cells were labeled for

TGF-β1 expression using an immunocytochemistry staining kit (cat. no. SA1022; Wuhan Boster Biological Technology Ltd., Wuhan, China) according to the manufacturer's instructions. Cells were fixed with 95% alcohol at room temperature for 15 min and dipped in 30% H<sub>2</sub>O<sub>2</sub> combined with 100% methanol (1:50 ratio) at room temperature for 30 min to block endogenous peroxidase activity. Cells were immersed in 5% bovine serum albumin blocking reagent at room temperature for 20 min. Following this, cells were treated with TGF-β1 antibodies (1:1,000; cat. no. BA0290) at 37°C for 1-2 h and biotinylated goat anti-rabbit IgG antibodies (1:500; cat. no. BA1003; both Wuhan Boster Biological Technology Ltd.) at room temperature for 20 min. Reagent SABC (Wuhan Boster Biological Technology Ltd.) was added at room temperature for 20 min. Cells were subsequently counterstained with hematoxylin at room temperature for 30 sec, dehydrated and observed under an optic microscope. For quantification of TGF-β1 expression in HBECs, images were captured of five randomly selected fields of view and analyzed using a medical image analysis and management system (MIAS version 4.1; Image Processing Center of Beihang University, Beijing, China) to assess the photodensity.

**Statistical analysis.** Data was presented as the mean ± standard deviation from the representative experiment. Each experiment was repeated four to six times. All data were processed using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). An unpaired Student's t-test was performed to determine the statistical difference between two groups and multiple comparisons were determined using one-way analysis of variance followed by Dunnett's t-test. Correlations were analyzed using Pearson's correlation analysis method. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effect of O<sub>3</sub> stimulation on cell morphology and proliferation.** The morphology of HBECs and HLFs cultured alone or co-cultured with or without O<sub>3</sub> stimulation was determined (Fig. 1). Results indicated the cells in culture demonstrated a normal shape and size individually (HBECs



were cuboidal-like shape and HLFs were spindle-like shape) and were organized in usual structures collectively (HBECs were in a relatively uniform monolayer and HLFs were in a hill-valley-like formation), respectively. When the proliferation of HBECs was evaluated using the MTT method, the OD values were not significantly different between O<sub>3</sub>-stimulated HBECs and their non-stimulated counterparts when cultured for up to 24 h ( $P>0.05$ ; Fig. 2). These results indicated that O<sub>3</sub> stimulation had no marked effect on cell morphology regardless of the cell type and culture approach; furthermore, there was no significant effect on the cell proliferation of HBECs.

**Co-culture with O<sub>3</sub>-stimulated HBECs enhances cell proliferation of HLFs.** To investigate the cell proliferation of Fibro, Co-culture and O<sub>3</sub> co-culture groups, the MTT assay was performed (Fig. 3). In comparison with the Fibro group, proliferation was significantly decreased in the Co-culture group ( $0.252\pm0.018$  vs.  $0.241\pm0.017$ , respectively;  $P<0.05$ ), which indicated that co-culture conditions without O<sub>3</sub> stimulation inhibited the proliferation of HLFs. However, cell proliferation of the O<sub>3</sub> co-culture group was significantly increased compared with the Fibro group ( $0.263\pm0.017$  vs.  $0.252\pm0.018$ , respectively;  $P<0.05$ ) and Co-culture group ( $0.263\pm0.017$  vs.  $0.241\pm0.017$ , respectively;  $P<0.01$ ), respectively. These findings suggested that co-culture with O<sub>3</sub>-stimulated HBECs significantly enhanced the proliferation of HLFs when compared with HLFs cultured alone. Furthermore, co-culture with O<sub>3</sub>-stimulated HBECs significantly reversed the inhibition of HLF proliferation when co-cultured with HBECs that were not stimulated with O<sub>3</sub>.

**Co-culture with O<sub>3</sub>-stimulated HBECs enhances collagen synthesis of HLFs.** As indicated in Fig. 4, the effects on collagen synthesis of HLFs co-cultured with O<sub>3</sub>-stimulated HBECs were similar to those indicated for cell proliferation. Notably, the Co-culture group exhibited significantly decreased collagen synthesis-associated hydroxyproline levels compared with the Fibro group ( $2.014\pm0.366$  vs.  $2.634\pm0.310$ , respectively;  $P<0.05$ ), which indicated that co-culture conditions without O<sub>3</sub> stimulation reduced the collagen synthesis capacity of HLFs. However, the levels of hydroxyproline from the O<sub>3</sub> co-culture group were significantly increased compared with the Co-culture group ( $2.919\pm0.407$  vs.  $2.014\pm0.366$ , respectively;  $P<0.05$ ). These results also suggested that co-culture with O<sub>3</sub>-stimulated HBECs may reverse the inhibition of HLF collagen synthesis due to co-culture with HBECs that were not stimulated with O<sub>3</sub>.

**Co-culture with O<sub>3</sub>-stimulated HBECs influences cytokine secretion in the supernatant of the co-culture system.** The concentration of major cytokines, including TGF- $\beta$ 1, TNF- $\alpha$  and PGE2, in the supernatant of the co-culture system with O<sub>3</sub>-stimulated HBECs were assessed (Fig. 5). Results indicated that the concentration of TGF- $\beta$ 1 remained high and continuously increased over the 24 h after co-culture. TNF- $\alpha$  concentration remained low and did not significantly vary over the 24-h time period. Unlike TGF- $\beta$ 1, the concentration of PGE2 was significantly increased to the maximum at 12 h compared with 6 h ( $P<0.01$ ) and plateaued thereafter. PGE2 concentration was significantly increased at 18 and 24 h compared with

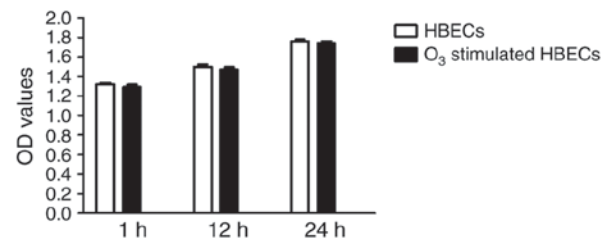


Figure 2. Effect of O<sub>3</sub> stimulation on the proliferation of HBECs cultured alone. The proliferation of HBECs was evaluated using the MTT method. The white bars represent HBECs cultured alone without O<sub>3</sub> stimulation (control) and the solid bars represent HBECs cultured alone with O<sub>3</sub> stimulation, respectively. Data are presented as the mean  $\pm$  standard deviation ( $n=4$ ). HBECs, human bronchial epithelial cells; O<sub>3</sub>, ozone; OD, optical density.

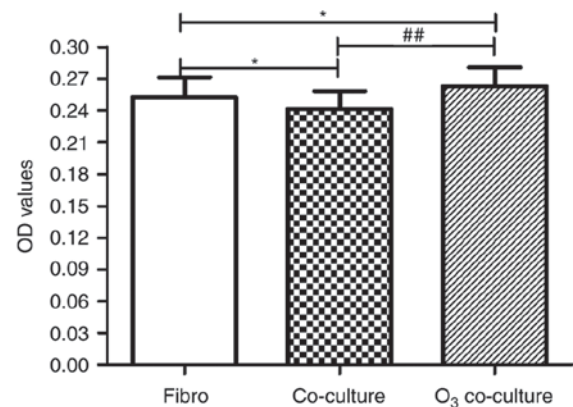


Figure 3. Effect of O<sub>3</sub>-stressed HBECs on HLF proliferation. HLF proliferation was assessed using the MTT method. Compared with the Fibro group, the proliferation of Co-culture group was significantly inhibited. However, in the O<sub>3</sub> co-culture group, the proliferation was significantly increased compared with the Fibro group and Co-culture group. Data are presented as the mean  $\pm$  standard deviation ( $n=18$ ). \* $P<0.05$ , \*\* $P<0.01$  as indicated. HBECs, human bronchial epithelial cells; HLFs, human lung fibroblasts; O<sub>3</sub>, ozone; OD, optical density; Fibro group, HLFs cultured alone; Co-culture group, HLFs co-cultured with HBECs without O<sub>3</sub>-stimulation; O<sub>3</sub> co-culture group, HLFs were co-cultured with O<sub>3</sub>-stimulated HBECs.

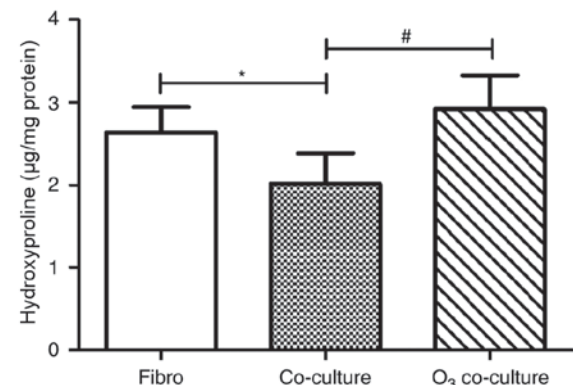


Figure 4. Effect of O<sub>3</sub>-stressed HBECs on HLF collagen synthesis. HLF collagen synthesis was measured according to the levels of hydroxyproline. Compared with the Fibro group, the hydroxyproline levels in the Co-culture group were significantly inhibited. However, in the O<sub>3</sub> co-culture group the hydroxyproline levels were significantly increased compared with the Co-culture group. Data are presented as the mean  $\pm$  standard deviation ( $n=4$ ). \* $P<0.05$ , # $P<0.05$  as indicated. HBECs, human bronchial epithelial cells; HLFs, human lung fibroblasts; O<sub>3</sub>, ozone; Fibro group, HLFs cultured alone; Co-culture group, HLFs co-cultured with HBECs without O<sub>3</sub>-stimulation; O<sub>3</sub> co-culture group, HLFs were co-cultured with O<sub>3</sub>-stimulated HBECs.

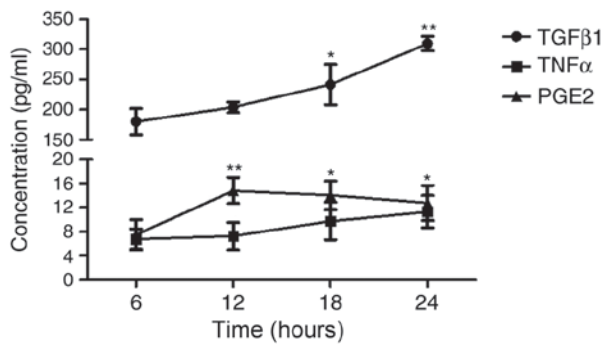


Figure 5. Time course of TGF- $\beta$ 1, TNF- $\alpha$  and PGE2 cytokine secretion in the supernatant of the co-culture system after human bronchial epithelial cells were stimulated with ozone. TGF- $\beta$ 1 and TNF- $\alpha$  secretion was measured using ELISA and PGE2 secretion was measured using a radioimmunoassay. The levels of TGF- $\beta$ 1 remained high and continued to gradually increase for up to 24 h. TNF- $\alpha$  levels were relatively low and did not vary significantly over the 24 h. However, the secretion of PGE2 increased significantly between 6 and 12 h and thereafter plateaued until 24 h. Data are presented as the mean  $\pm$  standard deviation (n=4). \*P<0.01, \*\*P<0.05 vs. 6 h. TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; PGE2, prostaglandin E2.

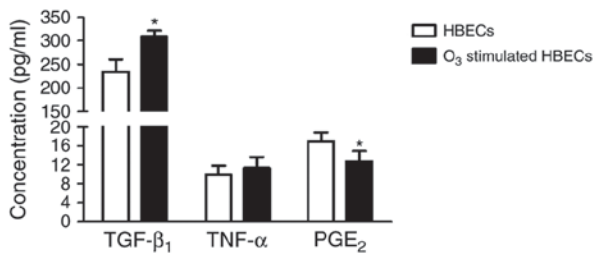


Figure 6. Effect of O<sub>3</sub> stimulation on the concentration of TGF- $\beta$ 1, TNF- $\alpha$  and PGE2 cytokines in HBECs cultured alone. The concentration levels of the cytokines TGF- $\beta$ 1 and TNF- $\alpha$  were measured using ELISA, and PGE2 was measured using a radioimmunoassay. The white bars represent HBECs cultured alone without O<sub>3</sub>-stimulation (control) and the filled bars represent HBECs cultured alone with O<sub>3</sub>-stimulation, respectively. Data are presented as the mean  $\pm$  standard deviation (n=4). \*P<0.05 vs. HBECs. TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; PGE2, prostaglandin E2; O<sub>3</sub>, ozone.

6 h (P<0.05), but no significant difference was identified when compared with 12 h.

When compared with HBECs alone, the O<sub>3</sub>-stimulated HBECs cultured for 24 h exhibited significantly increased TGF- $\beta$ 1 concentration (234.80 $\pm$ 25.55 vs. 309.47 $\pm$ 12.22 pg/ml, respectively; P<0.05; Fig. 6). However, the concentration of PGE2 was significantly decreased in O<sub>3</sub>-stimulated HBECs compared with HBECs alone (12.74 $\pm$ 2.19 vs. 16.97 $\pm$ 1.92 pg/ml, respectively; P<0.05) and no significant difference was indicated in the concentration of TNF- $\alpha$  between the experimental groups (P>0.05).

**Correlation between cytokine secretion and cell proliferation and collagen synthesis in HLFs.** In order to investigate whether cell proliferation or collagen synthesis was associated with the secretion of cytokines when HLFs were co-cultured with O<sub>3</sub>-stimulated HBECs, correlation analysis between HLF proliferation and hydroxyproline levels and cytokine secretion was performed (Fig. 7). Results indicated a significant positive correlation between the secretion of TGF- $\beta$ 1 from

the co-cultured system with O<sub>3</sub>-stimulated HBECs and cell proliferation (r=0.772, P=0.015) and collagen synthesis (r=0.758, P=0.018, Fig. 7A and B). Notably, no correlation was indicated between the cytokine secretion of TNF- $\alpha$  with cell proliferation or collagen synthesis of HLFs when co-cultured with O<sub>3</sub>-stimulated HBECs (Fig. 7C and D). However, a significant negative correlation was indicated between the cytokine secretion of PGE2 and cell proliferation (r=-0.783, P=0.013) and collagen synthesis (r=-0.817, P=0.007) of HLFs co-cultured with O<sub>3</sub>-stimulated HBECs.

**Effect of O<sub>3</sub> stimulation on TGF- $\beta$ 1 expression in HBECs.** Immunocytochemistry was conducted to investigate TGF- $\beta$ 1 expression levels in HBECs and quantitative expression analysis was performed (Fig. 8). Results indicated that the expression level of TGF- $\beta$ 1 in O<sub>3</sub>-stimulated HBECs cultured for 24 h was increased compared with HBECs alone (Fig. 8A). This difference was statistically significant (P<0.05; Fig. 8B). In the co-culture system of HBECs and HLFs, the expression level of TGF- $\beta$ 1 was similarly enhanced in the presence of O<sub>3</sub>-stimulated HBECs (data not shown).

## Discussion

Damage to the bronchial epithelium destroys the integrity of airway structure and function, leading to instability of the local microenvironment and dysfunction of the airways. These are initiative events of multiple airway diseases, including asthma and bronchitis (23). Repeated airway injury and abnormal repair may cause chronic airway inflammation, which eventually results in airway remodeling, as characterized by the activation and proliferation of fibroblasts and accumulation of the ECM (24). As one of the major structural cells of the airway submucosa, lung fibroblasts are known to contribute to the formation of subepithelial fibrosis, increase airway responsiveness and promote airway remodeling (25-27). Furthermore, lung fibroblasts have been acknowledged as the primary cells that produce ECM proteins to form collagen fibers, elastic fibers and reticular fibers, which are downstream components of the airway remodeling process (28). Although the function of lung fibroblasts has been extensively studied, it is not clear whether stress induced on the bronchial epithelium from the external environment, including air pollutants, may influence the submucosal lung fibroblasts to contribute to pathological airway remodeling. A useful model to explore this effect is the use of O<sub>3</sub> to stimulate the bronchial epithelium prior to examination of the cellular functions of adjacent fibroblasts not in direct contact of O<sub>3</sub> (29). O<sub>3</sub> is a common major component of air pollution and has been revealed in various studies to cause airway epithelial shedding, airway inflammation and airway hyperresponsiveness (16,30). Therefore, the aim of the present study was to co-culture HLFs with airway epithelial cells pre-stimulated with O<sub>3</sub> and subsequently measure the functional changes, including collagen synthesis, of the fibroblasts. Results of the present study indicated that, although O<sub>3</sub> stimulation seemed to cause no noticeable changes in cell morphology or proliferation of HBECs cultured for up to 24 h, functional changes in the co-culture system of O<sub>3</sub>-stimulated HBECs and HLFs were indicated. Specifically, in the co-culture group without O<sub>3</sub> stimulation, the proliferation of HLFs was significantly

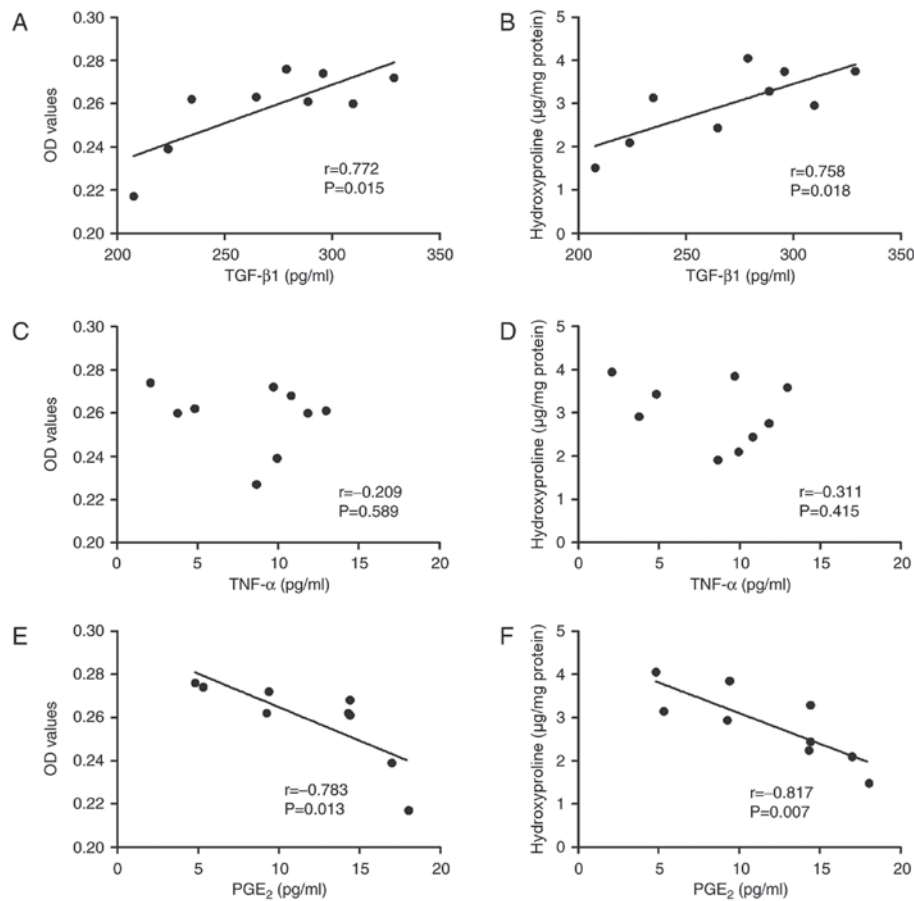


Figure 7. Correlation between cytokine concentration and cell proliferation and collagen synthesis in HLFs co-cultured with O<sub>3</sub>-stressed HBECs. (A and B) A positive linear correlation between proliferation of HLFs and the concentration of TGF- $\beta$ 1 in the co-culture supernatant. Collagen synthesis capacity in the culture supernatant was also positively correlated with TGF- $\beta$ 1 concentration ( $r=0.758$ ,  $P=0.018$ ). (C and D) There was no correlation between the proliferation and TNF- $\alpha$  concentration ( $r=0.209$ ,  $P=0.589$ ), nor between collagen synthesis and TNF- $\alpha$  concentration ( $r=-0.311$ ,  $P=0.415$ ). (E and F) A negative linear correlation between the proliferation and PGE<sub>2</sub> concentration of HLFs ( $r=-0.783$ ,  $P=0.013$ ) and between collagen synthesis and PGE<sub>2</sub> concentration ( $r=-0.817$ ,  $P=0.007$ ) was indicated. TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; O<sub>3</sub>, ozone; HBECs, human bronchial epithelial cells; HLFs, human lung fibroblasts; OD, optical density.

inhibited. However, co-culture of HLFs with O<sub>3</sub>-stressed HBECs resulted in significantly enhanced HLF proliferation, indicating that O<sub>3</sub> stimulation reversed co-culture-induced inhibition of cell proliferation. O<sub>3</sub> stimulation on HBECs had a similar effect on the collagen synthesis capacity of the HLFs in the *in vitro* co-culture model. These results suggest that O<sub>3</sub> stress on the airway epithelium may be associated with the pathogenesis of airway remodeling via its indirect effect on the submucosal structural cells, including the HLFs.

The bronchial epithelium is an important source of complex inflammatory mediators, including cytokines and chemokines (31). By secreting inflammatory mediators, HBECs may interact with interstitial fibroblasts and mediate the process of epithelial-mesenchymal transition or fibroblast-myofibroblast transition, therefore actively participating in asthmatic airway remodeling (32-34). In the present study, O<sub>3</sub> exposure upregulated the concentration of TGF- $\beta$ 1 in the supernatant of the co-culture system persistently for up to 24 h. Furthermore, O<sub>3</sub> stress also promoted TGF- $\beta$ 1 secretion in the HBEC supernatant and TGF- $\beta$ 1 expression in HBECs labeled by immunocytochemistry. In addition, correlation analysis suggested that O<sub>3</sub>-induced upregulation of TGF- $\beta$ 1 secretion was positively and linearly correlated with cell proliferation and collagen synthesis of HLFs

co-cultured with O<sub>3</sub>-stimulated HBECs. These results indicated that the increased TGF- $\beta$ 1 content may serve a key role in the proliferation and collagen synthesis of HLFs in the co-culture system. Furthermore, it is acknowledged that TGF- $\beta$ 1 may promote differentiation of fibroblasts into myofibroblasts, which secrete collagen and growth factors, including endothelin 1 and vascular endothelial growth factor, and then further promote proliferation of smooth muscle cells and vascular endothelial cells (35,36). Notably, it has also been reported that TGF- $\beta$ 1 induces mesenchymal transition of bronchial epithelial cells and thus promotes airway remodeling in asthma (37). Additionally, damaged epithelial cells may extend and amplify remodeling signals into a deeper layer of the mucous membrane (38). Such communication between epithelial cells and mesenchymal cells during asthma has been termed as epithelial mesenchymal trophic unit reactivation (38).

The present study indicated a low concentration of TNF- $\alpha$  was identified in the supernatant of the co-culture system with or without O<sub>3</sub> stimulation. TNF- $\alpha$  content was also not significantly correlated with HLF proliferation and collagen synthesis. However, the present study demonstrated that the synthesis of PGE<sub>2</sub> in the co-culture system with O<sub>3</sub>-stressed HBECs was significantly increased during the early stage and plateaued



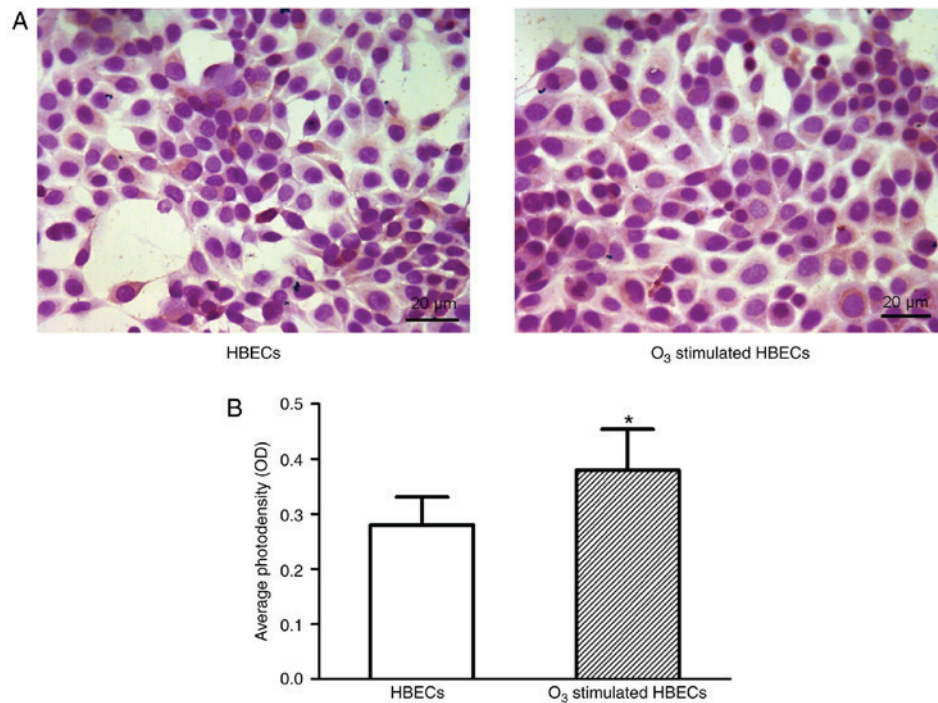


Figure 8. Representative images and corresponding quantitative expression level of TGF- $\beta$ 1 in HBECs with or without O<sub>3</sub> stimulation. HBECs were cultured alone without O<sub>3</sub> stimulation (control) or cultured alone with O<sub>3</sub> stimulation. (A) TGF- $\beta$ 1 in HBECs was labeled by immunocytochemistry and imaged by photomicroscope. (B) Expression level of TGF- $\beta$ 1 in HBECs was quantified according to the averaged photodensity of the images. Data are presented as the mean  $\pm$  standard deviation (n=4). \*P<0.05 vs. HBECs. HBECs, human bronchial epithelial cells; HLFs, human lung fibroblasts; O<sub>3</sub>, ozone; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

from 12-24 h. However, the secretion of PGE2 in O<sub>3</sub>-stimulated HBECs was significantly reduced compared with HBECs alone. In addition, correlation analysis indicated a negative linear correlation of PGE2 content with HLF proliferation and collagen synthesis. Therefore, the present findings suggested the regulatory effect of O<sub>3</sub>-stressed HBECs on HLF activity was associated with reduced PGE2 content in the airway tissues. Notably, PGE2 is a lipid mediator that may be derived from cell membrane phospholipids via the cyclooxygenase signaling pathway of arachidonic acid metabolism (39). PGE2 released from HBECs has been reported to relax airway smooth muscles, reduce the sensitivity of smooth muscles to contractile medium, inhibit the proliferation of smooth muscle cells and fibroblast activity and enhance the tolerance of lung epithelial cells to injury factors (23). These findings suggest that PGE2 may act as a negative feedback factor to counteract O<sub>3</sub>-induced proliferation and airway remodeling processes.

In the present study, the fact that the HBECs without O<sub>3</sub> stimulation in the co-culture system inhibited the proliferation of HLFs is conducive to maintaining the normal structure and function of the bronchial airways. However, co-culture with O<sub>3</sub>-stimulated HBECs with HLFs resulted in increased cell proliferation and collagen synthesis of the HLFs and increased secretion of TGF- $\beta$ 1. Considering the pro- and anti-inflammatory properties of TGF- $\beta$ 1 and PGE2 respectively, the present findings suggest that the possible mechanism involved in the effect of O<sub>3</sub> stimulation on the co-culture system may lie in the system's ability to maintain balance between pro- and anti-inflammatory factors. Under physiological conditions, the expression of anti-inflammatory factors, including PGE2, may

prevail in the system to inhibit the proliferation and collagen synthesis of fibroblasts via PGE2 receptor EP2 subtype binding and cAMP production, therefore maintaining the airway homeostasis (40,41). However, under a stressed state, such as with O<sub>3</sub> stimulation, the expression of pro-inflammatory factors, including TGF- $\beta$ 1, may be increased in the system and result in enhanced cell proliferation and collagen synthesis of fibroblasts, which may primarily occur through the regulation of p21/Waf1 gene transcription factors and the cyclin E-associated kinase (42). This may ultimately result to chronic and permanent airway remodeling *in vivo*.

In conclusion, the present findings suggest that O<sub>3</sub> stimulation of HBECs is a likely factor in promoting cell proliferation and collagen synthesis activity of HLFs via the regulation of pro- and/or anti-inflammatory cytokines, including TGF- $\beta$ 1 and PGE2. Such cellular and molecular functional associations between HBECs and HLFs in response to O<sub>3</sub> stimulation may provide an important connection between air pollution and airway remodeling in diseases such as asthma.

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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

YW, HO and LD designed the study. YW and MT performed the experiments. YW, HO and LD wrote the manuscript.

## Ethics approval and consent to participate

All experiments using *in vitro* cultured human bronchial epithelial cells and lung fibroblasts were approved by the Medical Ethics Committee of Changzhou University (Changzhou, China) according to Ethics Examination Methods on Human Related Biomedical Research issued by China's Health Ministry (approval ref. no. CCZUBME20150516YW).

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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