Chronic mucus hypersecretion in COPD and asthma

Tasena, Hataitip

CHAPTER 5

Airway mucus secretion in COPD-derived airway epithelial cells is promoted by fibroblast-epithelium crosstalk

Submitted

Hataitip Tasena[1,2]
Maarten van den Berge[2,3]
Alen Faiz[1,2,3]
Marjan Reinders-Luinge[1,2]
Machteld N Hylkema[1,2]
Wim Timens[1,2]
*Corry-Anke Brandsma[1,2]
*Irene H Heijink[1,2,3,]
(*both authors contributed equally)

[1]University of Groningen, University Medical Centre Groningen, Department of Pathology and Medical Biology, Groningen, the Netherlands.
[2]University of Groningen, University Medical Centre Groningen, Groningen Research Institute for Asthma and COPD, Groningen, the Netherlands.
[3]University of Groningen, University Medical Centre Groningen, Department of Pulmonary Diseases, Groningen, the Netherlands.

ABSTRACT

Chronic mucus hypersecretion (CMH) is a common feature in chronic obstructive pulmonary disease (COPD). One of the major factors contributing to CMH is higher mucin release from airway epithelium. We hypothesized that fibroblasts are involved in mucociliary differentiation and epithelial mucin production and release.

Primary bronchial epithelial cells (PBECs) from COPD patients with CMH were cultured at air-liquid interface (ALI) with and without primary airway fibroblasts (PAFs). MUC5AC and MUC5B release was measured in apical wash fluid. IL-6, CXCL8, and CCL20 release was measured in basal supernatant by ELISA. mRNA expression of mucociliary differentiation markers and cytokines was assessed by qPCR. Cross-sections of paraffin-embedded inserts were stained and semi-quantitatively scored for mucus-positive and ciliated cells using Alcian blue and acetylated α-tubulin immunohistochemistry, respectively.

Upon co-culture with PAFs, PBECs expressed higher MUC5B mRNA and secreted higher levels of MUC5AC and MUC5B proteins. These effects were accompanied by more pronounced mucous cell differentiation. Higher levels of IL-6 were secreted upon co-culture of PBECs and PAFs compared to mono-culture. PAFs, but not PBECs, expressed higher IL-6 mRNA upon co-culture. The use of anti-IL-6 neutralizing antibody in Calu-3 cultures abolished the increase in MUC5B mRNA upon co-culture with fibroblasts, while no change was observed for MUC5AC.

This study demonstrates that fibroblasts are involved in the regulation of mucous cell differentiation and epithelial mucin release, and that specifically MUC5B expression can be mediated, at least in part, by fibroblast-derived IL-6. These findings provide support for the notion that fibroblast-epithelium crosstalk contributes to CMH in COPD.

INTRODUCTION

Chronic mucus hypersecretion (CMH) is an important symptom in the patients with chronic obstructive pulmonary disease (COPD) and is associated with lower quality of life, an accelerated decline of lung function, more severe airflow obstruction, and an increased risk of exacerbations and mortality[1]. Currently, an effective CMH-targeted therapy is lacking, reflecting an urgent need to better understand the mechanisms underlying CMH pathophysiology.

CMH is usually characterized by the presence of chronic inflammation, cough and sputum expectoration[2,3]. Goblet cells present in the airway mucosa as well as mucus glands in the airway submucosa secrete heavily glycosylated proteins called mucins which are principal components of mucus. The most abundant gel-forming mucins found in human airways are MUC5AC and MUC5B[4]. Both are increased in COPD[6] and can be positively regulated by SAM Pointed Domain Containing ETS Transcription Factor (SPDEF)[6–8]. Furthermore, both goblet cell hyperplasia and ciliary dysfunction are thought to contribute to CMH in COPD[9,10]. Cilia on airway epithelium of COPD smokers are shorter than those of healthy smokers and non-smokers9, which can lead to ineffective mucus clearance.

While stromal cells in sub-epithelial layers do not produce mucus, they may play a critical role in CMH development by regulating differentiation of goblet or ciliated cells and/or regulation of mucus secretion. Various reports suggest that epithelial cells communicate with stromal cells, particularly fibroblasts, influencing the phenotypes of each other[11–14]. Previous studies using animal-derived cells suggest that fibroblasts can promote *in vitro* epithelial differentiation to closely resemble native tracheal epithelium[14,15]. In a rat model, more proliferation of basal cells as well as more differentiation into ciliated and MUC5AC-producing cells was observed upon co-culture with fibroblasts[16]. None of these studies assessed MUC5B production or identified the mediators driving this crosstalk. Moreover, it remains to be determined whether these findings are applicable to human cells and whether they are related to CMH in COPD. Recently, we reported that fibroblasts from COPD patients with CMH express higher levels of various receptors, including the IL-1 family receptor IL-1R1, which is involved in IL-6 and CXCL8 release[17]. These pro-inflammatory cytokines promote MUC5AC production in differentiated epithelial cells *in vitro*17. We demonstrated that CXCL8 secretion by fibroblasts can be induced by epithelial-derived IL-1α12 and that airway smooth muscle cells promote mucin secretion by bronchial epithelial cells via CCL20[18]. These findings led us to hypothesize that stromal cells, particularly airway fibroblasts, support the development of CMH in COPD by altering mucociliary differentiation and promoting epithelial mucin secretion.

In this study, we investigated whether co-culture of airway epithelial cells derived from COPD patients with CMH with airway fibroblasts leads to alterations in epithelial cell polarization, mucociliary differentiation, as well as MUC5AC and MUC5B expression and release. In addition, we further evaluated the underlying mechanism by assessing the release of specific pro-inflammatory cytokines, i.e. IL-6, CXCL8, and CCL20, and by using neutralizing antibodies in a Calu-3 cell line model.

METHODS

Isolation of primary bronchial epithelial cells and primary airway fibroblasts

Primary bronchial epithelial cells (PBECs) were isolated from explanted lungs from 3 COPD stage IV patients with CMH undergoing lung transplantation, as previously described[19]. Primary airway fibroblasts (PAFs) were isolated from 9 COPD stage IV patients (6 with CMH and 3 without CMH) as previously published[20]. The patient characteristics are described in Table 1. The presence of CMH was defined by the patients' clinical records. PBECs were grown in Keratinocyte Serum-Free Growth Medium (KSFM, Gibco, NY, USA) supplemented with 1% Penicillin (10,000 U/mL)/ Streptomycin (10,000 µg/mL) (P/S) (Gibco, California, USA), 1 µM isoproterenol, 25 ng/ml bovine pituitary extract (BPE, Gibco) and 2.5 µg/ml epidermal growth factor (EGF, Gibco). PAFs were grown in HAMs' F12 medium supplemented with 1% pennicilin/streptomycin, 1% L-glutamine (Lonza, Switzerland) and 10% fetal bovine serum (FBS, Sigma-Aldrich, Germany). Cells were passaged when 90% confluent. In all experiments, we used PBECs in passage 3 and PAFs in passage 4-6.

Co-culture of PBECs and PAFs at air-liquid interface

Air-liquid interface (ALI) culture of PBECs was performed as previously described17. Transwell inserts for 24-well plates (Corning®, NY, USA) were coated with 10 µg/ ml bovine serum albumin (BSA; Sigma-Aldrich, MO, USA), 10 µg/ml fibronectin (Sigma-Aldrich, MO, USA) and 30 µg/ml collagen (PureCol®, Advanced Biomatrix, San Diego, CA, USA) in Eagle's Minimum Essential Medium (EMEM, Lonza, Walkersville, MD, USA) before being used to culture PBECs. PBECs from each donor were seeded at a density of 75,000 cells/insert in ALI culture medium prepared by mixing DMEM (LONZA BE12-709F) and BEBM (Clonetics CC-3171) in 1:1 ratio supplemented with a set of BEGM Single Quots (Clonetics CC-4175) and 1.5µg/ml BSA (Sigma-Aldrich) and15 ng/ml retinoic acid (Sigma-Aldrich). After 4-5 days, the cells were air-exposed for 14 days (day 0-14) during which the basal medium was refreshed every 2-3 days. PAFs seeded in 24-well plates in HAMs' F12 medium until 70-80% confluent were co-cultured with PBECs on the inserts from day 7-14 using ALI culture medium (6 PAF donors per 1 PBEC donor). On day 0, day 7 and day 14, transepithelial electrical resistance (TEER) was measured using the Epithelial Volt-Ohm Meter (Millicel® ERS-2). At the end of day 14, 100 µl/insert of the wash medium was added to the apical compartment and incubated at room temperature for 5 minutes. This apical wash fluid was collected and incubated with 0.1% w/v Dithiothreitol (DTT) (Sputolysin®, Calbiochem, San Diego, CA, USA) at room temperature for 15-20 minutes before being centrifuged at 200 g for 5 minutes.

The supernatant was then collected and stored at -20 °C for mucin measurement. Next, the basal medium was collected and centrifuged at 200 g for 5 minutes, and the supernatant was collected and stored at -20 °C for cytokine measurements. The cells were collected for RNA isolation. Each condition was performed in duplicate, except for one experiment which was performed in triplicate to harvest inserts for immunohistochemistry (IHC) staining. An overview of our long-term co-culture model and the experimental design is illustrated in figure 1.

Table 1. Patient characteristics

donor	age	gender	pack-years	FEV1 (%predicted)	FEV1/FVC	CMH
E1	49	m	11	20	0.22	yes
E2	58	m	35	15	0.19	yes
E3	60	f	40	18	0.19	yes
F1	53	f	40	23	0.26	yes
F2	48	f	30	12	0.26	yes
F3	59	m	40	15	0.29	yes
F4	61	f	30	16	0.19	yes
F5	58	m	35	15	0.19	yes
F6	59	m	47	15	0.21	yes
F7	48	m	25	12	0.23	no
F8	61	f	70	22	0.25	no
F9	58	f	38	22	0.23	no

All patients are ex-smokers with COPD stage IV. E1-E3 are PBEC donors; F1-F9 are PAF donors; FEV1 is forced expiratory volume in 1 second; FVC is forced vital capacity; CMH is chronic mucus hypersecretion based on clinical records; m is male, f is female.

Blocking of IL-6 with neutralizing antibodies

To study the effects of fibroblast-derived IL-6 on epithelial mucin secretion, we used Calu-3 lung adenocarcinoma cells (known to produce mucus upon air exposure21) as a bronchial epithelial cell model to keep the epithelial component consistent. Calu-3 cells (ATCC® HTB-55™) were seeded at 165,000 cells/insert density in Transwell inserts for 24-well plates and submerged-cultured in DMEM-F12 medium supplemented with 1% non-essential amono acid (NEAA), 1% pennicilin/ streptomycin, 1% L-glutamine and 10% FBS until confluent. The cells were then air-exposed on the apical side for 10 days (day 0-10) during which the basal medium was refreshed every 2 days. From day 4 to day 10, the cells were co-cultured with 70-80%

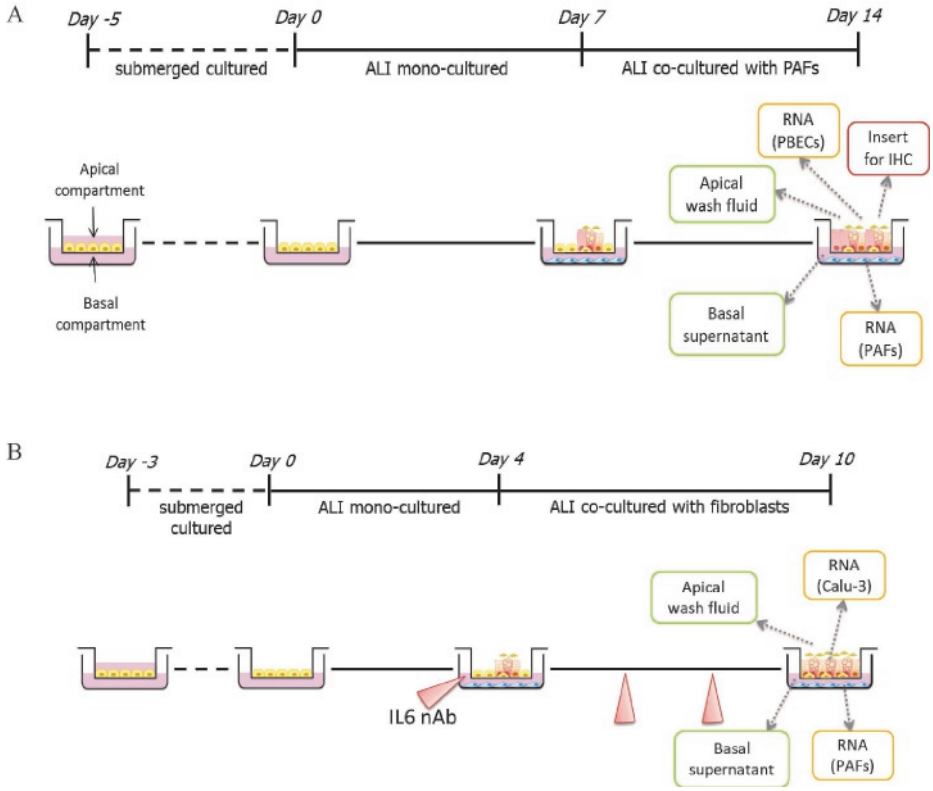

Figure 1. Experimental design. [A] ALI-culture of PBECs. PBECs were submerged-cultured for 5 days before air-exposed for 2 weeks (day 0-14), during which the medium was refreshed every 2-3 days. On day 7, PAFs on basal compartments were co-cultured with PBECs for 7 days. On day 14, apical wash fluid, basal supernatant and RNA from both cell types were collected. [B] ALI-culture of Calu-3 cells. Calu-3 cells were submerged-cultured for 3 days before air-exposed for 10 days, during which the medium was refreshed every 2 days. On day 4, PAFs on basal compartments were co-cultured with Calu-3 cells for the next 6 days, with or without the present of anti-IL-6 neutralizing antibody and IgG control antibody. On day 10, apical wash fluid, basal supernatant and RNA from both cell types were collected.

confluent PAFs from 3 COPD patients with CMH seeded on 24-well plates in the DMEM-F12 medium with or without 1 µg/ml anti-IL-6 neutralizing antibody (mabg-hil6-3, Invivogen, France) or IgG controls (mabg1-ctrlm, Invivogen). The experiment was repeated with PAFs from 3 other COPD patients with CMH, thus 6 PAF donors in total. On day 10, 100 µl/insert of Hank's Balanced Salt Solution (HBSS) (Lonza) was added to the apical compartment and incubated at room temperature for 5 minutes. This apical wash fluid was then collected and incubated with 0.1% w/v DTT at room

temperature for 15-20 minutes before being centrifuged at 200 g for 5 minutes. The supernatant was then collected and stored at -20 °C for mucin measurement. The cells were collected for RNA isolation. Each condition was performed in duplicate.

Reverse transcription and quantitative PCR (RT-qPCR)

Total RNA was collected and isolated using Tri Reagent® according to the manufacturer's protocol. To assess mRNA expression, total RNA was converted to cDNA using iScript™ cDNA Synthesis Kit (BioRad). qPCR was then performed using Taqman® assays (MUC5AC, ID: Hs00873651_Mh; MUC5B, Hs00861588_m1; SPDEF, Hs01026050_m1; FOXA2, Hs00232764_m1; FOXJ1, Hs00230964_m1; IL-6, HsHs00174131_m1; CXCL8, Hs00174103_m1; CCL20, Hs01011368_m1; IL-33, Hs04931857_m1; IL1RL1, Hs00545033_m1) and LightCycler® 480 Probes Master according to the manufacturer's guidelines (Roche, Switzerland). All mRNA expression was normalized to the expression of the reference genes: β2 microglobulin (B2M, Hs99999907_m1) and Peptidylprolyl Isomerase A (PPIA, Hs99999904_m1).

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of MUC5AC and MUC5B proteins were measured in apical wash fluid using ELISA kits for MUC5AC (SEA756Hu, USCN, China) and MUC5B (SEA684Hu, USCN, China) according to the manufacturer's protocols. Levels of IL-6, CXCL8, and CCL20 were measured in basal supernatants using ELISA kits (R&D Systems, Minnesota, USA) and C96 Maxisorp NUNC Immuno-plate (Sigma-Aldrich).

Immunohistochemical staining

Transwell membranes from the ALI-cultured inserts were fixed with formalin and embedded cross-sectionally in paraffin. Paraffin-embedded membranes were cut and double-stained with Alcian blue (CLIN-TECH, CI 42780, UK) for mucus-positive cells and acetylated-α-tubulin (Sigma-Aldrich, T7451, MO, USA) for ciliated cells. At least 5 sections per insert and experimental condition were semi-quantitatively scored for mucus positivity and ciliary differentiation by two independent researchers in a blinded manner. The scores ranged from 1, 2, and 3 representing lowest, moderate, and highest mucus positivity/epithelial differentiation. A mean score for each sample was then calculated.

Statistical analyses

One-sample Wilcoxon signed rank test was used to determine significant differences between mono-cultured PBECs or Calu-3 cells and co-cultured samples by assessing the fold change relative to mono-culture. Paired-samples Wilcoxon signed rank test was used to determine significant differences between mono-cultured PAFs and co-

cultured samples as well as between different conditions in co-cultured Calu-3 cells. Mann-Whitney U test was used for comparisons between changes induced by PAFs from patients with CMH and without CMH. Significant difference in the change of TEER from day 7 to day 14 of mono-cultured and co-culture PBECs was determined by one-sample Wilcoxon signed rank test. All statistical analyses were performed on GraphPad PRISM v7.

RESULTS

Fibroblasts promote mucous cell differentiation and epithelial mucus secretion

To investigate whether fibroblast-epithelium crosstalk promotes epithelial mucin secretion and alter mucociliary differentiation, PBECs were cultured at ALI in the presence and absence of PAFs. Firstly, we observed that co-cultured PBECs secreted significantly higher levels of MUC5AC and MUC5B proteins compared to mono-cultured cells (figure 2A), although MUC5AC level was not detectable in one of the three PBEC donors. Next, we investigated whether the changes in mucin secretion observed above were regulated at the transcriptional level and found that the expression of MUC5B mRNA was significantly higher in co-cultured PBECs (figure 2B). The effect of co-culture on MUC5AC mRNA expression varied among different PBEC donors and no significant change was observed (figure 2B). Expression of SPDEF, a transcription factor known to induce MUC5AC and MUC5B synthesis22, was upregulated in co-cultured PBECs (figure 2B) and correlated with both MUC5AC and MUC5B mRNAs (figure S1). Expression of Forkhead box protein A2 (FOXA2), a negative suppressor of MUC5AC gene expression, was not detected in any of the samples. The expression of Forkhead box protein J1 (FOXJ1), a transcription factor required for ciliated cell differentiation, did not change upon co-culture with fibroblasts (figure 2B). To determine whether mucin upregulation by PAFs from COPD patients with CMH was stronger than by PAFs from patients without CMH, we co-cultured PBECs from one donor with PAFs from three donors with CMH and three without CMH and found no significant differences in mucin protein secretion and gene expression (figure S2). In addition, we determined whether the higher mucin secretion upon co-culture was accompanied by alterations in mucociliary differentiation. We assessed TEER (an indicator of epithelial cell polarization), the presence of mucus positive cells, ciliated cells and epithelial cell differentiation. Upon air-exposure, we observed that PBECs increased their TEER from day 0 to day 7 indicating barrier formation and polarization, after which TEER levels stabilized. TEER was not affected by co-culture (figure S3). Semi-quantitative histological assessment of mucus-producing and ciliated cells revealed that the number of mucous cells significantly increased upon co-culture (figure 2C). Ciliated cells were only occasionally observed in the co-cultured epithelial layers. Furthermore, co-cultured PBECs tended to show more maturation, i.e. being taller and more multi-layered, compared to mono-cultured cells (figure 2D).

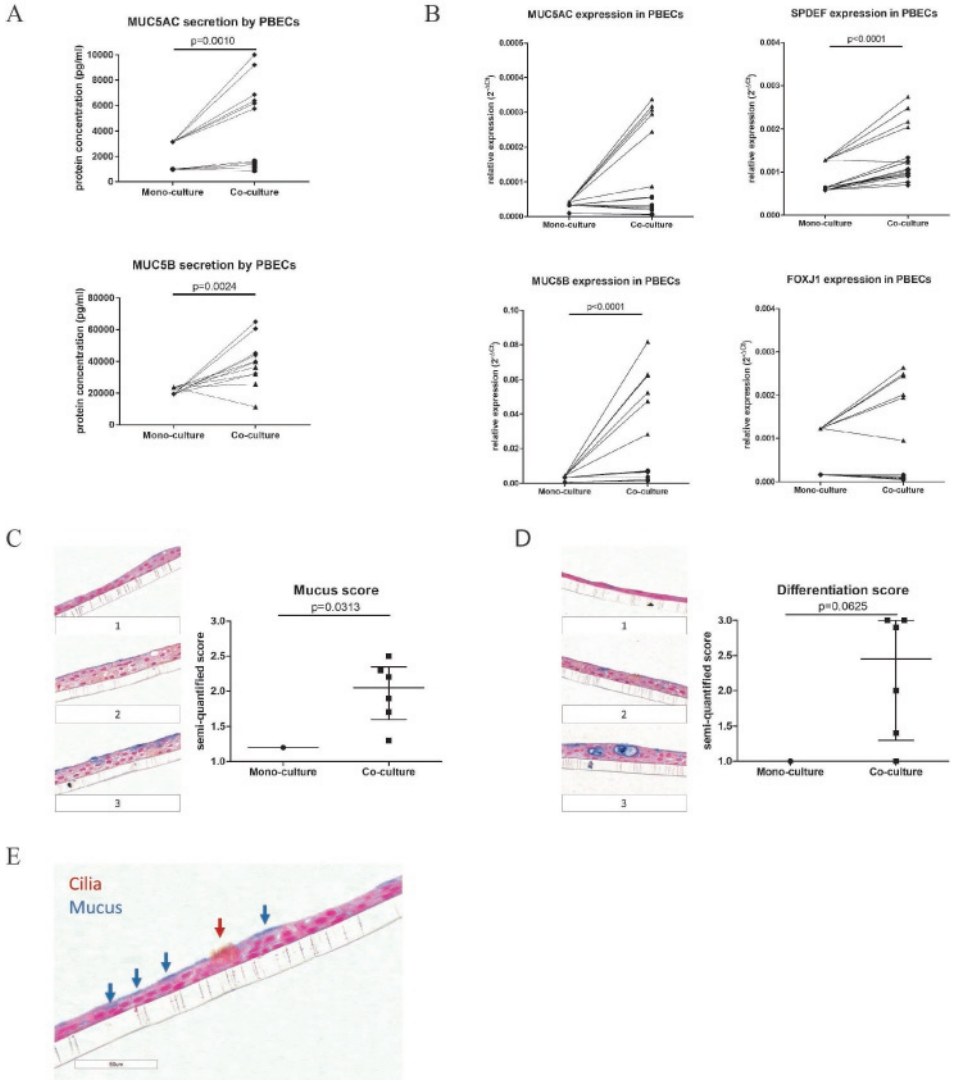

Figure 2. Fibroblasts promote epithelial mucin release and mucous cell differentiation. PBECs from each COPD patient with CMH were differentiated at ALI in the presence or absence of PAFs from 6 COPD patients. The experiment was repeated 3 times with different PBEC donors. [A] MUC5AC and MUC5B protein levels secreted by PBECs measured by ELISA. MUC5AC protein was not detectable in apical wash from PBEC donor 3, while MUC5B was not measured in PBEC donor 1 due to limited samples. Circles, diamonds, and triangles represent PBEC donor E1, E2, and E3, respectively. [B] mRNA expression of MUC5AC, MUC5B, SPDEF and FOXJ1 in PBECs measured by qPCR. Relative expression (2-dCt) normalized to the reference genes B2M and PPIA is shown. Expression of FOXJ1 in PBEC donor 1 was below detection limit. [C] Semi-quantitative analysis of mucus positivity determined by Alcian blue immunohistochemistry and [D] epithelial maturation determined by cell height and

layers. [E] example of a ciliated cell stained by acetylated α-tubulin immunohistochemistry. The score 1, 2, and 3 represent lowest, moderate, and highest mucus positivity/epithelial maturation, respectively. Mean scores of ≥ 5 inserts scored by two independent observers are depicted. Median±IQR is depicted. All significant differences were determined by one-sample Wilcoxon signed rank test.

Epithelial cells stimulate pro-inflammatory cytokine production by fibroblasts

To determine potential mediators of fibroblast-epithelium crosstalk, we assessed the concentration of IL-6, CXCL8 and CCL20 in basal supernatant samples. IL-6 secretion was increased upon co-culture when compared to both mono-cultured PBECs and mono-cultured PAFs (figure 3A). CXCL8 and CCL20 levels were higher upon co-culture when compared to mono-cultured PAFs but not compared to mono-cultured PBECs (figure 3A), suggesting that IL-6 is more likely to contribute to the changes observed upon co-culture in this model. To determine the source of IL-6 protein secreted upon co-culture, we assessed IL-6 mRNA expression and found that levels were higher in PAFs upon co-culture, but not in PBECs, indicating that PAFs are likely the source of the increased IL-6 levels upon co-culture (figure 3B). Therefore, we hypothesized that fibroblasts promote mucin expression and secretion by releasing IL-6.

IL-6 mediates the increase in MUC5B expression upon fibroblast-epithelium crosstalk

To determine if higher mucin expression and secretion upon co-culture was driven by IL-6 release, we used neutralizing antibodies in a co-culture model of Calu-3 epithelial cells with PAFs. Similar to our co-culture model with PBECs and PAFs, co-culture of Calu-3 with PAFs resulted in higher MUC5B mRNA expression and protein secretion as well as MUC5AC expression (figure 4 and S4A), while Calu-3 cells did not secrete detectable MUC5AC protein. When compared to untreated co-culture, IL-6 neutralization significantly suppressed the upregulation of MUC5B mRNA, but not MUC5AC (data not shown), by Calu-3 cells upon co-culture (figure 4). No difference in MUC5B secretion was observed between the untreated and anti-IL-6 antibody-treated co-cultures (figure 4). Treatment with the IgG control had no effect on MUC5B mRNA and protein (figure S4A). When compared to the IgG control, upregulation of MUC5B mRNA upon co-culture was significantly suppressed by anti-IL-6 antibody with a similar a trend for MUC5B protein secretion (figure S4B). In addition, a positive correlation between MUC5B expression and MUC5B protein secretion was observed (figure S4C).

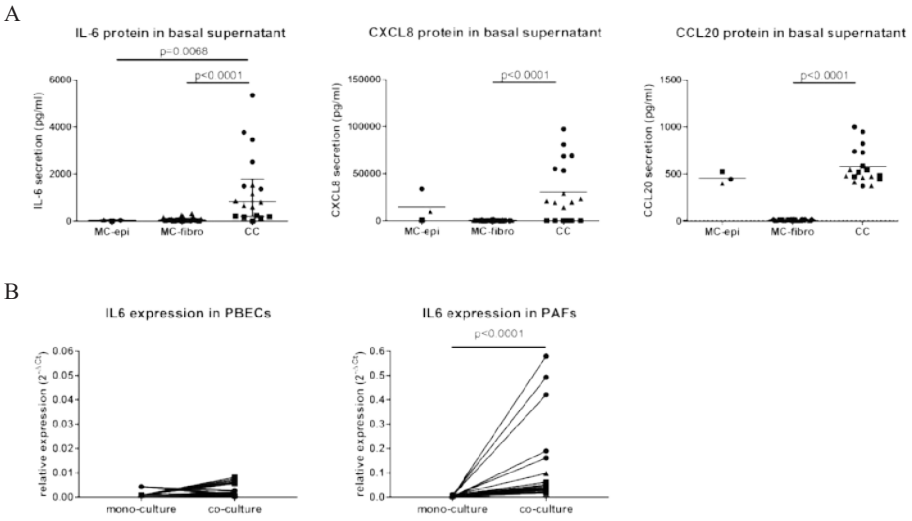

Figure 3. Epithelial cells stimulate pro-inflammatory cytokine production by fibroblasts. PBECs from 3 COPD patients with CMH were differentiated at ALI in the presence or absence of PAFs from 9 COPD patients. Basal supernatant and total RNA was collected for measurement of cytokine release and gene expression, respectively. [A] IL-6, CXCL8, and CCL20 protein secretion measured by ELISA and [B] IL-6 mRNA expression in PBECS and PAFs measured by qPCR. Significant difference was determined by one-sample Wilcoxon signed rank test or paired-samples Wilcoxon signed rank test.

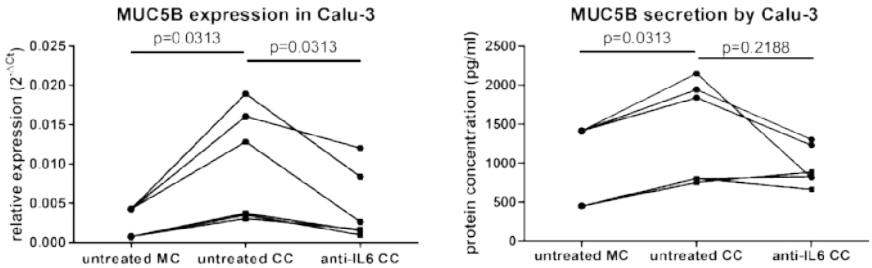

Figure 4. IL-6 neutralization suppresses fibroblast-induced MUC5B upregulation by epithelial cells. Calu-3 cells were co-cultured with PAFs from 6 COPD patients with CMH. MUC5B mRNA expression (left), MUC5B protein secretion (middle) and MUC5AC mRNA expression (right) is depicted comparing untreated mono-culture, untreated co-culture and anti-IL-6 treated co-culture. Significant difference was determined by paired-samples Wilcoxon signed rank test.

DISCUSSION

This study demonstrates that mucous cell differentiation and epithelial mucin expression and release in airway epithelial cells from COPD patients with CMH is promoted by COPD-derived airway fibroblasts, and that the increase in MUC5B expression can be mediated by IL-6. Our findings indicate the involvement of airway fibroblasts in the development of CMH in COPD at least in part through pro-inflammatory IL-6 secretion.

Our results show that co-culture with fibroblasts promotes mucous cell differentiation, contributing to increased production and release of mucins. With respect to morphology, the co-cultured epithelial cells appeared to be taller and more multilayered than the mono-cultured cells, which is in line with to the study by Goto et al.[15], showing that guinea pig-derived fibroblasts promote tracheal epithelial differentiation. Our findings show that this effect is also true for patient-derived cells, without the need of direct cell-cell contact, suggesting involvement of soluble factors in this crosstalk. Indeed, we observed that IL-6 may mediate the increase in MUC5B expression seen upon co-culture. In contrast to MUC5B, our findings suggest that MUC5AC expression is not mediated by IL-6. This is somewhat surprising, since previous studies showed that airway epithelial stimulation with IL-6 leads to higher levels of both MUC5AC and MUC5B[17,23]. Although both MUC5AC and MUC5B have been shown to increase in COPD[5], Kirkham et al. reported that MUC5B is the major mucin in sputum from COPD patients, with a higher MUC5B/MUC5AC protein ratio compared to smokers. MUC5B is also associated with lower lung function, suggesting that it is predominantly involved in COPD[24]. Therefore, especially the mechanisms involved in MUC5B secretion may be of interest for the treatment of CMH in COPD. Nevertheless, MUC5B is not as widely studied as MUC5AC and thus the molecular pathways regulating MUC5B production are less well known[25]. Both mucins share overlapping mechanisms such as those mediated by SPDEF[26], IL-33[27] and EGF[28,29], but a pathway that uniquely regulates either of them remains to be explored. In this study, we show that MUC5B expression and protein release in both COPD-derived bronchial epithelial cells and Calu-3 lung adenocarcinoma cells can be promoted by fibroblasts. As for MUC5AC release, the timing in Calu-3 may not have been optimal, as we did not detect MUC5AC protein here. Similarly, the timing for MUC5AC mRNA levels in the co-cultured primary cells may not have been optimal, explaining the lack of effect on MUC5AC mRNA expression upon co-culture. It might have been upregulated earlier, but as mRNA expression is a transient process, this change might have been receded at the end of the experiments, leaving only the change in MUC5AC protein levels to be observed. Our current findings do not render it likely that CCL20 and CXCL8 contribute to fibroblast-induced mucin secretion in our model. Nevertheless, this does not exclude a role for these cytokines

in CMH. In our other studies, we showed that CCL20 treatment increased mucus production in healthy airway epithelial cells (Chapter 8)[18] and that epithelial cell-fibroblast co-culture resulted in higher CXCL8 levels over mono-cultured epithelial cells[30].

Increased MUC5AC and MUC5B production[5,31] and goblet cell hyperplasia[10] are features commonly observed in COPD patients with CMH. In this study, we showed that co-culture with fibroblasts upregulated the transcription factor SPDEF in bronchial epithelial cells, likely promoting mucin expression and release. In our model, the fibroblasts supported epithelial maturation into more cuboidal cells that produced more mucus than those cells without the fibroblast support. Epithelial barrier formation and polarization is thought to be a prerequisite for epithelial differentiation[32]. Accordingly, we observed that TEER increased upon air exposure especially during the early stage of differentiation, and stabilized or slowly declined over time, consistent with the observation in other studies[21,29]. The lack of effect of co-culture on TEER levels can be explained by the fact that fibroblasts were co-cultured with the epithelial cells in the last week, when barrier formation and polarization was already established. Ciliated cells were only observed scarcely in co-cultured epithelial layers and no change in FOXJ1 expression was observed upon co-culture. There are various explanations for this lack of ciliary differentiation in our model. Firstly, the epithelial cells were air-exposed for 14 days before collecting RNA. This is the time when an increase in mucin expression is usually observed, but development of cilia is usually seen later, i.e. 28 days after air-exposure[33]. Secondly, we performed our experiments in a translational setting using PBECs from COPD patients with CMH, so deficiencies in the ability of these cells to differentiate towards ciliated cells may have existed. Lastly, it is possible that fibroblasts are more prone to drive differentiation into mucous cells rather than ciliated cells.

Although fibroblast-epithelium crosstalk also exists in healthy physiological conditions, it may be altered in pathological conditions. As we were interested in the mechanisms involved in CMH in COPD, we used epithelial cells and fibroblasts derived from COPD patients with CMH. We also used fibroblasts from COPD patients without CMH but did not observe different effects. Firstly, this may be due to the fact that epithelial cells were from COPD patients who already developed CMH and may be more susceptible to signals from fibroblasts, thus responding equally well to fibroblasts from both groups of donors. The other way round, signals from these epithelial cells may have abolished differences between fibroblasts from donors with and without CMH as previously observed[34]. Finally, since the sample size of donors without CMH was small, future studies including more fibroblast donors without CMH and epithelial cell donors without CMH are needed to clarify this point.

One of the major strengths of this study is that, for the first time, primary airway epithelial cells and fibroblasts derived from COPD patients were used for a

long-term air-exposed co-culture model to evaluate how fibroblasts are involved in CMH development by promoting mucous cell differentiation and epithelial mucin release. It is the *in vitro* setting that is, so far, most translational to patients in a context of CMH in COPD. Our findings provide novel insights into the mechanisms that regulate CMH in COPD and this can be of relevance to the identification of novel therapeutic strategies in the future.

REFERENCES

1. Burgel, P. R. Chronic cough and sputum production: A clinical COPD phenotype? Eur. Respir. J. 40, 4–6 (2012).
2. Ramos, F. L., Krahnke, J. S. & Kim, V. Clinical issues of mucus accumulation in COPD. Int. J. COPD 9, 139–150 (2014).
3. Burgel, P.-R. Chronic cough and sputum production: a clinical COPD phenotype? ´. Eur. Respir. J. 40, 4–6 (2012).
4. Kirkham, S., Sheehan, J. K., Knight, D., Richardson, P. S. & Thornton, D. J. Heterogeneity of airways mucus. Biochem. J. 546, 537–546 (2002).
5. Caramori, G. et al. Mucin expression in peripheral airways of patients with chronic obstructive pulmonary disease. Histopathology 45, 477–484 (2004).
6. Park, K. et al. SPDEF regulates goblet cell hyperplasia in the airway epithelium. J. Clin. Invest. 117, 978–988 (2007).
7. Yu, H., Li, Q., Kolosov, V. P., Perelman, J. M. & Zhou, X. Interleukin-13 Induces Mucin 5AC Production Involving STAT6/SPDEF in Human Airway Epithelial Cells. Cell Commun. Adhes. 17, 83–92 (2010).
8. Song, J. et al. Targeted epigenetic editing of SPDEF reduces mucus production in lung epithelial cells. Am. J. Physiol. Cell. Mol. Physiol. 312, L334–L347 (2017).
9. Hessel, J. et al. Intraflagellar transport gene expression associated with short cilia in smoking and COPD. PLoS One 9, (2014).
10. Saetta, M. et al. Goblet Cell Hyperplasia and Epithelial Inflammation in Peripheral Airways of Smokers with Both Symptoms of Chronic Bronchitis and Chronic Airflow Limitation. Am. J. Respir. Crit. Care Med. 161, 1016–1021 (2000).
11. Adamson, I. Y., Hedgecock, C. & Bowden, D. H. Epithelial cell-fibroblast interactions in lung injury and repair. Am. J. Pathol. 137, 385–392 (1990).
12. Osei, E. T. et al. Interleukin-1 α drives the dysfunctional cross-talk of the airway epithelium and lung fibroblasts in COPD. Eur. Respir. J. 48, 359–369 (2016).
13. Osei, E. T. et al. MiR-146a-5p plays an essential role in the aberrant epithelial-fibroblast crosstalk in COPD. Eur. Respir. J. 49, 1602538 (2017).
14. Albers, S., Thiebes, A. L., Gessenich, K. L., Jockenhoevel, S. & Cornelissen, C. G. Differentiation of respiratory epithelium in a 3-dimensional co-culture with fibroblasts embedded in fibrin gel. Multidiscip. Respir. Med. 11, (2016).
15. Goto, Y. et al. *In vitro* reconstitution of the tracheal epithelium. Am. J. Respir. Cell Mol. Biol. 20, 312–318 (1999).
16. Kobayashi, K. et al. Effect of Fibroblasts on Tracheal Epithelial Regeneration *in vitro*. Tissue Eng. 12, 2619–2628 (2006).
17. Spanjer, A. I. R. et al. A pro-inflammatory role for the Frizzled-8 receptor in chronic bronchitis. Thorax 71, 312–322 (2016).
18. Faiz, A. et al. Profiling of healthy and asthmatic airway smooth muscle cells following interleukin-1β treatment: A novel role for CCL20 in chronic mucus hypersecretion. Eur. Respir. J. 52, (2018).

19. Heijink, I. H. et al. Cigarette smoke-induced epithelial expression of WNT-5B: Implications for COPD. Eur. Respir. J. 48, 504–515 (2016).
20. Brandsma, C.-A. et al. Differential effects of fluticasone on extracellular matrix production by airway and parenchymal fibroblasts in severe COPD. Am. J. Physiol. Lung Cell. Mol. Physiol. 305, L582-9 (2013).
21. Grainger, C. I., Greenwell, L. L., Lockley, D. J., Martin, G. P. & Forbes, B. Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. Pharm. Res. 23, 1482–1490 (2006).
22. Lambrecht, B. N. & Hammad, H. The airway epithelium in asthma. Nat. Med. 18, 684–692 (2012).
23. Chen, Y. et al. Stimulation of Airway Mucin Gene Expression by Interleukin (IL) -17 through IL-6 Paracrine / Autocrine Loop *. 278, 17036–17043 (2003).
24. Kirkham, S. et al. MUC5B is the major mucin in the gel phase of sputum in chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 178, 1033–1039 (2008).
25. Theodoropoulos, G. & Carraway, K. L. Molecular signaling in the regulation of mucins. J. Cell. Biochem. 102, 1103–1116 (2007).
26. Rajavelu, P. et al. Airway epithelial SPDEF integrates goblet cell differentiation and pulmonary Th2 inflammation. J. Clin. Invest. 125, 2021–2031 (2015).
27. Ishinaga, H. et al. Interleukin-33 induces mucin gene expression and goblet cell hyperplasia in human nasal epithelial cells. Cytokine 90, 60–65 (2017).
28. Zhu, L. et al. Rhinovirus-induced major airway mucin production involves a novel TLR3-EGFR-dependent pathway. Am. J. Respir. Cell Mol. Biol. 40, 610–619 (2009).
29. Shaykhiev, R. et al. EGF shifts human airway basal cell fate toward a smoking-associated airway epithelial phenotype. Proc. Natl. Acad. Sci. U. S. A. 110, 12102–7 (2013).
30. Osei, E. T. et al. Interleukin-1α drives the dysfunctional cross-talk of the airway epithelium and lung fibroblasts in COPD. Eur. Respir. J. 48, 359–369 (2016).
31. Kesimer, M. et al. Airway Mucin Concentration as a Marker of Chronic Bronchitis. N. Engl. J. Med. 377, 911–922 (2017).
32. Gordon, S. et al. Non-Animal Models of Epithelial Barriers (Skin , Intestine and Lung) in Research , Industrial Applications and Regulatory Toxicology. Altern. to Anim. Exp. 32, 327–378 (2015).
33. Song, J. et al. Aberrant DNA methylation and expression of SPDEF and FOXA2 in airway epithelium of patients with COPD. Clin. Epigenetics 9, 42 (2017).
34. Spanjer, A. I. R. et al. A pro-inflammatory role for the Frizzled-8 receptor in chronic bronchitis. Thorax 71, 312–322 (2016).

SUPPLEMENTARY DATA

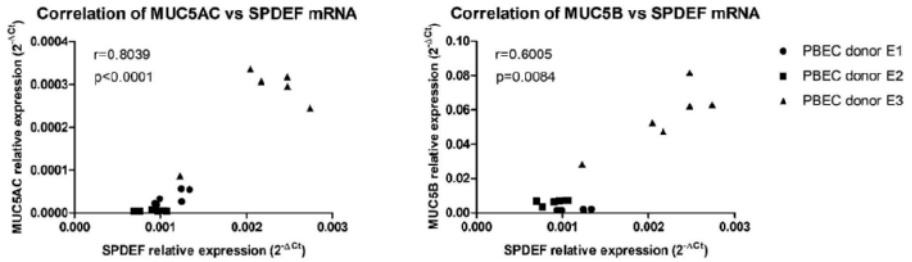

Figure S1. Correlations of mucin and SPDEF expression. PBECs from 3 COPD patients with CMH were differentiated at ALI in the presence or absence of PAFs from 9 COPD patients. Only co-cultured samples were analyzed. Significant correlation was determined by Spearman's rank correlation coefficient.

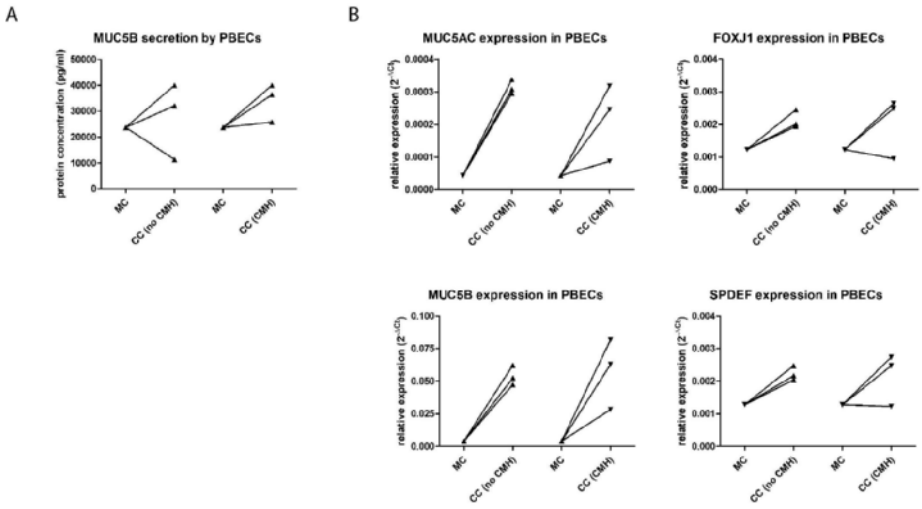

Figure S2. No difference between PAFs isolated from COPD patients with CMH and without CMH. PBECs from 1 COPD patient with CMH were differentiated at ALI in the presence or absence of PAFs from 3 COPD patients with CMH and 3 without CMH. [A] MUC5B secretion by PBECs. [B] Expression of mucociliary markers in PBECs. MUC5AC protein was not detected from these experiments. Significant difference between PAFs from the patients with and without CMH was determined by Mann-Whitney U test.

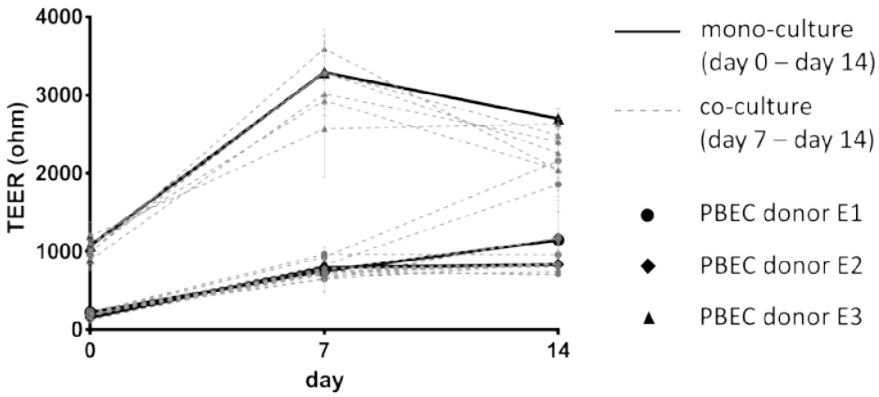

Figure S3. Transepithelial electrical resistance (TEER) of ALI-differentiated PBECs. PBECs were submerged-cultured for 5 days before air-exposed for 2 weeks with or without the presence of PAFs on day 7-14. No difference was observed between mono-cultured PBECs (black solid line) and co-cultured PBECs (grey dash line). Significant difference between mono-culture and co-culture was determined by one-sample Wilcoxon signed rank test.

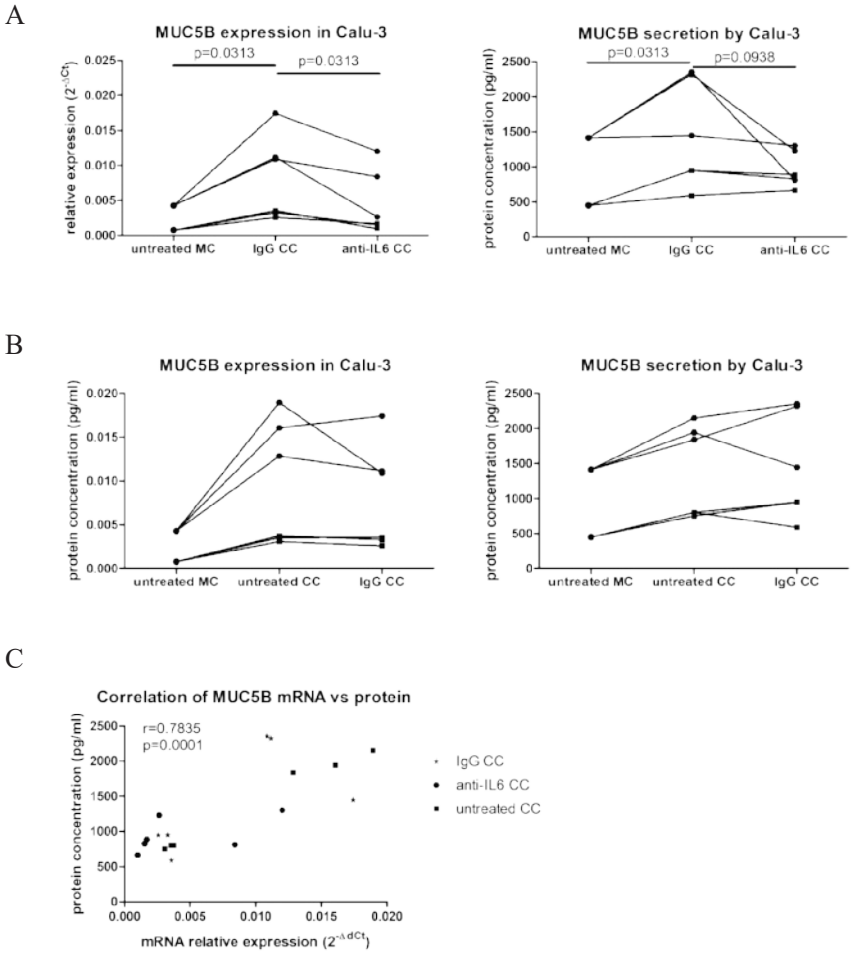

Figure S4. IL-6 neutralization abates fibroblast-induced mucin upregulation by epithelial cells. Calu-3 cells were co-cultured with PAFs from 6 COPD patients with CMH. Apical wash fluid and total RNA was collected for measurement of mucin release and gene expression, respectively. Significant difference between untreated mono-culture and IgG-treated co-culture was determined by one-sample Wilcoxon signed rank test. Significant difference between IgG-treated and anti-IL-6-treated co-culture was determined by paired-samples Wilcoxon signed rank test. Significant correlation between MUC5B mRNA and protein was determined by Spearman's rank correlation coefficient.