ORIGINAL ARTICLE

Epithelial to mesenchymal transition is increased in patients with COPD and induced by cigarette smoke

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ABSTRACT

Background Cigarette smoking contributes to lung remodelling in chronic obstructive pulmonary disease (COPD). As part of remodelling, peribronchiolar fibrosis is observed in the small airways of patients with COPD and contributes to airway obstruction. Epithelial to mesenchymal transition (EMT) appears to be involved in the formation of peribronchiolar fibrosis. This study examines the EMT process in human bronchial epithelial cells (HBECs) from non-smokers, smokers and patients with COPD as well as the in vitro effect of cigarette smoke extract (CSE) on EMT.

Methods HBECs from non-smokers (n=5), smokers (n=12) and patients with COPD (n=15) were collected to measure the mesenchymal markers α-smooth muscle actin, vimentin and collagen type I and the epithelial markers E-cadherin, ZO-1 and cytokeratin 5 and 18 by real time-PCR and protein array. In vitro differentiated bronchial epithelial cells were stimulated with CSE.

Results Mesenchymal markers were upregulated in HBECs of smokers and patients with COPD compared with non-smokers. In contrast, epithelial cell markers were downregulated. In vitro differentiated HBECs underwent EMT after 72 h of CSE exposure through the activation of intracellular reactive oxygen species, the release and autocrine action of transforming growth factor β_1, the phosphorylation of ERK1/2 and Smad3 and by the downregulation of cyclic monophosphate.

Conclusions The EMT process is present in bronchial epithelial cells of the small bronchi of smokers and patients with COPD and is activated by cigarette smoke in vitro.

Key messages

What is the key question?
► Are the bronchial epithelial cells from smokers and patients with COPD undergoing epithelial to mesenchymal transition (EMT)? Is cigarette smoke involved in bronchial EMT?

What is the bottom line?
► Phenotypic markers of EMT are observed in bronchial epithelial cells of small bronchi from smokers and patients with COPD. Cigarette smoke induces EMT in differentiated bronchial epithelial cells via release and autocrine action of TGF-β_1 as well as by enhancing oxidative stress. These results suggest that EMT could participate in the COPD remodelling process of small bronchi such as peribronchiolar fibrosis.

Why read on?
► The EMT process is activated in the small bronchi of smokers and patients with COPD as part of the chronic lung remodelling process. Cigarette smoke directly increases mesenchymal transition in differentiated human bronchial epithelial cells.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterised by airflow limitation that is progressive and not fully reversible. Cigarette smoking is considered the main risk factor for COPD and contributes to structural changes in the airways during COPD progression.[1] Bronchial epithelial cells are the first anatomical barrier exposed to noxious particles of cigarette smoke and are involved in the initiation of airway remodelling through the release of several proinflammatory mediators, goblet cell hyperplasia and mucus hypersecretion.[2] Other features that characterise COPD lung remodelling are the progressive loss of the alveolar wall, vascular remodelling with pulmonary hypertension and peribronchiolar fibrosis. As part of the fibrotic alterations in COPD, structural changes are observed primarily in the small airways (<2 mm in diameter), and the severity of the disease appears to be determined by thickening of the wall of the small airways caused by fibrosis and infiltration of inflammatory cells.[3] However, the exact factor responsible for producing the remodelled matrix as well as the role of activated epithelium in the remodelling process and in fibroblast activation/differentiation into myofibroblasts in small airway fibrosis in COPD is unclear.

It has been established that myofibroblast accumulation may occur as a consequence of resident fibroblast to myofibroblast transition, airway smooth muscle to myofibroblast transition, epithelial to mesenchymal transition (EMT) or by the recruitment of circulating fibroblastic stem cells (fibrocytes).[4] Depending on the precise physiological context, EMT can be induced either by environmental stresses/factors such as hypoxia[5] and reactive oxygen species (ROS)[6] or by extracellular mediators including transforming growth factor β_1 (TGF-β_1), fibroblast growth factor 2, connective tissue growth factor and insulin-like growth factor 2.[7] Among these, TGF-β_1, a multifunctional cytokine, has been shown to mediate EMT in vitro in several different cell lines including alveolar and bronchial epithelial cells and recently in

pseudostratified bronchial epithelial cells.[8] In vivo evidence for TGF-β_1-mediated EMT has also been reported, confirming the master regulation role of TGF-β_1.[9]

In epithelial cells, TGF-β_1-induced EMT is mediated by several downstream factors such as ROS generation, activation of mitogen-activated protein kinase and Smad signalling. Thus, anti-oxidant treatments[6] and other treatments directed at attenuating intracellular ROS, such as the increase in intracellular adenosine 3′,5′-cyclic monophosphate (cAMP),[10] effectively inhibit TGF-β_1-induced EMT.

It has recently been shown that cigarette smoke may induce EMT in the alveolar type II cell line A549[11] and the bronchial epithelial cell line BEAS2B,[12] but the effect of cigarette smoke on primary human bronchial epithelial cell (HBEC) to mesenchymal transition is currently poorly understood. In addition, evidence for EMT in the large airways of smokers and patients with COPD has been suggested by Sohal and colleagues, since cells included in the reticular basement membrane of large airways showed EMT markers.[13] However, whether EMT is present in the small airways of smokers and patients with COPD and whether cigarette smoke directly induces EMT in differentiated HBECs remains to be elucidated.

Using primary HBECs from small human bronchi of non-smokers, smokers and patients with COPD, we studied typical mesenchymal and epithelial cell markers of the EMT process. Furthermore, we determined whether cigarette smoke may induce EMT in differentiated air–liquid interface cultures as well as the potential signalling mechanisms involved.

METHODS

A more detailed version of the methods is given in the online supplement.

Patients

Human lung tissue from non-smokers (n=5), smokers (n=12) and patients with COPD (n=15) was obtained from patients undergoing surgery for lung carcinoma who gave informed consent, as previously described.[14] Inclusion and exclusion criteria are defined in the online supplement.

Isolation of primary bronchial epithelial cells

Primary HBECs were isolated and cultured from small pieces of human bronchi (0.5–1 mm internal diameter) as previously

Table 1 Clinical features of patients

	Non-smokers (n=5)	Smokers (n=12)	COPD (n=15)
Sex (female/male)	4/1	2/10	3/12
Age (years)	64 (56–72)	63 (52–76)	65 (54–74)
Tobacco consumption, pack-years	0	40 (33–46)	36 (28–43)
FEV$_1$, % predicted	102 (97–108)	91 (90–100)	71 (55–79)
FVC, % predicted	104 (93–108)	96 (92–106)	94(91–101)
FEV$_1$/FVC %	95 (91–101)	85 (77–91)	63 (54–68)
Pao$_2$, mm Hg	89 (85–93)	85 (81–90)	80 (78–83)
Paco$_2$, mm Hg	38 (36–41)	37 (35–40)	40 (38–42)

Data are shown as median (IQR).
Pack-year=1 year smoking 20 cigarettes per day.
COPD, chronic obstructive pulmonary disease; FEV$_1$, forced expiratory volume in 1 s; FVC, forced vital capacity; Pao$_2$, oxygen tension in arterial blood; Paco$_2$, carbon dioxide tension in arterial blood; % pred, % predicted.

outlined[15] and cultured for 1 week to analyse mRNA and protein expression (see online supplement for details).

Immunohistochemistry and immunofluorescence

Immunohistochemical analysis of α-smooth muscle actin (α-SMA), collagen type I, vimentin, NOX4, E-cadherin and ZO-1 was performed in human pulmonary tissue using mouse anti-human α-SMA (catalogue no A5228; Sigma), rabbit anti-human collagen type I antibody (catalogue no PA1-26204; Affinity Bioreagents), mouse anti-human vimentin (catalogue no V6389; Sigma), NOX4 (catalogue no NB110-58849; Novus Biologicals), mouse anti-human E-cadherin (catalogue no CM1681; ECM BioScience) and rabbit anti-human ZO-1 (catalogue no ab59720; Abcam). The scores of the staining intensity (0–3) and the percentage of immunoreactive cells (1–4 where 1 represents 0–25% cells positive, 2 represents 26–50% positive, 3 represents 51–75% positive and 4 represents 76–100% positive) were then multiplied to obtain a composite score ranging from 0 to 12. Secondary antibody anti-rabbit/mouse rhodamine/FITC was used for immunofluorescence analysis of primary bronchial epithelial cells. To detect F-actin distribution, phalloidin-tetramethylrhodamine isothiocyanate was employed (see online supplement for full details).

Culture of air–liquid interface bronchial epithelial cells

Differentiated HBECs for in vitro experiments were obtained from non-smoker patients and cultured in air–liquid interface until pseudostratified bronchial epithelium was obtained.[15] Details are described in the online supplement.

Preparation of cigarette smoke extract and transepithelial electrical resistance assay

Cigarette smoke extracts (CSE) were obtained as previously outlined[16] using a research cigarette (2R4F; Tobacco Health Research, University of Kentucky, Kentucky, USA). Transepithelial electrical resistance (TEER) was assayed using a millicell ERS-2 epithelial volt-ohm meter. Details of the TEER assay and the method of obtaining CSE, in vitro stimulation conditions as well as drug incubations are described in the online supplement.

Real-time PCR

Real-time PCR was performed as previously described.[17] Relative quantification of different transcripts was determined by the $2^{-\Delta\Delta Ct}$ method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control and with normalisation to the non-smoker group or control group as appropriate (see online supplement for further details).

Protein array and western blot analysis

Protein expression in lung tissue samples from non-smokers, smokers and patients with COPD was analysed with Zeptosens (Division of Bayer (Schweiz), Switzerland) protein array technology as previously outlined.[14] Western blot analysis was used to analyse β-catenin expression in the nucleus and cytoplasm (see online supplement for further details).

DCF fluorescence measurement of reactive oxygen species

2′,7′-Dichlorodihydrofluorescein diacetate (H$_2$DCF-DA, Molecular Probes, Nottingham, UK) was used to monitor the intracellular ROS in differentiated HBECs as previously described[14] (see online supplement for details).

Enzyme-linked immunosorbent assays for TGF-β_1, cAMP and MMP-9

Analyses of active TGF-β_1 and intracellular cAMP were performed using a Quantikine Human TGF-β_1 Immunoassay (R&D Systems, Madrid, Spain) and cAMP Biotrak enzyme immunoassay system (Amersham, London, UK) according to the manufacturers' instructions. Matrix metalloproteinase (MMP)-9 was determined using an assay kit for MMP-9 (R&D Systems, Nottingham, UK) according to the manufacturer's protocol (see online supplement for further details).

Statistics

Statistical analysis of results was carried out by parametric or non-parametric analysis as appropriate. Data from non-parametric analysis were expressed as median, IQR and minimum and maximum values. The Mann–Whitney U test or Kruskal–Wallis test followed by Dunn's post hoc test for multiple comparisons were used to compare mesenchymal and epithelial data from non-smokers, smokers and patients with COPD. Data from parametric analysis were expressed as mean (SE). Analysis of data from the in vitro mechanistic experiments in differentiated HBECs from non-smoker patients was performed by the Student t test or one-way or two-way analysis of variance followed by the Bonferroni post hoc test. Significance

was accepted when $p<0.05$. Full details are given in the online supplement.

RESULTS

EMT cell markers are increased in primary bronchial epithelial cells from smokers and patients with COPD

Primary HBECs were isolated from small bronchi of non-smokers, smokers and patients with COPD from uninvolved lung tissue during lobectomy/wedge resection for malignant lesions. The clinical characteristics of the patients are shown in table 1.

Under basal conditions, primary HBECs from smokers and patients with COPD showed a significant increase in mRNA and protein expression of typical mesenchymal markers α-SMA, vimentin and collagen type I (figure 1A–C). As with the mesenchymal markers, NOX4 mRNA and protein expression were also significantly upregulated in primary HBECs from smokers and patients with COPD (figure 1D). The analysis of epithelial markers revealed a significant downregulation of E-cadherin, ZO-1, KRT5 and KRT18 mRNA in patients with COPD but not in smokers, and a decrease in E-cadherin and ZO-1 protein expression in smokers and patients with COPD (figure 1E–H). In smokers and patients with COPD, phosphorylation of ERK1/2 was significantly increased, but an increase in Smad3 phosphorylation was only observed in cells from patients with
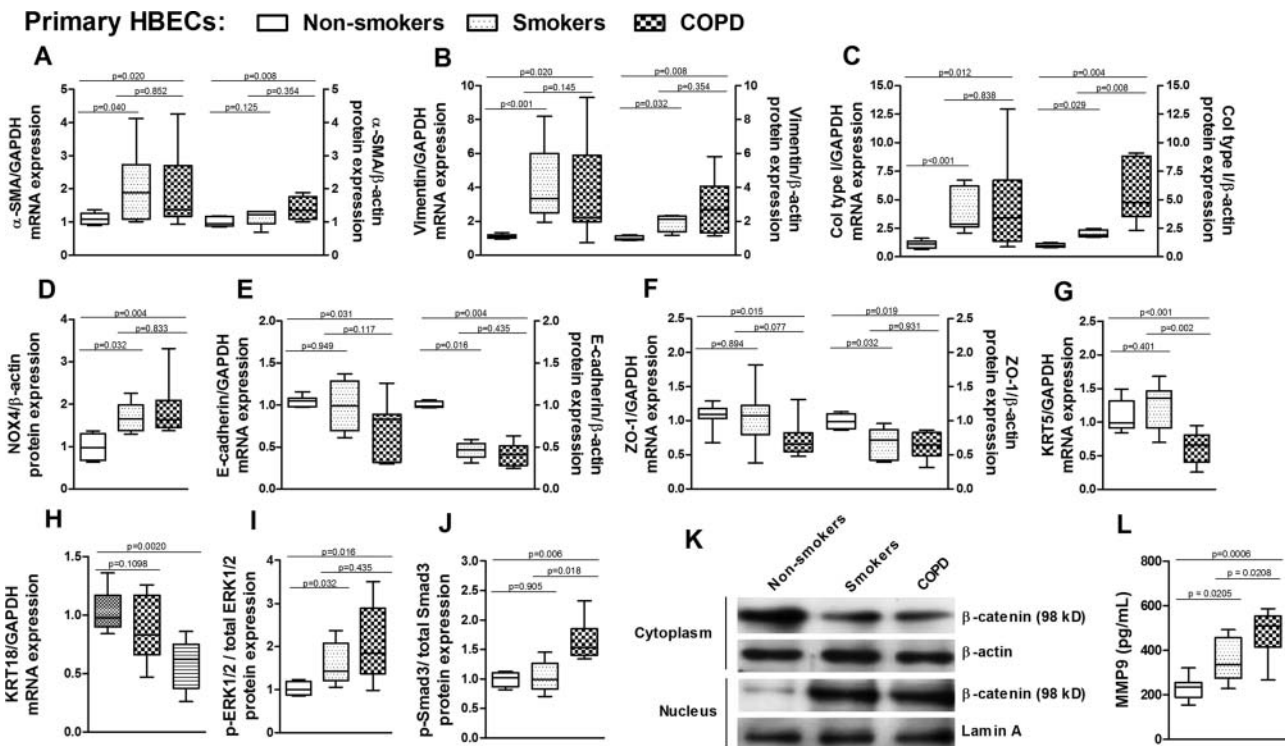
Figure 1 Expression of epithelial and mesenchymal markers in primary human bronchial epithelial cells (HBECs) from non-smokers, smokers and patients with chronic obstructive pulmonary disease (COPD). Primary HBECs were isolated from small bronchi of non-smokers (n=5), smokers (n=12) and patients with COPD (n=15) and total RNA and protein were obtained as described in the Methods section. mRNA and protein expression was determined by real-time PCR and protein array Zeptosens technology, respectively, with appropriate primers and specific antibodies. Basal expression of mRNA transcripts or protein of the mesenchymal markers (A) α smooth muscle actin, (B) vimentin, (C) collagen type I (Col type I) or (D) NOX4, as well as basal expression of the epithelial markers (E) E-cadherin, (F) ZO-1, (G) KRT5 or (H) KRT18 are shown. (I, J) Basal protein expression of phospho-Smad3 and phospho-ERK1/2. Data are expressed as the ratio to GAPDH for mRNA levels and to β-actin for protein levels and normalised to the non-smoker group. Phospho-Smad3 and phospho-ERK1/2 protein levels were expressed as the ratio to total ERK1/2 and Smad3, respectively, and normalised to the non-smoker group. (K) Representative images of western blot for cytoplasm and nuclear β-catenin with corresponding internal controls β-actin and lamin A. (L) Levels of matrix metalloproteinase (MMP)-9 were quantified by ELISA in primary culture cells following 24 h of cultures. Data are presented as box and whisker plots with median, IQR and minimum and maximum values. p exact values were obtained by the Kruskal–Wallis test and Dunn's post hoc test.

COPD (figure 1I,J). The transcription factor β-catenin was downregulated in the cytoplasm and upregulated in the nucleus of epithelial cells from smokers and patients with COPD (figure 1K). Furthermore, basal cell supernatants of 24 h cell cultures of patients with COPD showed higher MMP-9 release than those of non-smokers and smokers (figure 1L).

Phase contrast images showed that cells from smokers and patients with COPD had approximately 25% of cells with a spindle-shaped appearance characteristic of mesenchymal cells (figure 2). Expression of the tight junction (TJ) proteins E-cadherin and ZO-1 was visualised by immunofluorescence as a continuous line of fluorescence around the margin of the cells, and F-actin fibres were distributed at the margins of cells, between cell contacts in the non-smoker group. In contrast, cells from smokers and patients with COPD showed a reduction in E-cadherin and ZO-1 fluorescence intensity with sporadic and diffused expression. F-actin in cells from smokers and patients with COPD was homogeneously distributed as fibres in the cytoplasm. The mesenchymal markers collagen type I and vimentin were found to be upregulated in cells from smokers and patients with COPD and almost absent in cells from non-smokers (figure 2). The epithelial marker KRT5 was positively expressed in all HBECs from non-smokers, smokers and patients with COPD, although the intensity was lower in primary cultures from smokers and patients with COPD. Furthermore, positive KRT5 cells in smokers and patients with COPD were also positive for mesenchymal collagen I and vimentin positive cells (figure 2).

Immunohistochemical analysis of small airways revealed that α-SMA was immunostained in the submucosa of bronchi but not in bronchial epithelial cells from non-smokers (figure 3A, panel A). However, in both smokers and patients with COPD, α-SMA was markedly immunostained with a peribronchiolar location. Furthermore, α-SMA immunostaining was observed within the bronchial epithelium (figure 3A, panels F and K, black arrows). Vimentin immunostaining was absent in bronchial epithelial cells from non-smoker patients (figure 3A, panel B) but, in both smokers and patients with COPD, vimentin was located in basal epithelial cells (figure 3A, panels G and L). Collagen type I was increased in airway sections from smokers and patients with COPD, which correlates with the extracellular

matrix deposition found in peribronchiolar fibrosis.[3] NOX4 was weakly expressed in small lung sections from non-smokers, differing from smokers and patients with COPD who expressed NOX4 on bronchial epithelial cells as well as in bronchial infiltrated leucocytes (figure 3A, panels I and N). Epithelial ZO-1 and E-cadherin were markedly expressed in the bronchial epithelium of non-smokers (figure 3B, panels A and B), with lower expression in the bronchial epithelium of smokers and patients with COPD (figure 3B, panels D–H). Composite scores of immunohistochemistry showed higher values for α-SMA, vimentin, collagen type I and NOX4 in bronchial epithelial cells of smokers and patients with COPD compared with non-smoker patients and lower values for E-cadherin and ZO-1 (figure 3C).

EMT is induced by CSE in differentiated HBECs

To explore whether cigarette smoke may directly induce EMT in bronchial epithelial cells, we used an in vitro model of differentiated HBECs from non-smoker patients. CSE dose-dependently increased mesenchymal markers α-SMA and collagen type I, reaching a peak value at a concentration of 2.5% following 72 h of exposure (figure 4A; $p<0.05$). We therefore selected these time and concentration conditions of CSE to evaluate EMT markers in future experiments. In this respect, CSE at a concentration of 2.5% significantly increased mRNA transcripts and protein expression for the mesenchymal markers α-SMA, vimentin and collagen type I (figure 4B,C) while the epithelial markers E-cadherin, ZO-1, KRT5 and KRT18 were significantly downregulated (figure 4D,E). According to the loss of expression of TJ proteins, CSE (0.5–5%) dose-dependently decreased TEER (figure 4F) as a marker of loss of epithelial cell barrier. Furthermore, differentiated HBECs exposed to CSE adopted a flattened and elongated morphology characteristic of a myofibroblast-like phenotype. CSE-induced EMT markers were sustained for almost 7 days after the washout of CSE, which reflects the irreversibility of the EMT process (see figure C in online supplement).

CSE-induced EMT is partially mediated by the increase of ROS and downregulation of cAMP

Preincubation of differentiated HBECs with the antioxidants N-acetyl-ʟ-cysteine (NAC) and apocynin (APO) inhibited the increase of α-SMA, vimentin and collagen type I mRNA and
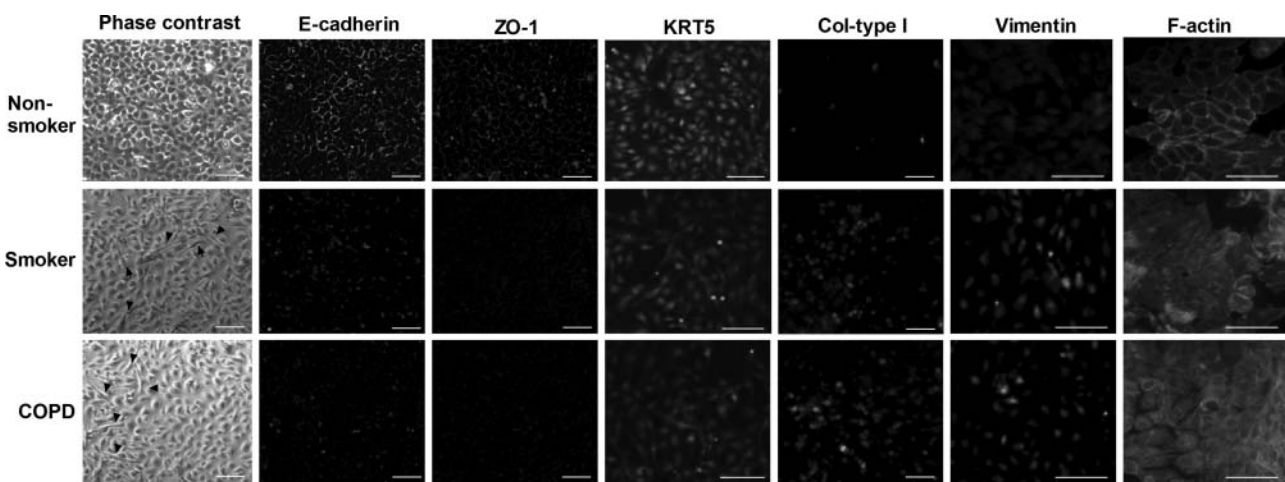
Figure 2 Small bronchial pieces from non-smokers (n=5), smokers (n=6) and patients with chronic obstructive pulmonary disease (COPD) (n=6) were placed on culture plates and bronchial epithelial cells grew around the explants. When the cells reached around 100% of confluence, phase contrast images and immunofluorescence staining of E-cadherin, ZO-1, KRT5 collagen type I (Col-type I) and vimentin were taken. F-actin fluorescent images were analysed with phalloidin-tetramethylrhodamine isothiocyanate. Representative images are shown (scale bar 20 μm). Black arrows show epithelial cells with spindle-shaped appearance. This figure is only reproduced in colour in the online version.
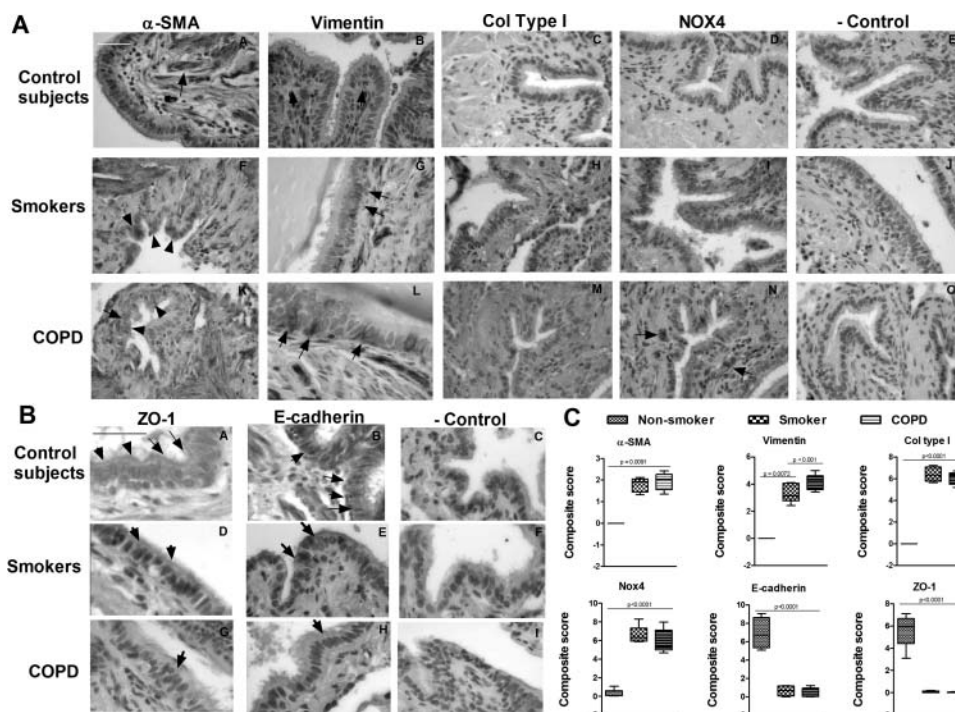
Figure 3 Immunohistochemistry of small airways. Small bronchial sections from non-smokers (n=4), smokers (n=10) and patients with chronic obstructive pulmonary disease (COPD) (n=10) were immunostained for (A) α smooth muscle actin (α-SMA), vimentin, collagen type I (Col type I), NOX4 (brown) and (B) epithelial ZO-1 and E-cadherin markers (brown). Black arrows show the main distribution of the positive immunostaining regions of the small bronchi. Representative immunohistochemistry images are shown (scale bar 45 μm). The control IgG isotype always gave a negative signal. (C) Box plot representing the composite score of α-SMA, vimentin, Col type I, NOX4, ZO-1 and E-cadherin markers across the bronchial epithelium in 10 slices per patient. Data are presented as box and whisker plots with median, IQR and minimum and maximum values. p exact values were obtained by the Kruskal–Wallis test and Dunn's post hoc test. This figure is only reproduced in colour in the online version.

protein expression as well as the downregulation of the epithelial markers E-cadherin, ZO-1, KRT5 and KRT18 induced by CSE (figure 5A–D). As observed for the antioxidants, the analogue of cAMP (dbcAMP) significantly inhibited the increase in mRNA and protein expression of the mesenchymal markers α-SMA, vimentin and collagen type I as well as the downregulation of the epithelial markers E-cadherin, ZO-1, KRT5 and KRT18 following CSE exposure (figure 5A–D). Phase contrast microscopic analysis of the differentiated HBEC culture surface and analysis of H&E-stained slide sections of multilayered HBECs showed that the cells underwent a morphological change from a cobblestone-like cell morphology to an elongated and spindle-like morphology, and reduced their cell–cell contact secondary to CSE exposure (figure 5E).

Interestingly, CSE 2.5% significantly decreased the intracellular cAMP content after 72 h, which was significantly inhibited by the antioxidants NAC and APO and the positive control dbcAMP (figure 6A), suggesting a role for ROS in cAMP downregulation. In this context, CSE 2.5% increased intracellular ROS after 30 min of exposure (figure 6B,C) and sustained this increase for the 72 h period (data not shown). The increase in ROS was accompanied by an increase in mRNA and protein expression of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase unit NOX4 after 72 h of exposure (figures 6D,E). Both ROS production and NOX4 expression induced by CSE were inhibited by dbcAMP, NAC and APO in differentiated HBECs (figure 6B–E).

TGF-β₁, ERK1/2 and Smad3 mediate CSE-induced EMT in differentiated HBECs

Stimulation with CSE 2.5% for 72 h significantly increased by approximately 2.5-fold the release of the active form of TGF-β₁

(figure 7A). Preincubation of differentiated HBECs with dbcAMP, NAC or APO completely suppressed the release of TGF-β₁ (figure 7A), suggesting a role for ROS and cAMP in mediating CSE-induced TGF-β₁ release. In other experiments, CSE 2.5% stimulation increased the phosphorylation of ERK1/2 and Smad3 which was significantly inhibited by anti-TGF-β₁ antibody (mAb-TGF-β₁) (figure 7B,C) suggesting an autocrine role for TGF-β₁. In parallel, preincubation of differentiated HBECs with dbcAMP, NAC or APO also inhibited CSE-induced ERK1/2 and Smad3 phosphorylation.

Finally, to analyse the autocrine effect of TGF-β₁ and its downstream pathways ERK1/2 and Smad3 in the CSE-induced EMT process, we preincubated cells with mAb-TGF-β₁, the ERK1/2 antagonist PD98058 or with the Smad3 inhibitor SIS3. All of them nearly suppressed CSE-induced upregulation of the mesenchymal markers α-SMA, vimentin and collagen type I (figure 8A), as well as downregulation of the epithelial markers E-cadherin and ZO-1 (figure 8B).

DISCUSSION

The main and novel results of the present study are that (1) primary HBECs of small bronchi from smokers and patients with COPD show an increase in the typical EMT expression profile and (2) in vitro stimulation of differentiated HBECs with CSE promotes EMT through the activation of intracellular ROS, the release of TGF-β₁, the phosphorylation of ERK1/2 and Smad3 as well as by the downregulation of cAMP. These new findings suggest that EMT may be induced directly by cigarette smoke, and that EMT is present in the small bronchi of smokers and patients with COPD, which may potentially
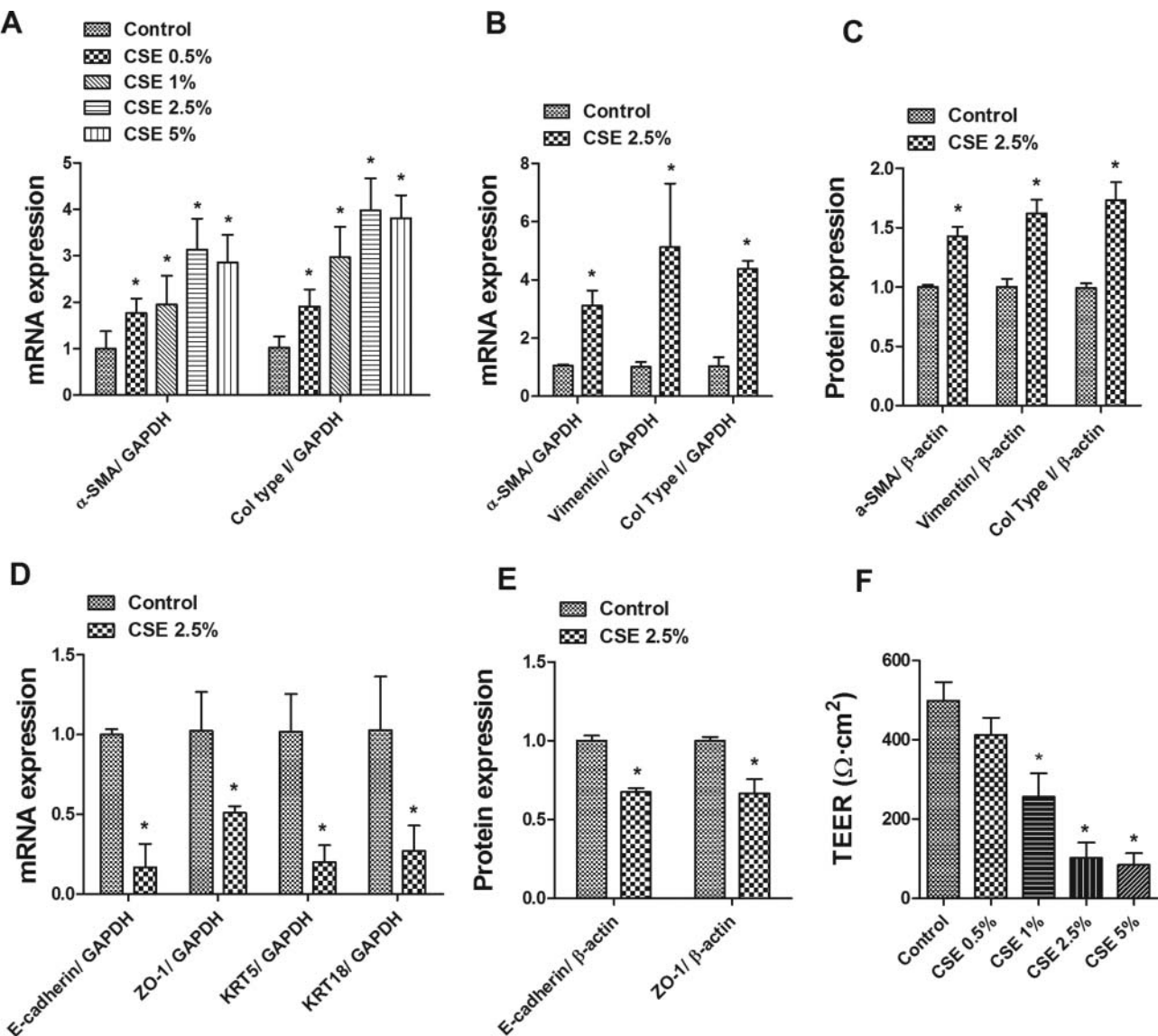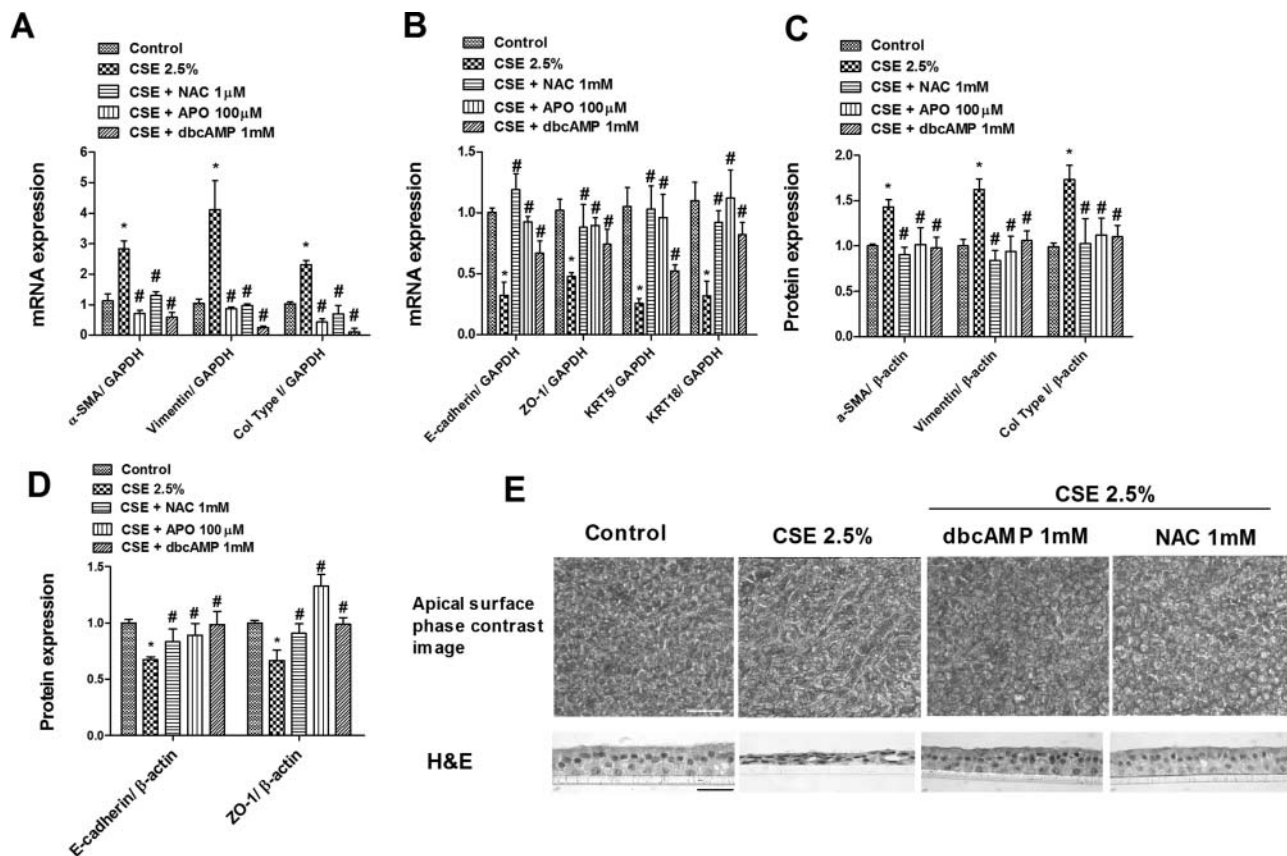
Figure 4 Cigarette smoke extract (CSE) induces epithelial to mesenchymal transition in differentiated human bronchial epithelial cells (HBECs). Differentiated HBECs were treated with 0.5–5% CSE or vehicle for 72 h and total RNA or cell lysates were obtained for real-time PCR (A, B, D) or protein array Zeptosens (C, E). CSE induced (A, B) mRNA transcripts and (C) protein expression of mesenchymal markers α smooth muscle actin, vimentin and collagen type I (Col type I). CSE significantly reduced (D) mRNA transcripts and (E) protein expression of epithelial markers E-cadherin, ZO-1, KRT5 and KRT18. Data are expressed as the ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for mRNA levels and to β-actin for protein levels and normalised to the solvent control group. (F) Development of transepithelial electrical resistance (TEER) in differentiated HBECs. TEER was measured after 72 h of CSE (0.5–5%) exposure. Results are expressed as mean (SE) of four independent experiments (one cell non-smoker population) per condition. Data analysed by one-way ANOVA followed by post hoc Bonferroni test (A) or Student t test (B–E). *$p < 0.05$ vs solvent controls.

contribute to the thickening of the wall of the small airways caused by fibrosis.

In the airways EMT has been implicated in several lung fibrotic disorders.[14] However, the hypothesis that lung bronchial fibroblasts may originate directly from HBECs as a result of EMT remains to be elucidated. In this respect, recently investigated in vivo biopsy specimens and ex vivo primary HBEC cultures from lung allograft recipients showed evidence of EMT.[18] In addition, Hackett and colleagues observed EMT in differentiated HBECs in response to TGF-β₁.[8] However, there is little knowledge regarding the potential for EMT to occur in COPD. In this regard, Sohal and colleagues recently observed that large airway biopsies from smokers and patients with COPD potentially undergo EMT.[13][19] However, the findings provided by

these studies were limited since the mesenchymal markers analysed (MMP-9, vimentin and S100A4) are also expressed in other cell types such as leucocytes and endothelial cells.[20][21] Furthermore, Sohal and colleagues studied EMT in large airways. Since bronchial remodelling in COPD, such as peribronchiolar fibrosis, is mainly located in small conducting airways (bronchioles <2 mm in diameter), and considering that large and small airways differ in their anatomical and pathophysiological characteristics,[22] previous findings need to be corroborated in small bronchi. In the present study we analysed the expression of mesenchymal markers broadly referenced in EMT studies such as the cytoskeleton proteins α-SMA and vimentin as well as the extracellular matrix protein collagen type I,[7] being α-SMA and collagen type I-specific for the fibroblast/
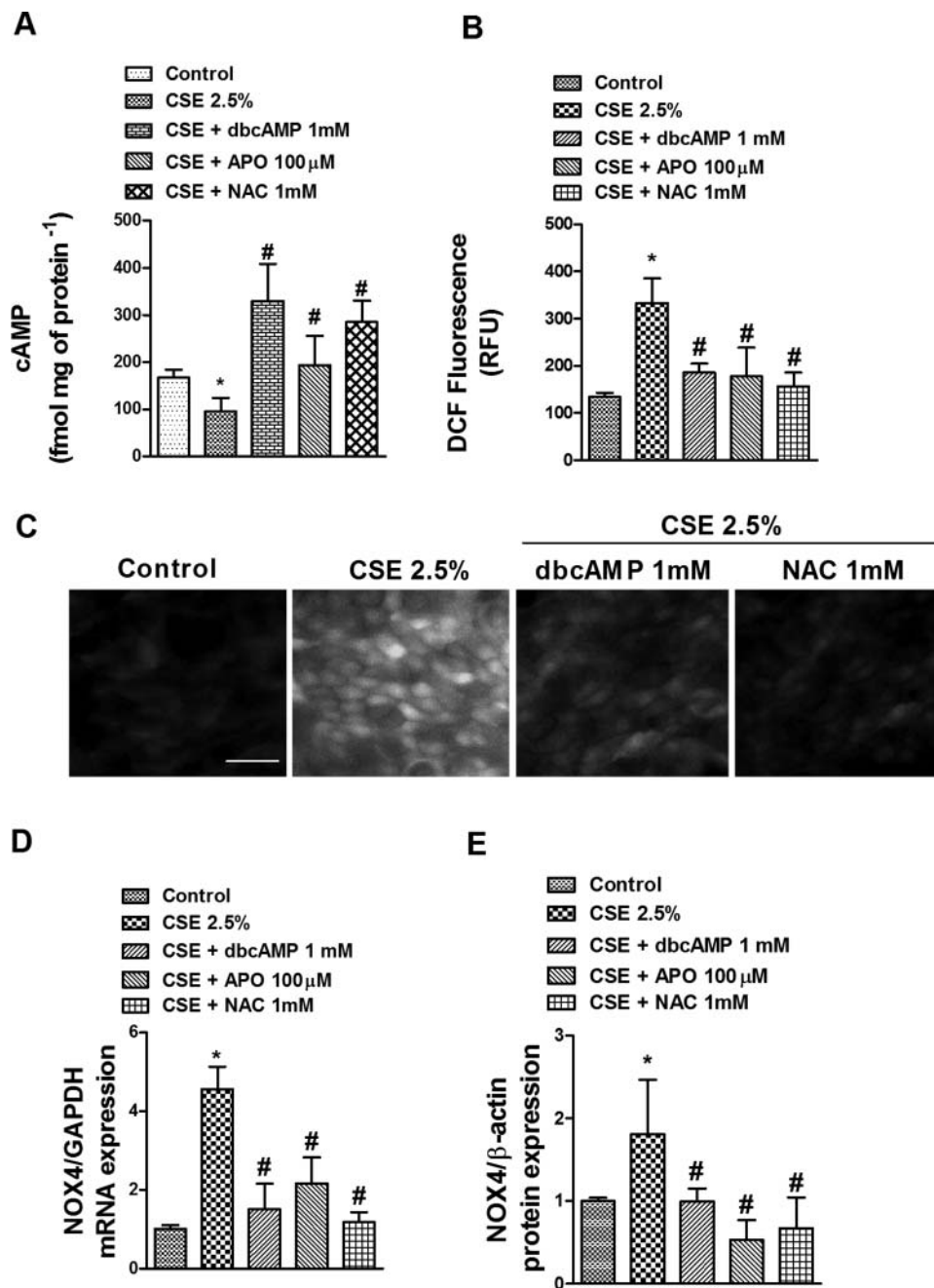
Figure 5 Cigarette smoke extract (CSE)-induced epithelial to mesenchymal transition is partially mediated by intracellular reactive oxygen species and inhibited by adenosine cyclic monophosphate (cAMP). Differentiated human bronchial epithelial cells (HBECs) were incubated with the antioxidants N-acetyl-ι-cysteine (NAC; 1 mM) or apocynin (APO; 100 μM), or with the cAMP analogue dibutyril cAMP (dbcAMP; 1 mM) for 30 min before CSE (2.5%) stimulation for 72 h. (A, B) Total RNA and (C, D) protein were isolated for real-time PCR and protein array Zeptosens analysis, respectively. CSE-induced upregulation of (A) mRNA and (C) protein expression of mesenchymal markers α smooth muscle actin, vimentin and collagen type I (Col type I) was inhibited by NAC, APO and dbcAMP. CSE-induced downregulation of (B) mRNA and (D) protein expression of epithelial markers E-cadherin, ZO-1, KRT5 and KRT18 was inhibited by NAC, APO and dbcAMP. Data are expressed as the ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for mRNA levels and to β-actin for protein levels and normalised to the solvent control group. Results are expressed as mean (SE) of n=4 (four cell non-smoker population) experiments per condition. Data analysed by two-way ANOVA followed by post hoc Bonferroni tests. *$p < 0.05$ vs solvent controls; #$p < 0.05$ vs CSE. (E) Morphological changes in differentiated HBECs after 72 h of exposure to 2.5% CSE showed a mesenchymal morphology with reduced cell–cell contact as assessed by phase contrast light microscopy (apical surface) and H&E section histology. Morphological changes were inhibited by NAC and dbcAMP. Representative images are shown (scale bar 40 μm). This figure is only reproduced in colour in the online version.

myofibroblast phenotype.[7] [23] Since EMT is characterised by the loss of epithelial cobblestone or cuboidal morphology and a marked reduction in epithelial E-cadherin, ZO-1 and cytokeratin expression, we also analysed the loss of expression of these epithelial markers as indicators of the EMT process. In this regard, positive KRT5 cells in cells from smokers and patients with COPD were also positive for mesenchymal collagen I and vimentin positive cells (figure 2). During the process of EMT some epithelial and mesenchymal cell markers are known to be co-expressed within individual cells,[24] which might explain why we have observed positive cells for both markers in HBECs from smokers and patients with COPD.

To our knowledge, this is the first report showing α-SMA in basal epithelial cells as a marker of human myofibroblasts in the small bronchi of smokers and patients with COPD. In this respect, recent work in a model of α-SMA-Cre/R26R mice of bleomycin-induced pulmonary fibrosis also showed upregulation of α-SMA in basal bronchial epithelial cells, supporting an in vivo EMT process in the bronchial epithelium.[25] Vimentin was also immunostained in basal epithelial cells from smokers and patients with COPD, as previously observed in large airways,[26]

and the expression of collagen type I was also upregulated in small bronchi of smokers and patients with COPD.

The epithelial markers E-cadherin and ZO-1 proteins were found to be downregulated in primary HBECs and the epithelium of small bronchi from smokers and patients with COPD. However, E-cadherin and ZO-1 mRNA expression was not decreased in smokers. These contradictory findings may be explained by E-cadherin and ZO-1 mRNA and protein expression being regulated at different levels. Thus, cell membrane E-cadherin and ZO-1 proteins as part of TJs between epithelial cells are degraded by different metalloproteinases released by the effect of oxidant agents such as cigarette smoke. In fact, it has been observed previously that ZO-1 is one means by which occludin is anchored to the cytoskeleton to maintain TJ. In the presence of oxidative stress, ZO-1:occludin association and E-cadherin is decreased and causes the diffuse cytoplasmic redistribution and degradation by metalloproteinases[27] [28] excluding transcriptional mechanisms. Another independent system is governed by transcriptional mechanisms in which the effect of some remodelling factors such as TGF-β₁ may reduce mRNA transcription of ZO-1 and E-cadherin.[29] The differences
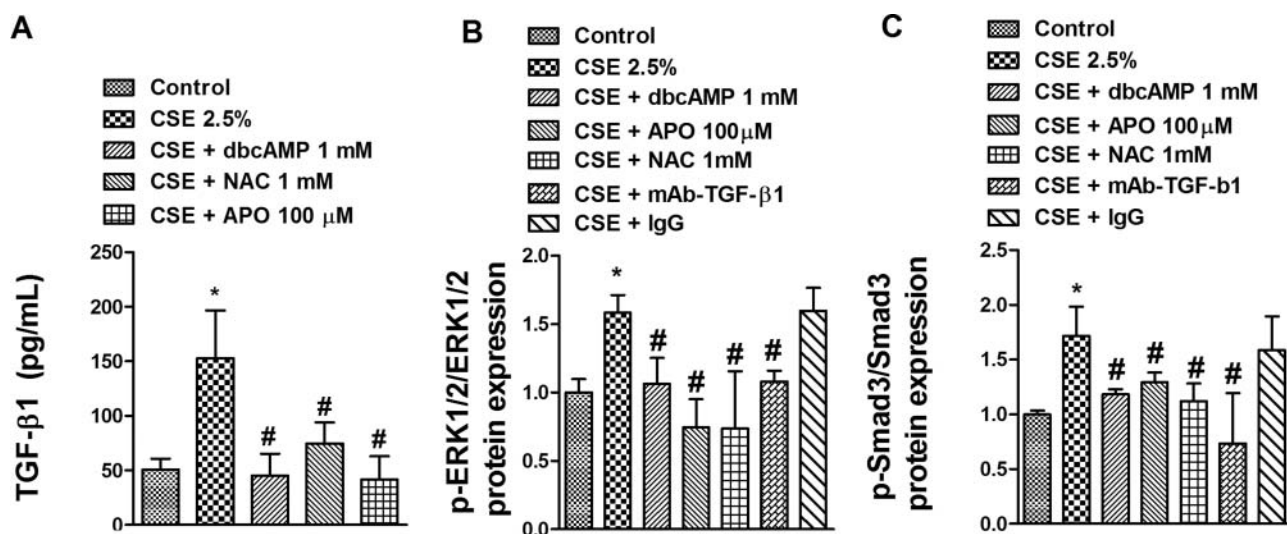
Figure 6 Cigarette smoke extract (CSE) reduces adenosine cyclic monophosphate (cAMP) intracellular levels and increases reactive oxygen species (ROS) and NOX4 expression in differentiated human bronchial epithelial cells (HBECs). Differentiated HBECs were incubated with the antioxidants N-acetyl-ʟ-cysteine (NAC; 1 mM) or apocynin (APO; 100 μM), or with the cAMP analogue dibutyril cAMP (dbcAMP; 1 mM) for 30 min before CSE (2.5%) stimulation for 72 h (A, D, E) or 30 min (B, C). (A) CSE-induced loss of intracellular cAMP was inhibited by NAC, APO and positive control dbcAMP. (B) ROS were determined by means of DCF fluorescence intensity after CSE stimulation in the presence or absence of NAC, APO or dbcAMP. (C) Representative images of positive DCF fluorescence in differentiated HBECs (scale bar 20 μm). (D, E) CSE induced mRNA and protein expression of NOX4 which were significantly inhibited by dbcAMP, APO and NAC (D, E). Data are expressed as the ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for mRNA levels and to β-actin for protein levels and normalised to the solvent control group. Results are expressed as means (SE) of n=4 (four cell non-smoker population) experiments per condition. Data analysed by two-way ANOVA followed by post hoc Bonferroni tests. *$p < 0.05$ vs solvent controls; #$p < 0.05$ vs CSE. RFU, relative fluorescence units. This figure is only reproduced in colour in the online version.

between mRNA and protein expression of ZO-1 and E-cadherin observed between smokers and patients with COPD could therefore be due to a predominant role of protein degradation in bronchial epithelial cells from patients with COPD because of a high level of metalloproteinase expression in their lung tissues.[30] Accordingly, we found higher basal levels of MMP-9 in cells from patients with COPD than in smokers.

Despite the novelty of our results, some limitations should be highlighted. In particular, both the primary cell culture and lung tissue analysed could be criticised. The loss of cell phenotype in prolonged culture is well described and could modify the results in primary HBECs. However, our primary ex vivo cell culture experiments were performed in HBECs that were grown around the explants of small bronchi until they reached approximately
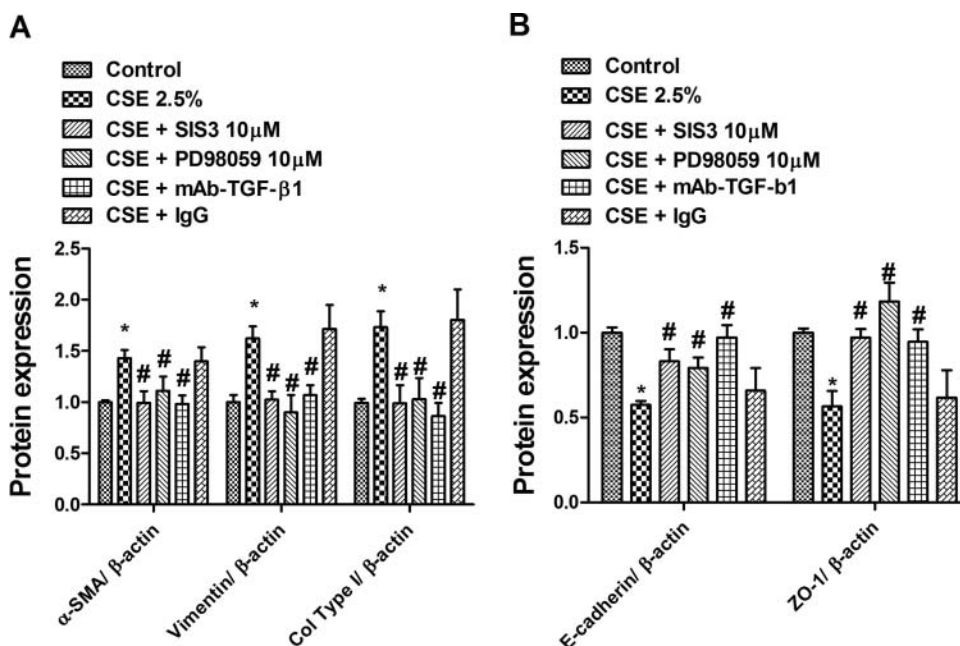
Figure 7 Cigarette smoke extract (CSE) increases secretion of transforming growth factor β_1 (TGF-β_1) and phosphorylation of ERK1/2 and Smad3 in differentiated human bronchial epithelial cells (HBECs). Differentiated HBECs were incubated with the antioxidants N-acetyl-L-cysteine (NAC; 1 mM) or apocynin (APO; 100 µM), or with the cyclic monophosphate (cAMP) analogue dibutyril cAMP (dbcAMP; 1 mM) for 30 min before CSE (2.5%) stimulation for 72 h. In panels B and C an anti-TGF-β_1 antibody (mAb-TGF-β_1; 4 µg/ml) and its negative control non-immune IgG were added for 30 min before CSE 2.5% stimulation for 72 h. Cell culture supernatant and protein content were extracted to quantify (A) TGF-β_1 release, (B) ERK1/2 phosphorylation and (C) Smad3 phosphorylation. (B, C) Phospho-Smad3 and phospho-ERK1/2 protein levels were expressed as the ratio to total ERK1/2 and Smad3, respectively, and normalised to the solvent control group. Results are expressed as mean (SE) of n=4 (four cell non-smoker population) experiments per condition. Data analysed by two-way ANOVA followed by post hoc Bonferroni tests. *$p < 0.05$ vs solvent controls; #$p < 0.05$ vs CSE.

100% of confluence, forming an epithelial cell layer (without passages and without trypsinisation) on KRT5 positive cells in order to prevent phenotypic changes, as previously outlined.[18]

On the other hand, the lung tissues studied were from patients undergoing lung surgery for lung carcinoma. Since EMT has been well characterised in epithelial lung malignant disease,[31] it

Figure 8 Cigarette smoke extract (CSE)-induced epithelial to mesenchymal transition is partially mediated by an autocrine transforming growth factor β_1 (TGF-β_1) effect and its downstream signalling phospho-ERK1/2 and phospho-Smad3. Differentiated human bronchial epithelial cells (HBECs) were incubated with the Smad3 inhibitor SIS3 (10 µM), the ERK1/2 inhibitor PD98059 (10 µM), the anti-TGF-β_1 antibody (mAb-TGF-β_1; 4 µg/ml) or its negative control non-immune IgG for 30 min before CSE (2.5%) stimulation for 72 h. After incubation periods, differentiated HBECs were lysed and protein array Zeptosens performed. (A) CSE-induced upregulation of α smooth muscle actin, vimentin and collagen type I (Col type I) mesenchymal markers was inhibited by SIS3, PD98059 and mAb-TGF-β_1. (B) CSE-induced downregulation of E-cadherin and ZO-1 epithelial markers was inhibited by SIS3, PD98059 and mAb-TGF-β_1. (A, B) Data are expressed as the ratio to β-actin for protein levels and normalised to the solvent control group. Results are expressed as means (SE) of n=4 (four cell non-smoker population) experiments per condition. Data analysed by two-way ANOVA followed by post hoc Bonferroni tests. *$p < 0.05$ vs solvent controls; #$p < 0.05$ vs CSE.

could be argued that the EMT expression profile is due to spread of tumour lesions. However, samples of distal lung, located as far away as possible from the tumour, were carefully checked histologically to exclude the presence of tumour.

Another limitation could be related to the fact that the observed increase in the expression of mesenchymal markers in the epithelium is related to the decline in lung function while increased mesenchymal markers are also observed in smokers with normal lung function. This apparent contradiction may be explained by the fact that remodelling is a set of processes affecting whole airways which differ between smokers and patients with COPD. Patients who are smokers show less goblet cell and submucosal gland hyperplasia than patients with COPD,[2] absence of loss of alveolar wall, less vascular remodelling[32] and less airway wall thickening and smooth muscle deposition around bronchi,[33] all contributing to lung function. Thus, although mesenchymal markers were upregulated in smokers with normal lung function, this may be considered as an initial step in small bronchial remodelling contributing to lung function over time together with the other remodelling processes.

Next we analysed possible mediators involved in the EMT process. A hypothesis generated from animal models is that cigarette smoke drives small airways remodelling by induction of profibrotic growth factors (eg, connective tissue growth factor, platelet-derived growth factor-β and TGF-β_1) from the airway wall.[34] These growth factors, which have also been implicated as inducers of EMT, could contribute to peribronchiolar fibrosis. Supporting this hypothesis, several in vitro studies have shown that components of cigarette smoke such as nicotine and benzo(a)pyrene induce EMT in immortalised airway epithelial cells.[35 36] In this study we first showed evidence that differentiated HBECs undergo EMT in response to cigarette smoke, as demonstrated by specific markers and phenotypic changes. Cigarette smoke contains a high number of ROS that may activate latent TGF-β_1 release.[37] Previous data support the hypothesis that cigarette smoke may release the active form of TGF-β_1, activating its downstream signalling Smad2/3 cascade in bronchial rat explants to promote small bronchi remodelling.[37] In addition, both cigarette smoke and TGF-β_1 also promote the activation of ERK1/2 signalling in HBECs,[38] suggesting a role for Smad3 and ERK1/2 phosphorylation in cigarette smoke-induced EMT. In this study we add new evidence to the mechanism by which CSE increases the release of the active form of TGF-β_1, the phosphorylation of Smad3 and ERK1/2, thus promoting the EMT process in differentiated HBECs. The release of TGF-β_1 and the downstream phosphorylation of Smad3 and ERK1/2 were activated by ROS generated by CSE since the antioxidants NAC and APO—as well as blocking TGF-β_1 with mAb-TGF-β_1—almost suppressed CSE-induced TGF-β_1 signalling and EMT. These in vitro findings are of clinical relevance since the phosphorylated forms of ERK1/2 and Smad3 were upregulated in primary HBECs from smokers and patients with COPD.

The importance of oxidant-mediated TGF-β release as well as ROS in fibrotic diseases has been previously documented.[39] In fact, myofibroblast transformation in lung fibrotic disorders following TGF-β_1 stimulation is partially mediated by the NADPH oxidase unit NOX4.[39] The plasmatic membrane unit NOX4 does not require interaction and activation by cytosolic regulatory subunits, so its activation is directly related to its expression.[40] In this study, CSE increased intracellular ROS as well as NOX4 expression in differentiated HBECs. Furthermore, NOX4 expression was upregulated in primary HBECs from smokers and patients with COPD and located on bronchial epithelial cells and leucocyte infiltrates in human small bronchi, confirming activation of oxidative stress in the tissues studied.

Considering the established role for cAMP as an inhibitor of ROS in epithelial cells as well as the subsequent EMT process,[10] we explored whether cAMP could modulate CSE-induced EMT in differentiated HBECs. In our in vitro model CSE induced the loss of cAMP, probably by an increase in the degrading cAMP phosphodiesterase 4 (PDE4) activity, as we recently reported in HBECs.[15] The addition of dbcAMP as an analogue of cAMP reduced ROS, TGF-β_1 secretion and their downstream signalling phospho-ERK1/2 and phospho-Smad3 as well as the EMT process. Modulation of cAMP levels could therefore be an appropriate therapy to attenuate EMT and small bronchi remodelling in patients with COPD.

A rational sequence of mechanistic events by which CSE induces EMT in HBECs therefore includes activation of the NADPH oxidase system and upregulation of the NADPH oxidase component NOX4 to produce intracellular ROS. Thus, ROS decrease cAMP levels through the increase in PDE4 activity. In a subsequent event, the increase in intracellular ROS together with the reduction in cAMP contributes to the release of TGF-β_1. TGF-β_1 activates Smad3 and ERK1/2, contributing to the increase in EMT.

In summary, our data provide evidence of an active EMT process in the small bronchi of smokers and patients with COPD which may potentially contribute to the thickening of the wall of the small airways. In vitro studies show that cigarette smoke may induce EMT, modulating the TGF-β_1 pathway as well as ROS and cAMP levels. Future treatments directed at these molecular pathways could potentially attenuate in vivo EMT and small bronchial remodelling of patients with COPD.

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Online data supplement

EPITHELIAL TO MESENCHYMAL TRANSITION IS INCREASED IN COPD PATIENTS AND INDUCED BY CIGARETTE SMOKE

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METHODS

Patients

A total of 5 non-smoker controls, 12 smokers and 15 COPD patients were included in the study. COPD patients were diagnosed according to the GOLD guidelines.[1] All lung tissues studied in this work were taken from uninvolved lung tissue during lobectomy/wedge resection for malignant lesions in the thoracic Surgery and Respiratory Unit, University General Hospital Consortium, Valencia, Spain, between 2009 and 2011. Samples of distal lung, located as far away as possible from the tumor, were chosen for the study. All pulmonary function tests were performed within 3 months before surgery. Clinical data of all patients was examined for possible co-morbidity and medication use. Inclusion criteria were defined as: 1) non-smokers, smokers and COPD patients free of symptoms of upper respiratory tract infection, and none received antibiotics perioperatively. 2) None of the patients received glucocorticosteroids, β2 adrenergics or anti-muscarinics during the 3 months before operation. After selection based on lung function, all lung tissue samples used for the study were checked histologically by using the following exclusion criteria: (1) presence of tumor, (2) presence of post stenotic pneumonia, and (3) fibrosis of lung parenchyma. The protocol was approved by the local research and independent ethics committee of the University General Hospital of Valencia. Informed written consent was obtained from each participant.

Isolation of Primary Bronchial Epithelial Cells

Isolation of human bronchial epithelial cells from small bronchi was assessed as previously outlined.[2] Small pieces of human bronchi (0.5-1mm internal diameter) were excised from microscopically normal lung areas, carefully dissected free from lung parenchyma and plated on collagen-coated culture dishes (10µg cm^{-2} rat type I collagen

(Sigma) in bronchial epithelial growth medium (BEGM, comprising bronchial epithelial basal medium (BEBM) supplemented with bovine pituitary extract 52 µg ml^{-1}, hydrocortisone 0.5 µg ml-^{1}, human recombinant epidermal growth factor (EGF) 25 ng ml^{-1}, epinephrine 0.5 µg ml^{-1}, transferrin 10µg ml^{-1}, insulin 5 µg ml^{-1}, retinoic acid 50nM, triiodo-L-thyronine 6.5 ng ml^{-1}, gentamycin 40 µg ml^{-1}, amphotericin B 50 ng ml^{-1}, bovine serum albumin 1.5µg ml^{-1} and retinoic acid 0.05 µM). Small bronchi were oriented with the epithelial layer to be in contact with the culture plate. After a period of ~1 week-12 days, bronchial epithelial cells were observed around bronchi and used to immunofluorescence analysis and to measure RNA and protein expression of epithelial and mesenchymal markers of different patients. The identity of the monolayer as bronchial epithelial cells was affirmed by morphological criteria and immunofluorescence for cytokeratin 5 (KRT5) as well as the later *in vitro* differentiation in air-liquid interface as pseudo-stratified bronchial epithelium with basal cells, ciliated cells, columnar and goblet cells. Cell viability was assessed by vital trypan blue exclusion analysis using the countness® automated cell counter (life technologies, Madrid, Spain). Cell viability was >98% in all cell cultures tested in this work.

Culture of Air Liquid Interface Bronchial Epithelial Cells

Differentiated HBECs for *in vitro* experiments were obtained from non-smoker patients. Primary HBECs were trypsinized and subpassaged on 12-well polyester Transwell inserts (Millipore) at 150 x 10^{3} cells per insert. Cells were left for 7 days submerged in BEGM/DMEM (1:1) culture medium. From day 7 the air-liquid interface culture was initiated by removing the medium from the upper well leaving the apical side of the cells exposed to air and changing the final epidermal growth factor (EGF) concentration

to 0.5 ng/ml (differentiation medium). In general air-liquid interface cultures were pursued until about 80-90% of cells were ciliated by microscopic inspection (about 3-4 weeks after initiation) before experiments were commenced.[2] Cells were maintained at 37°C with 5% CO_2 and medium changed every other day. At this stage a pseudo-stratified bronchial epithelium comprising basal cells, ciliated cells and goblet cells was obtained and considered as "differentiated".

Immunohistochemistry

For immunohistochemical analysis of human pulmonary tissue from non-smokers, smokers and COPD patients, tissue was fixed, embedded in paraffin, cut into sections (4-6 µm), and stained with haematoxylin, as reported previously.[3] The sections were incubated with mouse anti-human α-SMA (cat. nº: A5228; Sigma), rabbit anti-human collagen type I antibody (cat. nº: PA1-26204; Affinity Bioreagents), mouse anti-human vimentin (cat. nº: V6389; Sigma), NOX4 (cat. nº: NB110-58849; Novus Biologicals), mouse anti-human E-cadherin (cat. nº: CM1681; ECM BioScience) and rabbit anti-human ZO-1 (cat. nº: ab59720; Abcam) for 24 h at 4°C. A secondary anti-rabbit or anti-mouse antibody (1:100; Vector Laboratories, Burlingame, CA) with avidin-biotin complex/horseradish peroxidase was used for immunohistochemistry. The non-immune IgG isotype control was used as negative control. All stained slides were scored by a pathologist under a Nikon Eclipse TE200 (Tokio, Japan) light microscope and representative photographs taken (10 slices per patient). Staining intensity was analyzed in bronchial epithelium of small bronchi. Staining intensity for different antibodies was scored on a scale of 0–3 (0-negative, 1-weak, 2-moderate, 3-strong immunoreactivity). The percentage of cells positive for different antibodies within bronchial epithelium was scored on a scale of 1–4 as follows: 1: 0–25% cells positive; 2: 26–50% positive;

3: 51–75% positive; and 4: 76–100% positive. The score of the staining intensity and the percentage of immunoreactive cells were then multiplied to obtain a composite score ranging from 0 to 12.

The phenotype analysis of the *in vitro* cell culture of differentiated HBECs was assessed by contrast phase microscopy (Nikon eclipse TE200) and hematoxiline & eosin (H&E) stained of slide sections of paraffin embedded trans-well inserts.

Immunofluorescence

Basal HBECs from non-smokers, smokers and COPD patients were washed three times with PBS and fixed (4% paraformaldehyde, 30 min, at room temperature). After another three washes with PBS, HBECs were permeabilized (20 mM HEPES pH 7.6, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100), blocked (10% goat serum in PBS) and incubated with the primary antibodies mouse anti-human E-cadherin (cat. nº: CM1681; ECM BioScience) and rabbit anti-human ZO-1 (cat. nº: ab59720; Abcam), rabbit anti-human collagen type I antibody (cat. nº: PA1-26204; Affinity Bioreagents) or mouse anti-human vimentin (cat. nº: V6389; Sigma) overnight at 4°C, followed by secondary antibody anti-rabbit/mouse rhodamine/FITC- (1:100, Molecular Probes). Cells were visualized by epifluorescence microscopy (×200; Nikon eclipse TE200 inverted microscope, Tokyo, Japan).

F-actin staining

Cells were washed three times with PBS and fixed in a 3.7% formaldehyde-PBS solution for 10 min at room temperature. After two additional washes with PBS, cells were permeabilized with a solution of 0.1% Triton X-100 in PBS for 3–5 min and washed again with PBS. Phalloidin-tetramethylrhodamine isothiocyanate (0.2 µg/ml) was used to localize F-actin, as described by Cramer and coworkers.[4] Fluorescent dye

was diluted on blocking solution (1% BSA and 0.025% saponin in PBS) and added to coverslips for 40 min at room temperature. After three washes with PBS, coverslips were mounted on a microscopy slide with mounting media (mowiol; Calbiochem, La Jolla, CA). F-actin cytoskeleton imaging was performed with an epifluorescence microscope (Nikon TE200, Tokyo, Japan).

Preparation of Cigarette Smoke Extract

Cigarette smoke extracts (CSE) were obtained as previously outlined.[3,5] Briefly, the smoke of a research cigarette (2R4F; Tobacco Health Research, University of Kentucky, KY, USA) was generated by a respiratory pump (Apparatus Rodent Respirator 680; Harvard, Germany) through a puffing mechanism related to the human smoking pattern (3 puff/min; 1 puff 35 ml; each puff of 2 s duration with 0.5 cm above the filter) and was bubbled into a flask containing 25 ml of pre-warmed (37°C) BEGM/DMEM medium. The CSE solution was sterilized by filtration through a 0.22-µm cellulose acetate sterilizing system (Corning, NY). The resultant CSE solution was considered to be 100% CSE and was used for experiments within 30 min of preparation. CSE 10% corresponds approximately to the exposure associated with smoking two packs per day [6]. The quality of the prepared CSE solution was assessed based on the absorbance at 320 nm, which is the specific absorption wavelength of peroxynitrite. Stock solutions with an absorbance value of 3.0 ± 0.1 were used. To test for cytotoxicity from CSE, differentiated HBECs were treated with CSE concentrations of up to 10% for 72 h. No significant difference in the lactate dehydrogenase supernatant level (lactate dehydrogenase cytotoxicity assay; Cayman, Spain) was observed, compared with the control group.

In vitro Stimulation of Differentiated HBECs

For *in vitro* studies, differentiated HBEC were stimulated with CSE (0.5% to 5%) for the indicated times, replacing culture medium and stimulus every 24 h. Dibutyryl adenosine 3´-5´ cyclic monophosphate sodium salt (1 mM; dbcAMP; Sigma: catalogue n°. D0260),[2] N-acetyl-ι-cysteine (1mM; NAC; Sigma: catalogue n°. A-7250),[2] apocynin (described as NADPH oxidase inhibitor, 100µM; Sigma: catalogue n°. W508454),[2] SIS3 (described as Smad3 inhibitor, 10µM; Sigma: catalogue n°. S0447),[7] PD98059 (described as ERK1/2 inhbitor, 10 µM; Sigma: catalogue no. P215),[8] were added 30 min before stimulus. Monoclonal anti-human TGF-β1 mAb (4 µg/mL; anti-TGF-β1; R&D Systems, Madrid, Spain; catalogue n°. AB-246-NA) was added 30 min before stimulus to block the active form of TGF-β1 present in the culture supernatant as previously outlined.[9]

Both test compounds and CSE were added to the basolateral media (500µl) and at the apical surface (25µl). As manipulations at the apical surface may affect pseudo-stratified epithelium, all incubations with vehicle controls were run under identical conditions as with CSE and test compounds (for example identical volumes of medium were added to the apical surface throughout all conditions, same for the basolateral compartment). In control experiments where vehicles / medium (for test compounds / CSE) were added to the apical surface of differentiated HBECs over a maximum of 3 days (including daily replacement procedures, as indicated) the number of ciliated cells and expression of cilia markers were found to be not different from cultures of differentiated human bronchial epithelial cells in the absence of the manipulations at the apical surface.

Real Time RT-PCR

Total RNA was isolated from primary HBEC and differentiated bronchial epithelial cells in air liquid interface by using TriPure® Isolation Reagent (Roche, Indianapolis, USA). The integrity of the extracted RNA was confirmed with Bioanalizer (Agilent, Palo Alto, CA, USA). The reverse transcription was performed in 300 ng of total RNA with TaqMan reverse transcription reagents kit (Applied Biosystems, Perkin-Elmer Corporation, CA, USA). cDNA was amplified with specific predesigned by Applied Biosystems for α-SMA (Hs00559403_m1), α_1(I)-collagen (collagen type I; cat. nº: Hs00164004_m1), vimentin (cat. nº: Hs 00958116_m1), E-cadherin (cat. nº: Hs01023894_m1), zona occludens-1 (ZO-1; cat. nº: Hs01551861_m1), KRT5 (cat. nº: Hs00361185_m1), KRT18 (cat. nº: Hs02827483_g1) and NOX4 (cat. nº: Hs00276431_m1), and GAPDH (pre-designed by Applied Biosystems, cat. nº: 4352339E) as a housekeeping in a 7900HT Fast Real-Time PCR System (Applied Biosystem) using Universal Master Mix (Applied Biosystems). Relative quantification of these different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using GAPDH as endogenous control (Applied Biosystems; 4352339E) and normalized to non-smoker or control group.

Protein Array

Primary HBECs from non-smokers, smokers or COPD patients as well as differentiated HBECs in air liquid interface were lysed with CeLyA Lysis Buffer CLB1 (Zeptosens, Division of Bayer (Schweiz), Switzerland), incubated during 30 minutes at room temperature and centrifuged (5 min at 15,000xg) in order to remove debris. The supernatants were collected, frozen in liquid nitrogen and stored at -80ºC.

Protein concentration was determined using a Bradford-Coomassie Plus Assay Kit (Pierce). Protein content was adjusted to 2 mg/ml and samples were subsequently

diluted using spotting buffer (PBS + 10% DMSO + 5% Glicerol) to obtain four different protein concentrations corresponding to 100, 75, 50 and 25% (0.2 mg/ml, 0.15 mg/ml, 0.1 mg/ml and 0.05 mg/ml) of the primary spotting solution. The Nano-Plotter (GeSiM) impregnated the chips (Zeptosens) with drops (400 pl) of each dilution and these chips were blocked by nebulization with Blocking Buffer BB1 (Zeptosens) using the ZeptoFOG Blocking Station (Zeptosens).

For each of these four dilutions, duplicate spots were arrayed onto ZeptoMARK chips (Zeptosens) as single sample droplets of about 400 pl, using a Micro Pipetting System Nano-plotter[TM] (NP2.1, GeSiM, Großerkmannsdorf, Germany).

After spotting, the chips were dried for 1 h at 37ºC and blocked in an ultrasonic nebulizer (ZeptoFOG, Zeptosens) with CeLyA Blocking Buffer (BB1, Zeptosens). Blocked chips were rinsed with water (Milli-Q quality), dried and stored at 4ºC in the dark until further use. Antibody incubations were done in CeLyA Assay Buffer CAB1 based on BSA according to standard protocols (Zeptosens). The chips were assembled with chip fluidic structures in a ChipCARRIER (Zeptosens) and were incubated with primary antibodies (1:500 dilution in CAB1) overnight at room temperature. After rinsing the system with assay buffer, the chips were incubated with secondary fluorescence-labeled anti-species antibodies (Zenon Alexa Fluor 647 Rabbit IgG and mouse IgG Labeling Kit (cat. nº: Z25308 and Z25008, Molecular Probes) (1:500 dilution in CAB1) for 1h at room temperature. After rinsing the system with assay buffer to remove the excess secondary antibody, the fluorescence readout was performed with the ZeptoREADER instrument (Zeptosens), at an extinction wavelength of 635 nm and an emission wavelength of 670 nm. The fluorescence signal was integrated over a period of 1–10 s, depending on the signal intensity. Array images were stored as 16-bit TIFF files and analyzed with the ZeptoView Pro software package

(version 2.0, Zeptosens). Relative intensities were obtained by plotting net spot intensities against protein concentrations of the spotted samples determined by a Bradford assay as described above. Briefly, the eight data points for each sample were fitted using a weighted linear least squares fit.[10] The relative intensity was then interpolated at the median protein concentration. The SD calculated from the fit is indicative for the linearity of the dilution series. Subsequently the data were renormalized to correct for small variations in protein content using mouse anti-human β-actin (cat nº: A1978; Sigma), total rabbit anti-human ERK1/2 (1:1,000) antibody (monoclonal antibody; Cell Signalling, Boston, Massachusetts, USA; catalogue no. 4695) or total rabbit anti-human Smad3 (cat nº: 566414; Calbiochem) as internal standards. Primary antibodies used were mouse anti-human α-SMA (cat. nº: A5228; Sigma), rabbit anti-human collagen type I antibody (cat. nº: PA1-26204; Affinity Bioreagents), mouse anti-human vimentin (cat. nº: V6389; Sigma), mouse anti-human E-cadherin (cat. nº: CM1681; ECM BioScience), rabbit anti-human ZO-1 (cat. nº: ab59720; Abcam), mouse anti-human phospho-ERK1/2 (cat. nº: M-9692; Sigma), rabbit anti-human phospho-Smad3 (cat. nº: PS1023; Calbiochem), and NOX4 (cat. nº: NB110-58849; Novus Biologicals).

Western blot

Western blot analysis was used to detect changes in nuclear and cytoplasm β-catenin (98 kD) in HBECs of non-smokers, smokers and COPD patients. Nuclear and cytoplasm protein extraction was performed with the nuclear active motif extraction kit (Active Motif Europe, Rixensart, Belgium) according to the manufacturer's protocol. The Bio-Rad assay (Bio-Rad Laboratories Ltd., Herts, UK) was used (following manufacturer's instructions) to quantify the level of protein in each sample to ensure equal protein

loading. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to separate the proteins according to their molecular weight. Briefly, 10 µg proteins (denatured) along with a molecular weight protein marker, Bio-Rad Kaleidoscope marker (Bio-Rad Laboratories), were loaded onto an acrylamide gel consisting of a 5% acrylamide stacking gel stacked on top of a 10% acrylamide resolving gel and run through the gel by application of 100 V for 1 h. Proteins were transferred from the gel to a polyvinylidene difluoride membrane using a wet blotting method. The membrane was blocked with 5% Marvel in PBS containing 0.1% Tween20 (PBS-T) and then probed with a rabbit anti-human β-catenin (1:1,000) antibody (polyclonal antibody; Novus Biologicals, Cambridge, UK; cat n^o. NBP1-89989) and normalised to total mouse anti-human β-actin (1:1,000) antibody (monoclonal antibody cat n^o: A1978; Sigma) for cytoplasm protein expression and to lamin A (polyclonal antibody cat n^o: L1293; Sigma) for nuclear protein expression. The enhanced chemiluminescence method of protein detection using enhanced chemiluminescence reagents, ECL plus (Amersham GE Healthcare, Buckinghamshire, UK), was used to detect labelled proteins.

Transepithelial electrical resistance (TEER)

The transepithelial electrical resistance (TEER) was measured in differentiating cells using a millicell ERS-2 epithelial volt-ohm meter (Millipore, UK, Stevenage). Differentiated HBECs were incubated in presence or absence of CSE (0.5%-5%) for up to 72h. Medium was aspirated and replaced with 1mL in the basolateral and 0.5mL in the apical compartment. Cultures were equilibrated in the incubator for 30 minutes before measurement of TEER. Apical medium was then aspirated to restore ALI. TEER

of insert and medium alone was subtracted from measured TEER and $\Omega \cdot cm^2$ calculated by multiplying by the insert area.

DCF Fluorescence Measurement of Reactive Oxygen Species

2´, 7´-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Molecular Probes, UK) is a cell-permeable compound that following intracellular ester hydrolysis is oxidized to fluorescent 2´, 7´-dichlorofluorescein (DCF) by $O_2^{\cdot-}$ and H_2O_2, and can therefore be used to monitor intracellular generation of ROS.[11] To quantify ROS levels, polarized human bronchial epithelial cells were washed twice with PBS and incubated for 30 min with 50 µM H_2DCF-DA diluted in Opti-MEM in presence or absence of, dbcAMP or the antioxidants N-acetyl-ι-cysteine or apocynin. Then, cells were again washed twice with PBS to remove remaining H_2DCF-DA and stimulated with CSE for 30 min in presence or absence of the same drugs indicated before. Five randomly selected fields per condition were measured for fluorescent intensity using an epifluorescence microscope (Nikon Eclipse TE 200, Tokio, Japan) with filter set for FITC. Subsequent image capture and analysis was performed using Metafluor® 5.0 software (Analytical Technologies, US). Results were expressed as DCF fluorescence in relative fluorescence units.

Enzyme-Linked Immunosorbent Assays for TGF-β1, cAMP and MMP-9

Quantitative ELISA for TGF-β1 was performed with supernatants of differentiated HBECs following 72 h of stimulation with CSE in presence or absence of dbcAMP, N-acetyl-ι-cysteine or apocynin using quantikine human TGF-β1 immunoassay (R&D Sistems; catalogue no. 891124). To measure latent complexes of TGF-β1, activation was accomplished by acid treatment. Therefore, 50µl of cell culture supernatants were

treated with 10µl of 1 mol/l HCl, incubated for 10 min, and then neutralised with 10µl of 1.2 mol/l NaOH/0.5 mol/l HEPES.

Intracellular cAMP content was determined in differentiated HBEC as previously outlined.[12] Following incubations with CSE in presence or absence of dbcAMP or apocynin as indicated, culture medium was removed and the cells were washed with phosphate-buffered saline (PBS). Then, cells were lysed and the intracellular cAMP content was determined with the cAMP Biotrak enzyme immunoassay (EIA) system according to manufacturer´s instructions (Amersham, UK). Results were expressed as fmol cAMP per mg of protein (per insert). MMP-9 was determined by using commercially available enzyme-linked immunosorbent assay kit for MMP-9 (R&D Systems, Nottingham, UK) according to the manufacturer's protocol.

Statistics

Statistical analysis of results was carried out by parametric or non-parametric analysis as appropriate. $p < 0.05$ was considered statistically significant. Non-parametric tests were used to compare results from epithelial cells of non-smoker, smokers and COPD patients. In this case, data were displayed as medians, interquartile range and minimum and maximum values. When the comparisons concerned only 2 groups, between-group differences were analyzed by the Mann Whitney test. When comparisons concerned several groups (non-smokers, smokers and COPD data), a nonparametric one-way analysis of variance (Kruskal-Wallis test) was first performed. In the case of a global significant difference, between-group comparisons were assessed by the Dunn's post-hoc test, which generalizes the Bonferroni adjustment procedure.

In vitro mechanistic cell experiments (Figures 4-8 in main manuscript) were performed only in differentiated HBECs from non-smoker patients. In this case, results were

expressed as mean (SE) of n experiments since normal distribution for each data set was confirmed by histogram analyses and Kolmogorov–Smirnov test. In this case, statistical analysis was carried out by parametric analysis. Two-group comparisons were analysed using the two-tailed Student's paired t-test for dependent samples, or unpaired t-test for independent samples. Multiple comparisons were analysed by one-way or two-way analysis of variance followed by Bonferroni post hoc test. Statistical analysis was done on raw data considered as the gene expression corrected by the housekeeping GAPDH, protein expression corrected by the internal standards β-actin, total ERK1/2 and Smad3 as appropriate. Analysis of levels of cAMP, MMP-9, DCF fluorescence and TGF-β1 supernatants were performed on raw data.

RESULTS

Effect of chemicals on EMT markers in absence of CSE stimulus

Because, all tools used in this work to analyzed mechanistic pathways involved in CSE-induced EMT may modify the expression of mesenchymal or epithelial markers "per se", we measured the mRNA and protein expression after the drug exposure for 72h. Results showed no differences between the effect of NAC, dbcAMP, apocynin, SIS3, PD98059, mAb-TGF-β1 or isotype IgG alone at the concentrations assayed (Figure A)

Effect of CSE on cell viability

Differentiated HBECs were treated with different concentrations of CSE for up to 5% and during 72h. To test cell viability supernatant lactate dehydrogenase (LDH) levels were measured. We did not detect any differences in LDH levels respect non-treated cell group (Figure B). Furthermore, the combination of CSE 2.5% and different drug modulators did not modify significantly LDH (Figure B).

CSE-induced epithelial to mesenchymal transition in differentiated HBECs is maintained after CSE washout.

Differentiated human bronchial epithelial cells were culture in airway-liquid interface and stimulated with cigarette smoke extract (CSE) 2.5% for 3 days. Then CSE was retired and cells washed with fresh culture medium. Epithelial (E-cadherin) and mesenchymal (collagen type I (col type I)) markers were measured at the end of the stimulation period (at day 3) and at days 1, 3 and 7 after CSE washout. The changes of epithelial and mesenchymal markers induced by CSE 2.5% were maintained for at least 7 days after CSE washout.
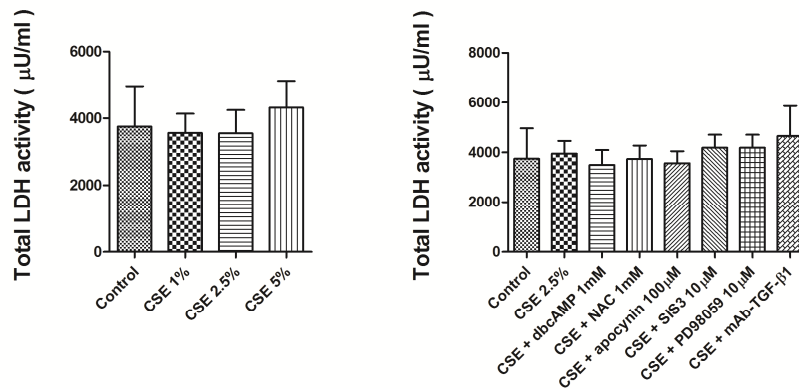
FIGURES AND FIGURE LEGENDS

Figure A
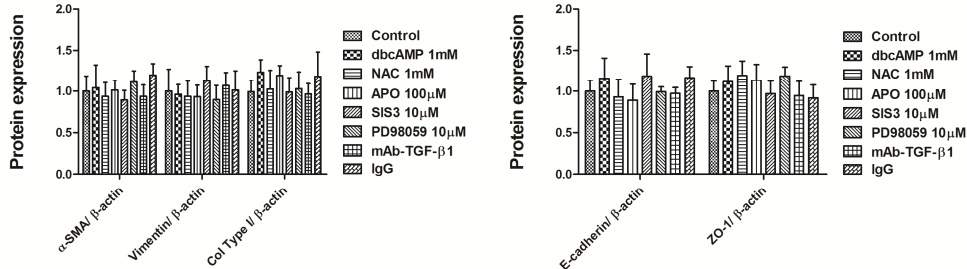
Figure legend A

The effect of NAC 1mM, dbcAMP 1mM, apocynin 100µM, SIS3 10µM, PD98059 10µM, mAb-TGF-β1 4 µg/mL or isotype IgG alone was compared with the solvent control for 72h of incubation in differentiated human bronchial epithelial cells. Protein expression of mesenchymal markers α-SMA, vimentin and collagen Type I were measured by protein array Zeptosens technology. . Data are expressed as the ratio to β-actin for protein levels and normalized to solvent control group. Results are expressed as means (SE) of $n = 4$ (one cell non-smoker population) experiments per condition. One-way ANOVA followed by post hoc Bonferroni tests. No differences were found respect solvent control.
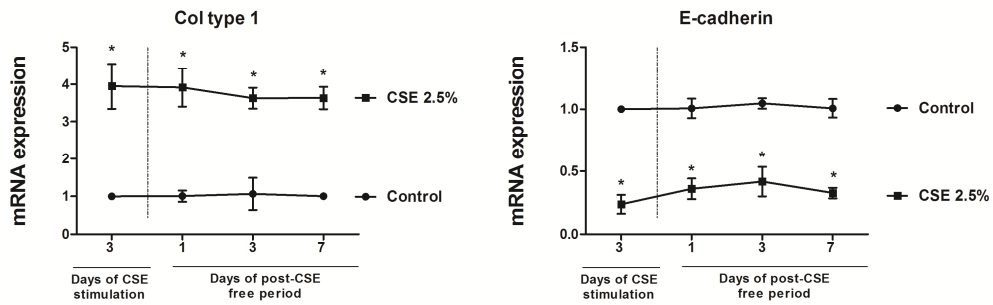
Figure B

Figure legend B

Effect of cigarette smoke extract (CSE) on epithelial cell viability. Differentiated human bronchial epithelial cells were exposed to growing concentrations to CSE or with CSE 2.5% in presence or absence of different drug modulators for 72h. Supernatant lactate dehydrogenase levels were measured by corresponding commercial kit. Results are expressed as means (SE) of $n = 4$ (one cell non-smoker population) experiments per condition. One-way ANOVA followed by post hoc Bonferroni tests. No differences were found respect solvent control.

Figure C

Figure legend C

Epithelial and mesenchymal markers persist once cigarette smoke is removed of the cell cultures. Differentiated human bronchial epithelial cells were culture in airway-liquid interface and stimulated with cigarette smoke extract (CSE) 2.5% for 3 days. Then CSE was retired and cells washed with fresh culture medium. Epithelial (E-cadherin) and mesenchymal (collagen type I (col type I)) markers were measured at the end of the stimulation period (at day 3) and at days 1, 3 and 7 after washing culture medium. The changes of epithelial and mesenchymal markers induced by CSE 2.5% were maintained for at least 7 days after CSE washout. Results are expressed as means (SE) of $n = 4$ (one cell non-smoker population) experiments per condition. One-way ANOVA followed by post hoc Bonferroni tests. *$p < 0.05$ related to solvent controls.

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