Online data supplement

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

Javier Milara,[1,2,3,*] Teresa Peiró,[3,4,*] Adela Serrano,[3,4] Julio Cortijo[1,3,4,5]

[1]Clinical research unit (UIC), University General Hospital Consortium, Valencia, Spain

[2]Department of Biotechnology, Universidad Politécnica de Valencia, Spain

[3]Research Foundation of General Hospital of Valencia, Spain

[4]Department of Pharmacology, Faculty of Medicine, University of Valencia, Spain

[4]CIBERES, Health Institute Carlos III, Valencia, Spain

*Both authors contributed equally to this work

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 ($10\mu g\ cm^{-2}$ 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. n°: A5228; Sigma), rabbit anti-human collagen type I antibody (cat. n°: PA1-26204; Affinity Bioreagents), mouse anti-human vimentin (cat. n°: V6389; Sigma), NOX4 (cat. n°: NB110-58849; Novus Biologicals), mouse anti-human E-cadherin (cat. n°: CM1681; ECM BioScience) and rabbit anti-human ZO-1 (cat. n°: 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[o]. D0260),[2] N-acetyl-ι-cysteine (1mM; NAC; Sigma: catalogue n[o]. A-7250),[2] apocynin (described as NADPH oxidase inhibitor, 100µM; Sigma: catalogue n[o]. W508454),[2] SIS3 (described as Smad3 inhibitor, 10µM; Sigma: catalogue n[o]. 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[o]. 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 n°: 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 n°: 566414; Calbiochem) as internal standards. Primary antibodies used were mouse anti-human α-SMA (cat. n°: A5228; Sigma), rabbit anti-human collagen type I antibody (cat. n°: PA1-26204; Affinity Bioreagents), mouse anti-human vimentin (cat. n°: V6389; Sigma), mouse anti-human E-cadherin (cat. n°: CM1681; ECM BioScience), rabbit anti-human ZO-1 (cat. n°: ab59720; Abcam), mouse anti-human phospho-ERK1/2 (cat. n°: M-9692; Sigma), rabbit anti-human phospho-Smad3 (cat. n°: PS1023; Calbiochem), and NOX4 (cat. n°: 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.
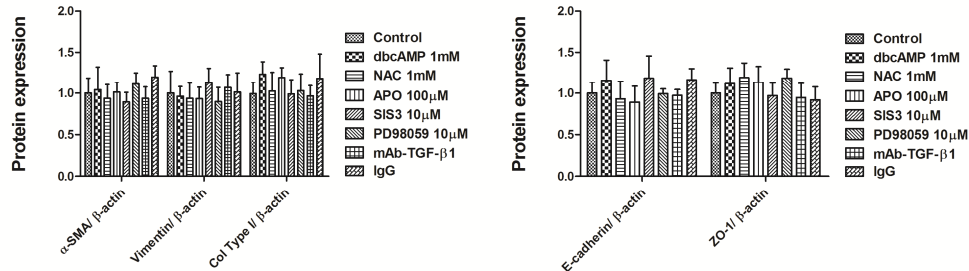
FIGURES AND FIGURE LEGENDS

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.
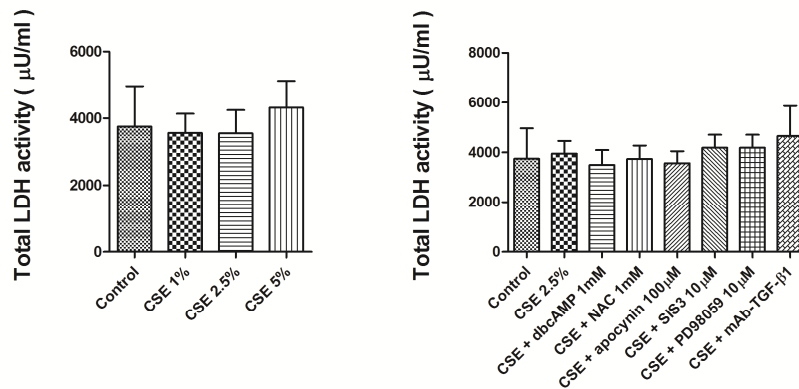
Figure B

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.
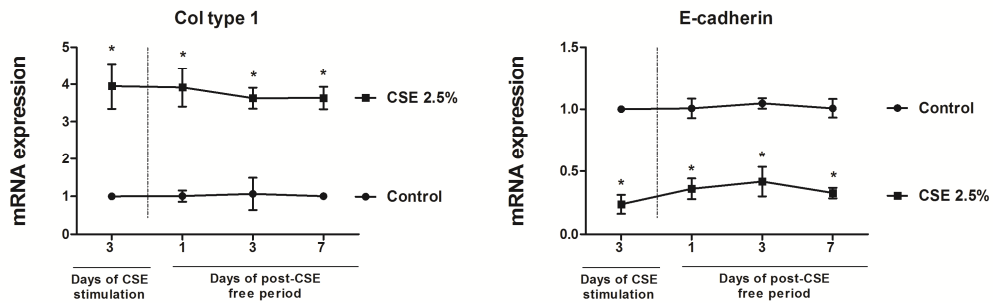
Figure C

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.

Online References

1 Rabe KF, Hurd S, Anzueto A, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med 2007; 176:532-555

2 Milara J, Armengot M, Banuls P, et al. Roflumilast N-oxide, a PDE4 inhibitor, improves cilia motility and ciliated human bronchial epithelial cells compromised by cigarette smoke in vitro. Br J Pharmacol 2012; 166:2243-2262

3 Ortiz JL, Milara J, Juan G, et al. Direct effect of cigarette smoke on human pulmonary artery tension. Pulm Pharmacol Ther 2010; 23:222-228

4 Cramer LP, Briggs LJ, Dawe HR. Use of fluorescently labelled deoxyribonuclease I to spatially measure G-actin levels in migrating and non-migrating cells. Cell Motil Cytoskeleton 2002; 51:27-38

5 Cortijo J, Mata M, Milara J, et al. Aclidinium inhibits cholinergic and tobacco smoke-induced MUC5AC in human airways. Eur Respir J 2011; 37:244-254

6 Su Y, Han W, Giraldo C, et al. Effect of cigarette smoke extract on nitric oxide synthase in pulmonary artery endothelial cells. Am J Respir Cell Mol Biol 1998; 19:819-825

7 Milara J, Navarro R, Juan G, et al. Sphingosine-1-phosphate is increased in patients with idiopathic pulmonary fibrosis and mediates epithelial to mesenchymal transition. Thorax 2012; 67:147-156

8 Milara J, Mata M, Serrano A, et al. Extracellular calcium-sensing receptor mediates human bronchial epithelial wound repair. Biochem Pharmacol 2010; 80:236-246

9 Milara J, Serrano A, Peiro T, et al. Aclidinium inhibits human lung fibroblast to myofibroblast transition. Thorax 2011

10 van Oostrum J, Calonder C, Rechsteiner D, et al. Tracing pathway activities with kinase inhibitors and reverse phase protein arrays. Proteomics Clin Appl 2009; 3:412-422

11 Trayner ID, Rayner AP, Freeman GE, et al. Quantitative multiwell myeloid differentiation assay using dichlorodihydrofluorescein diacetate (H2DCF-DA) or dihydrorhodamine 123 (H2R123). J Immunol Methods 1995; 186:275-284

12 Mata M, Sarria B, Buenestado A, et al. Phosphodiesterase 4 inhibition decreases MUC5AC expression induced by epidermal growth factor in human airway epithelial cells. Thorax 2005; 60:144-152