Airway Basal Stem/Progenitor Cells Have Diminished Capacity to Regenerate Airway Epithelium in Chronic Obstructive Pulmonary Disease

To the Editor:

Cigarette smoking, and the eventual development of chronic obstructive pulmonary disease (COPD) in susceptible individuals, is characterized by disordered differentiation of the small airway epithelium (SAE) (1, 2). The SAE is composed of four major cell types, the differentiated ciliated and secretory cells responsible for lung defense and the undifferentiated intermediate and basal cells that function as the stem/progenitor cells of the ciliated and secretory cells (3, 4). On the basis of the knowledge that hyperplasia of the basal cell population is the first biologic abnormality associated with smoking (5) and that the basal cells are the stem/progenitor cells of the SAE (4), we hypothesized that smoking and, to a greater extent, COPD are associated with basal cell "fatigue," resulting in reduced capacity of the basal stem/progenitor cells to successfully differentiate into a normal mucociliary epithelium. In addition, as DNA methylation is known to mediate renewal and differentiation capacities of adult tissue stem cells (6) and cigarette smoking is associated with changes in DNA methylation in lung cells (7, 8), we further hypothesized that basal cells from the SAE that fail to differentiate to a mucociliary epithelium have altered DNA methylation patterns compared with SAE basal cells capable of differentiating in a normal fashion.

To assess these hypotheses, we cultured primary basal cells from human SAE sampled by bronchoscopic brushing of 47 individuals (17 healthy nonsmokers, 14 healthy smokers, 16 COPD smokers) and assessed the capacity of the purified basal cells to differentiate in air–liquid interface (ALI) culture. The purified basal cells from each clinical phenotype were plated on type 4 collagen in a chamber well system, as previously described (9). After 2 days, culture media was removed from the apical chamber, exposing the cells to air and inducing differentiation over the course of 28 days (9, 10).

Because tight junction formation is associated with successful epithelial differentiation (11), transepithelial electrical resistance, a measure of tight junctional barrier integrity, was determined at Day 10 of ALI culture (Figure 1A). ALI cultures derived from SAE basal cells from smokers with COPD had significantly lower transepithelial electrical resistance compared with samples derived from healthy nonsmokers (smokers vs. nonsmokers, $P > 0.05$; COPD smokers vs. nonsmokers, $P < 0.003$; smokers vs. COPD smokers $P > 0.3$), which is consistent with previous observations that tight junction integrity is decreased and permeability is increased in the airway epithelium of smokers with and without COPD (12, 13). Furthermore, SAE basal cells from COPD smokers and, to a lesser extent, SAE basal cells from "healthy" smokers were limited in their ability to regenerate a fully differentiated epithelium under standard ALI model conditions, as assessed by pairwise comparison of survival curves using a log-rank test (Figure 1B; smokers vs. nonsmokers, $P > 0.09$; COPD smokers vs. nonsmokers, $P < 0.009$; smokers vs. COPD smokers, $P > 0.3$). Linking the inability to form stable junctional barrier to defective regenerative capacity, analysis revealed that ALI cultures that were able to survive to Day 28 had an average resistance of 1,669 $\Omega \cdot cm^2$ at Day 10, whereas cultures that were unable to survive to Day 28 had an average resistance of 367 $\Omega \cdot cm^2$ at Day 10 ($P < 8 \times 10^{-7}$, two-tailed t test). Further, we directly assessed the differentiation status of the ALI cultures that survived to Day 28 by performing immunofluorescence analysis of ciliated (β-tubulin IV) and secretory (secretoglobin 1A1, SCGB1A1) cell markers. We found similar numbers of ciliated cells in cultures from all three phenotypes, as evidenced by similar levels of β-tubulin IV staining; however, the number of SCGB1A1-expressing secretory cells was significantly lower in the ALI cultures derived from SAE basal cells of COPD smokers compared with nonsmoker samples ($P < 0.02$, two-tailed t test), and similar levels of SCGB1A1-expressing secretory cells were observed when comparing nonsmoker with healthy smoker samples and healthy smoker with COPD smoker samples ($P > 0.1$ nonsmoker vs. smokers; $P > 0.4$ smokers vs. COPD smokers; Figures 1C and 1D). These data demonstrate that the SAE-BC ALI cultures from patients with COPD who do survive to Day 28 have an altered differentiation phenotype with a decreased number of SCGB1A1-expressing secretory cells.

We have previously reported that smoking alters the DNA methylation profile of SAE samples obtained from bronchoscopic brushings (8), and other studies have demonstrated that smoking alters the methylation patterns of lung cell DNA (7, 14). On the basis of these observations, we hypothesized that DNA methylation of the basal cells of smokers and COPD smokers had been modified *in vivo* and that smoking-associated and smoking/COPD-associated changes in DNA methylation may underlie the altered capacity of SAE basal cells to generate normally differentiated airway epithelium (6). To assess this hypothesis, we quantified genomewide DNA methylation of the SAE basal cells. DNA was extracted from primary SAE basal cell cultures and assayed for methylation profiles, using the Illumina Infinium HumanMethylation450 array (San Diego, CA). On the basis of the observation that a subset of SAE basal cells from each smoking phenotype did not reach Day 28 on ALI, we compared the DNA methylation profiles of the original basal cell samples before they were placed on ALI culture. Comparing the 16 SAE basal cell samples that failed to normally differentiate to Day 28 on ALI (2 nonsmokers, 5 healthy smokers, 9 COPD smokers) with 30 samples that were successful (14 nonsmokers, 9 healthy smokers, 7 COPD smokers) identified 423 significant methylation probe sets with fold-change higher than 1.5 and $P < 0.05$ (Figure 2A), which is consistent with the concept that changes in DNA methylation may be one mechanism that differentiates SAE basal cells in their ability to regenerate, similar to adult stem cells in other tissues (6). Of the 423 significant probe sets identified in comparison of SAE basal cell samples that did not have the capacity to regenerate compared with SAE basal cells that

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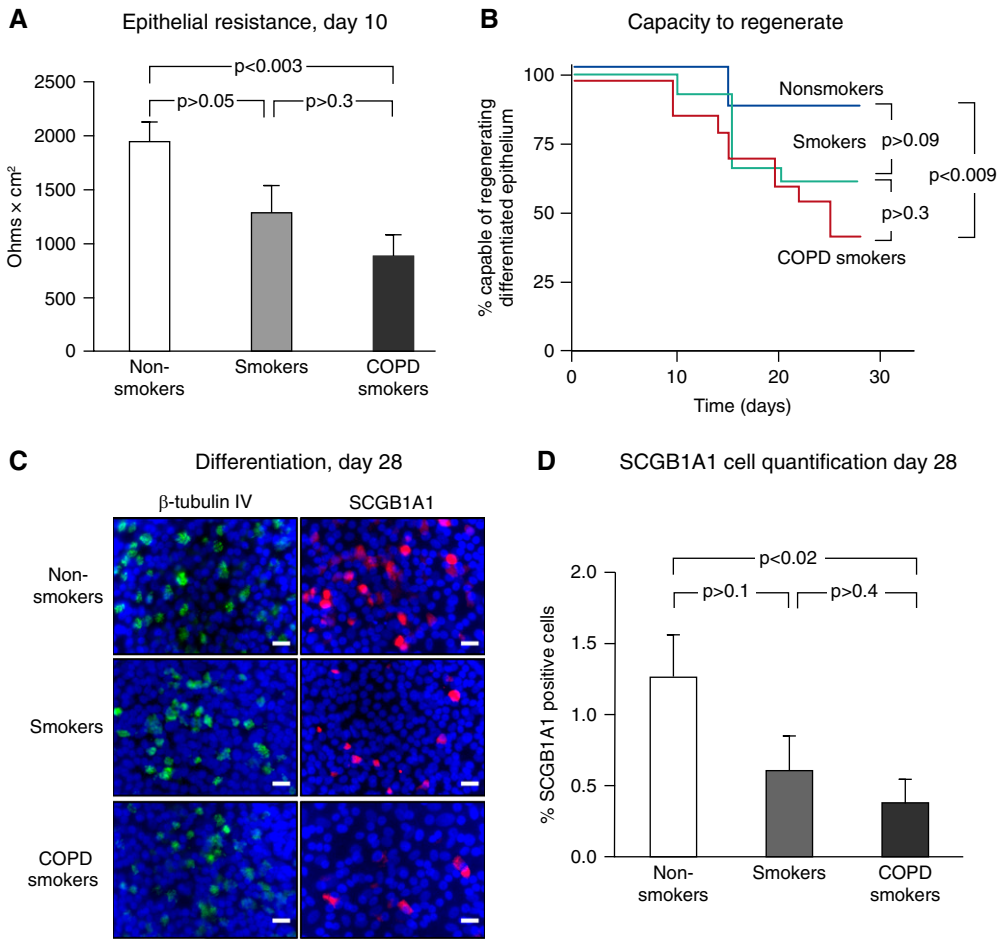

Figure 1. Capacity of airway basal cells from nonsmokers, smokers, and smokers with chronic obstructive pulmonary disease (COPD) to regenerate a mucociliary epithelium. The study cohort included n = 17 healthy nonsmokers, n = 14 healthy smokers, and n = 16 COPD smokers (9 Global Initiative for Chronic Obstructive Lung Disease [GOLD] I, 6 GOLD II, 1 GOLD III). All subjects were assessed by medical history, physical exam, routine blood tests, chest X-ray, and full lung function tests (including FVC, FEV_1, FEV_1/FVC, TLC, and diffusing capacity of carbon monoxide). Smoking was confirmed by urine tobacco metabolite analysis. All subjects had normal α1-antitrypsin levels and were HIV-negative. Healthy nonsmokers and healthy smokers had normal chest X-ray and lung function. Purified primary basal cells (BCs) were obtained by fiberoptic bronchoscopy and brushing of the small airway epithelium (SAE; 10th- to 12th-order bronchi) and were characterized as previously described (9, 10). The purified basal cells were seeded on air–liquid interface (ALI; 3×10^5 cells/cm^2, on 0.4-μm pore-sized Costar Transwell (New York, NY) insert precoated with type 4 collagen) and cultured for 28 days in medium (9, 10). Transepithelial electrical resistance was assessed at Day 10, and the ability to form a differentiated epithelium was assessed over the course of 28 days. (*A*) Transepithelial electrical resistance of SAE-derived BC cultured on ALI. Resistance ($\Omega \cdot$ cm^2) was measured at Day 10 of ALI culture. Data shown are the average transepithelial electrical resistance ± standard error. Significance was determined by a two-tailed *t* test (smokers vs. nonsmokers, $P > 0.05$; COPD smokers vs. nonsmokers, $P < 0.003$; smokers vs. COPD smokers, $P > 0.3$). (*B*) Capacity of SAE-derived BCs to regenerate a differentiated epithelium on ALI culture. Ordinate, percentage of total SAE-BC samples regenerating a differentiated epithelium on ALI culture; abscissa, time (in days) on ALI culture. Cultures that failed to fully differentiate to Day 28 on ALI exhibited spontaneous generation of holes in the cellular monolayer, followed by collapse and failure of the culture at 10–28 days. Pairwise comparison of survival curves was performed using a log-rank test (smokers vs. nonsmokers, $P > 0.09$; COPD smokers vs. nonsmokers, $P < 0.009$; smokers vs. COPD smokers, $P > 0.3$). To ensure that the number of samples did not affect the test statistic, an exact conditional test was used to compare the survival curves of different groups; this yielded similar *P* values (not shown). (*C*) Immunofluorescence analysis of ALI culture differentiation at Day 28. The ability of SAE-derived BC to differentiate on ALI at Day 28 was assessed by immunofluorescent staining for β-tubulin IV (ciliated cells, *green*), SCGB1A1 (secretory cells, *red*), and nuclei (*blue*); scale bars = 20 μm. (*D*) Quantification of differentiation of ALI Day 28 of SAE-BC cultures. The number of SCGB1A1-positive secretory cells from immunofluorescent staining was scored. Data are shown as percentage of total cells, average ± standard error. Statistics were calculated by two-tailed *t* test.

successfully regenerated to Day 28, 75 probe sets corresponded to unique genes that were hypermethylated, 202 probe sets corresponded to unique genes that were hypomethylated, and 71 probe sets corresponded to unique CpG island regions that were not associated with genes (27 hypermethylated, 44 hypomethylated). Unsupervised hierarchical clustering of the 423 methylation probe sets that were significantly different between the two groups with different capacity to differentiate showed that the methylation profile separated the two groups according to capacity to differentiate (Figure 2B).

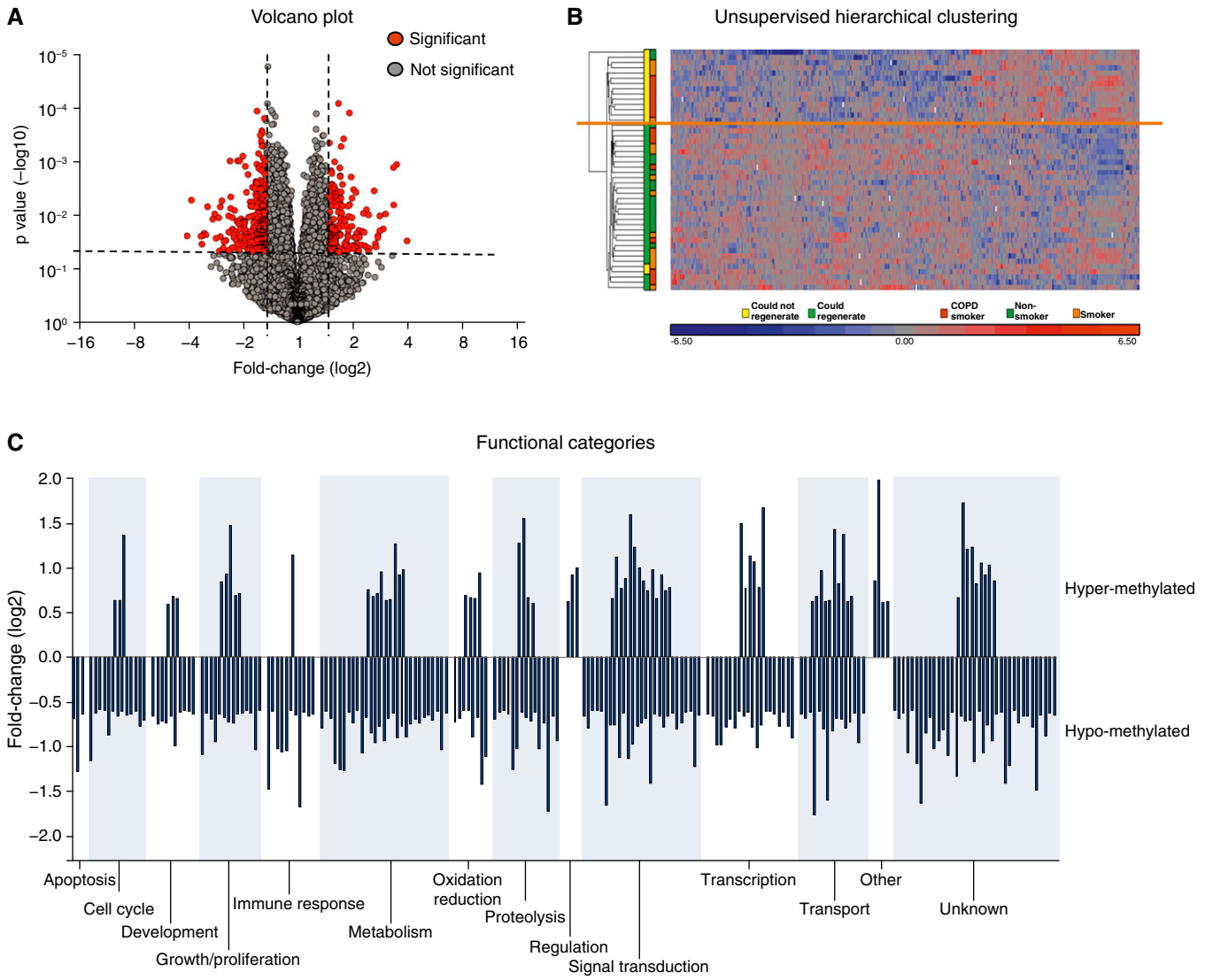

Figure 2. DNA methylation profile of small airway basal cells that could not regenerate a differentiated epithelium *ex vivo* compared with small airway basal cells that could regenerate a differentiated epithelium. Primary BCs from Figure 1 were assessed at time 0 before seeding on ALI culture. Genomewide DNA methylation data were assessed using the Illumina Infinium HumanMethylation 450 array. (*A*) Volcano plot, genomewide DNA methylation analysis of small airway epithelium (SAE)-derived basal cells (BCs) that did not have the capacity to regenerate a differentiated epithelium compared with BCs that did regenerate. Assessment was performed using logit transformed β values of more than 414,000 methylation sites and Illumina recommendations to eliminate probes with detection $P < 0.01$, as well as filtering out probes on X and Y chromosomes and probes not associated with genes, CpG islands, or shores. Ordinate, P value ($\log_{10}$); abscissa, fold change ($\log_2$). Red indicates probe sets significantly different ($P < 0.05$) with fold-change higher than 1.5 compared between samples that could not regenerate versus samples that could regenerate a differentiated epithelium; gray indicates nonsignificant probe sets. (*B*) Unsupervised hierarchical clustering, genomewide DNA methylation analysis of SAE-BC that could regenerate versus those that could not regenerate a differentiated epithelium over the course of 28 days in the ALI culture (423 probe sets, fold-change > 1.5; $P < 0.05$). Probe sets identifying hypermethylated regions are represented in *red*, hypomethylated in *blue*, and average in *gray*. Probe sets are represented horizontally and individual samples vertically. The phenotype of individual samples is shown vertically. The horizontal color legend represents the SAE-BC survival phenotype: *yellow*, ALI cultures that could not regenerate; *green*, ALI cultures that could regenerate and survive until Day 28. The smoking phenotype of samples is represented: *green*, healthy nonsmokers; *orange*, healthy smokers; *red*, COPD smokers. The *orange horizontal line* designates the major separation of clusters between SAE-BC samples that did not regenerate versus those that regenerated over the course of 28 days on ALI. (*C*) Functional categories of the 277 significant genes differentially methylated in SAE-BC that did not have the capacity to regenerate a differentiated epithelium versus SAE-BC that did have the capacity (fold-change > 1.5, $P < 0.05$). Shown are the fold-changes of the differentially methylated genes on a $\log_2$ scale. Ingenuity pathway analysis was performed on the 277 unique hyper- and hypomethylated genes.

To gain insight into what biological role the methylation differences might contribute, the methylation data were functionally annotated, using Gene Ontology and the Human Protein Reference Database (www.hprd.org; Figure 2C), with subsequent Ingenuity Pathway Analysis to determine the biological pathways represented in the 75 unique hypermethylated genes and the 202 unique hypomethylated genes. The top pathway represented in the Ingenuity Pathway Analysis of the 75 hypermethylated genes was "cellular growth and proliferation," with 11 molecules represented (BMP7, GRM3, IGFBP7, NPY, RPTOR, MAPK10, HEY1, MGP, PRKCE, MEIS1, and PRDM16; $P < 3 \times 10^{-4}$ to 5×10^{-2}).

In summary, these data provide *ex vivo* evidence that SAE basal cells from COPD smokers, and to a lesser extent SAE basal cells from "healthy" smokers, are limited in their ability to regenerate a fully differentiated epithelium. In addition, the methylation studies show that the DNA methylation profile of SAE basal cells that do not survive to Day 28 on ALI culture is significantly different than the profile of SAE basal cells that are able to successfully differentiate, suggesting that changes in DNA methylation acquired by basal cells of COPD smokers and healthy smokers *in vivo* may be one mechanism for the differences observed. We hypothesize that the lung microenvironment in individuals with COPD provide signals necessary to maintain the "COPD" epigenetic code within SAE basal cells, continuously supporting the disease state by altering the capacity of these stem/progenitor cells to maintain normally differentiated SAE. Therefore, even though the process of DNA methylation is reversible (15), the COPD lung environment might continuously stimulate the COPD DNA methylation profile that may determine abnormal SAE maintenance and regeneration. Thus, it is possible that studies focusing on modulating methylation profiles of SAE basal cells relevant to their capacity to regenerate normally differentiated mucociliary airway epithelium may lead to novel therapeutics to treat or prevent COPD. ■

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Early Intermittent Hypoxia Induces Proatherogenic Changes in Aortic Wall Macrophages in a Murine Model of Obstructive Sleep Apnea

To the Editor:

Obstructive sleep apnea (OSA) is a highly prevalent condition throughout the lifespan, affecting 2–10% of the general population at any given age. It is associated with an extensive array of cognitive, behavioral, metabolic, and cardiovascular morbidities