Exhaustion of Airway Basal Progenitor Cells in Early and Established Chronic Obstructive Pulmonary Disease

Moumita Ghosh¹, York E. Miller^{2,3}, Ichiro Nakachi⁴, Jennifer B. Kwon¹, Anna E. Barón⁵, Alexandra E. Brantley¹, Daniel T. Merrick⁶, Wilbur A. Franklin⁶, Robert L. Keith^{2,3}, and R. William Vandivier²

¹Division of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine, National Jewish Health, Denver, Colorado; ²COPD Program, Division of Pulmonary Sciences and Critical Care Medicine, Department of Medicine, and ⁶Department of Pathology, University of Colorado Anschutz Medical Campus, Aurora, Colorado; ³Veterans Affairs Eastern Colorado Healthcare System, Denver, Colorado; ⁴Division of Pulmonary Medicine, Department of Medicine, Keio University School of Medicine, Tokyo, Japan; and ⁵Department of Biostatistics and Bioinformatics, University of Colorado School of Public Health, Aurora, Colorado

Abstract

Rationale: Up to 40% of smokers develop chronic obstructive pulmonary disease (COPD) over a period that spans decades. Despite the importance of COPD, much remains to be learned about susceptibility and pathogenesis, especially during early, prediagnostic stages of disease. Airway basal progenitor cells are crucial for lung health and resilience because of their ability to repair injured airways. In COPD, the normal airway epithelium is replaced with increased basal and secretory (mucous) cells and decreased ciliated cells, suggesting that progenitors are impaired.

Objectives: To examine airway basal progenitor cells and lung function in smokers with and without COPD.

Methods: Bronchial biopsies taken from smokers at risk for COPD and lung cancer were used to acquire airway basal progenitor cells. They were evaluated for count, self-renewal, and multipotentiality

(ability to differentiate to basal, mucous, and ciliated cells), and progenitor count was examined for its relationship with lung function.

Measurements and Main Results: Basal progenitor count, self-renewal, and multipotentiality were all reduced in COPD versus non-COPD. COPD progenitors produced an epithelium with increased basal and mucous cells and decreased ciliated cells, replicating the COPD phenotype. Progenitor depletion correlated with lung function and identified a subset of subjects without COPD with lung function that was midway between non-COPD with high progenitor counts and those with COPD.

Conclusions: Basal progenitor dysfunction relates to the histologic and physiologic manifestations of COPD and identifies a subset that may represent an early, prediagnostic stage of COPD, indicating that progenitor exhaustion is involved in COPD pathogenesis.

Keywords: stem cells; cell self-renewal; multipotentiality; early diagnosis; disease susceptibility

In 1976, Fletcher and coworkers demonstrated that approximately 13% of susceptible smokers develop chronic obstructive pulmonary disease (COPD) (1).

Several large epidemiologic studies have extended their findings by showing that as many as 30–40% of susceptible smokers will develop COPD over a period that spans

decades (2–6). Recent data suggest that the prolonged period before COPD can be diagnosed is not benign, because approximately 50% of current/former

(Received in original form April 2, 2017; accepted in final form December 1, 2017)

Supported by grants to the University of Colorado Lung Cancer SPORE (Specialized Program of Research Excellence) from the National Cancer Institute (P50 CA058187), the Cancer Center Support Grant (P30 CA046934), and grants to individual investigators (Flight Attendant Medical Research Institute [FAMRI] grant 113259_YCSA, M.G.; NIH grants R01, HL129938-03, M.G., Y.E.M., and R.W.V.; VA Merit award CLN-005-085, R.L.K.; and FAMRI 150001F, R.W.V.).

Author Contributions: Study design and concept, M.G., R.W.V., and Y.E.M. Acquisition of data, M.G., R.W.V., J.B.K., I.N., and A. E. Brantley. Analysis and interpretation of data, M.G., R.W.V., Y.E.M., A. E. Barón, D.T.M., W.A.F., and R.L.K. Drafting of the manuscript, M.G., R.W.V., Y.E.M., D.T.M., W.A.F., and R.L.K. Critical revision of the manuscript for important intellectual content, M.G., R.W.V., Y.E.M., D.T.M., W.A.F., I.N., R.L.K., A. E. Barón, J.B.K., and A. E. Brantley. Statistical analysis, R.W.V. and A. E. Barón. Administrative, technical, or material support, M.G. and R.W.V. Study supervision, M.G., R.W.V., and Y.E.M.

Correspondence and requests for reprints should be addressed to: Moumita Ghosh, Ph.D., Department of Medicine, National Jewish Health, 1400 Jackson Street, Denver, CO 80206. E-mail: ghoshm@njhealth.org.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

The uncompressed videos are accessible from this article's supplementary material page.

Am J Respir Crit Care Med Vol 197, Iss 7, pp 885–896, Apr 1, 2018

Copyright © 2018 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201704-0667OC on December 6, 2017

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: Very little is known about the factors that contribute to chronic obstructive pulmonary disease (COPD) susceptibility and pathogenesis, and how they manifest during early COPD. Airway basal progenitor cells are critical for lung health and resilience. In COPD, the airway epithelium is altered, suggesting that airway basal progenitor dysfunction contributes to COPD pathogenesis. To test this, we measured basal progenitor number and function from subjects without and with COPD, and related these findings to lung function.

What This Study Adds to the

Field: Results show that airway basal progenitors are exhausted in COPD; that progenitor depletion relates to impaired lung function; and that progenitor depletion identifies a subset of people with lung function that may represent an early, prediagnostic stage of COPD. These findings indicate that progenitor exhaustion plays a role in COPD pathogenesis and suggest that a deeper understanding of this role has the potential to identify factors responsible for COPD susceptibility and resistance.

smokers with preserved lung function develop an early form of COPD characterized by increased respiratory symptoms, medication use, exacerbations, activity limitation, and airway disease (7, 8). To date, very little progress has been made to identify the factors that contribute to COPD susceptibility and how these factors manifest during early stages of disease, before diagnosis is possible using spirometry.

It is conceivable that COPD develops in susceptible smokers because of an aberrant response to repeated injury. In COPD, the airway epithelium is altered, leading to basal and mucous cell hyperplasia, loss of cilia, and ciliary dysfunction (9–16). Airway basal progenitor cells perform a critical role in the airway's response to injury by virtue of their abilities to self-renew, meaning that they are able to replenish themselves, and to differentiate into all cell types that normally populate their home tissue, including basal, secretory, and ciliated cells, a characteristic referred to as multipotentiality (17–21). These capacities of

basal progenitor cells to restore the airway to normal structure and function makes them indispensable for lung health and resilience (17, 22, 23). Importantly, the differentiation ability of airway basal cells is decreased in COPD (24–26).

Several studies, including our own, identified a subset of keratin (K) 5 expressing basal cells as the stem/progenitor cells for both human and mouse tracheobronchial epithelium (18, 20, 21, 27–33). During *in vitro* culture, basal progenitors form clones or spheres that replicate and differentiate into cell types that compose a normal epithelium (18, 21, 28). We used a cohort of current and former smokers to investigate how basal progenitor numbers and function differ in the airways of people with and without COPD, and whether they can be used to identify early airflow limitation in people without COPD. We found that COPD airways contained fewer basal progenitors and that their functional abilities were significantly perturbed. Furthermore, depletion of basal progenitors identified a group of subjects without COPD with intermediate lung function. We termed this impairment of basal progenitors as “exhaustion,” which may contribute to disease pathogenesis.

Methods

Detailed methods are provided in the online supplement.

Study Approval

This was a cross-sectional study to determine the role of airway basal progenitor cells on the pathogenesis of COPD. The Colorado Multiple Institutional Review Board approved this study.

Patient Population

This study leveraged two clinical studies examining premalignancy in the lung. The Biomarkers and Dysplastic Respiratory Epithelium Study (ClinicalTrials.gov identifier: NCT00900419) investigates biomarkers of premalignant respiratory epithelium, and the Pioglitazone for Lung Cancer Chemoprevention Study (ClinicalTrials.gov identifier: NCT00780234) examines the effect of pioglitazone on endobronchial histology. Both trials included subjects at increased risk for lung or upper aerodigestive malignancies. Subjects were included into the current study if they were greater than or equal to 40 years old, had greater than or equal to 10 pack-year

smoking history, and were undergoing baseline bronchoscopy. Subjects were excluded if they were undergoing active treatment for cancer or had a bleeding diathesis, active cardiac disease, or recent infection. Thirty-eight subjects were enrolled from the “Biomarkers” study and 12 were enrolled from the “Pioglitazone” study.

Subject Groups

Criteria established by the Global Initiative for Chronic Obstructive Lung Disease were used to categorize subjects as with COPD or without COPD (34). All subjects completed prebronchodilator pulmonary function testing and 82% completed post-bronchodilator testing. A combination of autofluorescence and white light bronchoscopy was used to collect endobronchial biopsies at the secondary (lobar) carinas.

Study Outcomes

Progenitor count was chosen as the primary study outcome, because it was the first assessment of basal progenitors that required the least manipulation, was completed within 7 days after the bronchoscopy, and therefore had the best possibility to mirror the *in vivo* phenotype. Secondary outcomes included progenitor self-renewal, multipotentiality, and measurements of lung function (i.e., airflows, lung volumes, and carbon monoxide diffusion). Lung function measurements were blinded until the biologic outcomes of progenitor count, self-renewal, and multipotentiality were complete.

Basal Progenitor Count

Basal progenitor count (referred to from here forward as progenitor count) was determined by numbers of clone-forming basal cells present in each biopsy. A schematic of the work-flow is presented in Figure 1A. Biopsies were digested with a mixture of dispase/collagenase/trypsin, followed by treatment with DNase and 1× red blood cell lysis buffer. Cell suspensions were washed with cold phosphate-buffered saline and total cell counts were determined using a hemocytometer. Ninety percent of the cell suspension was used to grow basal progenitor clones. Cells were plated in three wells of a 12-well plate that were precoated with irradiated 3T3 fibroblast feeders and were cultured in serum supplemented Epicult-B medium (20). Numbers of clones were counted under an inverted scope 7 days after bronchoscopy.

Figure 1. Airway progenitor counts in non-chronic obstructive pulmonary disease (COPD) and COPD airways. (A) Steps to grow and quantify clone-forming basal progenitors from airway biopsies. (B) Representative image of an endobronchial biopsy (arrow). (C) Cytopsin preparation of biopsy digest stained with antibodies to K5 (keratin 5) (green), K14 (keratin 14) (red), and DAPI (blue). K5/K14 dual positive cells are yellow. (D) Cytopsin stained with antibodies to Muc5b (mucin 5B) (green), FoxJ1 (Forkhead box protein J1) (red), and DAPI (blue). Scale bars are shown and colored arrowheads point to each cell type in C and D. (E–H) Pictures of clones generated by human airway basal progenitor cells. (E) Bright-field image, (F) K5 (green), (G) K14 (red), and (H) p63 (transformation protein 63) (red) and DAPI (blue, in panels F–H). Scale bars (70 μ m) are shown in E–H. (I) Progenitor counts (number of clones/ 10^3 epithelial cells) in non-COPD ($n = 31$) and COPD ($n = 19$). Horizontal lines show means $\pm$ SE. P value indicates results of Mann-Whitney test.

Ten percent of the cell suspension was used for cytopsin to determine the number and types of epithelial cells present in each biopsy. Two cytopsin were prepared and immunostained for basal (K5 and K14),

secretory (Muc5b), and ciliated cells (Foxj1) (Figures 1B–1D; see Figures E1A–E1H in the online supplement). The percent of epithelial cells per biopsy was determined by adding the %K5⁺, %Muc5b⁺, and

%Foxj1⁺ cells present in the biopsy. Detail of total cell yield from each biopsy, percent of epithelial cell types, and average clone count is provided in the online supplement (see Table E1 and Figure E1).

Self-Renewal and Multipotentiality

Self-renewal of basal progenitors was determined at clonal density following our published methods (14, 15, 17). Briefly, “generation 1” clones were harvested by trypsinization and plated in a 96-well plate coated with fibroblast feeders using a twofold dilution series that ranged from 1 to 128 cells/well. Cells were cultured for 7 days in Epicult-B medium and at Day 7 wells were scored for the presence or absence of clones. A linear regression analysis of log % negative wells versus cell input (35) was used to determine progenitor self-renewal.

Multipotentiality assays (i.e., differentiation abilities of clone cells) were performed in air-liquid interface cultures using the Pneumacult differentiation method following manufacturer’s instructions.

Immunostaining and Morphometry

Immunostaining of cytopins and Transwell membranes were performed following standard protocols as published before (20, 21, 27). Details of antibodies used, imaging, and morphometry is provided in the online supplement.

Statistical Analysis

Prism version 6.0 (GraphPad Software, Inc.) was used for statistical analyses. Characteristics of study participants at baseline were described and comparisons between COPD and non-COPD were made using a two-tailed Mann-Whitney test for continuous variables and chi-square test for dichotomous variables. All *in vitro* comparisons used a two-tailed Mann-Whitney test. Two-tailed Pearson correlations were used for all correlation analyses. For multiple comparisons, means were analyzed using a one-way ANOVA. When ANOVA indicated significance, a *post hoc* analysis was performed using the Tukey-Kramer multiple comparison test. The study addressed the hypothesis that airway basal progenitor numbers would be lower in COPD compared with non-COPD. No prior studies had examined basal progenitor counts in these two groups, so a prospective power analysis could not be performed. Thirty-one subjects without COPD and 19 subjects with COPD were enrolled. Based on a mean progenitor count of 40.1 for the non-COPD group and 15.3 for the COPD group, an SD of 29.8 for the non-COPD group and 7.2 for the COPD group, and a two-tailed α of 0.05, a

total of 31 subjects without COPD and 19 subjects with COPD resulted in more than 99% power to see a difference between groups.

Results

Airway Basal Progenitor Numbers and Function in COPD versus Non-COPD

To investigate whether basal progenitor cell malfunction contributes to airway

remodeling in COPD, we measured the ability of basal progenitors to make clones immediately after biopsies were collected from current/former smokers with and without COPD. Baseline clinical and demographic characteristics of subjects without COPD and subjects with COPD are shown in Table 1. Clones were composed entirely of K5, K14, and p63 expressing basal cells, and did not contain either secretory or ciliated cells (Figures 1E–1H). There were no differences in the cell

Table 1. Baseline Demographic and Clinical Characteristics of Study Subjects*

Characteristic	Non-COPD (n = 31)	COPD (n = 19)	P Value†
Age, yr	58.2 ± 9.2	60.2 ± 6.2	0.44
Male sex, n (%)	30 (96.8)	18 (94.7)	0.72‡
Race, n (%)			
White	24 (77.4)	17 (89.5)	0.28‡
Nonwhite	7 (22.6)	2 (10.5)	
Current smoker, n (%)	12 (38.7)	10 (52.6)	0.34‡
Smoking history, pack-year	42.6 ± 30.2	40.6 ± 20.1	0.33
Body mass index, kg/m ²	29.7 ± 6.3	26.0 ± 4.7	0.04
6-minute-walk distance, m	353 ± 53	358 ± 99	0.99
FEV ₁ , % of predicted value			
Prebronchodilator	85 ± 15	62 ± 18	<0.0001
Post-bronchodilator§	89 ± 16	72 ± 16	<0.0001
FEV ₁ , ml			
Prebronchodilator	3,135 ± 857	2,018 ± 556	<0.0001
Post-bronchodilator§	3,244 ± 981	2,350 ± 497	<0.01
FEV ₁ , % change in response to bronchodilator	7 ± 7	11 ± 9	0.06
FVC, % of predicted value			
Prebronchodilator	85 ± 13	80 ± 17	0.21
Post-bronchodilator§	88 ± 15	90 ± 17	0.74
FVC, % change in response to bronchodilator§	3 ± 7	9 ± 9	0.04
FEV ₁ /FVC ratio, %			
Prebronchodilator	76 ± 8	58 ± 9	<0.0001
Post-bronchodilator§	77 ± 5	61 ± 7	<0.0001
FEF _{25–75} , % of predicted value	92 ± 43	33 ± 14	<0.0001
FEF _{25–75} /FVC ratio, %	70 ± 30	27 ± 9	<0.0001
TLC, % of predicted value	105 ± 17	122 ± 29	0.09
RV, % of predicted value	140 ± 42	188 ± 60	<0.001
RV/TLC ratio, %	43 ± 9	50 ± 7	<0.01
DL _{CO} , % of predicted value	94 ± 22	76 ± 24	0.02
DL _{CO} /VA, % of predicted value	101 ± 20	81 ± 15	<0.01
GOLD stage, n (%)			
I	—	4 (21)	
II	—	12 (63)	
III	—	2 (11)	
IV	—	1 (5)	

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; FEF_{25–75} = forced expiratory flow, midexpiratory phase; GOLD = Global Initiative for Chronic Obstructive Lung Disease; RV = residual volume.

*Plus-minus values are means ± SD.

†Mann-Whitney test performed except where indicated.

‡Chi-square test.

§Post-bronchodilator spirometry was performed on 24 out of 31 subjects without COPD (i.e., 77%) and 17 out of 19 subjects with COPD (i.e., 89%). The prebronchodilator FEV₁/FVC ratio on the two subjects with COPD who did not receive post-bronchodilator testing was 33% and 55%, respectively.

||The GOLD stage ranges from stage I COPD, indicating mild disease, to stage IV COPD, indicating very severe disease.

composition of clones from subjects without COPD or subjects with COPD. Progenitor count (i.e., the number of clone-forming basal progenitors present in each biopsy) was determined. Comparisons of progenitor counts between biopsies showed that COPD airways contained 58% fewer basal progenitor cells than non-COPD (Figure 1I).

Self-renewal, an essential property of basal progenitors, was measured by quantifying the number of basal cells from “generation 1” clones that gave rise to “generation 2” clones (20, 21, 27). Comparisons between 13 COPD and 18 non-COPD airways showed that progenitor self-renewal was 82% lower in COPD versus non-COPD (Figure 2A). Clones generated by COPD progenitors were

visibly smaller than non-COPD clones (Figures 2B and 2C; see Figure E2). Self-renewal was further determined by serial passaging of clone cells at limiting dilution. In these analyses non-COPD clones could be passaged approximately five to seven times, whereas COPD clones could not be passaged more than two times. Taken together, the self-renewal of basal progenitors was severely impaired in COPD. Decreased self-renewal of basal progenitors from COPD could not be explained by induction of senescence, because only few senescent cells were present in COPD clones compared with non-COPD (see Figure E3).

To determine whether basal progenitors from COPD are multipotential, differentiation was examined during

air-liquid interface culture and differentiated membranes were immunostained for basal ($K5^+$), secretory ($Muc5b^+$), and ciliated (ACT^+) cells (21, 27). Quantification of each cell type showed that epithelium generated by COPD basal progenitors contained 1.5-fold greater numbers of basal cells, twofold greater numbers of mucous cells, and 88% fewer ciliated cells compared with non-COPD (Figures 2D–2H and Table E2; see Figure E4). Time-lapse imaging of the differentiated membranes further demonstrated loss of cilia in epithelium generated by COPD (Video 1) versus non-COPD progenitors (Video 2).

Differentiated membranes were also paraffin embedded, sectioned, and examined for the spatial distribution of

Figure 2. Self-renewal and multipotentiality of airway progenitor cells in non-chronic obstructive pulmonary disease (COPD) versus COPD. (A) Airway progenitor cell self-renewal in non-COPD ($n = 18$) and COPD ($n = 13$). (B and C) Representative images of basal cell clones made by non-COPD (B) and COPD (C) progenitors. Multipotentiality of progenitors was determined by measuring differentiation during air-liquid interface culture. Scale bars (70 μ m) are shown in B and C. (D–G) En face images of differentiated membranes from non-COPD progenitors (D and E) and COPD progenitors (F and G). In D and F, immunostaining was performed for K5 (keratin 5) (green) and DAPI (blue). In E and G, immunostaining was performed for Muc5b (mucin 5B) (red), acetylated tubulin (ACT) (green), and DAPI (blue). Arrowheads in G show mucus accumulation in the membranes from COPD progenitors. The scale bar in D applies to D–G. (H) Quantification of differentiated cell types using morphometry ($n = 10$ of each for non-COPD and COPD). Data presented as means $\pm$ SE. P values indicate results of Man-Whitney test.

Table 2. Baseline Demographic and Clinical Characteristics of the Non-COPD Cohort*

Characteristic	Non-COPD (High Progenitors) (n = 14)	Non-COPD (Low Progenitors) (n = 17)	P Value [†]
Age, yr	58.7 ± 9.6	57.9 ± 9.1	0.82
Male sex, n (%)	14 (100.0)	16 (94.1)	0.37 [‡]
Race, n (%)			
White	12 (85.7)	12 (70.6)	0.32 [‡]
Nonwhite	2 (14.3)	5 (29.4)	
Current smoker, n (%)	7 (50)	5 (29.4)	0.24 [‡]
Smoking history, pack-year	35.8 ± 33.5	46.3 ± 28.8	0.09
Body mass index, kg/m ²	27.4 ± 4.4	31.8 ± 7.0	<0.05
6-minute-walk distance, m	363 ± 62	345 ± 45	0.50
FEV ₁ , % of predicted value			
Prebronchodilator	94 ± 13	78 ± 12	<0.01
Post-bronchodilator [§]	98 ± 16	82 ± 12	<0.05
FEV ₁ , ml			
Prebronchodilator	3,465 ± 671	2,864 ± 916	0.10
Post-bronchodilator [§]	3,669 ± 823	2,989 ± 1,005	0.16
FEV ₁ , % change in response to bronchodilator [§]	5 ± 8	8 ± 6	0.27
FVC, % of predicted value			
Prebronchodilator	89 ± 13	81 ± 12	0.13
Post-bronchodilator [§]	94 ± 15	84 ± 14	0.14
FVC, % change in response to bronchodilator [§]	2 ± 6	4 ± 8	0.32
FEV ₁ /FVC ratio, %			
Prebronchodilator	80 ± 7	73 ± 7	0.01
Post-bronchodilator [§]	79 ± 4	75 ± 5	0.02
FEF _{25–75} , % of predicted value	116 ± 49	73 ± 25	<0.01
FEF _{25–75} /FVC ratio, %	82 ± 36	59 ± 21	0.02
TLC, % of predicted value	108 ± 18	102 ± 17	0.36
RV, % of predicted value	142 ± 53	139 ± 34	0.71
RV/TLC ratio, %	42 ± 11	44 ± 7	0.17
DL _{CO} , % of predicted value	98 ± 24	92 ± 20	0.42
DL _{CO} /VA, % of predicted value	105 ± 22	99 ± 19	0.29

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; FEF_{25–75} = mean forced expiratory flow between 25% and 75% of FVC; RV = residual volume.

*Values are means ± SD unless otherwise specified.

[†]Mann-Whitney test performed except where indicated.

[‡]Chi-square test.

[§]Post-bronchodilator spirometry was performed on 9 out of 14 non-COPD high-progenitor subjects (i.e., 64%) and 15 out of 17 non-COPD low-progenitor subjects (i.e., 88%).

different epithelial cells. Hematoxylin and eosin stained sections showed that airway basal progenitor derived epithelium recapitulated many of the histologic features of airways seen *in vivo* (10, 15, 17), including a normal mucocilliary epithelium for non-COPD (Figure 3A) and mucous cell hyperplasia for COPD (Figure 3B). Immunostaining and quantification for K5 and p63 (Figures 3C–3E; see Figure E5A) showed that there were 50% fewer K5/p63 dual positive cells in COPD epithelia compared with non-COPD. These dual positive cells were located on the basal layer of the epithelium, whereas K5 expressing cells in the upper layers of epithelium in COPD were p63 negative. Numbers of Scgb1a1-positive cells (i.e., nonmucous secretory cells) were not decreased in COPD versus non-COPD. In addition,

there was no significant difference in Ki67-positive cells between non-COPD and COPD (Figures 3F–3K; see Figures E5B and E5C). Together, these studies showed that multipotentiality of airway basal progenitor cells is decreased in COPD.

Airway Progenitor Cells and Lung Function

To examine whether loss of basal progenitors was related to lung function, correlations were performed with measures of lung function that relate to airflow (i.e., FEV₁, FEV₁/FVC, forced expiratory flow, midexpiratory phase [FEF_{25–75}], and FEF_{25–75}/FVC), air trapping (i.e., residual volume/TLC), and diffusion (i.e., DL_{CO} and DL_{CO}/VA). In the combined cohort, progenitor counts correlated with all four measures of airflow (Figures 4A–4D), but

did not correlate with measures of air trapping, diffusion, or pack-years of smoking (see Figure E6) ($P > 0.14$ for all). Current smoking did not affect progenitor counts (Figure 4E), but did strengthen the relationship between progenitor counts and airflow, air trapping, diffusion (Figures 4F–4L), and pack-years of cigarette smoke exposure (Figure 4M).

The ability of progenitor counts to correlate with measures of lung function related to the development of COPD (36–41) suggested that basal progenitors may contribute to disease pathogenesis. The distribution of progenitor counts for subjects with COPD and without COPD (Figure 1I) showed that there was substantial overlap between the two groups, but that all progenitor counts in the COPD group were less than 30. Based on this

Video 1. Two-dimensional time-lapse imaging of ciliary movement in Transwell membrane cultures generated by non-chronic obstructive pulmonary disease progenitors. The video was generated using an inverted AxioVert 200M microscope equipped with a long working distance $\times 20$ objective and Slidebook software (Intelligent Imaging Innovations, Inc.).

observation, we hypothesized that subjects without COPD with high progenitor counts (≥ 30) and subjects with COPD (< 30) represented two extremes in the lung function continuum, whereas subjects without COPD with low progenitor counts (< 30) that were equivalent to COPD represented an intermediate group. To explore this hypothesis, subjects without COPD were segregated into those with high progenitor counts (≥ 30) and low progenitor counts (< 30), and then examined for differences in baseline demographics and clinical characteristics, including lung function (Table 2 and Figure 5). Subjects without COPD with low progenitor counts had airflows that were lower than subjects with high progenitor counts, including FEV₁, FEV₁/FVC, FEF_{25–75}, and FEF_{25–75}/FVC (Table 2). No other significant differences were seen between the two groups other than body mass index (Table 2). Airflows for subjects

Video 2. Two-dimensional time-lapse imaging of ciliary movement in Transwell membrane cultures generated by chronic obstructive pulmonary disease progenitors. The video was generated using an inverted AxioVert 200M microscope equipped with a long working distance $\times 20$ objective and Slidebook software (Intelligent Imaging Innovations, Inc.).

without COPD with low progenitor counts were also midway between subjects without COPD with high progenitor counts and COPD (Figure 5). These results indicate that reduced airway progenitor counts relate to decreased lung function and thus, we termed these intermediate subjects as “early COPD.”

Discussion

Airway basal progenitor cells are critical for lung health and resilience, because of their ability to return injured airways to normal structure and function (17, 21, 22). In COPD, the cell repertoire of airway epithelium is altered suggesting that airway progenitor dysfunction contributes to COPD pathogenesis (9, 15). This study shows that airway basal progenitor number, self-renewal, and multipotentiality are all decreased in COPD; that depletion of basal progenitors relates to impaired lung function; and that progenitor depletion identifies a subset of people with lung function that may represent an early, prediagnostic stage of COPD. We describe this impairment as “exhaustion” of basal progenitors, similar to exhaustion of immune cells observed in chronic infections (42). Thus, improvement in basal progenitor function may have therapeutic value.

Airway basal progenitor function was examined in airway biopsies by measuring their numbers, ability to replicate, and capacity to differentiate into cell types that make up a normal airway. COPD airway biopsies contained fewer basal progenitor cells capable of producing a clone during *ex vivo* culture. When COPD airway biopsies did produce clones, they were smaller and had reduced self-renewal compared with non-COPD. Multiple studies have shown induction of cellular senescence in COPD (43–45), and thus in search for a mechanism responsible for decreased self-renewal, we looked at induction of senescence in basal progenitor clones. However, the results were far from conclusive; we identified few senescent cells in COPD clones compared with non-COPD, indicating that the mechanisms involved are more complex and require further research. No prior studies have examined airway basal progenitor cell numbers or self-renewal in COPD or in COPD disease models. Others have

shown a loss of hematopoietic and/or circulating endothelial progenitor cells in COPD (46, 47), suggesting that a systemic loss of progenitor cells may occur in COPD.

One of the provocative findings of this study is that COPD airway basal progenitors recapitulated various aspects of COPD airways (9–16) (e.g., basal and mucous cell hyperplasia and ciliated cell hypoplasia during air–liquid interface culture), whereas non-COPD progenitors produced a normal mucociliary epithelium. In this context it is also significant to mention that coimmunostaining of non-COPD and COPD epithelium with Muc5b and Muc5Ac, two major mucins of the airways, showed that both have comparable expression in subjects without COPD (see Figures E7A and E7B). In COPD, however, Muc5b and not Muc5Ac expression was higher for former smokers, whereas both were upregulated in current smokers (see Figures E7C–E7F). These data point to differences between smoke- and/or disease-induced changes affecting differentiation of basal progenitor cells in COPD. Together, our results indicate that COPD airway basal progenitors have been imprinted to create a “COPD-like” epithelium. The mechanisms underlying this are not known, but an understanding may aid reversal of the phenotype and restoration of a normal airway epithelium.

In contrast to our results, a study by Staudt and coworkers (26) observed no significant difference between airway basal cells from healthy smokers and smokers with COPD to generate a differentiated ciliated epithelium, although differentiation was decreased in COPD smokers compared with nonsmokers. The reasons for these differing results are not known. But it is important to note that they used an unselected population of basal cells (collected by small airway brushing) to differentiate at an air–liquid interface, whereas we only used clone-forming basal progenitor cells (Figures 1E–1H) for differentiation. Thus, it is plausible that use of a mixed population of basal cells may have masked a true loss of basal progenitor cell function.

Another intriguing observation was the finding that p63⁺ basal progenitors from COPD differentiate into two phenotypic populations: K5⁺/p63⁺ and K5⁺/p63[−] cells (Figures 3C–3E). This may have functional/therapeutic significance because

Figure 3. Spatial distribution of cells within cross-sections of differentiated epithelium. Differentiated membranes from air-liquid interface cultures were paraffin-embedded, sectioned, and stained. (A and B) Hematoxylin and eosin staining shows the histology of differentiated epithelia generated by non-chronic obstructive pulmonary disease (COPD) and COPD progenitors. Insets in A and B show magnified areas to demonstrate the presence or absence of cilia in non-COPD versus COPD epithelia. (C–K) Immunostaining and quantification of each cell type using morphometry. Name and color of antigens used are shown in respective images. Representative images from 10 non-COPD and COPD cultures are shown here. Data in E, H, and K are presented as means $\pm$ SE. All *P* values indicate results of Mann-Whitney test. Scale bars are shown in each image. K5 = keratin 5; Ki67 = marker of proliferation, MKi67; p63 = transformation protein 63; Scgb1a1 = secretoglobulin family 1A member 1.

Notch signaling pathway, necessary for differentiation of basal and parabasal p63⁺ cells, is downregulated in COPD (48, 49). Thus, manipulation of the Notch pathway can potentially restore basal progenitor function. These results also suggest that

there are subsets of K5⁺ basal cells in human airways with different differentiation capacities (30). We have previously described multipotential, bipotential, and unipotential basal cells in mouse airways where only multipotential

basal cells gave rise to basal, secretory, and ciliated cells; bipotential basal cells generated basal and secretory cells; and unipotential basal cells differentiated only to basal cells (27, 50). Pardo-Saganta and colleagues (51) have also described distinct

Figure 4. Airway progenitor counts and measurements of lung function in the combined and current smoker cohorts. (A–D) The combined cohort of current/ex-smokers with and without chronic obstructive pulmonary disease were examined by Pearson correlations to determine whether airway progenitor counts were related to measures of airflow, including FEV₁% predicted (A), FEV₁/FVC ratio (B), forced expiratory flow, midexpiratory phase (FEF_{25–75}) predicted (C), and FEF_{25–75}/FVC ratio (D). (E) Current and former smokers were examined for differences in airway progenitor counts. Data presented as means $\pm$ SE. *P* value indicates results of Mann-Whitney test. (F–L) Pearson correlations were performed to examine the relationship between airway progenitor counts and measures of lung function in current smokers, including FEV₁% predicted (F), FEV₁/FVC ratio (G), FEF_{25–75}% predicted (H), FEF_{25–75}/FVC ratio (I), RV/TLC ratio (J), DLCO % predicted (K), DLCO/VA ratio (L), and pack-years of cigarette smoke exposure (M). RV = residual volume.

differentiation fates for subpopulations of basal stem/progenitor cells following airway injury. Therefore, basal cell hyperplasia in COPD could be caused by the loss of multipotential basal progenitors and

generation of basal cells with limited differentiation capacities.

The impact of progenitor dysfunction on cell fate and airway structure seems to have important consequences, because it

relates to changes in pulmonary physiology that define COPD for clinicians. FEV₁ and FEV₁/FVC define COPD and are linked to the underlying genetic propensity to develop COPD (36, 38–41). FEF_{25–75} and

Figure 5. Relationship between progenitor counts and airflows in subjects with and without chronic obstructive pulmonary disease (COPD). (A–D) Current/ex-smoker subjects without COPD (non-COPD) were segregated into high and low progenitor groups and compared with COPD. All three groups were plotted and analyzed for differences based on (A) FEV₁ % predicted, (B) FEV₁/FVC ratio, (C) forced expiratory flow, midexpiratory phase (FEF_{25–75}) predicted, and (D) FEF_{25–75}/FVC ratio. One-way ANOVA $P < 0.001$ for A–D. Results of a Tukey-Kramer *post hoc* analysis are shown in each panel.

FEF_{25–75}/FVC also have heritability estimates for COPD that are as great as FEV₁ and FEV₁/FVC (52, 53), and have been suggested to describe a subgroup with increased susceptibility for COPD (53, 54). These measures of airflow obstruction reflect airway narrowing that largely occurs distal to the first or second generation of airways where biopsies were taken. Therefore, the fact that basal progenitor number correlated with multiple measures of airflow obstruction implies that large airway basal progenitors reflect distal/small airway dysfunction involved with COPD pathogenesis. Other investigators have similarly found that molecular changes in large airways are also seen in small airways (55). When the analysis was restricted to current smokers, the correlation between progenitor depletion and airflow obstruction became stronger, and the

relationship to air trapping, diffusion, and pack-years of smoke exposure became significant. These results are intriguing because they indicate that basal progenitor cells collected from the proximal airway reflect the pathobiology of processes that impact oxygen diffusion (DL_{CO} % predicted and DL_{CO}/VA). The relationship between progenitor counts and pack-years of smoking also implies that a dose–response relationship is present, at least in current smokers. Taken together, these results suggest a link between proximal airway progenitor dysfunction and COPD pathogenesis that is modified by cigarette smoke exposure, the most important cause of COPD in the developed world (34).

COPD develops slowly over decades as small airways narrow and disappear, causing lung function to decline (56–58). Despite the importance of this long period there is

no clinical or biologic definition of “early” or “pre” COPD. SPIROMICS and COPDGene researchers recently found that approximately 50% of current/former smokers with preserved lung function develop an early form of COPD with consequences that impact symptoms, therapy, and healthcare use (7, 8). Based on airway progenitor counts, our study identified three groups of subjects with normal, intermediate, and abnormal lung function. Because the three groups in our study were examined in cross-section and have the same mean age and smoke exposure history ($P > 0.56$ for both; one-way ANOVA), our data suggest the hypothesis that each group has a different susceptibility to develop COPD, which needs to be addressed in subsequent studies.

This study has several important limitations, including the fact that it was not prospectively designed to test the underlying hypotheses. Instead, we leveraged two clinical studies that assessed people at high risk to develop lung cancer, including those with a history of smoking or COPD, the groups of interest. To decrease potential bias researchers were blinded to COPD diagnosis and lung function results until biologic assays had been completed. Never-smokers were not included in our study because they were not included in the trials that we leveraged, and they were not essential to study the role of basal progenitors in disease susceptibility. The study was cross-sectional and could not examine the relationship between progenitor counts and lung function over time. Also, because the study was not prospectively designed to ask specific questions regarding symptoms and medication use, these data were not available to test whether a relationship exists between subjects without COPD with low progenitor counts and COPD-related symptoms and treatment. Because the clinical portion of the study was conducted at the Denver Veterans Affairs Medical Center, most subjects were white and male, limiting generalizability.

This study shows a relationship between basal progenitor cells, lung function, and the histologic manifestations of COPD, indicating that airway basal progenitor exhaustion is involved in disease pathogenesis. Future molecular studies are needed to establish how cigarette smoke leads to basal progenitor dysfunction in susceptible people, and the degree to which

this depends on host genetics or epigenetics. Studies also need to prospectively examine the association between low progenitor counts and accelerated lung function decline over time, and identify the clinical and/or biologic proxies of progenitor dysfunction so that those at risk can be identified before

the onset of disease. Ultimately, understanding the role of basal progenitor cells in COPD pathogenesis has the potential to improve the ability to detect, prevent, and treat disease early when the maximum benefit can be achieved, a goal shared by physicians and patients alike. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Brandy Bagwell for enrolling subjects and collecting biopsies and clinical data, and Mary Jackson for regulatory assistance.

References

- Fletcher CM, Peto R, Tinker C, Speizer RE. The natural history of chronic bronchitis and emphysema. An eight-year study of early chronic obstructive lung disease in working men in London. London: Oxford University Press; 1976.
- Løkke A, Lange P, Scharling H, Fabricius P, Vestbo J. Developing COPD: a 25 year follow up study of the general population. *Thorax* 2006;61:935–939.
- Rennard SI, Vestbo J. Natural histories of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2008;5:878–883.
- Krzyzanowski M, Jedrychowski W, Wysocki M. Factors associated with the change in ventilatory function and the development of chronic obstructive pulmonary disease in a 13-year follow-up of the Cracow Study. Risk of chronic obstructive pulmonary disease. *Am Rev Respir Dis* 1986;134:1011–1019.
- Lindberg A, Jonsson AC, Rönmark E, Lundgren R, Larsson LG, Lundbäck B. Ten-year cumulative incidence of COPD and risk factors for incident disease in a symptomatic cohort. *Chest* 2005;127:1544–1552.
- Anthonisen NR, Connett JE, Murray RP. Smoking and lung function of Lung Health Study participants after 11 years. *Am J Respir Crit Care Med* 2002;166:675–679.
- Woodruff PG, Barr RG, Bleecker E, Christenson SA, Couper D, Curtis JL, et al.; SPIROMICS Research Group. Clinical significance of symptoms in smokers with preserved pulmonary function. *N Engl J Med* 2016;374:1811–1821.
- Regan EA, Lynch DA, Curran-Everett D, Curtis JL, Austin JH, Grenier PA, et al.; Genetic Epidemiology of COPD (COPDGene) Investigators. Clinical and radiologic disease in smokers with normal spirometry. *JAMA Intern Med* 2015;175:1539–1549.
- puchelle e, Zahm JM, Tournier JM, Coraux C. Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2006;3:726–733.
- Rennard SI. Pathogenesis of COPD. *Clin Cornerstone* 2003;5:11–16.
- Gohy ST, Hupin C, Fregmilicka C, Detry BR, Bouzin C, Gaide Chevronay H, et al. Imprinting of the COPD airway epithelium for dedifferentiation and mesenchymal transition. *Eur Respir J* 2015;45:1258–1272.
- Heijink IH, Noordhoek JA, Timens W, van Oosterhout AJ, Postma DS. Abnormalities in airway epithelial junction formation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2014;189:1439–1442.
- Hessel J, Heldrich J, Fuller J, Staudt MR, Radisch S, Hollmann C, et al. Intraflagellar transport gene expression associated with short cilia in smoking and COPD. *PLoS One* 2014;9:e85453.
- Perotin JM, Adam D, Vella-Boucaud J, Delepine G, Sandu S, Jonvel AC, et al. Delay of airway epithelial wound repair in COPD is associated with airflow obstruction severity. *Respir Res* 2014;15:151.
- Randell SH. Airway epithelial stem cells and the pathophysiology of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2006;3:718–725.
- Yaghi A, Dolovich MB. Airway epithelial cell cilia and obstructive lung disease. *Cells* 2016;5:E40.
- Rock JR, Hogan BL. Epithelial progenitor cells in lung development, maintenance, repair, and disease. *Annu Rev Cell Dev Biol* 2011;27:493–512.
- Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, et al. Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc Natl Acad Sci USA* 2009;106:12771–12775.
- Rock JR, Randell SH, Hogan BL. Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. *Dis Model Mech* 2010;3:545–556.
- Ghosh M, Ahmad S, Jian A, Li B, Smith RW, Helm KM, et al. Human tracheobronchial basal cells. Normal versus remodeling/repairing phenotypes in vivo and in vitro. *Am J Respir Cell Mol Biol* 2013;49:1127–1134.
- Ghosh M, Helm KM, Smith RW, Giordanengo MS, Li B, Shen H, et al. A single cell functions as a tissue-specific stem cell and the in vitro niche-forming cell. *Am J Respir Cell Mol Biol* 2011;45:459–469.
- Weiss DJ, Chambers D, Giangreco A, Keating A, Kotton D, Lelkes PI, et al.; ATS Subcommittee on Stem Cells and Cell Therapies. An official American Thoracic Society workshop report: stem cells and cell therapies in lung biology and diseases. *Ann Am Thorac Soc* 2015;12:S79–S97.
- Crystal RG, Randell SH, Engelhardt JF, Voynow J, Sunday ME. Airway epithelial cells: current concepts and challenges. *Proc Am Thorac Soc* 2008;5:772–777.
- Crystal RG. Airway basal cells. The “smoking gun” of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2014;190:1355–1362.
- Shaykhiev R, Crystal RG. Early events in the pathogenesis of chronic obstructive pulmonary disease. Smoking-induced reprogramming of airway epithelial basal progenitor cells. *Ann Am Thorac Soc* 2014;11:S252–S258.
- Staudt MR, Buro-Auriemma LJ, Walters MS, Salit J, Vincent T, Shaykhiev R, et al. Airway basal stem/progenitor cells have diminished capacity to regenerate airway epithelium in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2014;190:955–958.
- Ghosh M, Smith RW, Runkle CM, Hicks DA, Helm KM, Reynolds SD. Regulation of tracheobronchial tissue-specific stem cell pool size. *Stem Cells* 2013;31:2767–2778.
- Hegab AE, Ha VL, Darmawan DO, Gilbert JL, Ooi AT, Attiga YS, et al. Isolation and in vitro characterization of basal and submucosal gland duct stem/progenitor cells from human proximal airways. *Stem Cells Transl Med* 2012;1:719–724.
- Borthwick DW, Shahbazian M, Krantz QT, Dorin JR, Randell SH. Evidence for stem-cell niches in the tracheal epithelium. *Am J Respir Cell Mol Biol* 2001;24:662–670.
- Engelhardt JF, Schlossberg H, Yankaskas JR, Dudus L. Progenitor cells of the adult human airway involved in submucosal gland development. *Development* 1995;121:2031–2046.
- Haji R, Baranek T, Le Naour R, Lesimple P, Puchelle E, Coraux C. Basal cells of the human adult airway surface epithelium retain transit-amplifying cell properties. *Stem Cells* 2007;25:139–148.
- Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *Am J Pathol* 2004;164:577–588.
- Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L643–L649.
- Global Initiative for Chronic Obstructive Lung Disease. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: 2017. 2017 [accessed 2017 Mar 25]. Available from: <http://goldcopd.org>.
- Taswell C. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J Immunol* 1981;126:1614–1619.

36. Cohen BH, Ball WC Jr, Brashears S, Diamond EL, Kreiss P, Levy DA, *et al*. Risk factors in chronic obstructive pulmonary disease (COPD). *Am J Epidemiol* 1977;105:223–232.
37. Celedón JC, Lange C, Raby BA, Litonjua AA, Palmer LJ, DeMeo DL, *et al*. The transforming growth factor-beta1 (TGFB1) gene is associated with chronic obstructive pulmonary disease (COPD). *Hum Mol Genet* 2004;13:1649–1656.
38. Kueppers F, Miller RD, Gordon H, Hepper NG, Offord K. Familial prevalence of chronic obstructive pulmonary disease in a matched pair study. *Am J Med* 1977;63:336–342.
39. Larson RK, Barman ML, Kueppers F, Fudenberg HH. Genetic and environmental determinants of chronic obstructive pulmonary disease. *Ann Intern Med* 1970;72:627–632.
40. McCloskey SC, Patel BD, Hinchliffe SJ, Reid ED, Wareham NJ, Lomas DA. Siblings of patients with severe chronic obstructive pulmonary disease have a significant risk of airflow obstruction. *Am J Respir Crit Care Med* 2001;164:1419–1424.
41. Silverman EK, Chapman HA, Drazen JM, Weiss ST, Rosner B, Campbell EJ, *et al*. Genetic epidemiology of severe, early-onset chronic obstructive pulmonary disease. Risk to relatives for airflow obstruction and chronic bronchitis. *Am J Respir Crit Care Med* 1998;157:1770–1778.
42. Yi JS, Cox MA, Zajac AJ. T-cell exhaustion: characteristics, causes and conversion. *Immunology* 2010;129:474–481.
43. Ito S, Araya J, Kurita Y, Kobayashi K, Takasaka N, Yoshida M, *et al*. PARK2-mediated mitophagy is involved in regulation of HBEC senescence in COPD pathogenesis. *Autophagy* 2015;11:547–559.
44. Mercado N, Ito K, Barnes PJ. Accelerated ageing of the lung in COPD: new concepts. *Thorax* 2015;70:482–489.
45. Tudor RM, Kern JA, Miller YE. Senescence in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2012;9:62–63.
46. Janssen WJ, Yunt ZX, Muldrow A, Kearns MT, Kloepper A, Barthel L, *et al*. Circulating hematopoietic progenitor cells are decreased in COPD. *COPD* 2014;11:277–289.
47. Palange P, Testa U, Huertas A, Calabrò L, Antonucci R, Petrucci E, *et al*. Circulating haemopoietic and endothelial progenitor cells are decreased in COPD. *Eur Respir J* 2006;27:529–541.
48. Mori M, Mahoney JE, Stupnikov MR, Paez-Cortez JR, Szymaniak AD, Varelas X, *et al*. Notch3-Jagged signaling controls the pool of undifferentiated airway progenitors. *Development* 2015;142:258–267.
49. Tilley AE, Harvey BG, Heguy A, Hackett NR, Wang R, O'Connor TP, *et al*. Down-regulation of the notch pathway in human airway epithelium in association with smoking and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2009;179:457–466.
50. Ghosh M, Brechbuhl HM, Smith RW, Li B, Hicks DA, Titchner T, *et al*. Context-dependent differentiation of multipotential keratin 14-expressing tracheal basal cells. *Am J Respir Cell Mol Biol* 2011;45:403–410.
51. Pardo-Saganta A, Law BM, Tata PR, Villoria J, Saez B, Mou H, *et al*. Injury induces direct lineage segregation of functionally distinct airway basal stem/progenitor cell subpopulations. *Cell Stem Cell* 2015;16:184–197.
52. Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R, *et al*. Interpretative strategies for lung function tests. *Eur Respir J* 2005;26:948–968.
53. DeMeo DL, Carey VJ, Chapman HA, Reilly JJ, Ginns LC, Speizer FE, *et al*. Familial aggregation of FEF(25-75) and FEF(25-75)/FVC in families with severe, early onset COPD. *Thorax* 2004;59:396–400.
54. DeMeo DL, Celedón JC, Lange C, Reilly JJ, Chapman HA, Sylvia JS, *et al*. Genome-wide linkage of forced mid-expiratory flow in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2004;170:1294–1301.
55. Steiling K, van den Berge M, Hijazi K, Florido R, Campbell J, Liu G, *et al*. A dynamic bronchial airway gene expression signature of chronic obstructive pulmonary disease and lung function impairment. *Am J Respir Crit Care Med* 2013;187:933–942.
56. Hogg JC. A pathologist's view of airway obstruction in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2012;186:v–vii.
57. Hogg JC, McDonough JE, Suzuki M. Small airway obstruction in COPD: new insights based on micro-CT imaging and MRI imaging. *Chest* 2013;143:1436–1443.
58. McDonough JE, Yuan R, Suzuki M, Seyednejad N, Elliott WM, Sanchez PG, *et al*. Small-airway obstruction and emphysema in chronic obstructive pulmonary disease. *N Engl J Med* 2011;365:1567–1575.