Airway Basal Cells

The "Smoking Gun" of Chronic Obstructive Pulmonary Disease

Ronald G. Crystal

Department of Genetic Medicine, Weill Cornell Medical College, New York, New York

Abstract

The earliest abnormality in the lung associated with smoking is hyperplasia of airway basal cells, the stem/progenitor cells of the ciliated and secretory cells that are central to pulmonary host defense. Using cell biology and 'omics technologies to assess basal cells isolated from bronchoscopic brushings of nonsmokers, smokers, and smokers with chronic obstructive pulmonary disease (COPD), compelling evidence has been provided in support of the concept that airway basal cells are central to the pathogenesis of smoking-associated lung diseases. When confronted by the chronic stress of smoking, airway basal cells become disorderly, regress to a more primitive state, behave as dictated by their inheritance, are susceptible to acquired changes in their genome, lose the capacity to regenerate the epithelium, are responsible for the major changes in the airway that characterize COPD, and, with persistent stress, can undergo malignant transformation. Together, these observations led to the conclusion that accelerated loss of lung function in susceptible individuals begins with disordered airway basal cell biology (i.e., that airway basal cells are the "smoking gun" of COPD, a potential target for the development of therapies to prevent smoking-related lung disorders).

Keywords: smoking; airway epithelium; basal cells; COPD; lung cancer

Chronic obstructive pulmonary disease (COPD), the third leading cause of death in the United States, is characterized by chronic obstruction to expiratory airflow that is not reversible by bronchodilators (1). The overwhelming cause of COPD is cigarette smoking, although inherited genetic variability plays a significant role in modulating the susceptibility of the lung to the stress of smoking (1, 2). For the past 40 years, the underlying concept of the pathogenesis of COPD has been that smoking induces damage to pulmonary structures directly and through chronic inflammation, resulting in the derangement of the airways and alveoli that manifests clinically as bronchitis and emphysema (1, 3). Although smoking affects to some degree all of the cell populations in the lung, in this Perspective, based on my Amberson lecture at the American Thoracic Society International Meeting in May 2014, I present evidence to support the concept that the key to understanding the early events in the pathogenesis of COPD is that smoking deranges the biology of the basal stem/progenitor cell population of airway epithelium (i.e., that airway basal cells are the "smoking gun" of COPD).

Early Lung Abnormalities Associated with Smoking

Our focus on the airway epithelium, and specifically on the basal cell population, is based on the classic histologic studies of smokers by Auerbach and colleagues (4) demonstrating that the earliest abnormalities associated with smoking are derangements of the airway epithelial architecture, with characteristic changes in the epithelial cell populations. These studies demonstrated that the first histologic change associated with smoking is hyperplasia of the airway epithelial basal cell population. Furthermore, although clinicians tend to think of the airway and alveolar disease of COPD as distinct entities, the work of Hogg and colleagues (5, 6) has shown that these pathologic processes are linked, with the initial development of emphysema centered around the early derangements of the airway epithelium of the small airways (i.e., bronchi of more than six generations).

The normal human airway epithelium is comprised of four major cells types, including ciliated, secretory, intermediate, and basal cells (Figure 1A). Ciliated and secretory cells are terminally differentiated cells central to pulmonary host defense, comprising the physical barrier that

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Correspondence and requests for reprints should be addressed to Ronald G. Crystal, M.D., Department of Genetic Medicine, Weill Cornell Medical College, 1300 York Avenue, Box 164, New York, New York 10065. E-mail: geneticmedicine@med.cornell.edu

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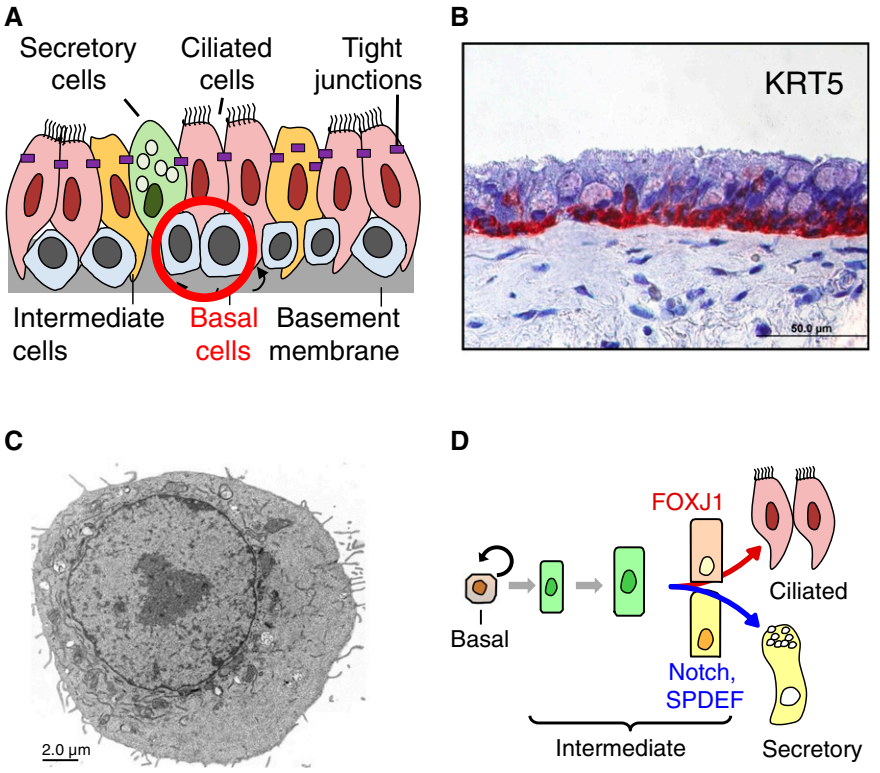

Figure 1. Basal cells and the airway epithelium in the normal human nonsmoker. (*A*) Schematic of the normal human airway epithelium. Basal cells represent 34% of the cell population in the trachea, decreasing generally to 10% in the small airways. Ciliated, secretory, and undifferentiated intermediate cells represent the other cell types. Tight junctions connecting the differentiated ciliated and secretory cells contribute to the integrity of the epithelial barrier. All of the epithelial cells are attached to the basement membrane. (*B* and *C*) Basal cells in the normal nonsmoker. (*B*) Human large airway epithelium. The basal cells are cuboidal, $KRT5^{+}$ cells lining the basement membrane. Hematoxylin and eosin; *bar* = 50 μm. (*C*) Transmission electron microscopy of a basal cell purified from the normal human airway epithelium. *Bar* = 2 μm. (*D*) Normal differentiation of the airway epithelium. The basal cell population contains stem/progenitor cells, which can self-renew and generate differentiated airway epithelium. During normal turnover and repair, the basal cells proliferate and differentiate, generating undifferentiated intermediate cells, which further differentiate into ciliated cells under the control of FOXJ1 and other transcriptional regulators shown and to secretory cells under the control of the Notch pathway. Generation of mucus-producing cells is governed by transcription factors SPDEF and FOXA3.

protects the airways and provides the mucociliary escalator function that clears the respiratory tract from inhaled pathogens, particulates, and other foreign material (7, 8). Basal cells are keratin 5–positive cuboidal cells that are tightly attached to the basement membrane (Figure 1B). Unlike the secretory and ciliated cells, basal cells are unremarkable in appearance, with a high nuclear to cytoplasmic ratio, a paucity of organelles, and scattered microvilli (Figure 1C). The proportion of basal cells in the airway epithelium is highest in the large airways and progressively decreases going down the tracheobronchial tree, representing an average of 34% in the trachea, 27% in the large airways, and 10% in the small airways (9, 10). Basal cells have a central and irreplaceable role: they function as progenitors of the ciliated and secretory cells (11, 12). Intermediate cells (also known as "parabasal" and "indetermined" cells) are located between the basal cells and differentiated cells and are thought to represent basal cell–derived precursors of ciliated and secretory cells (9, 13). Under physiological conditions—the normal adult human airway epithelium turns over relatively slowly, approximately every 1 to 4 months (14)—basal cells are relatively quiescent, and only few intermediate cells can be observed (9). However, in response to injury, such as with the stress of cigarette smoking, airway basal cells proliferate, form clonal patches, expand the pool of intermediate cells, and, under the influence of various factors present in the microenvironment, can either regenerate normally differentiated airway epithelium or, as often occurs in the airways of smokers, generate altered histologic phenotypes (4, 15–21).

The mechanisms that induce the basal cells to differentiate and what controls the specificity of differentiation to ciliated or secretory cells are only partially understood. The ratio of ciliated to secretory cells is tightly controlled at approximately 10 to 1 throughout the tracheobronchial tree, except for the most distal bronchioles, despite the fact that the proportions of basal cells are different going down the tracheobronchial tree and the secretory population changes from mucus-producing cells in the large airways to nonmucous secretory cells in the small airways (7, 18). Studies in mice, and to a lesser extent in humans, have shown that ciliated cell differentiation is governed by a network of transcription factors and regulators, including FOXJ1, multicilin, cyclin O, Myb, and RFX family proteins (22–30). Differentiation to the secretory lineage is mediated by the Notch pathway, whereas generation of mucus-producing cells largely depends on activation of transcription factors SPDEF and FOXA3 (12, 31–34) (Figure 1D). Although relatively little is known about the plasticity of human airway epithelial cells *in vivo*, evidence derived from mouse studies suggests the possibility that committed progenitors or even differentiated cells, in response to injury, may de-differentiate to cells expressing basal cell markers (35–37).

With chronic smoking and the development of COPD, there are characteristic derangements to the airway epithelium structure. The first abnormality is basal cell hyperplasia. This is followed by loss of ciliated cells, shorter cilia, mucus cell hyperplasia, squamous metaplasia (replacement of the normal differentiated cells with flat, squamous cells), and loss of cell junctions (with concomitant "leaky" epithelium) (4, 15–20, 38). Basal cell hyperplasia is seen throughout the airways of smokers, often underlying squamous metaplasia and secretory cell hyperplasia. It is based on these observations that my colleagues and I began focusing on the basal cells as the cell population central to the early abnormalities that initiate COPD.

Normal Human Basal Cells

Extensive studies of airway basal cells using animal models have led to many insights into their function (see References 12 and 39 for excellent reviews), but there has been little attention focused on human airway basal cells in health and disease. Although significant progress has been achieved regarding isolation, culture, and *in vitro* analysis of epithelial cells obtained from the human airways (40, 41), the "basal cell" identity of isolated cells had not been firmly established, and the cultures have been traditionally called primary "human bronchial epithelial cells". However, the contribution of individual cell populations and, particularly, airway basal cells, to the phenotype and functional properties of isolated human bronchial epithelial cells from healthy individuals and patients with lung disease remained unclear. We solved this problem by developing culture methods to isolate primary (not passaged) normal human airway basal cells from brushed airway epithelium (42) (Figure 2A). To accomplish this, flexible bronchoscopy is used to collect the cells by brushing. The cells are detached from the brush by flicking into culture media, disaggregated, and cultured in growth media (43). With periodic changes of the media to remove unattached cells, by 7 days the remaining cells are a pure culture of airway basal cells. Quantitative assessment of the cells by immunohistochemistry demonstrated that the cell population is >95% basal cells expressing the markers cytokeratin 5, p63, and CD151 but negative for the mesenchymal marker N-cadherin, the secretory cells markers mucin 5A and trefoil factor 3, the ciliated markers β-tubulin IV and dynein intermediate chain 1, and the neuroendocrine cell markers chromogranin A and calcitonin gene-related polypeptide α (*see* Figure 2A for examples) (42). As definitive proof that the isolated basal cells are indeed the stem/progenitor cells of the ciliated and secretory cells, the cultured basal cell population is seeded on type IV collagen on so-called "air–liquid interface" cultures, where the basal side of cells is exposed to growth media and the apical side is exposed to air (42). Over 28 days, the basal cells isolated from the airway brushes formed a complete differentiated airway epithelium, with progressive increase in the numbers of ciliated and secretory cells, tight junctions, and increased transepithelial resistance (Figure 2B).

Comparison of the transcriptome of normal human airway basal cells to the transcriptome of the entire differentiated human airway epithelium has permitted identification of the "human airway basal cells transcriptional signature," with >1,100 genes having >5-fold higher expression level in basal cells compared with the transcriptome of the differentiated epithelium (42). This basal cell signature is characterized by genes encoding growth factors, growth factor receptors, extracellular matrix components, G protein–coupled receptors, neuroactive ligands, and receptors and ion channels.

Normal basal cells not only have the ability to proliferate and generate differentiated secretory and ciliated cells, but they also secrete proteins that can influence their neighbor cells and have receptors that can be influenced by products from other cells (39, 44, 45). For example, assessment of the supernatants of cultured normal human airway basal cells demonstrates that these cells secrete a variety of growth factors that likely influence surrounding cells, including vascular endothelial growth factors A and C,

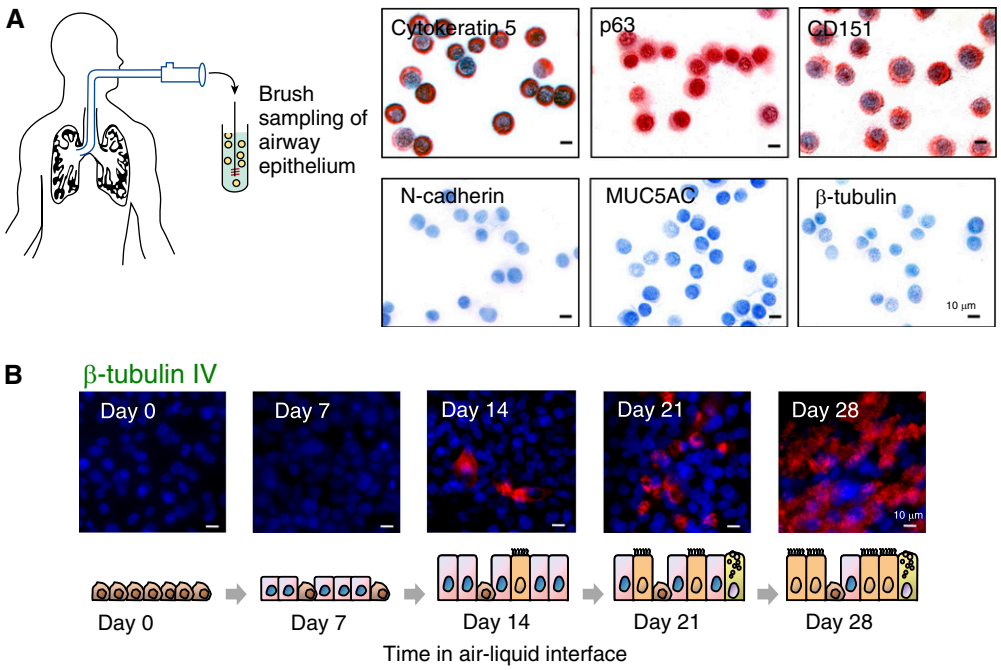

Figure 2. Isolation of basal cells from human the airway epithelium. (*A*) Basal cells are obtained from the airway epithelium using fiberoptic bronchoscopy and brushing (42). The purified basal cells were cytokeratin 5, p63, and CD151 positive but were negative for mesenchymal (N-cadherin), secretory (MUC5AC), or ciliated (β-tubulin) lineages. *Bar* = 10 μm. (*B*) Progenitor function of basal cells. The purified basal cells differentiate to ciliated and secretory lineages when placed in air–liquid interface cultures. Shown is evidence of the ciliated lineage (β-tubulin IV positive). *Bar* = 10 μm.

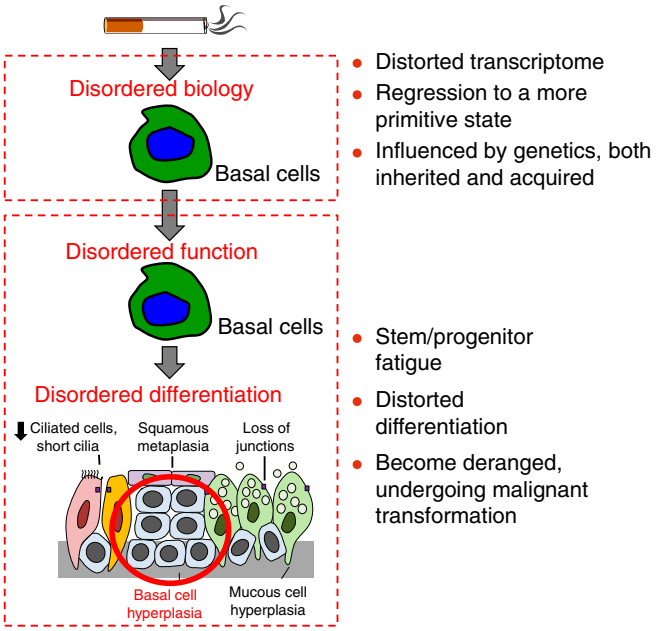

Figure 3. Abnormalities of the biology, function, and differentiation of the airway epithelium with cigarette smoking and the development of chronic obstructive pulmonary disease. The initial changes are in disordered biology, with a distorted transcriptome and regression to a more primitive state under the influence of inherited and acquired genetics. This evolves into disordered function and differentiation, with stem/progenitor cell fatigue, distorted differentiation, and, in some cases, malignant transformation. The first histologic abnormality associated with smoking is basal cell hyperplasia, followed by loss of ciliated cells, shorter cilia, mucus cell hyperplasia, loss of cell junctions, and squamous metaplasia.

angiopoietin, platelet-derived growth factors A and C, placental growth factor, bone morphogenetic proteins 1 and 2, transforming growth factors 1 and 2, fibroblast growth factors 2 and 11, endothelin, IL-1β and IL-8, and the Notch ligand Jagged. Furthermore, transcriptional analysis has demonstrated that normal human airway basal cells express receptors for epidermal growth factor, transforming growth factor, tumor necrosis factors, ephrin, leptin, vasopressin, histamine, serotonin, IL-1, and low-density lipoprotein. Together, these data suggest that basal cells can "talk" to and influence surrounding cells by secreting polypeptides and "listen" and respond to surrounding cells by expressing a variety of receptors (i.e., the basal cells play a central role in the biologic homeostasis of the airways far beyond that of being responsible for replenishing the differentiated cells). Indeed, various cytokines and growth factors, such as IL-1α, IL-33, and TGF-β, have been found to be up-regulated in airway basal cells of smokers and patients with COPD in association with airway remodeling (46–48), suggesting that, in addition to the ability to generate pathologic airway epithelial phenotypes through abnormal proliferation and/or differentiation, airway basal cells may contribute to disease pathogenesis by creating proinflammatory microenvironment and altering epithelial–mesenchymal interactions relevant to the pathogenesis of COPD.

Although the airway basal cells are conventionally considered a homogenous population of cuboidal cells attached to the basement membrane, there is increasing evidence that there are basal cell subsets. For example, whereas all basal cells express KRT5, only a subset expresses KRT14, with the KRT14+ cells likely representing an activated basal cells population that is proliferating and moving down the differentiation path (12, 49). Extensive data, including data generated in our laboratory, suggest a possibility that distinct subsets of basal cells may exist in the human airways based on the expression of integrins, different growth factor receptors, and components of the blood coagulation cascade (11, 39, 42, 49–51).

Consequences of Smoking on Basal Cell Biology

There is extensive data from our laboratory and others (43, 52–55) that cigarette smoking is associated with significant changes in the messenger RNA program of the airway epithelium, with, on average, hundreds of genes up- and down-regulated compared with that of the normal nonsmoker. Central to the concept that basal cells are the "smoking gun" of COPD was the observation by Ryan and colleagues (48) that the airway basal cells isolated from the airway epithelium of healthy smokers (with normal lung function and chest imaging) have a markedly different transcriptome than that of healthy nonsmokers, with 673 genes differentially expressed, primarily up-regulated. These up-regulated genes are in a variety of categories but are dominated by genes related to development, metabolism, signal transduction, transcription, and transport. These dramatic changes in gene expression were detectable even though the basal cells had been in primary culture for 1 week and thereby removed from the chronic stress of smoking. Although the mechanisms of these transcriptional differences are likely complex, we know that there are marked differences in methylation patterns of the small airway epithelium of healthy smokers compared with that of nonsmokers, mostly decreased methylation, consistent with the general up-regulation of the transcriptome program by smoking (56).

Among the basal cell genes up-regulated by smoking are several genes that had been thought to be a part of the human embryonic stem cell (hESC) molecular signature. Although not yet purified and characterized, it is very likely that a small subset of the basal cells are adult stem cells, which, by contrast to all other cells in the body that are derived from ESC, can self-renew and differentiate, but, compared with the latter, have limited differentiation repertoire (i.e., whereas ESCs are pluripotent, with the potential to differentiate into all human cell types, basal cells can only differentiate into secretory and ciliated cells). Although normal basal cells have suppressed the expression of many ESC-specific genes, the basal cells of smokers manifest a more "primitive" transcriptome phenotype, with the gene expression signature thought to be ESC

specific (57). Although this observation does not necessarily imply that smoking "reprograms" adult airway basal cells to ESCs, smoking-induced transcriptome modification, or "reprogramming," of airway basal cells made these cells remarkably similar to hESCs at the global gene expression level, a feature shared by a subset of lung carcinomas characterized by up-regulation of the same set of hESC genes, as in the airway basal cells of smokers (57).

Genetic Influence on Smoking-induced Deranged Basal Cell Biology

One important concept relating to the pathogenesis of COPD is that although the evidence is overwhelming that smoking markedly increases the risk for COPD, only 20% of smokers develop the disease (58). Genetics modulates this risk, with increasing evidence that genetic variability plays an important role in the risk for COPD (2). In this context, if basal cells play a central role in the pathogenesis of COPD, we hypothesized that the smoking-induced disordering of the biology of basal cells may represent one link between genetic susceptibility to COPD and the early disordered lung biology associated with smoking. To assess this hypothesis, we compared the location of the 673 basal cell smoking-dysregulated genes with known COPD risk loci (48). Interestingly, 25% of the smoking-dysregulated genes were localized to chromosome 19, with 13 of these genes localized to 19q13.2, a known COPD risk locus. Of these 13 genes which were up-regulated in basal cells of smokers, four (EGLN2, LTBP4, TGFB1, and NFKB1B) have been linked by genome-wide association studies or candidate gene studies to a risk for COPD (59–61). This observation provides a link between known genetic risks for COPD and airway basal cells, the airway cell population that exhibits the first histologic abnormalities associated with smoking.

Not all of the genetic influences on basal cell behavior are inherited; there may also be smoking-induced somatic variants that affect basal cell function. Because basal stem/progenitor cells are responsible for generating all airway epithelial cells, if correct, somatic variants induced by smoking in basal cells may have significant influence on the derangement of airway epithelial biology associated with smoking. Relevant to this concept, using massive parallel exome sequencing to compare the coding sequences in the DNA of airway basal cells of smokers compared with the same individual's exome sequences in blood DNA, preliminary data suggest a significantly higher somatic mutation score in smoker basal cells compared with their own blood (i.e., COPD could, in part, be an acquired genetic disease).

Transition from "Healthy" Smoking to COPD

The airway basal cells of the "healthy" smoker, with normal lung function and normal chest imaging, are clearly not "normal" at the biologic level, with marked changes in the transcriptional program, dictated in part by genetic variability and by epigenic modifications. There is ample evidence that the basal cell biology gets further deranged as the smoker transitions to clinically defined COPD.

The airway basal cells of individuals with COPD exhibit "stem/progenitor fatigue," with a loss of the capacity to regenerate a normal differentiated epithelium. Staudt and colleagues (62) demonstrated that when basal cells obtained from the small airway epithelium are placed on air–liquid interface and allowed to differentiate to a mucociliated epithelium over 28 days, 88% of samples isolated from healthy nonsmokers successfully regenerated airway epithelium, but only 64% of those from normal smokers and only 44% of those from smokers with COPD regenerated. Inability to form mechanically stable and properly differentiated airway epithelium by basal cells of smokers with COPD may underlie decreased host defense function and barrier integrity of the airway epithelium in patients with COPD, potentially leading to infection and inflammation, which contribute to progression of COPD as chronic disease. Although the mechanisms underlying this "stem/progenitor fatigue" must be complex, analysis of genome-wide DNA methylation of basal cell DNA from the small airway epithelium of normal nonsmokers, normal smokers, and smokers with COPD demonstrated marked differences. Comparison of the basal cell samples that failed to normally differentiate to the basal cell samples that were successful demonstrated 423 significantly different methylation probe sets (i.e., changes in methylation may be one mechanism that limits the ability of basal cells to regenerate a normal epithelium). Furthermore, the majority of the probe sets that differentiated the "successful" versus "not successful" basal cells were hypomethylated, consistent with the observed dominance of up-regulation of genes in basal cells isolated from smokers. Because the failure of the basal cells to regenerate a complete epithelium was observed *in vitro* over weeks after removal of the basal cells from the smoking stress *in vivo*, the data suggest that smoking and COPD create an environment in the airway epithelium inducing epigenetic changes in the basal cells, resulting in a reduced regenerative capacity of these cells.

In addition to the regenerative fatigue of the smoker and COPD smoker basal cells, basal cells have to contend with the stress of mediators induced in the local milieu by smoking. Two of these mediators are epidermal growth factor (EGF) and amphiregulin (AREG) (51, 63). EGF is a 6.4-kD protein involved in cell growth, proliferation, differentiation, and survival, whereas AREG is a 28-kD protein member of the EGF family, with similar functions as EGF. Both of these mediators function through the EGF receptor (EGFR), a member of the ErbB tyrosine kinase receptor family that is highly expressed on human airway basal cells (42, 64). Relevant to the disordered epithelium that characterizes COPD, smoking up-regulates the expression of EGF and AREG in the airway epithelium, exposing the EGFR on basal cells to chronic stimulation. *In vitro* studies with normal human airway basal cells differentiating on air–liquid interface have demonstrated that EGF induces squamous cell metaplasia and decreased airway epithelial resistance, whereas AREG induces basal cell hyperplasia, mucous cell hyperplasia, and shorter cilia and contributes to reducing airway epithelial resistance (i.e., together, EGF and AREG generate all of the pathologic features of the deranged epithelium that characterize COPD) (51, 63). Given that EGF and AREG are up-regulated in the airway epithelium of smokers and that both of these growth factors suppress integrity of the airway epithelial tight junctional barrier and normal differentiation, it is possible

that EGFR signaling driven my these mediators is central to the complex derangement of the normal airway epithelial architecture and its host defense and barrier function. Although there are undoubtedly other mediators that contribute to the deranged COPD airway epithelial differentiation, the EGF/AREG data provide a paradigm for understanding the central role that basal cells play in the pathogenesis of COPD, making the basal cell population a target for drug development to protect the lung from the stress of smoking.

Basal Cells and Lung Cancer

The evidence strongly supports the concept that, with the continued stress of smoking, airway basal cells are modified at the gene expression and functional levels and play a significant role in the pathogenesis of lung cancer, a disorder also caused primarily by smoking (i.e., with the continued stress of smoking, basal stem/progenitor cells can undergo malignant transformation, with specific "driver" mutations that lead to the development of bronchogenic carcinoma) (20). Fukui and colleagues (65) hypothesized that basal cells are the cell-of-origin of at least a subset of lung adenocarcinoma. Lung adenocarcinoma transcriptome data sets were assessed for their "basal cell signature," based on the identification of the human airway basal cell transcriptome by Hackett and colleagues (42). Transcriptome analysis of lung adenocarcinomas from three different data sets was categorized into basal cell "high" and "low" expressors. Assessment of the basal cell "high" adenocarcinomas demonstrated that they have a poor tumor grade, high frequency of vascular invasion, high frequency of KRAS mutations, suppression of ciliated and nonmucous secretory cell genes, and up-regulation of the epithelial–mesenchymal transition program. In all three data sets, representing together 318 lung adenocarcinomas, the individuals with adenocarcinomas in the airway basal cell "high expressor" group had a markedly shorter survival, typically by 50%. These data support the concept that basal cells are the potential cell-of-origin of these cancers and that these "basal cell–high" lung adenocarcinomas are much more aggressive. Several mechanisms might contribute to up-regulation of the airway basal cell transcriptome features in this "basal cell–high" subset of lung adenocarcinomas, including expansion of basal cell population or distinct subset(s) of basal cells in these tumors and the possibility of dedifferentiation or transdifferentiation of non–basal cell population(s) into cells expressing basal cell features in response to injury or oncogenic stress (66, 67). However, in all scenarios, acquisition of the basal cell–associated molecular phenotype seems to be critical for this subtype of lung adenocarcinoma in smokers.

How Does COPD Begin? The Basal Cell "Smoking Gun" Hypothesis

In 1977, Fletcher and Peto (68) reported on 792 working men in London followed with lung function studies over 8 years. They observed that most nonsmokers and many smokers never develop airflow obstruction but that in susceptible smokers smoking causes progressive, irreversible obstruction to expiratory airflow. In this Perspective, I have compiled evidence that, when confronted by the chronic stress of smoking, airway basal cells become disorderly, acquire a more primitive state, behave as dictated by their inheritance, are susceptible to acquired changes in their genome, lose the capacity to regenerate the epithelium, are responsible for the major changes in the airway epithelium that characterizes COPD, and, with persistent stress, might undergo malignant transformation and potentially become the cell-of-origin of a subset of smoking-associated lung carcinomas (Figure 3). Thirty-seven years later, using the advances in cell biology and 'omics technology, studies of airway basal cells in health and disease have provided a novel insight into the biologic mechanisms underlying the Fletcher and Peto observations (i.e., that airway basal cells are the "smoking gun" of COPD with the accelerated loss of lung function in susceptible smokers starting with disordered airway basal cell biology). As evidence mounts to support this concept, airway basal cells are an inviting target to prevent and treat smoking-related lung disorders. ■

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