

Epithelial Cell–Extracellular Matrix Interactions and Stem Cells in Airway Epithelial Regeneration

Christelle Coraux¹, Jacqueline Roux¹, Thomas Jolly¹, and Philippe Birembaut¹

¹INSERM, UMRS 903, CHU Hôpital Maison Blanche, Reims, France

In healthy subjects, the respiratory epithelium forms a continuous lining to the airways and to the environment, and plays a unique role as a barrier against external deleterious agents to protect the airways from the insults. In respiratory diseases such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), chronic bronchitis, or asthma, the airway epithelium is frequently remodeled and injured, leading to the impairment of its defense functions. The rapid restoration of the epithelial barrier is crucial for these patients. The complete regeneration of the airway epithelium is a complex phenomenon, including not only the epithelial wound repair but also the epithelial differentiation to reconstitute a fully well differentiated and functional epithelium. The regeneration implies two partners: the epithelial stem/progenitor cells and factors able to regulate this process. Among these factors, epithelial cells–extracellular matrix (ECM) interactions play a crucial role. The secretion of a provisional ECM, the cell–ECM relationships through epithelial receptors, and the remodeling of the ECM by proteases (mainly matrix metalloproteinases) contribute not only to airway epithelial repair by modulating epithelial cell migration and proliferation, but also to the differentiation of repairing cells leading to the complete restoration of the wounded epithelium. A better characterization of resident stem cells and of effectors of the regeneration process is an essential prerequisite to propose new regenerative therapeutics to patients suffering from infectious/inflammatory respiratory diseases.

Keywords: airway epithelium; regeneration; matrix metalloproteinases; stem cells

The lungs represent the most important surface of contact with the external milieu. In humans, it corresponds to an area of between 50 and 100 m². During normal breathing, the airways daily transport to the lung 10,000 liters of environmental air, which is frequently contaminated with a variety of pollutants, particles, bacteria, and viruses that are deposited in the airways. The respiratory epithelium, forming a continuous lining to the airways and to the environment, plays a unique role as a protective physical and functional barrier to external deleterious agents by elaboration of a series of defense mechanisms developed to protect the airways from the insults. The diversity of the cells composing the airway epithelium is adapted to assume these defense functions. The distribution of cell types within the epithelium varies along the airways. The epithelial surface of the cartilaginous airways (trachea, bronchi) is mainly composed of ciliated cells, which, with basal cells and a small percentage of goblet cells, form a pseudostratified columnar structure. The ciliated cells occupy the majority of the luminal surface, and basal

cells cover almost the totality of the basement membrane. This mucociliary epithelium extends into glandular ducts that emerge in submucosal glands composed of secretory mucous and serous cells (1). The distal noncartilaginous airways (bronchioles) are lined by an epithelium becoming more columnar and constituted by ciliated, basal, and secretory Clara cells. In the most distal bronchioles, only Clara cells are identified (2). The airway epithelial defense system is assumed by different functions, including features of the epithelium contributing to the epithelial barrier integrity, coordinated secretion and ciliary beating leading to an effective mucociliary clearance, and secretion of molecules with antibacterial, antioxidant, and antiprotease activities.

AIRWAY EPITHELIAL REGENERATION

Despite an efficient airway epithelial defense system, disruption of mucociliary clearance due to inhaled particles or pathogens, or caused by infectious and/or inflammatory respiratory diseases such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), chronic bronchitis, or asthma, leads to more or less extensive changes in the architecture of the airway walls, leading in turn to the impairment of the epithelial defense functions. The lesions can vary from the epithelial structure remodeling with generation of basal and/or goblet cell hyperplasia and/or squamous metaplasia, or the loss of the epithelial integrity by disruption of the intercellular junctions, partial shedding of epithelial cells with some basal cells still attached to the basement membrane, or complete denudation of the basement membrane (Figure 1).

Airway Epithelial Regeneration: a Complex Process

To restore its functions, the airway epithelium has to rapidly repair the injuries and regenerate its structure *ad integrum*. The regeneration process is a complex phenomenon that quickly starts after the lesion occurred. Sequentially, epithelial cells at the wound edge dedifferentiate, acquiring a phenotype called “repair cells” (3), spread, then migrate to cover the denuded area. In animal models, it has been demonstrated that re-epithelialization of the injured basement membrane is due to cell migration rather than cell proliferation at first. After migration, epithelial cells in the repairing area start to proliferate. At this step, although the wound is closed, the epithelial integrity is still not restored (4, 5). Then, the repairing epithelium forms a transitory squamous metaplasia followed by a progressive redifferentiation to restore a pseudostratified and functional mucociliary epithelium (6).

Recent studies have examined, using a global approach, the program that mediates regeneration of the human airway epithelium *in vivo* and *in vitro*. *In vivo*, this genetic program has been defined using fiberoptic bronchoscopy and mechanical brushing to denude the airway epithelium of normal individuals, followed by sequential sample of the same regions at Days 7 and 14 after injury, and microarray analysis to compare relative mRNA levels of the samples to that of resting noninjured epithelium. At Day 7, among the 1,196 significantly modulated genes, the repair transcriptome is dominated by cell cycle, signal

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Correspondence and requests for reprints should be addressed to Christelle Coraux, Ph.D., INSERM UMRS 903, Centre Hospitalier Universitaire Maison Blanche, 45 rue Cognacq Jay, 51092 Reims Cedex, France. E-mail: christelle.coraux@univ-reims.fr

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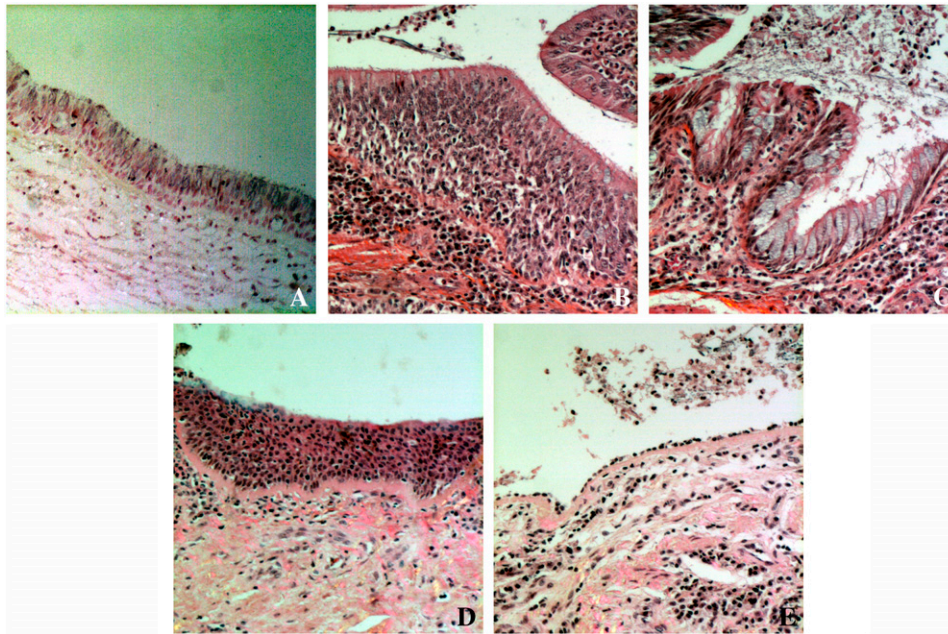


Figure 1. Airway epithelial remodeling and injuries. In patients suffering from infectious/inflammatory diseases, the airway epithelium exhibits frequent areas of (B) basal cell and (C) goblet cell hyperplasia, (D) squamous metaplasia, and (E) epithelial shedding with basal cells still attached to the basement membrane (E). (A) Nonremodeled airway epithelium.

transduction, metabolism and transport, and transcription genes, the majority of differentially expressed cell cycle genes belonging to the G2 and M phases, suggesting that the proliferating cells are relatively synchronized 7 days after injury. At Day 14 after injury, the transcription profile is similar to that of resting noninjured airway epithelium (7). *In vitro*, Ross and coworkers have grown bronchial epithelial cells at the air-liquid interface over a 28-day period to identify genes involved in mucociliary differentiation. They identified over 2,000 genes that displayed at least statistically significant twofold changes in expression during time course, among them genes involved in cell adhesion, immunity, transport, and cilia formation. In particular, they have highlighted networks containing genes involved in transforming growth factor (TGF)- β , WNT/ β -catenin, and epidermal growth

factor (EGF) receptor pathways, suggesting potential roles for these families in airway epithelia (8).

The cellular and molecular factors involved in the repair and regeneration of the airway epithelium are numerous. During this process, the airway epithelium has the ability to modulate the wound healing and reconstitution of the epithelial barrier, mainly through the secretion of extracellular matrix proteins, and interactions with and remodeling of the secreted migratory provisional extracellular matrix. Besides releasing pro-inflammatory cytokines and chemokines, the surviving epithelial cells and the cells of the epithelial environment secrete factors contributing to airway repair and regeneration, including modulators of cell migration, remodeling of the extracellular matrix (ECM), and cell proliferation and differentiation (9).

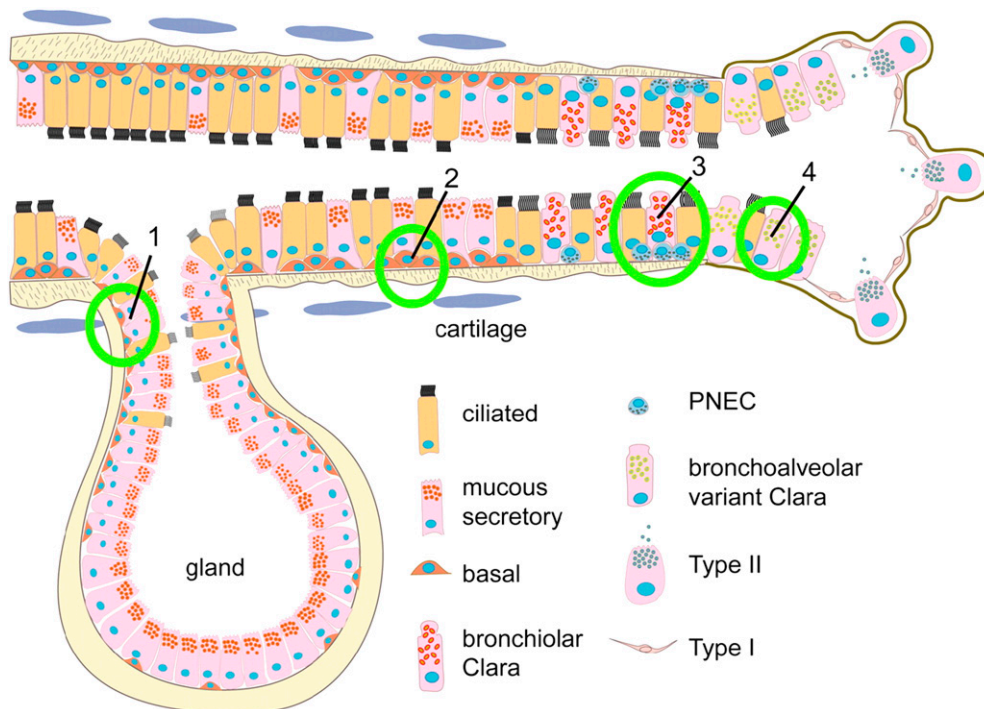


Figure 2. Representation of reported stem cell niches in the animal airways. (1) Basal cells in the glandular ducts. (2) Basal cells in the surface airway epithelium in the intercartilaginous areas. (3) Pollutant-resistant Clara cells in neuroepithelial bodies. (4) Pollutant-resistant Clara cells at the bronchioalveolar duct junctions. Reprinted by permission from Reference 53.

Epithelial Cell–ECM Interactions Modulate Wound Repair

The re-epithelialization process involves several steps, including spreading and migration of cells at the wound edge into the denuded surface and cell proliferation to repopulate the wound site. Interactions between epithelial cells themselves and with ECM may be of primary importance in directing repair of injury.

Matrix proteins and epithelial cell receptors. These processes are mediated by ECM constituents, ligands for cellular receptors such as integrins. Each integrin is composed of an α and a β subunit, the heterodimers mediating cell–cell interactions and cell–ECM adhesion to one or more matrix proteins. The use of blocking antibodies directed against integrin subunits has shown that β_1 -integrins are necessary to the rapid migration of airway epithelial cells on type IV collagen and laminin-1 and -2, and that α_2 -, α_3 -, and α_6 -integrin subunits are directly involved in epithelial cell migration on type IV collagen (10). After airway epithelial damage, epithelial cells at the edge of injury appear to flatten and migrate on the provisional matrix of the wound, including the inflammatory glycoproteins fibronectin and vitronectin as well as components of the basement membrane such as laminin and type IV collagen. Matrix proteins can stimulate migration of airway epithelial cells, in particular the cellular fibronectin (11). *In vivo* and *in vitro*, fibronectin is deposited at the airway cell–matrix interface during the wound repair process. The incubation of wounded cultures with anti-fibronectin–blocking antibodies impairs the wound closure, and $\alpha_5\beta_1$ -integrins, receptors of fibronectin, are up-regulated in the migrating cells of the repairing area (12, 13). Moreover, fibronectin variants involved in airway mucosa wound repair are age-dependently differentially expressed (14), and during its polymerization in the ECM, the fibronectin exposes biologically active matricryptic sites, among them the III-1 site that exhibits the stimulatory role of small airway epithelial cell motility (15). As nonintegrin receptors, carbohydrates on the epithelial cell surface play an important role in cell–cell and cell–substrate interactions. Cell surface N-glycosylation, particularly terminal fucosylation, has a functional role in airway epithelial repair process (16): inhibition of α -dystroglycan binding to laminin attenuates cell migration and spreading after mechanical injury (17), and blockade $\alpha_{1,6}$ -fucose (18) or sialyl Lewis^x (19) prevents epithelial wound repair.

Matrix metalloproteinases. During airway epithelial cell spreading and migration to re-epithelialize the wounded area, cells interact with ECM proteins through focal and primordial contacts that enable the anchorage by which the cells can exert traction on the matrix. These contacts are transient structures: cell movement implies the formation of new sites of adhesion to the ECM at the front of the migrating cells and the release of adhesion sites at the back of these cells. MMPs are directly involved in airway epithelial wound repair, especially in the remodeling of the provisional matrix secreted by repairing cells. MMP-9 (gelatinase B) plays a key role in the migration of bronchial epithelial cells during wound healing. MMP-9 is overexpressed by basal migratory cells at the wound edge, and addition to the culture medium during the repair process of a monoclonal antibody known to inhibit MMP-9 activation, or prevention of its activation, results in decreased speed of wound closure or its complete abolition (20, 21). MMP-3 and MMP-11, also called stromelysin 1 and 3, are exclusively expressed by repairing basal airway epithelial cells that also expressed the mesenchymal marker vimentin, suggesting that stromelysins are involved in epithelial cell migration and ECM remodeling during wound healing and that migrating repairing cells acquire a mesenchymal phenotype necessary for cell migration (22). Unlike most MMPs, MMP-7 is constitutively expressed by airway epithelial cells, where it functions in host

defense by activating the latent form of defensins (23). However, in case of airway epithelial injury, MMP-7 is overexpressed by repairing cells, epithelial migration is reduced by a hydroxamate inhibitor of MMP catalytic activity, and re-epithelialization in tracheas from MMP-7–null mice is essentially blocked (24). MMP-7 could also act during epithelial repair by mediating the shedding of the ectodomain of E-cadherin required for epithelial repair (25). Together, these data highlight the crucial role of MMP-7 in airway epithelial wound repair. Beside their involvement in cell migration during airway epithelial wound closure, MMPs could also modulate the repair process by influencing cell proliferation. Membrane type 1 matrix metalloproteinase (MT1-MMP) is a protease produced by airway epithelial cells. The impaired airway epithelial repair in MT1-MMP–knockout mice after naphthalene treatment has been recently reported, MT1-MMP being required for the proliferative response in distal airway epithelial cells through keratinocyte growth factor (KGF) receptor, but not epidermal growth factor (EGF) receptor signaling pathway (26). The involvement of MMP-2 and MMP-9 in cell proliferation has also been demonstrated, as tracheal cartilage–conditioned medium–derived MMP2 and -9 inhibit respiratory epithelial cell attachment to type I collagen and impair their proliferation, whereas addition of MMP inhibitors to cartilage culture conditioned media does not inhibit epithelial cell growth (27).

Endogenous and exogenous modulators of epithelial cell–ECM interactions. After injury, airway epithelial cells are able to produce effectors of cell migration modulating cell–ECM interactions. The production of matrix molecules, and particularly fibronectin, is influenced by inflammatory mediators secreted in case of epithelial wound, such as transforming growth factor (TGF)- β or tumor necrosis factor (TNF)- α (28, 29). The matrix production by epithelial cells seems to be probably influenced by mediators released from epithelial cells themselves as well as from the inflammatory cells of the airways (30). Moreover, airway epithelial sheet migration over matrix-coated dishes is impaired by treatment with TGF- β due to the enhancement of cell adhesion via integrins (31) and $\alpha_v\beta_8$ integrin-activated TGF- β_1 delays the degree of wound closure (32). On the other hand, an inducing role for TGF- β_1 on airway epithelial wound repair has also been demonstrated by addition of TGF- β_1 that speeds epithelial repair through up-regulation of MMP-2 (33).

Some exogenous factors are able to modulate airway epithelial wound healing. NO, at low concentration, promotes airway epithelial cell migration and wound repair, associated with increased localized expression and activation of MMP-9 in cells at the wound edge, the NOS-derived NO contributing to airway epithelial repair through mechanisms dependent and independent of the cGMP-dependent protein kinase. Moreover, inhibition of NOS completely abolishes the action of NO (34). Purinergic receptor stimulation by extracellular ATP is also a critical determinant of epithelial cell migration and repair after injury, and is associated with activation of ERK1/2 and MMP-9 requiring the activation of Duox1 (35). Pollution plays also a critical role in airway epithelial wound repair. Indeed, diesel exhaust particles (DEP) exposure reduces airway epithelial wound repair in a dose-dependent manner, not only by altering cell proliferation, but also by inducing a reduction in the expression of α_3 - and β_1 -integrin subunit as well as of the hyaluronic acid nonintegrin receptor CD44, and a decreased production of MMP-1 leading to increased cell–ECM adhesion capacity (36). Airway infections alter the wound healing process. For example, *P. aeruginosa* virulence factors, especially elastase, impede airway epithelial wound repair by slowing the cell migration velocity due to the altered actin cytoskeleton

polymerization in the lamellipodia of cells at the wound edge, and by causing an imbalance between pro- and activated forms of MMP-2 via its overactivation and the decrease of its specific tissue inhibitor of metalloproteinases (TIMP)-2 expression (37).

Epithelial Cell-ECM Interactions Modulate Cell Differentiation

After injury, the complete regeneration of the airway epithelium implies, not only re-epithelialization of the denuded basement membrane, but also the reestablishment of the epithelial integrity and the cell redifferentiation to restore the airway epithelial functions. While the involvement of airway epithelial cell-ECM interactions during the wound repair has been extensively studied, their modulation of the epithelial differentiation is poorly documented. Some rare data have suggested that, in addition to their involvement in epithelial cell migration and wound closure, MMPs could be involved in cell differentiation. In particular, MMP-7 and MMP-9 influence airway epithelial reconstitution by modulating the mucociliary differentiation in an *in vivo* model of airway epithelial regeneration. In a humanized xenograft model allowing recapitulation of the sequence of cellular events leading to the regeneration of the airway epithelial barrier (i.e., epithelial cell adhesion, spreading, migration, proliferation, and differentiation), expression and secretion of epithelial MMP-7 and -9 progressively increase during the regeneration time course and are localized at the apical surface of the well-regenerated airway epithelium. More interestingly, incubation of epithelial cells with inhibitors of these MMPs during the regeneration process leads to a default of mucociliary differentiation and to the generation of a remodeled airway epithelium exhibiting squamous metaplasia with areas of basal cell hyperplasia, demonstrating the crucial role of MMP-7 and MMP-9 in the human airway epithelial mucociliary differentiation (38). In the same xenograft model using noninfected CF cells, the airway epithelial regeneration is delayed as mucociliary differentiation is obtained 1 week later than in non-CF grafts, and leads to the generation of a remodeled airway epithelium with pronounced height increase and basal cell hyperplasia, this CF regeneration process being associated with a deregulated pattern of expression of MMP-7, MMP-9, TIMP-1, and IL-8 (39).

RESIDENT STEM/PROGENITOR CELLS

The repair of injuries and the regeneration of the epithelial structure involve stem and progenitor cells. Stem cells are quiescent undifferentiated cells able to divide slowly but indefinitely under particular conditions such as injury, to self-renew for the entire lifespan and to give rise to less proliferative committed daughter cells, called transient amplifying cells or progenitor cells, which will differentiate to form all the cell types of the tissue. Much evidence supports the presence of stem and/or progenitor cells in the airways: although the proliferation index is less than 0.2% in healthy human bronchial epithelium, it greatly increases in some respiratory diseases such as CF (40). Moreover, some experiments showed the ability of airway epithelial cells to reform the complete array of tracheal/bronchial epithelial cells after epithelial wound (3). Despite these results, airway epithelial stem cells are not well characterized, particularly in humans.

Although the concept of a single stem cell common to the epithelium of the whole respiratory organ has been proposed (41), it is now suggested that each subdivision of the lung possesses its own stem cell (Figure 2). In tracheas and bronchi of rodents, basal and secretory cells were first considered as candidate for stem or progenitor cells status due to their proliferation capacity and ability to reconstitute in xenograft models,

after their dedifferentiation into a similar highly proliferative phenotype called "poorly differentiated cells," a pseudostratified mucociliary airway epithelium (42) developing a subepithelial respiratory gland network (43). Although basal and secretory cells appear to be involved in epithelial restitution after injury in animal models, it is now suggested that only basal cells represent the stem cell compartment of the airway epithelium. Basal cells exhibit clonal growth as well as a greater colony-forming efficiency than secretory cells (44). In mouse after epithelial damage, slow-cycling long-term bromodeoxyuridine (BrdU) label-retaining cells are identified as basal cells in the glandular ducts and in foci near cartilage-intercartilage junctions in the surface airway epithelium, leading to the evidence of stem cell niches (45), and are composed of subsets capable of either multipotent or unipotent differentiation leading to the restoration of a fully differentiated airway epithelium (46). In humans, few data are available. In human fetal tracheas, both basal cells and suprabasal cells are endowed with a similar potential to regenerate a fully differentiated airway epithelium after isolation and seeding in a humanized xenograft model in SCID mice (47). Different results have been obtained in human adult airway epithelium. We have recently demonstrated that, in contrast to the observation made in fetal tracheas, only purified adult basal cells are able to reconstitute a fully mature and functional airway epithelium, secretory cells being incapable of adhering, proliferating, or regenerating the epithelium (48), suggesting that adult secretory cells are more mature than fetal secretory cells and have lost their progenitor potential.

In the terminal bronchioles, 15% of proliferating airway epithelial cells are Clara cells, and in the respiratory bronchioles, this number increases up to 44% (49). The nature of the bronchiolar epithelial stem cells has been studied exclusively in animal models, especially in mouse. Although CCSP-expressing cells (Clara cells) and calcitonin gene-related peptide (CGRP)-expressing cells (neuroendocrines cells) localized in neuroepithelial bodies are able to proliferate after bronchiolar epithelial injury, only a subset of pollutant-resistant Clara cells deficient in cytochrome P450 2F2, localized at these particular areas as well as at the bronchioalveolar junctions, are able to reconstitute the bronchiolar airway epithelium and can be considered as stem cells of the bronchioles (50, 51). More recently, the concept of "one stem cell for each lung subdivision" has been questioned: Kim and coworkers have isolated at the bronchioalveolar duct junction an epithelial population resistant to bronchiolar and alveolar damages, able to proliferate after injury *in vivo* and to give rise, not only to Clara cells, but also to type I and type II pneumocytes *in vitro*, highlighting their stem cell properties of both bronchiolar and alveolar epithelia (52). Although the nature of stem/progenitor cells in rodent bronchiolar airway epithelium is well documented, it remains to be clearly determined in humans.

WAYS FOR THE STUDY OF HUMAN AIRWAY EPITHELIAL REGENERATION

Lesions of the airway epithelium are invalidating for patients with infectious/inflammatory respiratory diseases. They lead to a more or less rapid decline of their respiratory capacity. The fast and complete restoration of the airway epithelial structure and functions is essential for these patients. Considering the constant increasing number of people suffering from respiratory troubles, it is important to better understand the airway epithelial regeneration process to propose efficient therapies for these people. A variety of animal models (dogs, rabbits, rats, mice, etc.) have been developed to analyze the repair process of the airway epithelium after injury from different sources (oxidants, bacterial or viral infection, mechanical injury, etc.). These animal models

highlight a common process of epithelial repair and regeneration, including sequential steps of spreading and migration of the basal cells neighboring the wound, dedifferentiation of repairing cells, active mitosis followed by epithelial squamous metaplasia, and progressive redifferentiation with a final step of ciliogenesis and goblet cell differentiation representative of the complete regeneration of a mucociliary epithelium. Nevertheless, histologic differences exist between human and animal airways, raising doubts as to the relevance of the latter as models for the study of the human airway epithelium regeneration. For example, mouse tracheal epithelium is mainly composed of ciliated cells and Clara cells, the latter being present only in human distal bronchiolar airways, whereas only few submucosal gland cells are identified at the upper tracheal level in mice when they are identified in human upper and lower airways.

The regeneration process of the human airway epithelium is a complex phenomenon, partly elucidated by the use of animal models. However, some histologic differences between human airways and those of other animal species led to the development of humanized xenograft models in immunodeficient mice to characterize the cellular and molecular events involved in the reconstitution of a fully mature and functional human airway epithelium, as well as to try to identify the stem and progenitor cells implicated in this process. These xenograft models will allow a better knowledge of the mechanisms involved in airway epithelium regeneration and may help to develop regenerative therapeutics, allowing the reconstitution of a functional airway epithelium in numerous respiratory diseases such as asthma, COPD, CF, and chronic bronchitis.

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