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## Regeneration of airway epithelial cells to study rare cell states in Cystic Fibrosis

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**Abstract:**

Pathological remodeling of the airway epithelium is commonly observed in cystic fibrosis (CF). Thus, tissue repair is critical to restore integrity and maintenance of the epithelial barrier function. Epithelial repair is a multi-step process initiated by progenitor cell migration into the injured area, proliferation, and re-differentiation into all of the cell types that contribute to the function of a normal airway epithelium. Recent technological advances applied to relevant animal and cell injury models have helped in understanding the complexity of progenitor cell differentiation. This short review will introduce the current knowledge of the mechanisms regulating airway epithelial cell (AEC) regeneration and repair, with a focus on the specification of two rare cell types/states: ionocytes and deuterosomal cells.

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**Introduction:**

Different reparative strategies involving basal stem cells play a central role in airway epithelium regeneration. Characterized by a relatively slow turnover, the pseudostratified airway epithelium can be rapidly reconstituted after episodes of infection, inflammation or injury that are commonly observed in respiratory diseases, including CF (1,2). The airway epithelium is not only constituted of basal cells (BCs), club/secretory cells (SCs, predominantly found in bronchioles), goblet cells (GCs) and multiciliated cells (MCCs), but also of less frequent cell types including ionocytes, neuroendocrine, tuft, and intermediate progenitor cells like deuterosomal cells (Figure 1). These cell types/states, which all arise from self-renewing and multipotent BCs, contribute to tissue homeostasis and function of the airway epithelium (3-9).

Lineage tracing in mouse models subjected to different airway epithelial injuries has established the dynamics and plasticity of mouse AEC differentiation during tissue repair. It is becoming clear that improper tissue repair may contribute to destructive chronic lung diseases (10). In this context, several lines of evidence point to an intrinsic defect in the repair process of the CF airway epithelium. Increased BC proliferation was observed on lung sections from CF patients (11), on CF airway epithelial regeneration in a humanized nude mouse xenograft model (12) and in primary cultures of human CF AECs after mechanical injury (13). CFTR-deficient airway epithelial cell lines (14) and primary cultures of human CF AECs (15) undergoing repair after injury showed delay in the kinetics of wound closure, which could be partially rescued with CFTR modulators (16,17). It is thought that, in the absence of exogenous inflammation, the regenerated CF epithelium is remodeled, exhibits basal cell hyperplasia, and shows a delay in ciliated cell differentiation.

The combination of relevant animal and cell models (CF pigs, CF human AEC primary cultures) and new powerful methods of single-cell gene expression are likely to shed new lights on the impact of CFTR deficiency on the deregulated mechanisms during regeneration and repair of the CF airway epithelium. The aim of this short review is to briefly describe the different

1 steps of AEC differentiation and emphasize two recently described populations of cells that  
2 are relevant for CF, namely pulmonary ionocytes and deuterosomal cells.  
3

#### 4 **Cell lineage in regenerating AECs:**

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6 The task of maintaining and repairing the airway epithelium falls mainly on BCs (Figures 1 and  
7 2). Basal cells are a heterogeneous population of cells expressing TRP63 (transformation  
8 related protein 63) and KRT5 (cytokeratin 5), a subset of which express KRT14. The Yes-  
9 associated protein (YAP) is critical for BC maintenance by interacting with TRP63; YAP  
10 overexpression has been shown to enhance proliferation, BC self-renewal, and to block  
11 terminal AEC differentiation (18,19). YAP interacts with Smad proteins of the BMP (bone  
12 morphogenetic protein) and TGF- $\beta$  signaling pathways, which are known to regulate the  
13 expression of genes involved in proliferation and development (20-22).  
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20 Upon injury, which exposes BCs to the luminal surface, receptors for growth factors are  
21 unmasked, triggering their activation and initiating their migration and proliferation (23). This  
22 phase of newly proliferating and migrating cells, or blastema (containing suprabasal and early  
23 progenitor cells), is paralleled by cell differentiation and lineage decisions (Figures 1 and 2).  
24 Some BCs appear to function as classic multipotent stem cells, while others are thought to be  
25 progenitors already committed to a ciliated or secretory fate (5,6). Several key signaling  
26 pathways regulate the continuous transition between proliferation and differentiation. First,  
27 AEC proliferation self-perpetuates until closure of the wound; wound closure triggers signals  
28 to stop proliferation via mechanisms that involve Notch and PPAR $\gamma$  signaling (24). The  
29 expression of Notch3 in some daughter cells coincides with the generation of early progenitors  
30 (25,26), while early differentiation is dependent on Notch1 and Notch2 signaling to give rise to  
31 SCs (27-29). Although Notch signaling appeared unaltered between CF and non-CF repairing  
32 AECs, deficient PPAR $\gamma$  signaling was shown in CF cells, which in turn may affect the fine-  
33 tuning mechanisms coordinating the transition between proliferation and differentiation within  
34 the blastema (30).  
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46 It is now clearly established that SCs can act as facultative progenitors to give rise to  
47 GCs and MCCs (31). According to Watson and collaborators, SCs are short-lived and give rise  
48 to the dominant MCC population in well-differentiated airway epithelia (6). Late differentiation  
49 to MCCs is achieved through cell division arrest and Notch pathway inhibition in response to  
50 increased expression of miR-34/449 (32). Of note, attenuation of BMP activity seems also  
51 necessary for the specification of MCCs (20). The differentiation of other cell types, including  
52 mucous cells, ionocytes, and deuterosomal cells, is also influenced by the Notch/BMP/PPAR $\gamma$   
53 interplay (7,9,33). Because ionocytes are highly enriched in CFTR (7,8) and deuterosomal  
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cells represent an immature population of MCCs (33), they may well contribute to the intrinsic remodeling of the CF airway epithelium.

### **Ionocytes:**

The levels and distribution of CFTR expression and activity across the airway epithelium have been investigated by numerous laboratories. However, a detailed understanding remains incomplete at least in part due to a lack of 'gold standard' reagents for detecting CFTR. This large body of work has led to the general view that CFTR is expressed at low levels in ciliated cells (34), although early studies using *in situ* hybridization identified rare, CFTR-high expressing cells in both the airway surface epithelium and the submucosal gland ducts (35).

Recently, single-cell RNA-seq profiling of primary mouse and human AECs uncovered a rare population of CFTR-high expressing cells, accounting for approximately 1% of the epithelium (7,8). These cells also uniquely expressed the *Foxi1* transcription factor, multiple subunits of the vacuolar ATPase (V-ATPase) proton pump, as well as other ion channels. This expression signature is found in ionocytes in teleost fish gills and skin (36) and *Xenopus* larval skin (37,38), as well as in intercalated cells in the mammalian kidney, forkhead-related (FORE) cells in the inner ear, and clear cells in the epididymis (39), and therefore were termed "pulmonary ionocytes".

Despite their paucity, pulmonary ionocytes express approximately 54% of the *Cftr* transcripts in the mouse trachea (8). Furthermore, functional studies measuring channel activity with Ussing chambers found that ionocytes account for 60% of the mean channel current in primary human AEC cultures (7). Together, these data indicate that pulmonary ionocytes are a major source for CFTR activity. Lineage tracing in mice indicates that BCs can give rise to pulmonary ionocytes (8), and immunofluorescence analysis identified Krt5<sup>+</sup>Foxi1<sup>+</sup> cells in mouse tracheae (7), suggesting that this differentiation occurs without an intermediate state (Figure 1). Differentiated air-liquid interface cultures derived from human airway BCs also contain pulmonary ionocytes, and FOXI1 overexpression is sufficient to drive the ionocyte transcriptional program in this system (7).

The identification and characterization of the pulmonary ionocyte raises intriguing new possibilities for CF therapy. Identifying the signaling pathways that drive ionocyte production may lead to strategies for increasing the pool of ionocytes, and ultimately CFTR activity, for patients with partial loss of function *CFTR* mutations. Alternatively, gene therapy approaches, either gene transfer or gene editing strategies, may need to focus on targeting the ionocytes, or the pool of basal progenitors that will give rise to ionocytes. Finally, previous studies in *Xenopus* have demonstrated a defect in ciliogenesis in ionocyte-depleted embryos (37). These data suggest a role for ionocytes in airway remodeling and necessitate careful assessment of their abundance in diseased tissues.

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2 **Deuterosomal cells:**

3 Multiciliogenesis, the complex process leading to the formation of mature MCCs, involves early  
4 E2F-driven transcriptional events, in possible partnership with several geminin-related factors,  
5 such as GMNC and MCIDAS, resulting in the expression of MYB, FOXJ1, RFX2, and RFX3,  
6 which collectively regulate the expression of a large body of effectors contributing to the  
7 formation of multiple motile cilia. A key step of this process corresponds to the massive  
8 biogenesis of centrioles in a limited time window, which mostly occurs through an acentriolar  
9 structure named the deuterosome.

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15 Single-cell RNA-sequencing has recently been used to reveal lineage hierarchies in the  
16 airway epithelium. Data analysis has revealed the expression of many molecules involved in  
17 early steps of multiciliogenesis in a specific group of cells, intermediate between secretory and  
18 MCCs (Figure 1). This specific population, entitled deuterosomal cells (33), expresses FOXJ1  
19 but differs from mature MCCs by a unique expression pattern with transcripts that are also  
20 expressed in cycling BCs. Specific markers include *DEUP1*, which expresses a key component  
21 of deuterosomes, but also *FOXN4*, *PLK4*, *CCNO* and *CEP78*. This population is also  
22 characterized by a re-expression of cell cycle markers, such as *CDK1*, *CKS1-2*, *PTTG1*,  
23 *CENPF*, *CENPU*, *CENPW*, probably in line with their requirement for the biosynthesis of  
24 hundreds centrioles.

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32 Deuterosomal cells express specifically *CDC20B*, the host gene of miR-449abc, which  
33 was recently shown as a key regulator of centriole amplification (33). *CDC20B* is required for  
34 centriole release and subsequent cilia production in mouse and *Xenopus* multiciliated cells. It  
35 interacts with PLK1, a kinase known to coordinate centriole disengagement with the protease  
36 Separase in mitotic cells. Strikingly, over-expression of Separase rescues centriole  
37 disengagement and cilia production in *CDC20B*-deficient MCCs. This work revealed the  
38 shaping of deuterosome-mediated centriole production in vertebrate multiciliated cells, by  
39 adaptation of canonical and recently evolved cell cycle-related molecules. Detailed analysis of  
40 *CDC20B* revealed the existence of an alternatively spliced isoform, which is more highly  
41 expressed and likely corresponding to the major source of miR-449abc (32,40,41). Gene set  
42 enrichment of the deuterosomal population suggests an association with processes such as  
43 cilium assembly, centrosome maturation and cell-cycle mechanisms. Genes involved in  
44 mitochondrial membrane constitution are also enriched, suggesting an increased number of  
45 mitochondria in these cells.

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1 and *SAP30*, two known transcriptional co-repressors, and of *DYRK1A*, an inhibitor of the  
2 intracellular domain of Notch. *HES6*, an inhibitor of Notch pathway, is also enriched in  
3 deuterosomal cells. Finally, miR-449abc, which is known to alter the Notch pathway (32), is  
4 upregulated in deuterosomal cells. Another characteristic of these cells is the activation of the  
5 TFG- $\beta$  pathway, with an increased expression of target genes such as *SERPINE1*, *CTGF*,  
6 *ATF3*, *TGFBR3* or *IRF7* (9). This is consistent with the known role of TGF- $\beta$  signaling in the  
7 regulation of cilia length (42).  
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### 11 **Summary and future directions:**

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13 Single-cell experiments have highlighted novel cell states, pulmonary ionocytes and  
14 precursors of MCCs in human AECs. Many questions remain to be addressed: 1) what is the  
15 abundance and distribution of the pulmonary ionocyte throughout the respiratory tract and is it  
16 altered in CF? 2) Does the ionocyte have region-specific roles in CFTR-mediated lung  
17 homeostasis, as for example, in the ducts of the submucosal glands? 3) Does FOXI1 regulate  
18 CFTR expression in other tissues? 4) Are the deuterosomal cells altered during constant  
19 renewal of MCCs in the injured CF airway epithelium, in which massive multiplication of  
20 centrioles has to take place? 5) What is the contribution of other rare cell types/states (e.g.,  
21 pulmonary neuroendocrine cells, tuft cells, mucous ciliated cells) to the homeostasis of the  
22 normal and CF airway epithelium? Future experiments using advanced profiling technologies,  
23 detailed histological analysis, and careful functional studies will be crucial for answering these  
24 and other questions, which will help to inform the next wave of CF therapeutic development.  
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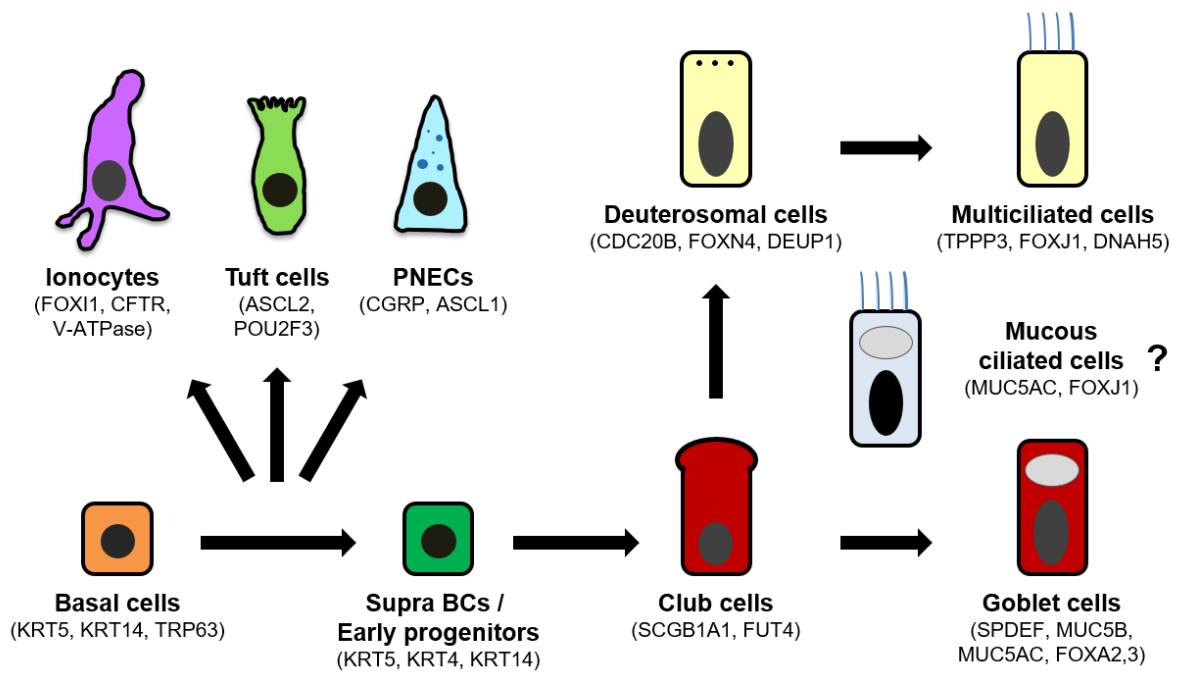
52 **Conflict of Interest Statement:** PB, AC, and MC do not have any conflicts of interest relevant to  
53 this publication. ABJ and LWP are employees of Novartis Institutes of BioMedical Research. ABJ is a  
54 stockholder in Novartis.  
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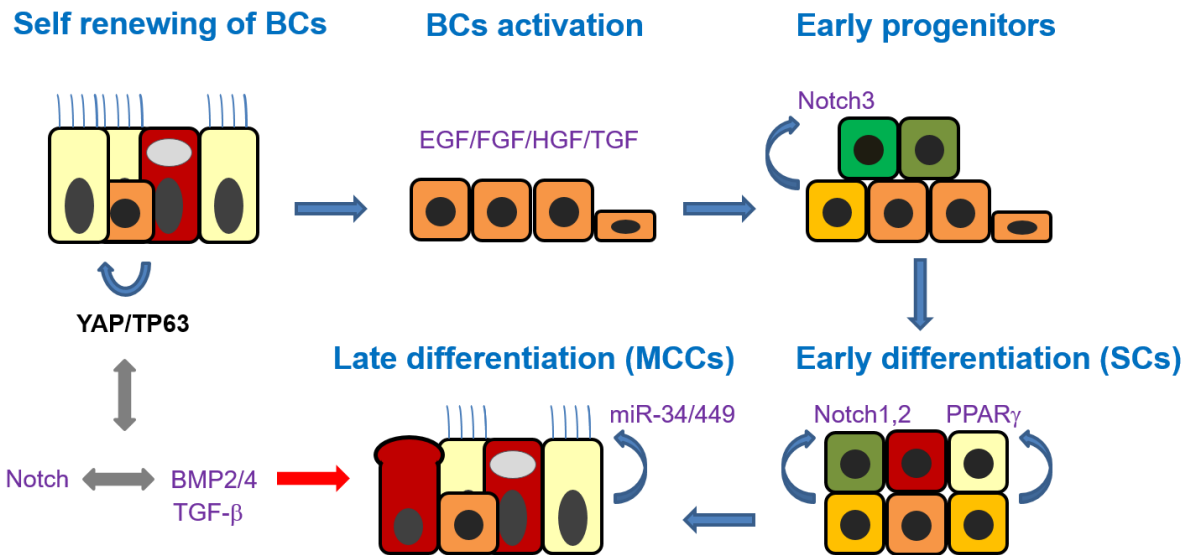
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**Figure 1:** Lineage hierarchy of the airway epithelium.



**Figure 2:** Main steps of the airway epithelium regeneration and their regulation.

## **\*Conflict of Interest statement**

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### **Conflict of interest statement**

PB, AC, and MC do not have any conflicts of interest relevant to this publication. ABJ and LWP are employees of Novartis Institutes of BioMedical Research. ABJ is a stockholder in Novartis.