Secretory Cells Dominate Airway CFTR Expression and Function in Human Airway Superficial Epithelia

Kenichi Okuda¹, Hong Dang^{1*}, Yoshihiko Kobayashi^{2*}, Gianni Carraro^{3*}, Satoko Nakano¹, Gang Chen¹, Takafumi Kato¹, Takanori Asakura¹, Rodney C. Gilmore¹, Lisa C. Morton¹, Rhianna E. Lee¹, Teresa Mascenik¹, Wei-Ning Yin¹, Selene Margarita Barbosa Cardenas¹, Yvonne K. O'Neal¹, Caroline E. Minnick¹, Michael Chua¹, Nancy L. Quinney¹, Martina Gentzsch¹, Carlton W. Anderson⁴, Andrew Ghio⁵, Hirotohi Matsui⁶, Takahide Nagase⁷, Lawrence E. Ostrowski¹, Barbara R. Grubb¹, John C. Olsen¹, Scott H. Randell¹, Barry R. Stripp³, Purushothama Rao Tata², Wanda K. O'Neal¹, and Richard C. Boucher¹

¹Marsico Lung Institute/Cystic Fibrosis Research Center and ⁴Center for Gastrointestinal Biology and Disease, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; ²Department of Cell Biology, School of Medicine, Duke University, Durham, North Carolina; ³Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, California; ⁵Clinical Research Branch, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Chapel Hill, North Carolina; ⁶Center for Respiratory Disease, National Hospital Organization Tokyo Hospital, Kiyose, Tokyo, Japan; and ⁷Department of Respiratory Medicine, The University of Tokyo, Tokyo, Japan

ORCID ID: 0000-0001-9341-2730 (K.O.).

Abstract

Rationale: Identification of the specific cell types expressing CFTR (cystic fibrosis [CF] transmembrane conductance regulator) is required for precision medicine therapies for CF. However, a full characterization of CFTR expression in normal human airway epithelia is missing.

Objectives: To identify the cell types that contribute to CFTR expression and function within the proximal-distal axis of the normal human lung.

Methods: Single-cell RNA (scRNA) sequencing (scRNA-seq) was performed on freshly isolated human large and small airway epithelial cells. scRNA *in situ* hybridization (ISH) and single-cell qRT-PCR were performed for validation. *In vitro* culture systems correlated CFTR function with cell types. Lentiviruses were used for cell type-specific transduction of wild-type CFTR in CF cells.

Measurements and Main Results: scRNA-seq identified secretory cells as dominating CFTR expression in normal human large and, particularly, small airway superficial epithelia,

followed by basal cells. Ionocytes expressed the highest CFTR levels but were rare, whereas the expression in ciliated cells was infrequent and low. scRNA ISH and single-cell qRT-PCR confirmed the scRNA-seq findings. CF lungs exhibited distributions of CFTR and ionocytes similar to those of normal control subjects. CFTR mediated Cl[−] secretion in cultures tracked secretory cell, but not ionocyte, densities. Furthermore, the nucleotide-puriner regulatory system that controls CFTR-mediated hydration was associated with secretory cells and not with ionocytes. Lentiviral transduction of wild-type CFTR produced CFTR-mediated Cl[−] secretion in CF airway secretory cells but not in ciliated cells.

Conclusions: Secretory cells dominate CFTR expression and function in human airway superficial epithelia. CFTR therapies may need to restore CFTR function to multiple cell types, with a focus on secretory cells.

Keywords: CFTR; airway secretory cells; ionocytes; single-cell RNA sequencing

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At a Glance Commentary

Scientific Knowledge on the

Subject: The pathogenesis of cystic fibrosis (CF) lung disease reflects abnormal ion transport caused by CFTR (CF transmembrane conductance regulator) dysfunction. Molecular strategies for CFTR correction require precise identification of cellular targets. Despite substantial efforts, including recent single-cell RNA sequencing (scRNA-seq) studies that identified a CFTR-rich ionocyte, a comprehensive characterization of CFTR expression and function in human airway epithelia is still missing.

What This Study Adds to the

Field: This study, using scRNA-seq technologies coupled with novel and more sensitive molecular methods, provides a comprehensive description of *CFTR*-expressing cell types in normal human conducting airways. scRNA-seq identified secretory cells as dominating *CFTR* expression in normal human large and, particularly, small airway superficial epithelia, followed by basal cells. Ionocytes expressed the highest CFTR levels per cell but were rare, whereas expression in ciliated cells was low and infrequent. Single cell-based qRT-PCR and RNA *in situ* hybridization data confirmed the scRNA-seq findings. CFTR-mediated Cl^- secretory function was correlated with secretory cell types but not ionocytes. Importantly, secretory cells in CF airway epithelia, but not ciliated cells, were capable of CFTR-mediated Cl^- secretion after transduction with wild-type CFTR. CFTR therapies likely will need to restore CFTR function in airway secretory cells.

The pathogenesis of cystic fibrosis (CF) pulmonary disease includes abnormal mucociliary clearance (MCC). Absent or abnormal CFTR (CF transmembrane conductance regulator) function disrupts airway surface liquid (ASL) homeostasis, producing dehydrated mucus and failure of MCC (1–3). Questions remain pertaining to specific links between abnormal ion and fluid transports and CF lung disease, including which cell types within the human lung express CFTR. Resolving this issue is important, as molecular strategies for CFTR correction, including RNAs (4) and CRISPRs (clustered regularly interspaced short palindromic repeats) (5, 6), require precise identification of cellular targets.

Previous studies using immunohistochemistry and RNA *in situ* hybridization (ISH) in fresh tissues or primary cultures focused on CFTR expression and function in large airways, specifically the tracheobronchial region. These studies described CFTR in superficial epithelial ciliated cells (7), rare CFTR “hot” (highly expressing) cells (7), and nonciliated cells in submucosal gland (SMG) ducts (8). Recently, single-cell RNA (scRNA) sequencing (scRNA-seq) identified a rare cell type expressing high levels of *CFTR*, called the ionocyte, as the dominant CFTR-expressing cell in tracheobronchial regions (9, 10). However, subsequent scRNA-seq data (11, 12) suggested that other cell types, including the secretory cells and *KRT8*⁺ intermediate cells, for example, maybe the dominant tracheobronchial *CFTR*-expressing cell types. The key missing components of the description of CFTR expression in the lung are 1) CFTR expression in the “small” (distal bronchiolar) airways, that are believed to be the primary initiating site of CF lung disease (13–24) and 2) evidence describing the relative roles of other CFTR-expressing cell types versus ionocytes in the regulation of airway epithelial Cl^- and fluid secretory rates.

We used transbronchoscopically obtained brush-biopsied cells, normal and CF freshly excised human lungs, and *in vitro* cell culture systems to characterize the cellular distribution of *CFTR* within the proximal–distal airway axis. We used complementary methods and cohorts, including scRNA ISH, single-cell qRT-PCR (scqRT-PCR), and functional measurements, to identify the cells that regulate CFTR and, hence, MCC. Some of the results of this study have been previously reported in the form of abstracts (25, 26).

Methods

Methods are available in the online supplement.

Results

Identification of Airway Epithelial Cell Types in Human Large and Small Airways

Drop-Seq scRNA profiling was performed on human airway samples, including 1) fresh, large airway epithelial (LAE) (main bronchial) cells obtained via bronchoscopy from healthy volunteers ($n = 4$) and 2) small airway epithelial (SAE) (bronchiolar, diameter < 2 mm) cells obtained from nonsmoker transplant donors ($n = 3$) by microdissection (see Figure 1A and Table E1 in the online supplement). A total of 28,041 cells were analyzed, with 16 clusters identified, and labels were assigned on the basis of canonical cell-type markers (Figures E1A–E1AC). Eleven conducting airway epithelial cell clusters (16,488 LAE and 9,831 SAE cells) were further analyzed (3 ciliated clusters, 3 secretory clusters, 2 basal [suprabasal and basal] clusters, 1 ionocyte/neuroendocrine [NE] cluster, 1 cycling/deuterosomal cluster, and 1 undefined cell cluster) (Figures 1B–1D). Both LAE and

Author Contributions: S.H.R. provided human lung samples. A.G. provided the bronchoscopic bronchial brushing biopsy. Y.K. and P.R.T. performed Drop-Seq single-cell RNA sequencing. G. Carraro and B.R.S. performed 10x Genomics single-cell RNA sequencing. K.O., T.K., T.A., R.C.G., R.E.L., and C.E.M. performed immunostainings and RNA *in situ* hybridization. N.L.Q., M.G., and B.R.G. performed Ussing chamber experiments. T.M. and M.C. performed confocal microscopic imaging. S.N., S.M.B.C., Y.K.O.N., and C.E.M. analyzed morphometry and quantified images. C.W.A. performed single-cell qRT-PCR. L.C.M., W.-N.Y., L.E.O., and J.C.O. generated constructs to transduce wild-type CFTR (cystic fibrosis transmembrane conductance regulator) in cystic fibrosis cells. H.D. contributed to bioinformatic and statistical analysis. G. Chen, H.M., and T.N. provided guidance and feedback to the overall work. W.K.O.N. and R.C.B. designed and supervised the project. K.O., W.K.O.N., and R.C.B. drafted and finalized the manuscript.

Correspondence and requests for reprints should be addressed to Richard C. Boucher, M.D., Marsico Lung Institute/Cystic Fibrosis Research Center, University of North Carolina at Chapel Hill, 125 Mason Farm Road, 7008 Marsico Hall, Chapel Hill, NC 27599 7248. E-mail: richard_boucher@med.unc.edu.

This article has a related editorial.

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

Figure 1. Single-cell RNA sequencing for normal human large airway epithelial (LAE) and small airway epithelial (SAE) cells. (A) Schematic of airway locations where LAE and SAE cells were collected for Drop-Seq single-cell RNA sequencing. (B) Clustering of combined LAE and SAE cells into 11 conducting airway epithelial cell types by UMAP. (C) Pie charts depicting the cell-type composition among LAE or SAE cells. (D) Heat map depicting the average expression levels per cell-type cluster for the top 10 differentially expressed genes in each cluster against all other cells as a group; $n = 4$ for LAE cells and $n = 3$ for SAE cells. 1L, 2L, and 3L = LAE ciliated or secretory 1, 2, and 3, respectively; 1S, 2S, and 3S = SAE ciliated or secretory 1, 2, and 3, respectively; B = basal; BL = LAE basal cells; BS = SAE basal cells; Cycl/Deut = cycling/deuterosomal; L = LAE cells; NE = neuroendocrine; S = SAE cells; SB = suprabasal; SL = LAE suprabasal cells; SS = SAE suprabasal cells; UMAP = uniform manifold approximation and projection.

SAE cells contributed to the major conducting airway epithelial clusters, with SAE cells exclusively expressing reported small airway markers (*SFTPB*, *SCGB3A2*) (27, 28) (Figures E1D and E1E). Clustering was consistent with previously published scRNA-seq data performed on human healthy airways (29, 30). Sample-specific quality metrics for scRNA-seq data analysis, cell-type assignment, and characteristics of each cluster are described in the online supplement (Figures E2 and E3 and Table E2).

Identification of Major Cell Types Expressing CFTR Transcripts in Human Airway Epithelia

The contribution of each cell type to the total detected *CFTR* transcript expression was quantitated in both LAE and SAE cells. Among the 26,319 airway epithelial cells studied, 3.6% met the criteria for *CFTR*⁺ expression (Table E3). Among the *CFTR*⁺ cells, secretory cells were the most common cell type expressing *CFTR*, followed by basal cells (Figures 2A–2C), collectively accounting for approximately 80% of *CFTR*⁺ cells. *CFTR* expression in *SCGB1A1*⁺ secretory cells was confirmed by RNA ISH in the normal human SAE region (Figure 2D). Among the basal cell types, *CFTR*⁺ cells were more frequent in the suprabasal cluster than in the basal cluster in all airway regions (Figure 2C). Ionocyte/NE cells, reflecting their rarity, accounted for approximately 4.3% of all *CFTR*⁺ cells, whereas ciliated cells, despite their abundance, accounted for only approximately 5.6%. The undefined and cycling/deuterosomal clusters contained the remaining *CFTR*⁺ cells.

On the basis of calculations of the average expression level of *CFTR* per cell among the *CFTR*⁺ cells in each cluster (Table E4), the ionocyte/NE cluster exhibited the highest average per cell *CFTR* expression in both LAE and SAE cells, as reported previously (9, 10). There were no significant differences in *CFTR* expression levels between the LAE and SAE cells among any clusters (Figures 2E and 2F).

Next, the contribution of each cell cluster to total *CFTR* expression was calculated (Figure 2G). Secretory cell clusters dominated the total detected *CFTR* expression in LAE and SAE cells, followed by basal cell clusters. The relatively low ionocyte/NE contribution to the total *CFTR* expression in the LAE and SAE cells reflected the trade-off between high-per-cell expression and rarity. Note that SAE and

LAE ionocyte/NE clusters differed in important aspects. First, the SAE ionocyte/NE cluster exhibited a lower *CFTR*⁺ cell frequency/cluster (34.4%) than the LAE cluster (48.9%) (Table E3). Second, the ionocyte/NE cluster in SAE cells exhibited distinct molecular signatures, with more NE genes (*CALCA*, *CHGB*) and fewer ionocyte-associated genes (*ASCL3*, *STAP1*, *CEL*) than exhibited in LAE cells (Figures E4A and E4B). Consistent with these findings, GRP-expressing NE bodies were frequently identified in normal human terminal bronchioles (Figures E4C and E4D). Collectively, the SAE ionocyte/NE cluster contained more NE cells and fewer ionocytes than the LAE ionocyte/NE cluster.

As a replicate cohort, large airway (bronchial) and distal lung macrodissected specimens were obtained from the same donor ($n = 8$) and sequenced by 10x Genomics (10x) scRNA-seq (Figures 3A and 3B) (31), yielding nine conducting airway epithelial cell clusters (11,688 LAE and 4,955 SAE cells (Figures 3C and 3D). Using prediction scores (32), each 10x scRNA-seq cluster was assigned to one Drop-Seq scRNA-seq cluster (Figure E5). Consistent with Drop-Seq scRNA-seq results, the rank order for cell types contributing to total *CFTR* expression by 10x scRNA-seq was 1) secretory, basal, ionocyte/NE, cycling/deuterosomal, and ciliated cells in LAE cells and 2) secretory, basal, cycling/deuterosomal, ionocyte/NE, and ciliated cells in SAE cells (Figures 3E–3I and Tables E5 and E6). Because LAE cells were isolated from the main bronchi that contained SMGs, one of the secretory 1 subclusters (secretory 1-ii) contained a fraction of SMG cells characterized by SMG-serous cell markers (*LYZ* and *DMBT1*) (11, 33) (Figure E6). Note that the *CFTR* expression was not identified in those cells expressing SMG markers (Figure E6). Collectively, the two scRNA-seq data sets identified the secretory cell as the highest contributor to the total *CFTR* expression in LAE regions and, particularly, SAE regions, with ionocytes contributing relatively less to *CFTR* expression in both regions.

Relationship of CFTR to MUC5AC and MUC5B

It is not clear whether *CFTR* and mucin secretory functions reside in the same airway cell type (34–39). Analyses of *CFTR*⁺ cells for mucin gene expression revealed that 38.2% of LAE cells and 70.6% of SAE cells expressing *CFTR* coexpressed

the secretory mucins *MUC5B* and/or *MUC5AC* (Figures E7A and E7B). RNA ISH in normal human LAE regions also revealed colocalization of *CFTR* with *MUC5B*⁺/*SCGB1A1*⁺ secretory cells (Figure E7C). Collectively, these data demonstrate that airway secretory cells expressed secretory mucins and *CFTR* in LAE regions and, especially, in SAE regions.

Validation of scRNA-seq CFTR Expression Data in Human Airway Epithelia

Validation of scRNA-seq data is important, especially for low-abundance transcripts. Therefore, we used complementary and more sensitive methods, specifically scRNA ISH and sqRT-PCR, to identify *CFTR* transcripts. First, RNA ISH fluorescent assays were performed on freshly dissociated brush biopsy main bronchial (large) and microdissected bronchiolar (small) epithelial cells. *CFTR* transcript expression and colocalization with airway epithelial markers, including secretory (*SCGB1A1*), ciliated (*FOXJ1*), basal (*KRT5*), and ionocyte (*FOXI1*) cell markers, were measured (Figures 4A and E8). Of 11,648 LAE cells and 22,459 interrogated SAE cells, 6,373 (54.7%) and 8,721 (38.8%) cells expressed *CFTR* in LAE and SAE cells, respectively (Figures E8B and E8C); these values were approximately 10 times higher than those detected in scRNA-seq (Table E3 and E5). Analyses of the frequency of *CFTR* transcript expression revealed that the majority of *SCGB1A1*⁺ (~90%) and more than half of *KRT5*⁺ (~69%) cells coexpressed *CFTR* in both LAE and SAE cells, with similar levels of *CFTR* intensity between regions (Figures 4B–4D). A smaller proportion of *FOXJ1*⁺ cells coexpressed *CFTR* with lower *CFTR* intensity. *FOXI1*⁺ ionocytes were identified in the cell preparations but were rare, particularly in SAE cells (LAE region: 100 cells vs. SAE region: 16 cells). Most ionocytes coexpressed *CFTR* with relatively high intensity (Figure 4D).

Second, sqRT-PCR was performed on 264 bronchial cells obtained from two healthy donors by bronchoscopic-brush biopsy, followed by single-cell sorting (Figures 4E and E9A). Among the 146 cells passing quality checks, 102 (69.9%) cells expressed *CFTR*. This frequency was higher than in scRNA-seq but was consistent with the scRNA ISH data. The 146 cells were assigned to secretory (*SCGB1A1*⁺), basal

Figure 2. Characterization of *CFTR* (cystic fibrosis transmembrane conductance regulator)-positive cell types in normal human large airway epithelial (LAE) and small airway epithelial (SAE) cells. (A) Cellular distribution of *CFTR*⁺ cells on the combined LAE and SAE cells by uniform manifold approximation and projection (UMAP). (B) Regional source of *CFTR*⁺ cells superimposed on the UMAP. (C) Pie charts depicting the proportion of each cell type identified among all *CFTR*⁺ LAE or SAE cells. (D) Representative fluorescent RNA *in situ* hybridization images showing colocalization of *CFTR* (green) within *SCGB1A1*⁺ (white) cells in the normal human bronchiolar epithelium. Scale bar, 20 μ m. (E) Violin plots depicting *CFTR* expression in *CFTR*⁺ conducting airway epithelial cells of LAE or SAE regions. The difference between regions was compared using the nonparametric two-sided Wilcoxon rank-sum test. (F) Violin plots depicting *CFTR* expression in *CFTR*⁺ cells per each cell type in LAE or SAE cells. (G) Pie charts depicting the contribution of each cell type to total *CFTR* expression in LAE or SAE cells; $n = 4$ for LAE cells and $n = 3$ for SAE cells. B = basal; Cycl/Deut = cycling/deuterosomal; NE = neuroendocrine; NS = not significant; SB = suprabasal.

Figure 3. *CFTR* (cystic fibrosis transmembrane conductance regulator)-expressing cell types in normal human large airway epithelial (LAE) and small airway epithelial (SAE) cells in an independent replicate cohort. (A) Schematic of airway locations where LAE and SAE cells were collected for 10x Genomics single-cell RNA sequencing. Matched LAE and SAE cells were obtained from eight donors. (B) Regional source of conducting airway epithelial cells on the uniform manifold approximation and projection (UMAP). (C) Clustering of combined LAE and SAE cells into nine conducting airway epithelial cell types by UMAP. Each cluster was assigned to one of the Drop-Seq single-cell RNA-sequencing cell clusters (Figure 1) on the basis of the prediction score as shown in Figure E5. (D) Pie charts depicting the cell-type composition among LAE or SAE cells. (E) Cellular distribution of *CFTR*⁺ cells on the combined LAE and SAE cells by UMAP. (F) Pie charts depicting the proportion of each cell type identified among *CFTR*⁺ LAE or SAE cells. (G) Violin plots depicting *CFTR* expression in *CFTR*⁺ conducting airway epithelial cells of LAE or SAE regions. The difference between regions was compared using the nonparametric two-sided Wilcoxon rank-sum test. (H) Violin plots depicting *CFTR* expression in *CFTR*⁺ cells per each cell type in LAE or SAE cells. (I) Pie charts depicting the total contribution of each cell type to *CFTR* expression in LAE or SAE cells. For definition of abbreviations, see Figure 2.

Figure 4. Validation of *CFTR* (cystic fibrosis transmembrane conductance regulator) expression in freshly excised normal human large airway epithelial (LAE) and small airway epithelial (SAE) cells by single-cell RNA *in situ* hybridization (ISH) and single-cell qRT-PCR (scqRT-PCR). (A) Cytospins are obtained from large and small airways. RNA ISH colocalizes *CFTR* in *FOXI1*⁺ cells in the large airway (arrow) and *SCGB1A1*⁺ cells in the small airway (arrowheads) but does not colocalize *CFTR* in *FOXJ1*⁺ cells in either region. Scale bars, 10 μ m. (B) Histograms depicting the intensity of *CFTR* expression in each marker-positive cell identified by single-cell RNA ISH. The number of *CFTR*⁺ dot signals was quantified in 200 cells expressing *SCGB1A1*, *KRT5*, or *FOXJ1* for each donor. Histograms of *FOXI1*⁺ cells depict the intensity of *CFTR* expression in 100 and 16 *FOXI1*⁺ cells identified in large and small airway samples, respectively. *FOXI1*⁺ cells were identified in two of five donors in the small airway samples. (C) Frequency of *CFTR*⁺ cells among each cell type identified by single-cell RNA ISH. (D) Quantification of the intensity of *CFTR* expression among each cell type. The average number of dot signals for *CFTR* expression was quantitated in *CFTR*⁺ cells among each cell type. For B–D, $n = 5$. (E) Schematic depicting an experimental layout of scqRT-PCR of freshly brushed main bronchial epithelial cells. (F) Frequency of *CFTR*⁺ cells among each cell type identified by scqRT-PCR. The cell-type assignment is shown in Figure E9. (G) Expression of *CFTR* and *SLC12A2* in each cell type identified by scqRT-PCR. $n = 146$ cells from two donors. (C, D, and G) Histogram bars and error bars represent the mean $\pm$ SD. FACS = fluorescence-activated cell sorter; L = large airway cells; S = small airway cells.

(*KRT5*⁺), ciliated (*FOXJ1*⁺) cells, or cells expressing multiple cell-type markers (Figures E9B and E9C and E10). *FOXI1*⁺ ionocytes were not identified, reflecting their rarity. *CFTR* expression was detected in the

majority of *SCGB1A1*⁺ secretory cells and approximately half of the *KRT5*⁺ basal cells (Figures 4F and 4G), consistent with the scRNA ISH data (Figure 4C). Notably, *CFTR* transcripts, as well as the *SLC12A2*

$\text{Na}^+ \cdot \text{K}^+ \cdot 2\text{Cl}^-$ cotransporter, which mediates sustained Cl^- secretion (40), were not detected in *FOXJ1*⁺ ciliated cells (Figure 4G). *CFTR* transcripts were also detected in cells expressing multiple cell-type markers.

Figure 4. (Continued).

Intraregional FOXI1 and CFTR Expression in Normal and CF Human Lungs

To characterize intraregional pulmonary distributions of *CFTR* expression and ionocytes, RNA ISH signals for *CFTR* and *FOXI1* were quantitated in histologic sections from 10 normal excised lungs (Table E7). *CFTR* was consistently expressed within normal airway superficial epithelia from the trachea to terminal bronchioles (Figures 5A–5C). In contrast, the number of *FOXI1*⁺ ionocytes decreased from the trachea to the terminal bronchioles. The discordance between *CFTR* expression and ionocyte numbers in the distal airways indicates a lesser contribution of *FOXI1*⁺ ionocytes to *CFTR* expression in the distal airways, consistent with SAE-based scRNA-seq and scRNA ISH data. Immunohistochemistry detected *CFTR* protein in apical membranes of *FOXI1*⁺ ionocytes in SMG ducts and superficial epithelia in the large airways (Figure E11A), as well as dome-shaped nonciliated cells, which morphologically corresponded to secretory club cells in the distal (bronchiolar) airways (Figure E11B).

To investigate whether the loss of *CFTR* and/or CF-associated lung infection and inflammation affect *CFTR* or *FOXI1*

expression in airway epithelia, RNA ISH studies of *CFTR* and *FOXI1* were performed in nine excised CF lungs (Tables E7 and E8). *FOXI1* expression was similar to that of normal control subjects in large airways (bronchi) and, as in normal control subjects, waned in distal airways (Figure 5D). In contrast, the consistent expression of *CFTR* was detected throughout CF conducting airways. Immunohistochemistry identified abnormal intracellular *CFTR* protein expression in ionocytes in CF airways (Figure E11C), consistent with previous reports (41).

Is CFTR Expressed in Nonionocyte Epithelial Cells Functional?

To relate *CFTR*-expressing cell types to Cl[−] secretory function, matched human LAE and SAE cell culture systems (42) were used to measure regional *CFTR* Cl[−] secretory rates (Figures E12A and E12B). Fully differentiated SAE cultures retained distal airway characteristics, such as *SFTPB* and *SCGB3A2* expression, which distinguished SAE cultures from LAE cultures (Figures 6A and E12C and Table E9) (42). Like the scRNA-seq data on freshly excised human SAE and LAE cells, the expression of *CFTR* was similar in both airway regions (Figure 6A). In contrast, the number of *FOXI1*⁺ ionocytes and ionocyte markers (*FOXI1*,

ASCL3, *ATP6V1B1*) were reduced in SAE cultures compared with LAE cultures (Figures 6A–6C and E12D). Morphologic evaluation of both LAE and SAE cultures revealed localization of *CFTR* within *SCGB1A1*⁺ secretory cells (Figures 6D–6F).

In Ussing chamber functional studies, SAE cells exhibited robust *CFTR*-mediated Cl[−] secretory responses to forskolin that matched that of LAE cells (Figure 6G). The response to the *CFTR* inhibitor-172 was also similar in both SAE and LAE cultures (Figure 6H). Thus, SAE cells exhibited *CFTR* Cl[−] secretory currents similar to those of LAE cells, which correlated to *CFTR* expression in *SCGB1A1*⁺ secretory cells (Figure 6A), not in *FOXI1*⁺ ionocytes.

Nucleotides Regulate CFTR Activation in Secretory, Not Ionocyte, Airway Epithelial Cells

CFTR chloride secretion is regulated *in vivo* in part by the concentrations of purine nucleotides in ASL (43–45). Triphosphate nucleotides, including ATP and uridine triphosphate (UTP), activate *CFTR* via P2RY2 receptors to maintain ASL homeostasis (46). It is notable that scRNA-seq and sqRT-PCR revealed significant *P2RY2* expression in secretory and suprabasal cells, without detectable

Figure 5. Intraregional *CFTR* (cystic fibrosis [CF] transmembrane conductance regulator) and *FOXI1* expression in normal and CF human lungs detected by RNA *in situ* hybridization (RNA ISH). (A) Regional distribution of *CFTR* and *FOXI1* transcript localization in normal human airways. The *FOXI1*⁺ cells with high intensity were identified in the submucosal gland duct and main bronchial epithelium (arrows). (i) Submucosal gland ducts. (ii) Main bronchi. (iii) Segmental bronchi. (iv) Bronchioles. (v) Terminal bronchioles. Scale bars, 25 μ m. (B) Morphometric quantification of RNA ISH *CFTR* expression and *FOXI1*⁺ cells in normal human airway superficial epithelia. The histogram with error bars represents the mean $\pm$ SD of the proportion of the *CFTR*-stained area within the corresponding superficial airway epithelial area (left y-axis). The red dashed line with error bars shows the mean $\pm$ SD of the number of *FOXI1*⁺ cells in the superficial airway epithelia normalized to the length of BM (right y-axis); $n = 10$. (C) Comparison of *CFTR* expression (left) and frequency of *FOXI1*⁺ cells (right) between normal human large airway superficial epithelia versus small airway superficial epithelia. Red circles in the large airway epithelial (LAE) category represent mean values of the *CFTR* expression (left) or the number of *FOXI1*⁺ cells (right) of the trachea and main, segmental, and distal bronchi, and blue circles in the small airway epithelial (SAE) category represent those of bronchioles and terminal bronchioles from the 10 distinct subjects. These 10 subjects were the same as shown in B. Histogram bars and error bars represent the mean $\pm$ SD; NS by linear mixed-effect models. (D) Morphometric quantification of RNA ISH *CFTR* expression and *FOXI1*⁺ cells in human CF airway superficial epithelia. $n = 9$. Note that the trachea and main bronchi were not available from the resected CF lungs. *** $P < 0.001$. BM = basement membranes; NS = not significant.

Figure 6. CFTR (cystic fibrosis transmembrane conductance regulator) expression in secretory cells is associated with Cl^- secretory function. (A) Comparison of gene expression between *in vitro* large airway epithelial (LAE) cells versus small airway epithelial (SAE) cells, measured by bulk RNA sequencing; $n = 10$. (B) Representative whole-mount immunohistochemistry images for CCSP (green), CFTR (yellow), and FOXI1 (white) in LAE and SAE cells. FOXI1⁺ ionocytes (arrowheads) were identified in the middle level of the z-axis (Middle-Z). The nuclei and tight junction were stained with Hoechst and Phalloidin, respectively. Scale bars, 50 μm . (C) Quantification of the number of FOXI1⁺ ionocytes in LAE

expression in ionocyte/NE cells (Figures 7A and 7B and Figure E13), a finding confirmed by scRNA ISH of dissociated cultured human LAE cells (Figures 7C and 7D).

Ussing chamber experiments were designed to test whether the differential expression of P2RY2 in lumen-facing secretory cells, but not ionocytes, had a functional correlate. Luminal addition of the P2RY2 agonist UTP to LAE cultures, in the presence of a TMEM16A blocker (Ani9) (47), stimulated an increase in short circuit currents (I_{sc}), which was blocked by CFTR inhibitor-172 (Figures 7E and 7F). The UTP-stimulated I_{sc} were not detected in the CF epithelium. Collectively, these data demonstrate that the UTP-activated Cl^- secretory current was mediated by secretory cells coexpressing P2RY2 and CFTR. Importantly, UTP-stimulated I_{sc} were restored in CF cells treated with ellexacaftor–tezacaftor–ivacaftor (Figure 7G).

Secretory Cells Are Competent to Correct CFTR Function in CF Cells

It is important to target the correct cell type for successful molecular therapy of CF. Our data that describe the secretory cell as the predominant CFTR-expressing cell type in human airways predicts that restoring CFTR function in this cell type will restore CFTR function in mixed-cell-population CF cell cultures. The rCCSP (rat CCSP) promoter (48) was used to drive wild-type CFTR expression in human CF secretory cells. For comparison, the hFOXJ1 (human FOXJ1) promoter mediated CFTR expression in ciliated cells (49).

Quantitative PCR revealed expression of human *CFTR* complementary DNA (cDNA) in cultures infected with lentiviral vectors containing the rCCSP or hFOXJ1 promoter (Figure E14A). Notably, CF cells infected with the vector carrying wild-type CFTR driven by the rCCSP promoter restored forskolin-activated, CFTR-mediated Cl^- secretory responses in CF cultures to levels similar to those of control non-CF cultures (Figure E14B). In contrast, CF cells infected with lentiviral vectors containing the hFOXJ1

promoter driving wild-type CFTR failed to restore CFTR-mediated Cl^- secretion. The responses to the CFTR inhibitor-172 paralleled the forskolin responses in rCCSP versus hFOXJ1–CFTR–expressing CF cells (Figure E14C). These data indicate that CF airway epithelial secretory cells, not ciliated cells, have the capacity to express transduced wild-type CFTR functionally.

Discussion

It is necessary for an understanding of CF pathogenesis and guiding precision-medicine therapies to identify the cell types expressing CFTR in all airway regions, including the disease-initiating and most severely affected small airway region (13–24).

Multiple approaches were used to characterize *CFTR* expression per airway cell type. scRNA-seq in two cohorts, using different platforms, identified the major *CFTR*⁺ airway epithelial cell types. *CFTR*⁺ cells represented only a minor fraction of total cells, specifically 4–6%, interrogated in the Drop-Seq and 10x scRNA-seq cohorts. However, *CFTR*⁺ cells were distributed widely among multiple clusters (Figures 2 and 3). Secretory cells were the most common *CFTR*⁺ cells, followed by basal cells, ciliated cells, and ionocytes, respectively. Ionocytes expressed the highest levels of *CFTR* per cell, which is consistent with previous reports (9, 10), and this was followed by similar levels of *CFTR* intensity in the other cell types. Combining these two features (cell numbers and *CFTR* levels) produced a rank order for the total *CFTR* expression in the human superficial airway epithelia of secretory cells, basal cells, ionocytes, and ciliated cells, respectively.

Because scRNA-seq methods have limited sensitivity for low-abundance transcripts (50), including *CFTR*, we used novel scRNA ISH quantitative imaging and single cell–sorted qRT-PCR techniques to extend the scRNA-seq findings. The percentage of cells expressing *CFTR* using these more sensitive methods was found to be over

40%, compared with approximately 4–6% for scRNA-seq (Figure 4). Thus, scRNA-seq greatly underestimated the frequency of *CFTR*⁺ cells. This effect was most profound (>10× underestimation) in lower-expressing cell types, including secretory and suprabasal cells. *CFTR* expression was only underestimated twofold, specifically by around 96% in cytospin RNA ISH versus by around 43% in scRNA-seq, in the higher-expressing ionocyte population. The expression of *CFTR* in secretory cells covering a large fraction of the airway surface may assure the local control of airway surface hydration required for mucus clearance.

Intraregional variation of the ionocyte marker *FOXI1* versus *CFTR* expression was detected in quantitative RNA ISH studies of excised normal human lungs. *CFTR* was homogeneously expressed throughout large and small airway superficial epithelia. In contrast, *FOXI1* expression was highest in SMG ducts and waned in the superficial epithelium along the proximal–distal axis. These data, coupled with the scRNA-seq data indicating that ionocytes are present with reduced frequency in small airways (Figures 2 and 3), and the absence of SMGs in small airways, suggest that most *CFTR* expression in SAE regions is mediated not by ionocytes but by secretory cells. Similar regional *CFTR* and ionocyte expression patterns were observed in CF lungs. These findings in diseased CF lungs are important because precision-medicine approaches will by necessity be performed in people with CF who have lung disease.

The scRNA data describing dominant *CFTR* expression in secretory cells raised the question of whether CFTR functions as a Cl^- secretory channel in this cell type. This question was addressed via multiple approaches. First, we asked whether CFTR expression in secretory cells was associated with Cl^- secretory function. SAE cultures expressed similar levels of *CFTR* and secretory cell marker transcripts, but reduced levels of ionocyte marker transcripts and protein, compared with LAE cultures

Figure 6. (Continued). and SAE cells normalized to the unit area; $n = 3$. (D) *In vitro* LAE and SAE cells probed for *SCGB1A1* and *CFTR* (arrowheads) by RNA *in situ* hybridization (ISH). Scale bars, 10 μm . (E) Single-cell RNA ISH images of dissociated *in vitro* LAE and SAE cells. Scale bars, 10 μm . (F) Frequency of *CFTR*⁺ cells among *SCGB1A1*⁺ LAE and SAE cells identified by single-cell RNA ISH. The number of *CFTR*⁺ cells was quantitated in 200 *SCGB1A1*⁺ cells per donor; $n = 6$. (G and H) ΔI_{sc} in response to Fsk (G) and subsequent I-172 (H) measured by Ussing chambers; $n = 15$. The histogram bars and error bars represent the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, and NS by linear mixed-effect models (A, C, G, and H). ΔI_{sc} = changes in short circuit currents; Fsk = forskolin; I-172 = *CFTR* inhibitor-172; NS = not significant.

Figure 7. Nucleotides regulate CFTR (cystic fibrosis [CF] transmembrane conductance regulator) activation via P2RY2 receptor expressed in secretory airway epithelial cells. (A) Heat map showing expression of the selected genes identified in the combined large airway epithelial (LAE) cells and small airway epithelial cells by Drop-Seq single-cell RNA sequencing. (B) Expression of P2RY2 transcripts per each cell in each cell type identified by single-cell qRT-PCR. The single-cell qRT-PCR was performed on the same cells shown in Figures 4E–4G. (C) Representative single-cell

(Figures 6A–6C and E12D). In Ussing chambers, SAE cultures exhibited CFTR-mediated Cl^- secretory responses that matched those of LAE cultures, demonstrating a correlation between secretory cell, but not ionocyte, CFTR expression and function in the two airway regions. Other groups have used Notch signaling inhibitors to associate reductions in secretory cell numbers with reductions in CFTR secretion rates, consistent with our findings (9, 51). Second, we asked whether secretory cells expressed signaling pathways required for the physiologic regulation of CFTR Cl^- secretion in human airways. scRNA-seq analyses demonstrated that secretory cells, but not ionocytes, express the P2RY2 purinoceptor that regulates CFTR activity and ASL volume in functioning airway epithelia *in vivo* (45). The Ussing-chamber studies demonstrated a functional relationship between purinoceptor activation and CFTR-mediated Cl^- secretion in secretory cells. Third, we asked whether CF secretory cells were competent for the expression of Cl^- secretory activity after transduction with wild-type CFTR. Both hFOXJ1 and rCCSP promoters drove wild-type CFTR transcript expression in CF cultures, but only rCCSP transduction of wild-type CFTR restored Cl^- secretory responses to forskolin (Figure E14). These data parallel our previous studies of transgenic overexpression of wild-type CFTR in CF mice that revealed that CFTR transduced by rCCSP, but not by hFOXJ1, restored CFTR-dependent Cl^- secretion (48, 52). These findings, coupled with the coexpression of all epithelial sodium channel subunits in secretory cells, suggest that the secretory cell is a dominant ion-transporting cell in the human airway.

Basal cells were the next most highly expressing CFTR cell type. It was notable that the suprabasal cell cluster that expressed CFTR most highly was the cluster that expressed secretory cell genes and KRT8 (11) (Figure E2D), suggesting this cell type may be transitioning to secretory cell differentiation and function. With respect to ciliated cells, the absence of CFTR and the SLC12A2 $\text{Na}^+ \cdot \text{K}^+ \cdot 2\text{Cl}^-$ cotransporter expression in single ciliated cells as defined by scqRT-PCR (Figure 4G), coupled with the failure to restore for CFTR function by ciliated cell-specific transduction of wild-type CFTR *in vitro* (Figure E14) and *in vivo* (48, 52), support a conclusion that mature ciliated cells

provide little *CFTR*-mediated Cl^- secretion. Finally, ionocytes appear to express the requisite RNAs for Cl^- transport (e.g., *CFTR* and *SLC12A2*) but do not express the *P2RY2* (Figure 7) required for regulation of airway surface hydration *in vivo*. These data, coupled with the small ionocyte contribution to the total percentage of airway epithelial *CFTR* expression, argue for no or small ionocyte contributions to basal superficial epithelial ASL volume homeostasis. These findings are in agreement with the findings of the studies by Seibold and colleagues, which revealed no loss of Cl^- secretory function in LAE cells where ionocytes were depleted by CRISPR-targeting of *FOXI1* (11). We speculate that the rare ionocyte cell type may either 1) perform sensing and/or acute response functions, particularly in the proximal airways, a region exposed to hyperosmotic environmental stresses (53), or 2) render SMG secretions mildly hypotonic by NaCl but not by fluid absorption (54).

Our studies also have implications for molecular therapies for CF lung disease. First, *CFTR* is widely expressed in a common cell type that lines airway surfaces, specifically the secretory cells. Recent data indicate that secretory cells can, particularly in the small airways, enter a proliferative basal cell population, suggesting that targeting the lumen-facing accessible secretory cells may confer both short-term and long-term correction (55, 56). Second, *CFTR* is expressed in basal cells that may be moving into a secretory cell lineage. This finding suggests that 1) targeting basal cells may be an attractive strategy to ultimately populate the lumen-facing secretory cell pool and 2) “ectopic” expression of *CFTR* in basal cells by mRNA or cDNA expression strategies may not produce deleterious off-target effects.

Third, with respect to the need to target ionocytes, data from human subjects with *FOXI1* mutations describe hereditary deafness and renal tubular acidosis, but not lung disease, as phenotypes (57), suggesting that targeting ionocytes for treatment of CF lung disease may not be necessary. Note that targeting basal cells, however, may also repopulate ionocyte populations, as recently suggested by Plasschaert and colleagues (9).

There are limitations to our studies. First, our unsupervised clustering algorithms did not separate cycling cells from deuterosomal cells, and ionocytes from NE cells, respectively, reflecting similar gene signatures between the two cell types (11, 29). Cell clustering is heavily influenced by defined parameters in clustering algorithms, specifically the resolution of cell-state stratification in scRNA-seq experiments. It remains challenging to simultaneously stratify cell types and different substates in each cell type (58). Our clustering algorithms provided subclusters in major airway epithelial cell types, including secretory, ciliated, and basal cells, which enabled us to study whether there were specific subsets expressing higher *CFTR* within the major cell types. Our data indeed suggest that the secretory 1 subcluster, which was characterized by the mucin secretory cell markers, specifically *SCGB1A1*, *MUC5B*, *MUC5AC*, and *RAB3D* (Figures E2A and E2E), dominated total *CFTR* expression among the three secretory subclusters. With the advent of more efficient and unbiased clustering algorithms that have been explored (58, 59), improvements in clustering methodologies are anticipated. Second, it is challenging to completely remove doublets from cell partitioning into oil droplets in the high-throughput

scRNA-seq microfluidics modalities. This technique’s difficulty is coupled with difficulties in computationally distinguishing doublets, composed of two distinct cell types, from transitional cells expressing multiple cell-type markers in the downstream bioinformatic analysis (60). Our secretory 3 subcluster in the Drop-Seq scRNA-seq data demonstrated both secretory and ciliated cell signatures (Figure E2A), suggesting the possibility that this cluster contained doublets composed of secretory and ciliated cells. However, recent scRNA-seq studies argue that there is a transitional cell type expressing secretory and ciliated cell markers *in vivo* and *in vitro* in human airway epithelia (29, 30, 61). Importantly, because the contribution of the transitional secretory 3 subcluster to *CFTR* expression was small (~5.3%) among the secretory subclusters, the doublet issue does not alter our conclusion that the secretory cell is the dominant *CFTR*-expressing cell type in the airway superficial epithelium.

In summary, our data suggest that *CFTR* is widely expressed in the major lumen-facing cells of the lung, notably secretory cells. These data suggest a heretofore unanticipated complexity in function for the secretory cell with respect to maintaining the homeostasis of the well-hydrated mucus that covers airway surfaces. Importantly, these data provide a roadmap for molecular therapeutic approaches that emphasizes targeting secretory cell types for the short-duration restoration of *CFTR* function on airway surfaces and/or targeting secretory and/or basal cells for long-term correction. ■

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Figure 7. (Continued). RNA *in situ* hybridization image showing colocalization of *P2RY2* with *SCGB1A1*⁺ secretory cells (arrows), but not in *FOXI1*⁺ ionocytes (arrowhead) of *in vitro* LAE cells. Scale bar, 10 μm . (D) Quantification of the number of dot signals of *P2RY2* expression in *SCGB1A1*⁺ or *FOXI1*⁺ cells in the dissociated LAE cells identified by single-cell RNA *in situ* hybridization. A total of 800 *SCGB1A1*⁺ cells (200 cells per donor) and 110 *FOXI1*⁺ cells (27.5 ± 19.8 cells per donor) were analyzed for *P2RY2* expression; $n = 4$. (E) Representative I_{sc} measured in non-CF and CF LAE cells by Ussing chambers. (F) Quantification of responses to uridine triphosphate (UTP) and I-172 in the presence of Ani9 in non-CF and CF LAE cells; $n = 4$ donors. (G) Quantification of responses to UTP in the presence of Ani9 in well-differentiated CF LAE cells with or without elxacaftor-tezacaftor-ivacaftor (triple-therapy) treatment; $n = 4$ CF donors, including two F508del/F508del, one F508del/R347P, and one F508del/G330X subject. The same symbol colors were used to indicate the inserts obtained from the same CF donor. The histogram bars and error bars represent the mean $\pm$ SD. A comparison between the two groups was performed using linear mixed-effect models with a donor as a random-effect factor (D and G) or Wilcoxon rank-sum test (F). The statistical comparison was performed after log transformation in D. * $P < 0.05$ and ** $P < 0.01$. Amil = amiloride; Ani9 = 2-(4-chloro-2-methylphenoxy)-N-[(2-methoxyphenyl)methylideneamino]-acetamide; CTL = control; Cycl/Deut = cycling/deuterosomal; ΔCt = change in cycle threshold; I-172 = *CFTR* inhibitor-172; I_{sc} = short circuit currents; NE = neuroendocrine; Veh = vehicle.

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