Ontogeny and Biology of Human Small Airway Epithelial Club Cells

Wu-Lin Zuo^{1*}, Sushila A. Shenoy^{1*}, Sheng Li^{2‡}, Sarah L. O'Beirne^{1,3}, Yael Strulovici-Barel¹, Philip L. Leopold¹, Guoqing Wang¹, Michelle R. Staudt^{1§}, Matthew S. Walters^{1§}, Christopher Mason^{2,4}, Robert J. Kaner^{1,3}, Jason G. Mezey^{1,5||}, and Ronald G. Crystal^{1,3||}

¹Department of Genetic Medicine, ²Institute for Computational Biomedicine, ³Department of Medicine, and ⁴Department of Physiology and Biophysics, Weill Cornell Medical College, New York, New York; and ⁵Department of Biological Statistics and Computational Biology, Cornell University, Ithaca, New York

Abstract

Rationale: Little is known about human club cells, dome-shaped cells with dense cytoplasmic granules and microvilli that represent the major secretory cells of the human small airways (at least sixth-generation bronchi).

Objectives: To define the ontogeny and biology of the human small airway epithelium club cell.

Methods: The small airway epithelium was sampled from the normal human lung by bronchoscopy and brushing. Single-cell transcriptome analysis and air-liquid interface culture were used to assess club cell ontogeny and biology.

Measurements and Main Results: We identified the club cell population by unbiased clustering using single-cell transcriptome sequencing. Principal component gradient analysis uncovered an ontologic link between KRT5 (keratin 5)⁺ basal cells and SCGB1A1 (secretoglobin family 1A member 1)⁺ club cells, a hypothesis verified by demonstrating *in vitro* that a pure population of human KRT5⁺ SCGB1A1[−] small airway epithelial basal cells differentiate into SCGB1A1⁺KRT5[−] club cells on air-liquid interface culture. Using SCGB1A1 as the marker of club cells, the single-cell analysis identified novel roles for these cells in host defense, xenobiotic metabolism, antiprotease, physical barrier function, monogenic lung disorders, and receptors for human viruses.

Conclusions: These observations provide novel insights into the molecular phenotype and biologic functions of the human club cell population and identify basal cells as the human progenitor cells for club cells.

Keywords: club cell; principal component gradient analysis; small airway epithelium

At a Glance Commentary

Scientific Knowledge on the Subject: Club cells, the major secretory cells of the human small airways, have been extensively studied in the murine lung, but little is known regarding the biology or ontogeny of club cells in the human airways.

What This Study Adds to the Field: Analysis of the club cell transcriptome, in addition to demonstrating that human club cells play a major role in host defense, revealed that human club cells express genes responsible for most of the monogenic lung disorders, including cystic fibrosis and alpha-1 antitrypsin deficiency, and receptors for a variety of viruses that cause human lung infections.

(Received in original form October 27, 2017; accepted in final form June 6, 2018)

*These authors contributed equally to this study.

‡Current address: The Jackson Laboratory for Genomic Medicine, Farmington, Connecticut.

§Current address: University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma.

||These authors contributed equally as senior investigators for this study.

Supported in part by NIH grants R01HL107882 and R01HL1189541, and the NIH Shared Instrumentation Grant (S10RR027699) for Shared Resources. S.A.S. was supported in part by NIH grant T32HL094284.

Author Contributions: Drafting manuscript, W.-L.Z., S.A.S., S.L.O'B., Y.S.-B., and R.G.C. Data interpretation, W.-L.Z., Y.S.-B., S.A.S., G.W., P.L.L., M.S.W., and M.R.S. Data analysis, W.-L.Z., S.A.S., S.L., Y.S.-B., and J.G.M. Lead physicians, R.J.K. and S.L.O'B. Contributed to design and execution, S.L.O'B., G.W., C.M., and J.G.M.

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, NY 10065. E-mail: geneticmedicine@med.cornell.edu.

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

Am J Respir Crit Care Med Vol 198, Iss 11, pp 1375–1388, Dec 1, 2018

Copyright © 2018 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201710-2107OC on June 6, 2018

Internet address: www.atsjournals.org

The human small airway epithelium (SAE) is comprised of basal, ciliated, mucin-secreting (goblet cells), and club cells (1–4). The club cells are the major secretory cell type, representing approximately 20% of the human SAE (5, 6). Club cells have a characteristic apical dome shape, microvilli, dense cytoplasmic granules, extensive smooth endoplasmic reticulum and mitochondria, and uniquely express SCGB1A1 (secretoglobin family 1A member 1; also called CC10 [club cell 10-kDa protein], CC16, or uteroglobin [3, 7, 8]), a member of the secretoglobin family with antiinflammatory properties (9–12). In the murine lung, SCGB1A1⁺ club cells function in host defense, barrier maintenance, metabolism of xenobiotics, and as the progenitor of ciliated and other secretory cells (13–20). In humans, other than a proposed role in host defense (see Table E1 in the online supplement), little is known about the biology of the club cell and its ontogeny.

Taking advantage of SCGB1A1 as a unique marker for club cells and KRT5 (keratin 5) as a unique marker for airway basal cells (BC) (4, 7, 21–23), and our ability to sample the SAE of the normal human lung by bronchoscopy and brushing (24, 25), we have used single-cell transcriptome analysis to characterize the ontogeny and biology of human SAE club cells. The data demonstrate that in humans, the SAE club cell is derived, at a minimum, from KRT5⁺ BC. Importantly, the single-cell analysis uncovered a markedly expanded role for the human club cell in host defense, including immune function, expression of antibacterial proteins, metabolism of xenobiotics, antiprotease defense, and physical barrier functions. The data suggest a potential role for human club cells in most lung hereditary disorders, and in infectious lung diseases, via expression of receptors for known human lung pathogens.

Methods

The SAE (10th- to 12th-generation bifurcation) was obtained by bronchoscopy from three healthy nonsmokers (25). Single-cell capture and RNA sequencing was performed using the Fluidigm system. Two types of analyses were performed: unsupervised analysis to assess cell populations in human SAE, and semiunsupervised, multivariate analysis to gain insight into the

ontogeny of the human club cell; and supervised analysis to assess the biology of the human club cell. The supervised analysis took advantage of SCGB1A1 as a unique marker for club cells, KRT5 for BC, DNAI1 (dynein axonemal intermediate chain 1) for ciliated cells, and MUC5AC for mucin-secreting cells (5, 7, 22, 23, 26). Categories of genes were determined using a variety of databases and the literature. Airway epithelial BC air-liquid interface (ALI) culture was performed using primary

purified BC. TaqMan and morphologic studies (transmission electron microscopy [TEM], immunohistochemistry) were done using conventional methods. See the METHODS in the online supplement for details.

Results

Club cells are identified by anti-SCGB1A1 immunohistochemistry (Figures 1A and E1A–E1C). With fiberoptic bronchoscopy

Figure 1. Source of club cells for analysis. SCGB1A1⁺ club cells were recovered from the normal human small airway epithelium (SAE) by bronchoscopy and brushing. (A and C) SCGB1A1⁺ immunohistochemistry, red stain; scale bars, 20 μm. (A) Source of club cells; SCGB1A1⁺ cells in the SAE in a section of normal lung. (C) Example of SCGB1A1⁺ cells recovered by brushing the SAE. B and D are the negative controls for A and C, respectively. (E and F) Transmission electron microscopy assessment of human SAE club cells recovered by brushing. Shown is a club cell with characteristic dense granules. (E) Whole club cell; scale bar, 2 μm. (F) Enlarged image of the granules; scale bar, 0.2 μm.

and brushing, SCGB1A1⁺ club cells were easily sampled (Figures 1C and E1D–E1F). TEM analysis of the brushed cells included cells with classic club cell features, such as 0.3- to 1.0- μ m dense granules and smooth

endoplasmic reticulum in the apical cytoplasm, and 1:1 to 2:1 cytoplasmic to nuclear ratio (Figures 1E and 1F).

Single-cell sequencing was used to assess the transcriptome of SAE cells

brushed from three healthy individuals. After quality filtering, 157 samples, each representing a single cell, were selected for analysis (Figure 2). The single-cell transcriptomes were assessed by principal

Figure 2. Unsupervised clustering of the 157 single cells from human small airway. Small airway epithelium was recovered by bronchoscopy, treated with trypsin to generate single cells, and mechanically filtered to select 10- to 17- μ m cells. A total of 157 cells pooled from three healthy individuals were sequenced. (A) Principal component analysis (PCA) of 157 human small airway single-cell transcriptomes using the genes in Figure E11 as the input dataset. Four different clusters were identified by the unsupervised clustering: cluster 1, red dots, 47 single cells; cluster 2, blue dots, 28 single cells; cluster 3, green dots, 67 single cells; cluster 4, purple dots, 15 single cells. (B) Expression of basal cell marker KRT5 in the small airway single cells in PCA plots. (C) Expression of club cell marker SCGB1A1 in the small airway single cells in PCA plots. In B and C, blue represents the relative expression of markers shown above the figures. (A–C, top row) y-axis is principal component 1 (PC1), and x-axis is PC2. (A–C, bottom row) y-axis is PC1, and x-axis is PC3. (D) Boxplots show the gene expression of basal cell marker KRT5 (left) and club cell marker SCGB1A1 (right) in the unsupervised clusters 1–4. The colors in the boxplot coordinated with the colors of clusters 1–4 in A. TPM = transcripts per kilobase million.

component analysis. The unsupervised clustering identified four different cell populations with signature genes (Figure 2 and Table E2). Clusters 1, 3, and 4 highly expressed KRT5 (BC), SCGB1A1 (club), and DNAI1 (ciliated), respectively (Figures 2B–2D and Table E2). Immunofluorescence staining using the cluster 3 (club cells) signature genes (Table E2) MUC5B (Figure E2A), MUC1 (Figure E2B), and PIGR (Figure E2C) showed that each colocalized with SCGB1A1 in the normal SAE. The cluster 1 (BC) signature gene KRT15 colocalized with KRT5 (Figure E2E). Cluster 2 cells representing nonepithelial cells expressing CCL5 (C-C motif chemokine ligand 5), CD3g (CD3g molecule), and CCR5 (C-C motif chemokine receptor 5) were associated with fibroblasts, macrophages, and T cells, respectively (Table E2) (27–29).

From the principal component analysis, cluster 4 (ciliated cells) was clearly separated from the other clusters, whereas cluster 3 (club cells) and cluster 1 (BC) seemed to be closely connected (Figures 2A–2C). A subset population in cluster 3 (club cells) expressed the BC marker KRT5 (Figures 2B–2D), and a subset of cluster 1 (BC) cells expressed the club cell marker SCGB1A1 (Figures 2C and 2D), that is, there seemed to be a close relationship between BC and club cells. Consistent with this concept, assessment of the SAE in normal lung showed separate KRT5⁺ and SCGB1A1⁺ cells, but also cells expressing both KRT5 and SCGB1A1 (Figure 3A). Likewise, analysis of the cells brushed from the SAE revealed a subgroup of cells expressing both the BC marker KRT5 and the club cell marker SCGB1A1, consistent with the concept that there is a population of intermediate KRT5⁺SCGB1A1⁺ cells (Figure 3B). The KRT5⁺ only cells were small and flat and had a small cytoplasm/nucleus ratio, whereas the KRT5⁺SCGB1A1⁺ dual-positive cells had a spindle shape and a larger cytoplasm/nucleus ratio. Quantification of the proportions of the KRT5⁺ and SCGB1A1⁺ cells from the brushed SAE demonstrated: 5.5 ± 2.0% KRT5⁺SCGB1A1[−], 4.1 ± 1.1% KRT5⁺SCGB1A1⁺, and 24.2 ± 4.4% KRT5[−]SCGB1A1⁺ (Figure 3C). Of note, the three nonsmoker samples used in single-cell RNA sequencing were involved in the KRT5⁺/SCGB1A1⁺ quantification. In these three samples, 3.3 ± 1.0% cells

Figure 3. Additional evidence of the close relationship between human small airway epithelium (SAE) basal and club cells using *in vivo* staining. (A and B, top) Immunofluorescence staining of the normal SAE using the club cell marker SCGB1A1 (red) and basal cell marker KRT5 (green). (A and B, bottom) Relative negative controls for the staining above. (A) Lung sections with SAE. (B) SAE recovered by

were $KRT5^{+}SCGB1A1^{-}$, and $22.0 \pm 5.8\%$ cells were $KRT5^{-}SCGB1A1^{+}$.

To assess the ontogeny of club cells in the human SAE and the possible link between the basal and club cell transcriptomes, we used principal component gradient analysis (PCGA) of cluster 3 (club cells) and cluster 1 (BC) to describe the relative position of each cell within the multivariate spectrum of the basal and club cell transcriptomes. PCGA demonstrated a gradient of expression of both SCGB1A1 and KRT5 markers (Figure 4C, black arrows) with the single-cell gradient value determined by its position along this expression gradient. These expression gradients (Figures 4A and 4B, arrows) were parallel but had opposite directions, suggesting a continuous spectrum of cell types with BC at one extreme and club cells at the other. This suggests that either the BC is the precursor of the club cell or *vice versa*. However, it was not possible to determine the directionality of these cell type transitions from the transcriptome data alone. Most of the signature genes in clusters 1 and 3 were significantly correlated with the principal component gradient, including SCGB1A1 and KRT5 (Figures 4D and 4E). A total of 188 genes were significantly positively correlated, and 192 genes had a significant negative correlation (Table E3). Of these, 33 positively correlated genes had significantly increased expression in club cells and 16 negatively correlated genes had significantly increased expression in BC (Table E3).

Based on the single-cell transcriptome analysis, the *in vivo* immunohistochemistry data, and the knowledge that BC are the progenitor cells of human airway ciliated and mucus-producing cells (22, 23, 30), we hypothesized that BC in human SAE are the stem/progenitor cells of club cells. To prove this, highly purified human small airway BC ($99.0 \pm 1.1\%$ $KRT5^{+}SCGB1A1^{-}$) (Figures E3 and E4) were used to initiate ALI cultures to assess the ability of human small airway BC to differentiate into club cells. mRNA levels of the BC marker KRT5 mRNA dramatically decreased during BC

differentiation on the ALI (Figure 5A). In contrast, mRNA levels of SCGB1A1 increased significantly as the BC differentiated (Figure 5B). Expression of other BC signature genes (CCND2 [cyclin D2], FAT2 [FAT atypical cadherin 2], and PDGFA [platelet-derived growth factor subunit A]) decreased as BC differentiation progressed, whereas the expression of club cell signature genes, such as PIGR, MUC5B, and XBP1 (X-box-binding protein 1), increased (Figures 5A and 5B and Table E2).

Consistent with the mRNA data, immunofluorescence assessment of expression of proteins characteristic of BC (KRT5) decreased over time in ALI culture (Figures 5C and E5D). In contrast, the expression of proteins characteristic of club cells (SCGB1A1) appeared at Day 7 in ALI culture, and the proportion of $SCGB1A1^{+}$ cells increased during ALI culture (Figures 5C and E5D). Interestingly, most ($\sim 90\%$) of the $SCGB1A1^{+}$ cells were $KRT5^{+}$ at an early stage (Day 7) of BC differentiation, and $SCGB1A1^{+}KRT5^{+}$ cells were gradually replaced by $SCGB1A1^{+}KRT5^{-}$ cells in the middle (Day 14)/later (Day 28) stages in ALI culture (Figures E5D and E5E). In addition, the club cell markers MUC5B and SLPI expression dramatically increased over time along with SCGB1A1 (Figures 5D and 5E). The negative control subjects are shown in Figures E5A–E5C. Finally, TEM analysis of the ALI cultures demonstrated cells at Day 0 with characteristics of BC (Figures E4D–E4H), but at the end of ALI culture (Day 28), cells were identified with the classic morphologic characteristics of club cells (short microvilli, electron-dense granules) (Figures 5F–5M).

Besides differentiation into club cells, human SAE BC also differentiate into β -tubulin IV $^{+}$ ciliated cells and MUC5AC $^{+}$ mucin-secreting cells (Figures E6A and E6B). Of note, the ciliated cell marker β -tubulin IV weakly expressed in a subset of $SCGB1A1^{+}$ cells (Figure E6A), whereas most of the MUC5AC $^{+}$ mucin-secreting goblet cells were $SCGB1A1^{+}$ (Figure E6B).

To further study the transcriptome and biology of club cells, a supervised analysis

was conducted based on the known markers of different cell types in SAE (SCGB1A1 for club cells, KRT5 for BC, MUC5AC for mucin-producing cells, DNAI1 for ciliated cells) (Figure 6). The bias toward club and BC was caused by the use of a mechanical filter (10–17 μ m) to select the cells for analysis.

Prior knowledge regarding the biology of human SAE club cells is based on assessment of human lung sections using morphologic techniques to assess candidate genes (Table E1). The single-cell transcriptome analysis markedly increased the knowledge of club cell biology, including an interesting assortment of genes coding for transcription factors, receptors, transporters, secreted proteins, intracellular antioxidants, ion channels, and structural elements (Tables E4 and E5). Other than SCGB1A1, highly expressed club cell genes included SLPI (a potent antiserine protease inhibitor), PIGR (suggesting a role for club cells in IgA transport), many mitochondria-encoded genes (suggesting high metabolic activity), and many ribosomal genes. Interestingly, of the 12 genes previously reported to be expressed by human club cells, single-cell analysis confirmed the identity of only six (Table E1); it is unclear whether this relates to the sensitivity and/or specificity of the single-cell transcriptome methodology, or lack of cell specificity in prior studies.

Single-cell transcriptome analysis of genes expressed by club cells distinguished SAE club cells from basal, ciliated, and mucin-secreting cells (Table E6). In addition to SCGB1A1, there were seven genes uniquely expressed by club cells: C16orf89 (chromosome 16 open reading frame 89; thyroid development), CLDN22 (contributing to barrier function), GNPAT1 (glucosamine-phosphate N-acetyltransferase 1; glycan biosynthesis), UBL4A (ubiquitin-like 4A; an endoplasmic reticulum ubiquitin), CCDC68 (coiled-coil domain-containing 68; implicated as a tumor suppressor), EPSTI1 (epithelial stromal interaction 1; extracellular matrix

Figure 3. (Continued). brushing. Nuclei are stained with DAPI (blue). In addition to $KRT5^{+}SCGB1A1^{-}$ (basal cells) and $SCGB1A1^{+}KRT5^{-}$ (club cells), there are also $KRT5^{+}SCGB1A1^{+}$ putative “intermediate” cells in the lung sections and cells recovered by brushing. Scale bar, 20 μ m. (C) Immunofluorescence staining quantification of the percentage of $KRT5^{+}SCGB1A1^{-}$ basal cells, $KRT5^{+}SCGB1A1^{+}$ putative intermediate cells, and $SCGB1A1^{+}KRT5^{-}$ club cells in the cell populations recovered by brushing of the SAE. The data represent assessment of a total of 4,691 cells from nine healthy nonsmokers (mean $\pm$ SEM). *P* values are shown in the figure. n.s. = not significant.

Figure 4. Ordering of expression of small airway epithelium single cells using a semisupervised gradient analysis. Principal component (PC) analysis was performed on the 114 single cells from unsupervised clusters 1 and 3. Principal component analysis plots are colored by (A) KRT5 expression, (B) SCGB1A1 expression, and (C) pseudotime gradient values. The red arrows shown in A and B were fit using linear regression; specifically, each marker gene's expression was regressed on the first four PCs. The black arrow in C shows the direction of the pseudotime gradient, which was constructed by averaging the slopes of the red lines shown in A and B and assigning direction arbitrarily. The gradient analysis demonstrates that the KRT5⁺ and SCGB1A1⁺ cell types are related but cannot distinguish direction (i.e., KRT5⁺→SCGB1A1⁺ or SCGB1A1⁺→KRT5⁺). (D and E) Examples of genes significantly correlated with the gradient values. The blue curves show three-parameter sigmoid functions fit to each gene. (D) SCGB1A1⁺. (E) KRT5⁺. TPM = transcripts per kilobase million.

interactions), and OSBPL7 (oxysterol-binding protein-like 7; intracellular lipid receptor; Table E7). We further did PCGA of these seven genes on the unsupervised cluster 1 (BC) and cluster 3 (club cells). All these seven genes showed a similar trend to SCGB1A1 (Figure E7), and three (C16orf89, CLDN22, and GNPAT1) of these seven genes showed a significant correlation with

principal component gradient ($P < 0.05$; Table E3).

The most commonly expressed club cell transcription factors have broad roles in transcriptional regulation (HMGB1 [high-mobility group box 1], FOS [Fos proto-oncogene], BTF3 [basic transcription factor 3], EGR1 [early growth response 1], ATF4 [activating

transcription factor 4], and ZNF286A [zinc finger protein 286A]). Another group of transcription factors included broadly expressed transcription factors with defined roles, such as XBP1 (endoplasmic reticulum stress response, regulation of major histocompatibility class II genes), SOX4 (SRY-box 4; cell fate regulator), and NFE2L2 (nuclear factor, erythroid 2-like 2;

Figure 5. *In vitro* demonstration that basal cells (BCs) are the progenitor cells for club cells in the human small airway epithelium (SAE). Highly purified BCs isolated from the SAE of healthy individuals (see Figures E3B–E3F) were placed on air–liquid interface (ALI) on type IV collagen-coated transwells and cultured for 28 days. The BCs initiating the cultures were $99.0 \pm 1.1\%$ KRT5⁺ and 0% SCGB1A1⁺ (see Figures E4A and E4C). (A and B) TaqMan PCR assessment of SAE BC differentiation in ALI cultures over 28 days. BC and club cell characteristic genes were based on the single-cell transcriptome

antioxidant protein regulator; Table E8). Club cells expressed several transcription-related genes more commonly than the other three major SAE cell types, including FOS, XBP1, ATF4, HEY1 (hes-related family bHLH transcription factor with YRPW motif 1), and FOSB; these genes may play a role in club cell differentiation and/or unique biologic functions (Table E9).

Analysis of club cell-enriched pathways demonstrated high metabolic activity (oxidative phosphorylation, mitochondrial function, and mTOR [mammalian target of rapamycin] signaling), production of large quantities of secreted proteins (unfolded protein response), contribution to SAE structure (regulation of actin-based motility, epithelial adherens junction signaling, integrin signaling, and actin nucleation), hypoxia sensing, and xenobiotic metabolism (aryl hydrocarbon receptor signaling; Table E10). The single-cell transcriptome analysis of SCGB1A1⁺ “pure” human club cells demonstrated some heterogeneity (Figure E8), consistent with evidence of club cell heterogeneity in the murine lung (31–35).

Analysis of the transcriptome of the pure SCGB1A1⁺ club cells markedly added to the concept that human SAE club cells play a role in lung host defense (Tables 1 and E1). The club cell transcriptome identified several novel host defense-related club cell functions, including 1) chemoattractant cytokines (CXCL17, dendritic cells; CXCL1 and CXCL6, neutrophils); 2) complement components (complement component 3, complement regulatory protein, and complement decay accelerating factor); 3) genes with immune-related functions (β_2 -microglobulin, polymeric immunoglobulin Fc receptor, and toll-like receptors); 4) antibacterial proteins (lysozyme, lipocalin 2, and BPIFB1 [LPLUNC1]); 5) xenobiotic metabolism;

6) antiproteases, including alpha-1 antitrypsin and secretory leukocyte peptidase inhibitor; 7) barrier function; and 8) cell surface mucins.

Surprisingly, the transcriptome analysis suggested a potential role of club cells in the pathogenesis of lung-related human monogenic hereditary disorders (Table 2). The club cells express genes that, when mutated, are causative of cystic fibrosis, primary ciliary dyskinesia, hereditary bronchiectases, alpha-1 antitrypsin deficiency, Birt-Hogg-Dubé syndrome, cutis laxa, lymphangioleiomyomatosis, Loeys-Dietz syndrome, and fibrotic lung diseases.

It is known that the influenza virus infects club cells via sialic acid on the cell surface (36), but otherwise the club cells have not been implicated in the pathogenesis of human lung infections. Interestingly, single-cell transcriptome analysis identified that club cells express known viral receptors, including group C adenoviruses and respiratory pathogens associated with lung disease in immunocompromised individuals (Tables 3 and E11).

In addition to the SCGB1A1⁺KRT5[−]MUC5AC[−]DNAI1[−] “pure” club cells, consistent with immunohistochemistry staining of brushed SAE, an “intermediate cell” population expressing both marker genes (KRT5⁺SCGB1A1⁺MUC5AC[−]DNAI1[−]) was identified at the transcriptome level. The transcriptome analysis also identified SCGB1A1⁺MUC5AC⁺KRT5[−]DNAI1[−] cells, and immunofluorescence staining identified SCGB1A1⁺MUC5AC⁺ cells (Figure E9A). These cells may be a newly identified SAE cell type, or a cell in transition to “pure” club cells and/or MUC5AC⁺ “pure” mucin-producing cells. Consistent with the concept that these cells are a specific cell type, TEM analysis of the brushed SAE identified a unique cell population with mucin-secreting (goblet-

Figure 6. Analysis of small airway epithelium single-cell transcriptomes of healthy individuals. Venn diagram of the single cells based on the expression of cell markers of the major cell populations in human small airway epithelium. Shown are the numbers of single cells expressing the markers SCGB1A1 (club cells), KRT5 (basal cells), DNAI1 (ciliated cells), and MUC5AC (mucin-secreting cells). The numbers of “pure” and various combinations of MUC5AC⁺, SCGB1A1⁺, KRT5⁺, and DNAI1⁺ cells are indicated.

like) morphology, club cell-like electron-dense granules, and mucin-producing cell characteristic large light staining granules (Figure E9C). Finally, transcriptome analysis also identified SCGB1A1⁺DNAI1⁺ cells (Figure 6), suggesting the possibility of an ontologic link between club cells and ciliated cells, as observed in the murine small airways (18).

Based on the findings that human small airway BC are stem/progenitor cells for club cells, we evaluated the transcriptomic differentiation program from BC to club cells. Using the single-cell transcriptome data and cell type definitions based on expression of specific markers, the analysis identified KRT5⁺ only BC, SCGB1A1⁺ only club cells, and KRT5⁺SCGB1A1⁺ “intermediate cells” (Figures 3 and 6). Transcriptome analysis identified 35 genes with significantly higher expression in club cells compared with BC and 19 genes significantly enriched in BC compared with club cells (Figure 7 and Table E12). Further

Figure 5. (Continued). analysis of signature genes that characterize cluster 1 and 3 cells (see Table E2). (A) BC signature genes: KRT5, CCND2, FAT2, and PDGFA. (B) Club cell signature genes: SCGB1A1, PIGR, MUC5B, and XBP1. (C) Immunofluorescence staining of BC marker KRT5 (green) and club cell marker SCGB1A1 (red) in sections of the ALI culture at Days 0, 7, 14, and 28. Over time, the KRT5⁺ cells disappear, and the SCGB1A1⁺ cells appear. Nuclei are stained with DAPI. Scale bar, 20 μ m. (D) Immunofluorescence top staining of additional club cell marker MUC5B in the SAE derived from BC at Days 7, 14, and 28 in ALI culture. Top row, Day 7; middle row, Day 14; bottom row, Day 28. Left column, SCGB1A1 (green); middle column, MUC5B (red); right column, overlapping of SCGB1A1 and MUC5B. The appearance over time of the MUC5B⁺ cells tracks with SCGB1A1⁺ cells. Nuclei are stained with DAPI (blue). Scale bar, 20 μ m. (E) Similar to D, but with SCGB1A1 and SLPI. The relative negative control subjects for C–E are shown in Figures E5A–E5C. (F–M) Transmission electron microscopic assessment of the airway epithelium in ALI culture at Day 28. The Day 28 cultures included cells with the characteristic features of club cells, including microvilli (F–H and L), dense cytoplasmic granules (F–K), and abundant smooth endoplasmic reticulum (K and M). Scale bar, 1 μ m. SLPI = secretory leukocyte peptidase inhibitor.

Table 1. Host Defense Genes Expressed by Human Club Cells*

Category	Gene Symbol	Gene Name	% Cells [†]	Mean Expression (log ₂ TPM) [‡]
Antiinflammation Cytokines	SCGB1A1 [§]	Secretoglobulin, family 1A, member 1 (uteroglobin)	100.0	17.5
	CXCL17	Chemokine (C-X-C motif) ligand 17	94.6	12.0
	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, α)	73.0	8.9
	CXCL6	Chemokine (C-X-C motif) ligand 6	56.8	7.4
Immune function	C3	Complement component 3	81.1	9.9
	CD46	Complement regulatory protein	78.4	6.4
	CD55	Complement decay accelerating factor	81.1	7.2
	B2M	β -2-Microglobulin	94.6	12.3
	PIGR	Polymeric immunoglobulin receptor	83.8	13.1
	TLR5	Toll-like receptor 5	48.6	5.3
Antibacterial	LCN2	Lipocalin 2	86.5	10.6
	LYZ	Lysozyme	56.8	10.6
	BPIFB1	BPI fold containing family B, member 1	62.2	13.9
Xenobiotic metabolism	GSTP1	Glutathione S-transferase pi 1	86.5	11.7
	RDH10	Retinol dehydrogenase 10 (all-trans)	78.4	8.5
	AKR1C1	Aldo-keto reductase family 1, member C1	73.0	7.0
	DHRS9	Dehydrogenase/reductase (SDR family) member 9	67.6	7.9
	CYP2F1	Cytochrome P450, family 2, subfamily F, polypeptide 1	81.1	11.1
	MGST1	Microsomal glutathione S-transferase 1	83.8	9.8
	CYP4B1	Cytochrome P450, family 4, subfamily B, polypeptide 1	81.1	8.7
	POR [§]	P450 (cytochrome) oxidoreductase	64.9	5.9
	CYP2B6	Cytochrome P450, family 2, subfamily B, polypeptide 6	29.7	6.1
	GSTT1 [§]	Glutathione S-transferase theta 1	45.9	7.2
Antiprotease	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	24.3	4.8
	SLPI [§]	Secretory leukocyte peptidase inhibitor	94.6	15.6
	WFDC2	WAP four-disulfide core domain 2	91.9	13.3
	SERPINF3	Serpin peptidase inhibitor, clade B (ovalbumin), member 3	78.4	13.5
	CST3	Cystatin C	83.8	6.9
Barrier function	CTNNA1	Catenin (cadherin-associated protein), β 1, 88 kDa	94.6	7.5
	CXADR	Coxsackie virus and adenovirus receptor	78.4	5.8
	CLDN1	Claudin 1	43.2	5.2
	CLDN4	Claudin 4	56.8	6.1
	OCLN	Occludin	45.9	7.4
	TFF3	Trefoil factor 3	73.0	10.4
	MUC1	Mucin 1, cell surface associated	83.8	8.7
	MUC5B	Mucin 5B, oligomeric mucus/gel-forming	54.1	6.3
	ITGAV	Integrin, α V	29.7	5.3
	ITGB1	Integrin, β 1	73.0	6.2

Definition of abbreviation: TPM = transcripts per kilobase million.

*Genes with defense-related function expressed in SCGB1A1⁺KRT5⁻DNAI1⁻MUC5AC⁻ single cells with mean log₂TPM >1 and expressed in $\geq$ 20% of the club cells.

[†]Percentage of club cells with gene expression (log₂TPM >1) out of 37 total.

[‡]Mean expression among the club cells expressing that gene (i.e., mean expression for all 37 club cells/% club cells expressing that gene).

[§]Defense-related genes previously associated with human club cells (see Table E1).

evidence of an ontologic link between KRT5⁺ and SCGB1A1⁺ cells came from transcriptome comparison of pure KRT5⁺ cells, putative “intermediate” KRT5⁺ SCGB1A1⁺ cells, and pure SCGB1A1⁺ cells. A comparison of the genes expressed by pure KRT5⁺ cells versus pure SCGB1A1⁺ cells demonstrated that, within

each functional category, the two cell types had significantly different levels of expression (Figure 7A and Table E12). KRT5⁺SCGB1A1⁺ cells expressed “intermediate” levels of the KRT5⁺ BC “signature genes” (Figure 7B). Likewise, KRT5⁺SCGB1A1⁺ cell expression of SCGB1A1⁺ club cell “signature genes”

was intermediate between the KRT5⁺ SCGB1A1⁻ BC (low expression) and KRT5⁻SCGB1A1⁺ club cells (high expression) (Figure 7C). Finally, analysis of the transcriptome of KRT5⁺SCGB1A1⁺ “intermediate cell” genes identified the genes expressed by both basal and club cells (Table E13).

Table 2. Club Cell Expression of Genes Associated with Monogenic Hereditary Lung Disorders*

Disease Category	Disease	Gene Symbol	Gene Name	% Cells [†]	Mean Expression (log TPM) [‡]
Bronchiectasis	Cystic fibrosis	CFTR	Cystic fibrosis transmembrane conductance regulator	32.4	5.6
	Primary ciliary dyskinesia	OFD1	Oral-facial-digital syndrome 1	67.6	6.5
		SPAG1	Sperm associated antigen 1	27.0	5.6
		C21orf59	Chromosome 21 open reading frame 59	35.1	3.9
		DNAH5	Dynein, axonemal, heavy chain 5	29.7	4.3
	Other bronchiectasis	SCNN1A	Sodium channel, non-voltage-gated 1 α subunit	89.2	5.8
		SCNN1B	Sodium channel, non-voltage-gated 1 β subunit	62.2	5.5
SCNN1G		Sodium channel, non-voltage-gated 1 γ subunit	35.1	4.5	
Cystic	Alpha-1 antitrypsin deficiency	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antitrypsin), member 1	24.3	4.8
	Birt-Hogg-Dubé syndrome	FLCN	Folliculin	51.4	2.9
	Cutis laxa	LTBP4	Latent transforming growth factor β -binding protein 4	32.4	4.7
	Lymphangioleiomyomatosis	TSC1	Tuberous sclerosis 1	43.2	4.6
		TSC2	Tuberous sclerosis 2	37.8	6.9
	Loeys-Dietz syndrome	SMAD3	SMAD family member 3	48.6	5.4
TGFBR1		Transforming growth factor, β receptor 1	24.3	5.4	
Fibrosis	Familial fibrosis	MUC5B	Mucin 5B, oligomeric mucus/gel-forming	54.1	6.3
	Fibrosis and hypothyroidism	NKX2-1	NK2 homeobox 1	29.7	5.3
	Hermansky-Pudlak syndrome	AP3B1	Adaptor-related protein complex 3, β 1 subunit	56.8	4.4
		CD63	CD63 molecule	81.1	10.1
		HPS3	Hermansky-Pudlak syndrome 3	24.3	5.1
		HPS4	Hermansky-Pudlak syndrome 4	24.3	4.5
		HPS5	Hermansky-Pudlak syndrome 5	27.0	5.7

Definition of abbreviation: TPM = transcripts per kilobase million.

*Known monogenic lung disorders listed by type of lung disease associated with mutations of the listed genes; see Tilley and coworkers (51) for details.

[†]Percentage of club cells with gene expressed ($\log_2$ TPM >1) out of 37 total.

[‡]Mean $\log_2$ TPM gene expression in subset of 37 SCGB1A1⁺KRT5⁻DNAI1⁻MUC5AC⁻ single cells with $\log_2$ TPM >1 (mean expression for all 37 club cells/% club cells expressing that gene).

Discussion

The epithelium monolayer covering the small airways is comprised of ciliated, mucin-secreting, basal and club cells (2–5). In contrast to the murine lung, where club cells function as stem/progenitor cells for the differentiated ciliated and mucin-secreting cells (16–20) and are known to contribute to lung host defense (15, 20), the understanding of the biology of club cells in the human SAE is based only on a few morphology-based candidate gene studies, and little is known about the ontogeny of small airway club cells in humans. Taking advantage of the ability to sample club cells from the SAE of healthy nonsmokers by bronchoscopy and brushing, single-cell RNA sequencing of the brushed cells

enabled identification of the lineage of human club cells and characterization of the biologic function of these cells.

Club cells have a characteristic apical dome shape, microvilli, dense cytoplasmic granules, extensive endoplasmic reticulum and mitochondria, and uniquely express SCGB1A1 (3, 7–9, 11, 12). Taking advantage of SCGB1A1 as a club cell-specific marker and KRT5 as a BC-specific marker (4, 7, 21–23), we used single-cell transcriptome analysis to characterize human SAE club cells. In addition to characterizing transcription factors, receptors, transporters, and ion channels that play a role in human club cell function, the single-cell analysis uncovered a markedly expanded role for the human club cells in host defense. In

addition to SCGB1A1, which has anti-inflammatory properties (9, 11, 12), club cells express cytokines that attract dendritic cells and neutrophils, a variety of mediators with immune functions, including complement components, the polymeric immunoglobulin receptor, and several toll-like receptors, as well as genes encoding proteins with antibacterial function. As in murine club cells (37), human club cells express genes that function in xenobiotic metabolism. It has been known from immunohistochemical studies that human club cells express the secretory leukocyte peptidase inhibitor (38), but, surprisingly, transcriptome analysis demonstrated that human club cells also express alpha-1 antitrypsin, the most important antineutrophil elastase inhibitor (39).

Table 3. Viral Receptor Genes Expressed in Human Small Airway Epithelium Club Cells*

Gene Symbol	Gene Name	Viruses Causing Human Lung Infection	% Cells [†]	Mean Expression (TPM) [‡]
CD55	CD55 molecule, (Cromer blood group)	Coxsackie viruses A21, B1, B3; human echoviruses 6, 7, 11, 12, 20, 21	81.1	7.2
CD46	CD46 molecule	Adenovirus subgroup B2, measles virus, human herpes virus 6	78.4	6.4
CXADR	Coxsackie virus and adenovirus receptor	Adenovirus type C, coxsackievirus B1-B6	78.4	5.8
ITGAV	Integrin subunit α V	Adenovirus type C; coxsackievirus A9, B1; human herpes virus 8; herpes simplex 1; human parechovirus 1	29.7	5.3
ITGB1	Integrin subunit β 1	Human echoviruses 1,8; cytomegalovirus; Epstein-Barr virus; human parvovirus B19	73.0	6.2
ITGB5	Integrin subunit β 5	Adenovirus type C	29.7	4.7
NECTIN1	Nectin cell adhesion molecule 1	Herpes simplex virus 1, 2	21.6	4.5
NECTIN2	Nectin cell adhesion molecule 2	Herpes simplex virus 1	21.6	5.4
NECTIN4	Nectin cell adhesion molecule 4	Measles virus	64.9	6.5
DPP4	Dipeptidyl-peptidase 4	Human coronavirus (Erasmus Medical Center)	54.1	5.6
TNFRSF14	Tumor necrosis factor receptor superfamily, member 14	Herpes simplex virus 1, 2	48.6	5.2
SIVA1	SIVA1 apoptosis-inducing factor	Coxsackievirus B3	35.1	6.7
IDE	Insulin-degrading enzyme	Varicella-zoster virus	29.7	4.9
EFNB2	Ephrin-B2	Hendra virus; Nipah virus	24.3	4.0

Definition of abbreviation: TPM = transcripts per kilobase million.

*Viral receptor genes expressed in SCGB1A1⁺KRT5⁺DNAI1⁺MUC5AC⁺ small airway epithelium club cells; see Table E11 for further details, including references to the literature regarding the specific virus receptors and the links of these viruses to human disease.

[†]Percent of club cells with gene expressed ($\log_2$ TPM > 1) out of 37 total.

[‡]Mean $\log_2$ TPM gene expression in subset of 37 SCGB1A1⁺KRT5⁺DNAI1⁺MUC5AC⁺ single cells with $\log_2$ TPM > 1.

Human club cells express several genes that function in cell–cell and cell–matrix interactions and express cell surface mucins, suggesting that the club cell plays an important role in SAE barrier function.

Transcriptome analysis demonstrated that club cells express several ion channel genes, including epithelial Na⁺ channel, voltage-dependent anion channels, Cl[−] channels, and water channels, suggesting that club cells might actively regulate ionic balance across the epithelium. Analysis of the transcription factors expressed by the club cells identified HES1 (hes family bHLH transcription factor 1), the downstream effector of the notch pathway, as one of the most highly expressed transcription factors (40). Other transcription factors highly expressed by club cells include XBP1, a canonical transcription factor that participates in endoplasmic reticulum stress (41), and CREB3L1 (cAMP-responsive element-binding protein 3–like 1), a transcription factor induced by ER stress that contributes to the unfolded protein response, extracellular matrix production, and antiviral defense (42, 43). Interestingly, the

“pure” club cells, which only express SCGB1A1 and are negative for other major epithelial cell–type markers, have a unique transcriptome expressing C16orf89, CLDN22, GNPAT1, UBL4A, CCDC68, EPSTI1, and OSBPL7. However, these genes are only expressed in the subset of the “pure” club cells, suggesting the heterogeneity of the club cells. This concept is also supported in the PCGA, where only part of these genes showed a significant correlation.

Human club cells express genes implicated in most of the human lung hereditary disorders. Included among these genes are the causative genes for cystic fibrosis (CFTR) and alpha-1 antitrypsin deficiency (SERPINA1), and other rare, lung-related hereditary disorders. Because the SAE makes up most of the surface area of the human tracheobronchial tree (1), club cells compose 20% of the SAE (5), and the levels of expression of these genes in the club cells are substantial, if there are mutations in these genes, club cells may possibly contribute substantially to the pathogenesis of these disorders. Relative functional study of these genes in club cells remains to be defined in future studies.

Also of interest, the transcriptome data demonstrated that the human club cells express receptors for several respiratory tract viruses, particularly those associated with increased risk for infection in conditions associated with decreased host response. Consistent with the concept that human small airway club cells might be involved in the pathogenesis of acquired human lung disorders, Xu and coworkers (44) used single-cell transcriptome analysis of lung biopsy samples to assess the pathogenesis of idiopathic pulmonary fibrosis, identifying a goblet/club cell population that is altered in this disorder.

Although the ultrastructural features of human and mouse club cells are similar (45), the localization of club cells in the human and mouse airway epithelium is different. In the murine lung, club cells line all of the conducting airways, including nasal (46), whereas in humans, club cells are only present in the small airways (5). In the mouse, club cells function as the stem/progenitor cells of ciliated cells (18) and mucin-secreting (“goblet”) cells (16, 17, 19). Also, club cells can dedifferentiate into p63⁺ BC in damaged lung parenchyma (47). In the mouse trachea, club cells are

Figure 7. Cell type-specific genes that characterize the differentiation program from basal cells (BC) to club cells. Comparison of gene expression of pure club cells versus pure BC to identify club cell and BC “signature” genes. (A) Volcano plot showing differential gene expression between the 37 KRT5[−]SCGB1A1⁺ club cells and 29 KRT5⁺SCGB1A1[−] BC. Genes with significantly higher expression in BC are indicated in red, and genes significantly higher in club cells are indicated in blue. See Table E12 for a list of significant genes. (B and C) BC, red; intermediate cells, purple; club cells, blue. (B) Box plot examples of BC signature genes in SCGB1A1[−]KRT5⁺ BC, KRT5⁺SCGB1A1⁺ intermediate cells, and KRT5[−]SCGB1A1⁺ club cells. (C) Box plot examples of club cell signature genes in SCGB1A1[−]KRT5⁺ BC, KRT5⁺SCGB1A1⁺ intermediate cells, and KRT5[−]SCGB1A1⁺ club cells.

the transiently amplifying cell population that increase the capacity of self-renewal and multilineage differentiation only during injury (18). Instead of club cells, mouse tracheal BC function as stem/progenitor cell population to self-renew and give rise to club and ciliated cells (18).

BC represent 5–10% of the cells in the human SAE, and club cells represent approximately 20% (5, 48). To identify the relationship between human SAE BC and club cells *in vivo*, inspired, in part, by the Monocle Pseudotemporal Ordering Algorithm (49), we used PCGA to link high-dimensional transcriptional profiles and key cell type markers of basal and club cells. The data revealed a novel expression gradient of both the club cell marker SCGB1A1 and the BC marker KRT5 in these cell populations. In addition, using the transcriptome data and immunohistochemical staining of brushed human SAE, we identified an intermediate cell population positive for both the BC marker KRT5 and the club cell marker SCGB1A1. The gradient expression of both markers and the presence of intermediate cell types bridging BC and club cells suggests a transition between the two cell types and provides an important clue to the lineage of these cell types in the human SAE. This finding was verified using ALI *in vitro*, demonstrating that pure human small airway KRT5⁺SCGB1A1[−] BC differentiate into typical club cells. Quantification of the SCGB1A1⁺ cells in ALI demonstrated that the percentage of SCGB1A1⁺ cells increased during BC differentiation, reaching 21% at Day 28, similar to the proportion of SCGB1A1⁺

club cells (~20–25%) *in vivo* (5). The percentage of KRT5⁺ in ALI culture at Day 28 (35.8%) was higher than *in vivo* (~5–10%) (48), suggesting the complexity of the airway epithelium *in vivo*. Of note, the proportion of SCGB1A1⁺KRT5⁺ “intermediate” cells decreased, whereas the proportion of SCGB1A1⁺KRT5[−] cells increased in the middle/late time (Days 14 and 28) of ALI culture. SCGB1A1⁺KRT5⁺ “intermediate” cells may not be the obligatory step, but definitely are one of the critical intermediate cell populations during human BC differentiation into club cells. It is possible that SCGB1A1⁺KRT5⁺ cells may exclusively differentiate to club cells with greater proportion and at a greater rate. A small subset of SCGB1A1⁺ cells were not KRT5⁺ at Day 7 of ALI culture. These cells might be the “early mature club cells” generated from KRT5⁺SCGB1A1⁺ cells before Day 7 of ALI culture, or another club cell subpopulation generated from KRT5⁺ BC through another pathway not expressing KRT5. Also, we observed that KRT5⁺ SAE BC could differentiate into other cell types (e.g., ciliated cells and mucin-secreting goblet cells) other than club cells, indicating that BC potential is heterogeneous, with multiple capabilities for differentiation.

Although BC are the progenitor cell of club cells in the human SAE, it is also possible that human club cells can function as stem/progenitor cells as in the murine lung. Immunohistochemical staining has shown that club cells contribute significantly to the proliferating population of epithelial cells in the human SAE (5), suggesting the human club cells likely can self-renew. Based on the single-cell PCGA, the

direction of the gradient marker gene expressions is arbitrary, and it is possible that human club cells could differentiate back to BC. Lineage tracer studies in mouse support the hypothesis that SCGB1A1⁺ secretory cells can dedifferentiate into functional basal/stem cells (47, 50). Interestingly, several more “intermediate” cell populations were identified by single-cell RNA-seq (e.g., a subset of SCGB1A1⁺ cells are positive for the ciliated cell marker DNAIL1), suggesting the possibility that, like in the mouse (18), human club cells might also differentiate into ciliated cells and *vice versa*. Furthermore, transcriptome analysis suggests that human club cell might have an ontologic relationship with mucin-secreting cells based on the coexpression of club cell marker SCGB1A1 and mucin-secreting goblet cell marker MUC5AC. Consistent with this analysis, TEM of cells brushed from the human SAE identified a cell with both large, round secretory and electron-dense granules. Because goblet cell hyperplasia is one of the common features for chronic obstructive pulmonary disease and many other lung diseases, in the stress of smoking or other factors, the mucin-secreting-like club cells may serve as a newly identified or the “intermediate” precursor to differentiate to mature disease-related goblet cells. ■

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

Acknowledgment: The authors thank N. Mohamed for help in preparing this manuscript and the Electron Microscopy & Histology Core of Weill Cornell Medicine for electron microscopic services.

References

- Weibel ER. Morphometry of the human lung. New York: Academic Press; 1963.
- Crystal RG, Randell SH, Engelhardt JF, Voynow J, Sunday ME. Airway epithelial cells: current concepts and challenges. *Proc Am Thorac Soc* 2008;5:772–777.
- Lumsden AB, McLean A, Lamb D. Goblet and Clara cells of human distal airways: evidence for smoking induced changes in their numbers. *Thorax* 1984;39:844–849.
- Rock JR, Randell SH, Hogan BL. Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. *Dis Model Mech* 2010;3:545–556.
- Boers JE, Ambergen AW, Thunnissen FB. Number and proliferation of Clara cells in normal human airway epithelium. *Am J Respir Crit Care Med* 1999;159:1585–1591.
- Clara M. Zur Histobiologie des Bronchialepithels [On the histobiology of the bronchial epithelium]. *Z Mikrosk Anat Forsch* 1937;41:321–347.
- Singh G, Katyal SL. Clara cells and Clara cell 10 kD protein (CC10). *Am J Respir Cell Mol Biol* 1997;17:141–143.
- Rokicki W, Rokicki M, Wojtacha J, Dzelijli A. The role and importance of club cells (Clara cells) in the pathogenesis of some respiratory diseases. *Kardiochir Torakochirurgia Pol* 2016;13:26–30.
- Widdicombe JG, Pack RJ. The Clara cell. *Eur J Respir Dis* 1982;63:202–220.
- Umland TC, Swaminathan S, Singh G, Warty V, Furey W, Pletcher J, et al. Structure of a human Clara cell phospholipid-binding protein-ligand complex at 1.9 Å resolution. *Nat Struct Biol* 1994;1:538–545.
- Gamez AS, Gras D, Petit A, Knabe L, Molinari N, Vachier I, et al. Supplementing defect in club cell secretory protein attenuates airway inflammation in COPD. *Chest* 2015;147:1467–1476.
- Tokita E, Tanabe T, Asano K, Suzuki H, Rubin BK. Club cell 10-kDa protein attenuates airway mucus hypersecretion and inflammation. *Eur Respir J* 2014;44:1002–1010.

13. Jones KG, Holland JF, Foureman GL, Bend JR, Fouts JR. Xenobiotic metabolism in Clara cells and alveolar type II cells isolated from lungs of rats treated with beta-naphthoflavone. *J Pharmacol Exp Ther* 1983;225:316–319.
14. Devereux TR. Alveolar type II and Clara cells: isolation and xenobiotic metabolism. *Environ Health Perspect* 1984;56:95–101.
15. Wang SZ, Rosenberger CL, Bao YX, Stark JM, Harrod KS. Clara cell secretory protein modulates lung inflammatory and immune responses to respiratory syncytial virus infection. *J Immunol* 2003;171:1051–1060.
16. Park KS, Korfhagen TR, Bruno MD, Kitzmiller JA, Wan H, Wert SE, et al. SPDEF regulates goblet cell hyperplasia in the airway epithelium. *J Clin Invest* 2007;117:978–988.
17. Chen G, Korfhagen TR, Xu Y, Kitzmiller J, Wert SE, Maeda Y, et al. SPDEF is required for mouse pulmonary goblet cell differentiation and regulates a network of genes associated with mucus production. *J Clin Invest* 2009;119:2914–2924.
18. Rawlins EL, Okubo T, Xue Y, Brass DM, Auten RL, Hasegawa H, et al. The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. *Cell Stem Cell* 2009;4:525–534.
19. Tompkins DH, Besnard V, Lange AW, Wert SE, Keiser AR, Smith AN, et al. Sox2 is required for maintenance and differentiation of bronchiolar Clara, ciliated, and goblet cells. *PLoS One* 2009;4:e8248.
20. Reynolds SD, Malkinson AM. Clara cell: progenitor for the bronchiolar epithelium. *Int J Biochem Cell Biol* 2010;42:1–4.
21. Crystal RG. Airway basal cells. The “smoking gun” of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2014;190:1355–1362.
22. Hackett NR, Shaykhiev R, Walters MS, Wang R, Zwick RK, Ferris B, et al. The human airway epithelial basal cell transcriptome. *PLoS One* 2011;6:e18378.
23. Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, et al. Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc Natl Acad Sci USA* 2009;106:12771–12775.
24. Hackett NR, Butler MW, Shaykhiev R, Salit J, Omberg L, Rodriguez-Flores JL, et al. RNA-Seq quantification of the human small airway epithelium transcriptome. *BMC Genomics* 2012;13:82.
25. Harvey BG, Heguy A, Leopold PL, Carolan BJ, Ferris B, Crystal RG. Modification of gene expression of the small airway epithelium in response to cigarette smoking. *J Mol Med (Berl)* 2007;85:39–53.
26. Hovenberg HW, Davies JR, Carlstedt I. Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. *Biochem J* 1996;318:319–324.
27. Allison TJ, Winter CC, Fournié JJ, Bonneville M, Garboczi DN. Structure of a human gammadelta T-cell antigen receptor. *Nature* 2001;411:820–824.
28. Eyman D, Damodarasamy M, Plymate SR, Reed MJ. CCL5 secreted by senescent aged fibroblasts induces proliferation of prostate epithelial cells and expression of genes that modulate angiogenesis. *J Cell Physiol* 2009;220:376–381.
29. Tyner JW, Uchida O, Kajiwarra N, Kim EY, Patel AC, O’Sullivan MP, et al. CCL5-CCR5 interaction provides antiapoptotic signals for macrophage survival during viral infection. *Nat Med* 2005;11:1180–1187.
30. Gomi K, Arbelaez V, Crystal RG, Walters MS. Activation of NOTCH1 or NOTCH3 signaling skews human airway basal cell differentiation toward a secretory pathway. *PLoS One* 2015;10:e0116507.
31. Reynolds SD, Giangreco A, Power JH, Stripp BR. Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. *Am J Pathol* 2000;156:269–278.
32. Hong KU, Reynolds SD, Giangreco A, Hurley CM, Stripp BR. Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. *Am J Respir Cell Mol Biol* 2001;24:671–681.
33. De Proost I, Pintelon I, Brouns I, Kroese AB, Riccardi D, Kemp PJ, et al. Functional live cell imaging of the pulmonary neuroepithelial body microenvironment. *Am J Respir Cell Mol Biol* 2008;39:180–189.
34. Guha A, Vasconcelos M, Zhao R, Gower AC, Rajagopal J, Cardoso WV. Analysis of Notch signaling-dependent gene expression in developing airways reveals diversity of Clara cells. *PLoS One* 2014;9:e88848.
35. Majka SM, Beutz MA, Hagen M, Izzo AA, Voelkel N, Helm KM. Identification of novel resident pulmonary stem cells: form and function of the lung side population. *Stem Cells* 2005;23:1073–1081.
36. Heaton NS, Langlois RA, Sachs D, Lim JK, Palese P, tenOever BR. Long-term survival of influenza virus infected club cells drives immunopathology. *J Exp Med* 2014;211:1707–1714.
37. Fanucchi MV, Murphy ME, Buckpitt AR, Philpot RM, Plopper CG. Pulmonary cytochrome P450 monooxygenase and Clara cell differentiation in mice. *Am J Respir Cell Mol Biol* 1997;17:302–314.
38. De Water R, Willems LN, Van Muijen GN, Franken C, Fransen JA, Dijkman JH, et al. Ultrastructural localization of bronchial antileukoprotease in central and peripheral human airways by a gold-labeling technique using monoclonal antibodies. *Am Rev Respir Dis* 1986;133:882–890.
39. Crystal RG. Augmentation treatment for α 1 antitrypsin deficiency. *Lancet* 2015;386:318–320.
40. Kageyama R, Ohtsuka T, Kobayashi T. The Hes gene family: repressors and oscillators that orchestrate embryogenesis. *Development* 2007;134:1243–1251.
41. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 2001;107:881–891.
42. Denard B, Seemann J, Chen Q, Gay A, Huang H, Chen Y, et al. The membrane-bound transcription factor CREB3L1 is activated in response to virus infection to inhibit proliferation of virus-infected cells. *Cell Host Microbe* 2011;10:65–74.
43. Vellanki RN, Zhang L, Volchuk A. OASIS/CREB3L1 is induced by endoplasmic reticulum stress in human glioma cell lines and contributes to the unfolded protein response, extracellular matrix production and cell migration. *PLoS One* 2013;8:e54060.
44. Xu Y, Mizuno T, Sridharan A, Du Y, Guo M, Tang J, et al. Single-cell RNA sequencing identifies diverse roles of epithelial cells in idiopathic pulmonary fibrosis. *JCI Insight* 2016;1:e90558.
45. Smith MN, Greenberg SD, Spjut HJ. The Clara cell: a comparative ultrastructural study in mammals. *Am J Anat* 1979;155:15–30.
46. Pack RJ, Al-Ugaily LH, Morris G. The cells of the tracheobronchial epithelium of the mouse: a quantitative light and electron microscope study. *J Anat* 1981;132:71–84.
47. Zheng D, Yin L, Chen J. Evidence for Scgb1a1(+) cells in the generation of p63(+) cells in the damaged lung parenchyma. *Am J Respir Cell Mol Biol* 2014;50:595–604.
48. Boers JE, Ambergen AW, Thunnissen FB. Number and proliferation of basal and parabasal cells in normal human airway epithelium. *Am J Respir Crit Care Med* 1998;157:2000–2006.
49. Trapnell C, Cacchiarelli D, Grimsby J, Pokharel P, Li S, Morse M, et al. The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat Biotechnol* 2014;32:381–386.
50. Tata PR, Mou H, Pardo-Saganta A, Zhao R, Prabhu M, Law BM, et al. Dedifferentiation of committed epithelial cells into stem cells in vivo. *Nature* 2013;503:218–223.
51. Tilley AE, Staudt MR, Salit J, Van de Graaf B, Strulovici-Barel Y, Kaner RJ, et al. Cigarette smoking induces changes in airway epithelial expression of genes associated with monogenic lung disorders. *Am J Respir Crit Care Med* 2016;193:215–217.