ORIGINAL ARTICLE

# Localization of Secretory Mucins MUC5AC and MUC5B in Normal/Healthy Human Airways

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## Abstract

**Rationale:** MUC5AC and MUC5B are the predominant gel-forming mucins in the mucus layer of human airways. Each mucin has distinct functions and site-specific expression. However, the regional distribution of expression and cell types that secrete each mucin in normal/healthy human airways are not fully understood.

**Objectives:** To characterize the regional distribution of MUC5B and MUC5AC in normal/healthy human airways and assess which cell types produce these mucins, referenced to the club cell secretory protein (CCSP).

**Methods:** Multiple airway regions from 16 nonsmoker lungs without a history of lung disease were studied. MUC5AC, MUC5B, and CCSP expression/colocalization were assessed by RNA *in situ* hybridization and immunohistochemistry in five lungs with histologically healthy airways. Droplet digital PCR and cell cultures were performed for absolute quantification of *MUC5AC/5B* ratios and protein secretion, respectively.

**Measurements and Main Results:** Submucosal glands expressed MUC5B, but not MUC5AC. However, MUC5B was also extensively expressed in superficial epithelia throughout the airways except for the terminal bronchioles. Morphometric calculations revealed that the distal airway superficial epithelium was the predominant site for MUC5B expression, whereas MUC5AC expression was concentrated in proximal, cartilaginous airways. RNA *in situ* hybridization revealed *MUC5AC* and *MUC5B* were colocalized with *CCSP*-positive secretory cells in proximal superficial epithelia, whereas *MUC5B* and *CCSP*-copositive cells dominated distal regions.

**Conclusions:** In normal/healthy human airways, MUC5B is the dominant secretory mucin in the superficial epithelium and glands, with distal airways being a major site of expression. MUC5B and MUC5AC expression is a property of CCSP-positive secretory cells in superficial airway epithelia.

**Keywords:** airway mucins; MUC5AC; MUC5B; club cells; distal airways

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## At a Glance Commentary

**Scientific Knowledge on the Subject:** MUC5AC and MUC5B are the major gel-forming mucins in the mucus layer of human airways. Each mucin has a distinct regional expression. However, a systematic study of the site- and cell-specific expression of MUC5B and MUC5AC in normal/healthy human airways is not available.

**What This Study Adds to the Field:** This study provides a comprehensive description of secretory mucin expression in the normal/healthy human lung essential for understanding how abnormal regulation of secretory mucin expression contributes to the pathogenesis of mucoobstructive lung diseases. RNA *in situ* hybridization and immunohistochemistry characterized regional MUC5AC and MUC5B expression and cell types expressing these mucins along the proximal–distal axis of the normal/healthy human lung. MUC5B is the dominant secretory mucin in not only submucosal glands but also superficial airway epithelia. The distal airways are a major site of MUC5B expression and constitute a source of MUC5B production equivalent in importance to submucosal glands. The predominant region for MUC5AC production is the proximal (cartilaginous) airway superficial epithelium. MUC5B and MUC5AC production are properties of club cell secretory protein–expressing cells in both proximal and distal superficial airway epithelia.

Mucociliary clearance is a critical innate defense system for maintenance of lung health. Failed mucus clearance is a common feature of mucoobstructive lung diseases, yet how abnormalities in mucus properties produce intrapulmonary mucus accumulation remains unclear. Part of this uncertainty relates to the absence of a comprehensive formulation that describes all elements of mucus transport in health.

MUC5AC and MUC5B are the predominant gel-forming mucins in human airways (1–4). The classic paradigm describes MUC5AC secretion in human airways as a feature of superficial epithelial goblet cells, whereas MUC5B is predominantly secreted from submucosal glands (SMG) (2, 5–8). However, previous data generated from mice have demonstrated that the superficial epithelial secretory club cells in large and small airways produce Muc5b, which is rapidly secreted in the absence of Alcian Blue and periodic acid–Schiff (AB-PAS)-definable mucin granules or goblet cell morphology (9–13). Moreover, studies with Muc5b knockout mice demonstrated that Muc5b was required for normal lung defense and mucociliary clearance (14), despite the fact that SMG in the mice are restricted to the proximal trachea (15).

Recent studies of normal and diseased human lungs suggest the murine paradigm may also be pertinent to the human lung. Most mucoobstructive diseases (e.g., cystic fibrosis and chronic obstructive pulmonary disease) are associated with small airway mucus plugs that are composed of MUC5B and MUC5AC (16–18). Importantly, recent studies of normal and diseased tissues have established the presence of MUC5B expression in superficial epithelia, suggesting distal airway superficial epithelia may locally secrete the MUC5B associated with plugging (19–21). Of particular relevance to small airway MUC5B expression is the discovery of the link between MUC5B overexpression in peripheral airways and idiopathic pulmonary fibrosis (IPF) (22, 23).

These findings led us to perform a comprehensive study of region- and cell-specific expression of MUC5B and MUC5AC with an emphasis on comparisons of large airways with superficial epithelia and SMG versus small airways lined exclusively with superficial epithelia. To achieve this aim, multiple complementary approaches, including RNA *in situ* hybridization (ISH), immunohistochemistry, droplet digital PCR (ddPCR) quantification, and primary cell cultures, were used to quantitate the regional distribution of MUC5B and MUC5AC expression along the proximal–distal axis of the normal/healthy human lung. Based on murine data (10–14), we also explored the hypothesis that club cell secretory protein (CCSP) (also termed secretoglobin 1A member 1)-expressing secretory cells produce MUC5B, and MUC5AC, in human superficial airway epithelia. Some of the results of this study have been previously reported in the form of abstracts (24, 25).

## Methods

For further details on the applied methods, *see* the online supplement.

### Subjects and Tissue Collection

Lungs that provided airways for study were obtained from "normal" subjects, defined as no history of cigarette smoking or lung disease, who had been maintained on mechanical ventilation less than or equal to 7 days as potential lung transplant donors. Sixteen consecutive lungs (May 2016–March 2018) that were not eligible for transplant were studied. Table E1 in the online supplement shows demographics of the subjects studied. The protocol for lung dissection was approved by University of North Carolina Institutional Review Board. *MUC5B* promoter variant rs35705950 genotypes were determined using TaqMan (Life Technologies) genotyping (26).

### Tissue Preparation, Immunohistochemistry, and RNA ISH

Excised lungs, obtained from 10 of the 16 normal subjects studied, were dissected to obtain tracheas, primary bronchi, segmental bronchi, and lung parenchyma containing distal bronchi and bronchioles (*see* Figure E1). Tissue specimens were fixed, 5-μm sections cut, and serial sections stained by hematoxylin and eosin (H&E) and AB-PAS. Because mechanical ventilation can induce goblet cell metaplasia and/or hyperplasia (GMH) in lungs of normal subjects (i.e., a ventilator-induced unhealthy state) (27–31), airways were histologically assessed for "health" by a pathologist. Five of the 10 lungs were selected as histologically "healthy" as defined by volume densities occupied by mucin-expressing cells in an airway epithelial region. We term these five lungs without airway GMH "normal/healthy." This definition of normal/healthy is presented in detail in the online supplement. *MUC5AC*, *MUC5B*, and *CCSP* mRNA localization was assessed by RNA ISH using RNAscope (Advanced Cell Diagnostics) (*see* Figure E2) (32). Protein localization

was assessed by immunohistochemistry as described (33). Antibodies and RNA ISH probes are shown in Table E2.

### Morphometric Analysis

The airways were classified as: *1*) trachea; *2*) primary bronchi; 3) segmental bronchi; *4*) distal bronchi (>2 mm in diameter); *5*) proximal bronchioles (1–2 mm in diameter); *6*) distal bronchioles (<1 mm in diameter); and *7*) terminal bronchioles, based on airway diameters and morphology. All histologic sections were digitally scanned using an Olympus VS120 microscope. Table E3 shows the number of airways and the lengths of basement membrane studied for each airway region. Volume densities for mucous glycoproteins, MUC5AC, MUC5B, and CCSP mRNAs and proteins within airway superficial epithelia, obtained from AB-PAS staining, RNA ISH, or immunohistochemistry, were quantified by morphometric methods (13, 34–36) (*see* Figure E3). The number of cells expressing target genes was manually counted to assess gene colocalization and normalized to airway basement membrane lengths (*see* Figure E4).

Calculations of region-specific total mucins and *CCSP* mRNA–stained volumes, using Weibel stereologic parameters (37, 38), were performed as described in the METHODS section of the online supplement (*see* Table E4).

### Absolute Quantification of *MUC5AC* and *MUC5B* Transcript Copy Numbers in Human Airway Epithelia

Designated airway regions, including trachea, segmental bronchi, bronchioles, and peripheral lung parenchyma obtained from 6 of the 16 normal subjects studied, were examined for absolute quantification of *MUC5AC* and *MUC5B* transcript copy numbers by ddPCR (Bio-Rad) (39).

### Large and Small Airway Epithelial Cell Culture

Matched large and small airway epithelial (LAE and SAE, respectively) cells, isolated from four lifelong nonsmoker lungs, were expanded using a modified conditional reprogramming cell method (40, 41) to generate well-differentiated air–liquid interface cultures (*see* Figure E5) (42).

### Mass Spectrometry

Quantification of MUC5B, MUC5AC, and surfactant protein B (SFTPB) proteins in apical washes of LAE and SAE cell cultures was performed by mass spectrometry (43, 44).

### Statistical Analysis

Statistics were performed using R version 3.5.1 (R Foundation for Statistical Computing). Comparison between two groups was performed by Wilcoxon rank sum test and comparison between three or more groups was performed by Kruskal-Wallis test, followed by pairwise Wilcoxon rank sum test for *post hoc* analysis. A $P$ value of less than 0.05 was considered significant.

## Results

### The Regional Distribution of Mucous Glycoproteins in Superficial Airway Epithelium

AB-PAS staining to quantitate airway regional variations in superficial epithelial mucous glycoproteins, which include

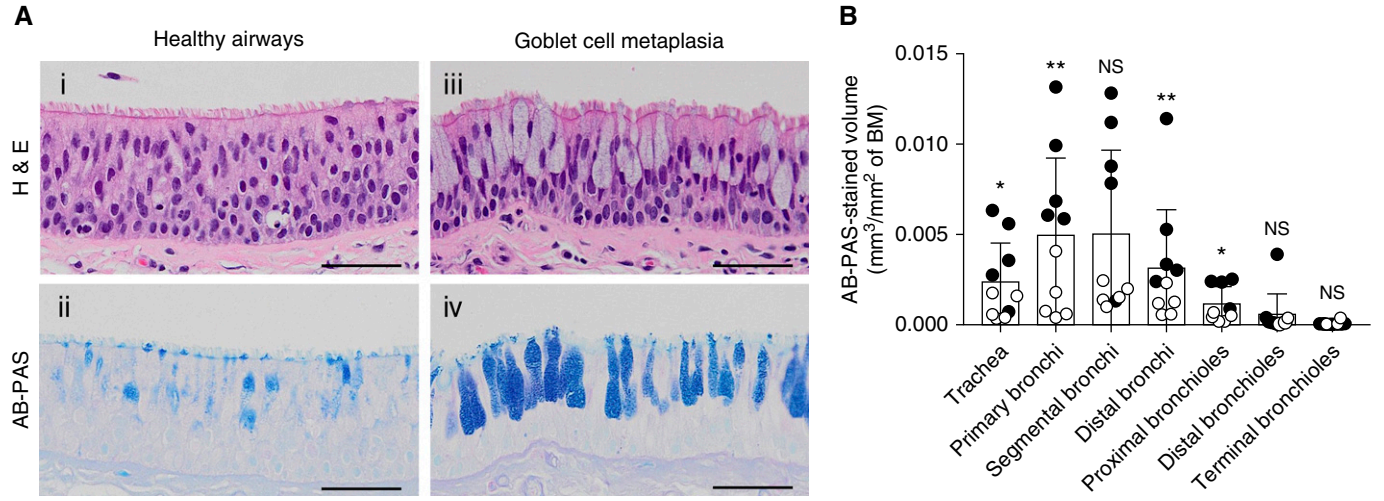


**Figure 1.** Regional distribution of mucus glycoproteins in superficial airway epithelia from 10 subjects with no prior lung disease history. (*A*) Hematoxylin and eosin and Alcian Blue and periodic acid–Schiff (AB-PAS) staining of the superficial epithelium of primary bronchi in (*i* and *ii*) a subject with histologically healthy airways versus (*iii* and *iv*) a subject with airway goblet cell metaplasia. (*B*) Quantification of AB-PAS–positive mucus glycoproteins in airway superficial epithelium of different airway regions. AB-PAS–stained volume densities in airway superficial epithelium were quantified ($n = 10$). Each circle represents AB-PAS–stained volume density for each airway region obtained from subjects with histologically healthy airways (open circles; $n = 5$; *see Ai* and *Aii*) and subjects with airway goblet cell metaplasia and/or hyperplasia (solid circles; $n = 5$; *see Aiii* and *Aiv*). Mean AB-PAS–stained volume densities in subjects with histologically healthy airways were compared with those in subjects with airway goblet cell metaplasia and/or hyperplasia by Wilcoxon rank sum test. Each circle in distal bronchi and bronchioles represent mean values of the AB-PAS–stained volume densities from multiple airways per subject. No proximal bronchiole was available in one of the five subjects with airway goblet cell metaplasia and/or hyperplasia. Histogram bars and error bars depict mean ± SD from the 10 subjects. $^*P < 0.05$ and $^{**}P < 0.01$. BM = basement membrane; H&E = hematoxylin and eosin; NS = not significant. Scale bars = 50 μm.

MUC5B and MUC5AC, was performed in 10 lungs (Figure 1). Airway GMH, defined as an AB-PAS–stained volume density greater than 0.005 $mm^3/mm^2$ per airway superficial epithelial region (36), was identified in one or more airway regions in 5 of the 10 subjects. AB-PAS–stained volume densities in subjects with airway GMH were generally greater than those in subjects with histologically healthy airways, particularly in larger proximal airways (Figure 1B). Based on the exclusion criteria, the five subjects without GMH in any airway region were selected for the RNA ISH and immunohistochemistry study of

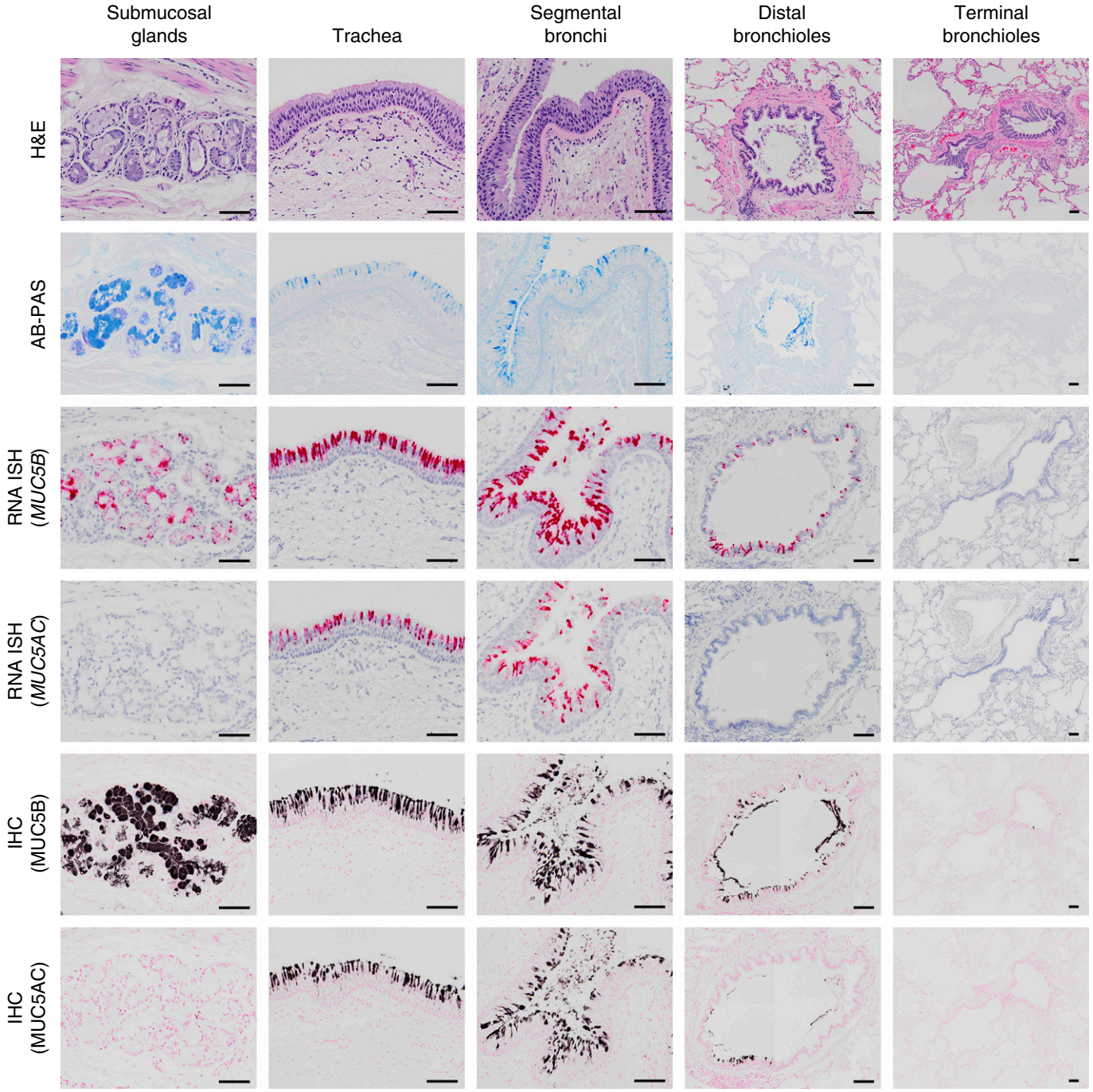


**Figure 2.** Regional distribution of MUC5B and MUC5AC mRNA and protein localization in normal/healthy human airways. Serial sections from five different regions of airways from one normal/healthy lung were stained by hematoxylin and eosin and Alcian Blue and periodic acid–Schiff, and probed for MUC5B and MUC5AC by RNA *in situ* hybridization and immunohistochemistry. Scale bars = 40 μm. For details on the methods of image acquisition, *see* Quantitation of mucous glycoproteins, MUC5AC, MUC5B, and CCSP in AB-PAS, immunohistochemistry and RNA ISH in the online supplement. AB-PAS = Alcian Blue and periodic acid–Schiff; H&E = hematoxylin and eosin; IHC = immunohistochemistry; ISH = *in situ* hybridization.

mucin expression as representative of normal/healthy lungs.

### The Regional Distribution of MUC5B and MUC5AC in Normal/Healthy Human Airways

RNA ISH and immunohistochemistry revealed extensive MUC5B, but not MUC5AC, mRNA, and protein localization in SMG (Figure 2). Both RNA ISH and immunohistochemistry demonstrated significantly greater stained volume densities for MUC5B, and MUC5AC, in the superficial epithelium of larger cartilaginous airways, including the trachea and bronchi, compared with bronchioles (Figures 2 and 3; *see* Figure E6). Notably, robust MUC5B, but not MUC5AC, mRNA, and protein staining, were detected in the distal bronchioles. In the terminal bronchioles, neither MUC5B nor MUC5AC was detected.

Two approaches were used to verify the RNA ISH and immunohistochemistry data suggesting that MUC5B is the dominant mucin in bronchioles. First, the relative sensitivities of the RNA ISH *MUC5B* versus *MUC5AC* probes were tested by comparing RNA ISH with ddPCR signals in pellets derived from clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9–modified A549 cells expressing *MUC5AC* and *MUC5B*, *MUC5AC* only, or *MUC5B* only. Data from three independent experiments revealed that the *MUC5B* probe exhibited 2.1 ± 0.3-fold increased sensitivity compared with *MUC5AC* (*see* Figure E7). Adjusting the RNA ISH data by this factor did not alter the conclusion that *MUC5B* was the dominantly expressed mucin in bronchioles. Second, sections from large versus small airways were obtained from six lungs, and ddPCR of superficial epithelia from each region

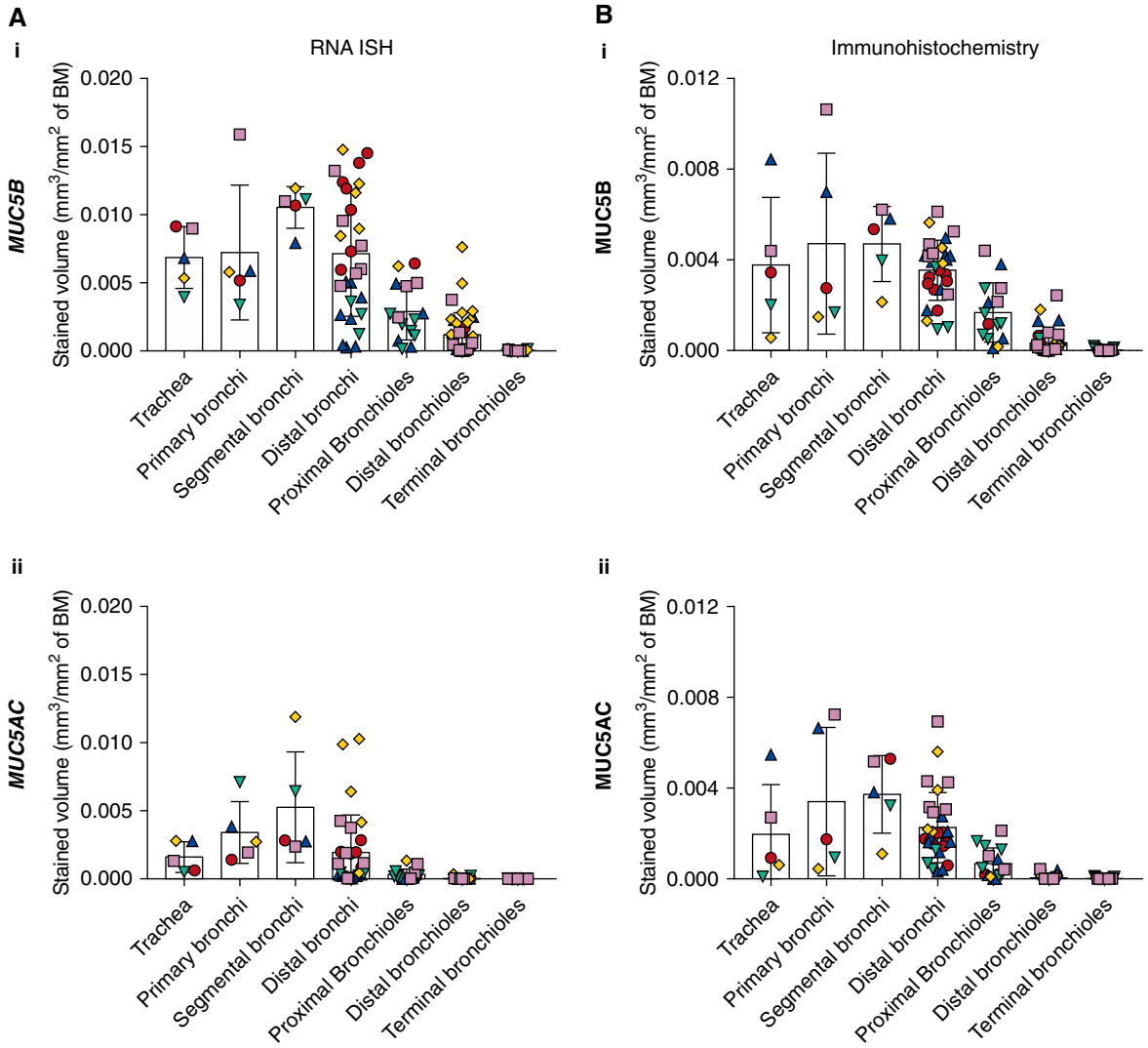


**Figure 3.** Quantification of MUC5B and MUC5AC mRNAs and proteins in normal/healthy human airway superficial epithelia. (*A*) *MUC5B* (*i*) and *MUC5AC* (*ii*) mRNA–stained volume densities in airway superficial epithelium from normal/healthy lungs were quantified ($n = 5$). (*B*) MUC5B (*i*) and MUC5AC (*ii*) protein–stained volume densities by immunohistochemistry were also quantified ($n = 5$). Histogram bars and error bars represent mean ± SD. Symbols represent the five distinct subjects. For distal bronchi and bronchioles, more than one airway per region was examined per subject. BM = basement membrane; ISH = *in situ* hybridization.

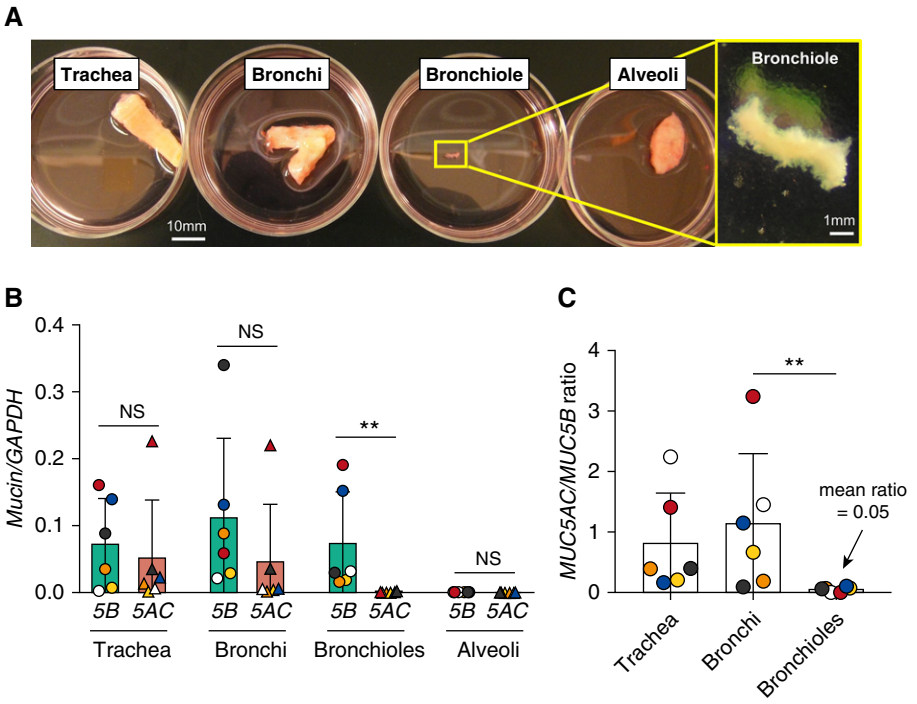


**Figure 4.** Droplet digital PCR quantification of *MUC5B* and *MUC5AC* transcript copy numbers in freshly isolated human airway epithelium from subjects with no prior lung disease history. (*A*) Examples of dissected airway tissues including a trachea, bronchus, bronchiole, and peripheral lung parenchyma. (*B*) Absolute transcript copy numbers in different airway regions for *MUC5B* (circles) and *MUC5AC* (triangles). (*C*) *MUC5AC*/*MUC5B* ratios in each airway region. Measurements in *B* and *C* were performed by droplet digital PCR, with absolute *MUC5B* or *MUC5AC* transcript copy numbers normalized to *GAPDH* and shown as target/*GAPDH* ratios. Histogram bars and error bars represent mean ± SD. $n = 6$. Different symbol colors indicate results from six distinct individual subjects. $^{**}P < 0.01$ by Wilcoxon rank sum test. NS = not significant.

was performed to compare *MUC5B* with *MUC5AC* transcript copy number in each airway region (Figure 4A). Absolute quantification of *MUC5B* and *MUC5AC* transcripts revealed that *MUC5B* normalized to *GAPDH* (*MUC5B*/*GAPDH*) was not different from *MUC5AC*/*GAPDH* in the larger cartilaginous airways, including trachea and bronchi. In contrast, *MUC5B*/*GAPDH* was significantly greater than *MUC5AC*/*GAPDH* in bronchioles (Figure 4B). Based on the *MUC5AC*/*MUC5B* ratio (Figure 4C), *MUC5B* transcript copy number was 20-fold higher than *MUC5AC* in bronchioles, consistent with RNA ISH/immunohistochemistry data.

### What Cell Type Expresses *MUC5B* and *MUC5AC* mRNAs in Normal/Healthy Human Large and Small Airways?

Based on mouse airway data (10–14), $CCSP^+$ secretory cells were identified as candidates for secretory mucin expression in normal/healthy human airways. Both RNA ISH and immunohistochemistry demonstrated widespread superficial epithelial localization of CCSP mRNA and protein from the trachea to the terminal bronchioles (Figures 5A–5D; *see* Figure E8). With respect to SMG, *CCSP* mRNAs were expressed in the ciliated ducts but not collecting ducts nor acini (Figures 5E and 5F). In contrast, *MUC5B* mRNAs were localized throughout all SMG epithelial structures. Quantification of *CCSP* mRNA–stained volume densities revealed robust expression in the segmental, distal bronchi, and bronchioles compared with trachea or primary bronchi (Figure 5G).

Quantitative colocalization studies were performed using fluorescent RNA ISH in five normal/healthy lungs (Figure 6). Typical goblet cells, characterized by AB-PAS–positive large secretory vesicles filling the cytoplasm, were rarely identified in superficial epithelia of these subjects (Figure 6Bii). In contrast, SMG consistently exhibited mucous cells with AB-PAS–definable large mucin granules (Figure 6Aii).

Human airway cells expressing *MUC5AC*, *MUC5B*, *CCSP* mRNAs, or a combination of these markers were classified into the seven possible types (*see* Table E5). Four distinct, region-specific dominant cell types were identified among the seven possible types. First, $CCSP^-/MUC5B^+/MUC5AC^-$ cells were routinely found in SMG yet rarely, if at all, in superficial epithelia. Second, $CCSP^+/MUC5B^+/MUC5AC^+$ cells were the dominant cell type in primary bronchial superficial epithelia. In this region, H&E and AB-PAS staining identified two nonciliated epithelial cell types: a nonciliated epithelial cell that had an AB-PAS–stained apical bulge protruding into the airway lumen; and a cell with apical cell membranes at the same height as those of adjacent ciliated cells without an apical bulge (Figures 6Bi and 6Bii). $CCSP^+/MUC5B^+/MUC5AC^+$ cells exhibited morphologies similar to both cell types (Figure 6Bvii; *see* Figure E9). The number of $CCSP^+/MUC5B^+/MUC5AC^+$ cells dramatically decreased in the bronchioles, and virtually disappeared in the terminal bronchioles, paralleling the decreased volume densities of *MUC5AC* mRNA and protein (Figure 3). Third, $CCSP^+/MUC5B^+/MUC5AC^-$ cells were the dominant cell type in the distal bronchiolar superficial epithelium. H&E staining identified nonciliated epithelial cells with dome-shaped apical bulges, which morphologically corresponded to the $CCSP^+/MUC5B^+/MUC5AC^-$ cells (Figures 6Ci and 6Cvii). Finally, although a small number of $CCSP^+/MUC5B^+/MUC5AC^-$ cells were detected, $CCSP^+/MUC5B^-/MUC5AC^-$ cells were the dominant cell type in the terminal bronchioles. Of note, $CCSP^+/MUC5B^-$ cells in superficial epithelia of terminal bronchioles uniquely expressed *SFTPB*, which is known as a distal airway marker (*see* Figure E10) (45).

### Primary Human SAE Cells Secrete MUC5B Protein *In Vitro*

Based on the reported distinct progenitor cell properties of large versus distal airway epithelial cells (40, 45, 46), we used an SAE versus LAE cell culture technique to test whether MUC5B protein was locally produced by small airway epithelia as predicted by *in vivo* RNA ISH and immunohistochemistry data. H&E staining revealed monolayer epithelia in SAE cell

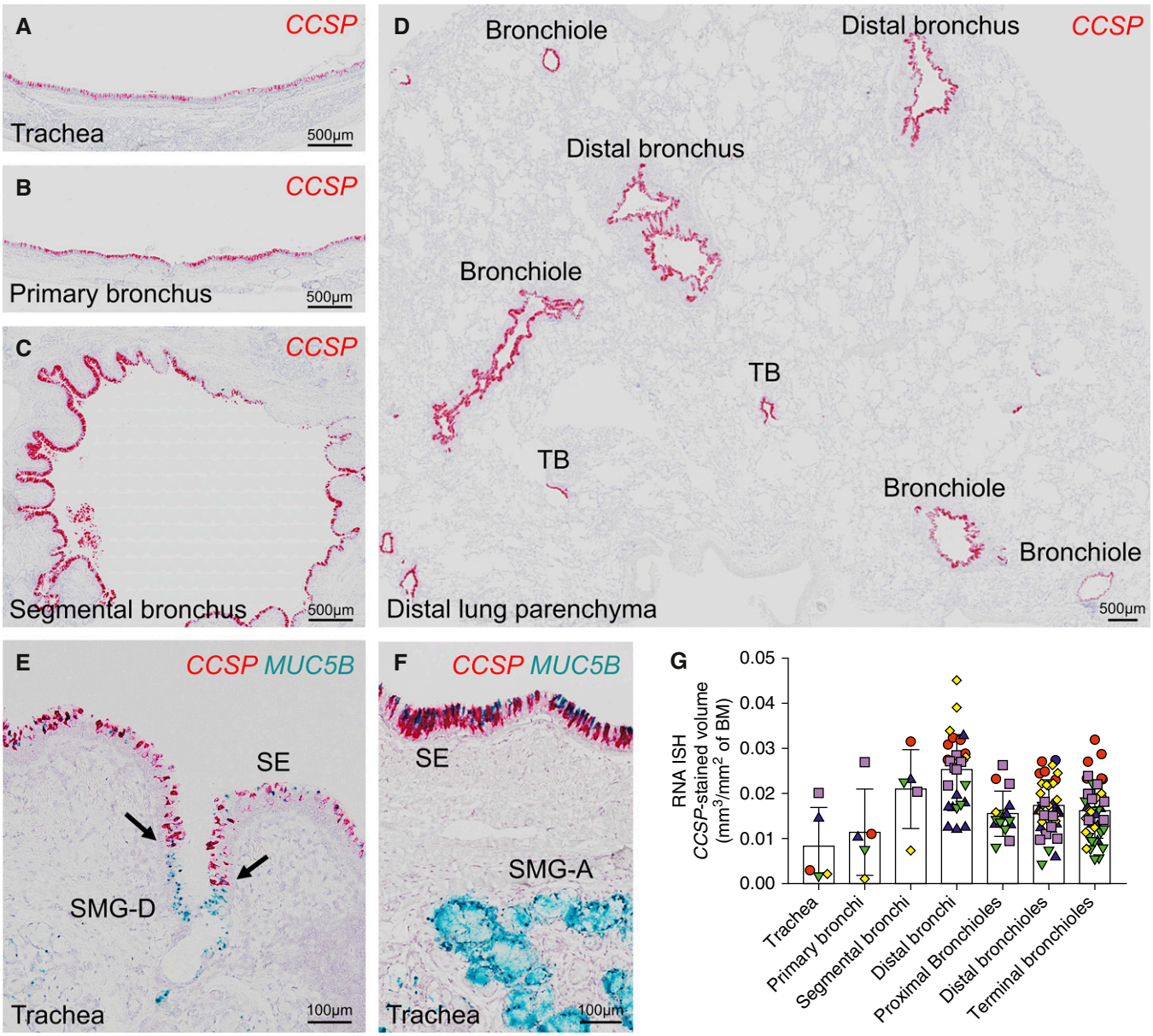


**Figure 5.** Regional distribution of *CCSP* mRNA localization in normal/healthy human airways. Representative *CCSP* mRNA localization from one normal/healthy lung stained by RNA *in situ* hybridization (ISH) in (*A*) trachea, (*B*) primary bronchus, (*C*) segmental bronchus, and (*D*) distal lung parenchyma containing distal bronchi and bronchioles. (*E*) *CCSP* and *MUC5B* mRNA localization stained by RNA ISH in normal/healthy tracheal submucosal gland ducts. *CCSP* (red) mRNA signals stop in the middle of the submucosal gland ducts (arrows) with *MUC5B* (teal) mRNA signals being expressed throughout the submucosal gland ducts. (*F*) *CCSP* and *MUC5B* mRNA localization costained using RNA ISH in the normal/healthy tracheal superficial epithelium and submucosal glands acini. (*G*) Quantification of *CCSP* mRNA–stained volume densities in normal/healthy human airway superficial epithelium ($n = 5$). *CCSP* mRNA–stained volume densities by RNA ISH were quantified. Histogram bars and error bars represent mean ± SD. Symbols represent the five distinct subjects. For distal bronchi and bronchioles, more than one airway per region was examined per subject. BM = basement membrane; CCSP = club cell secretory protein; SE = superficial epithelium; SMG-A = submucosal glands acini; SMG-D = submucosal gland ducts; TB = terminal bronchiole.

cultures versus stratified multilayers in LAE cells (Figures 7Ai and 7Aii), consistent with *in vivo* morphologic features of large versus small airway epithelia in human lungs (47, 48). As a distal airway marker (45), SFTPB was detected by quantitative PCR and mass spectrometry in SAE but not LAE cultured cells (Figures 7B and 7D). These findings indicate that our *in vitro* SAE cell culture model retained characteristics of distal airways distinguishable from large airways.

RNA ISH showed *MUC5B* mRNA localization in *CCSP* mRNA$^{+}$ nonciliated secretory cells in both LAE and SAE cells (Figures 7Aiii–7Avi). Both Western blotting and mass spectrometry identified MUC5B protein in apical washes of SAE cells (Figures 7C and 7E), although less than secreted by LAE cells. In apical washes obtained from SAE cells, the concentration of MUC5B protein (6.04 ± 5.95 pmol/ml) was 9.2-fold higher than MUC5AC (0.66 ± 0.20 pmol/ml) (Figure 7F).

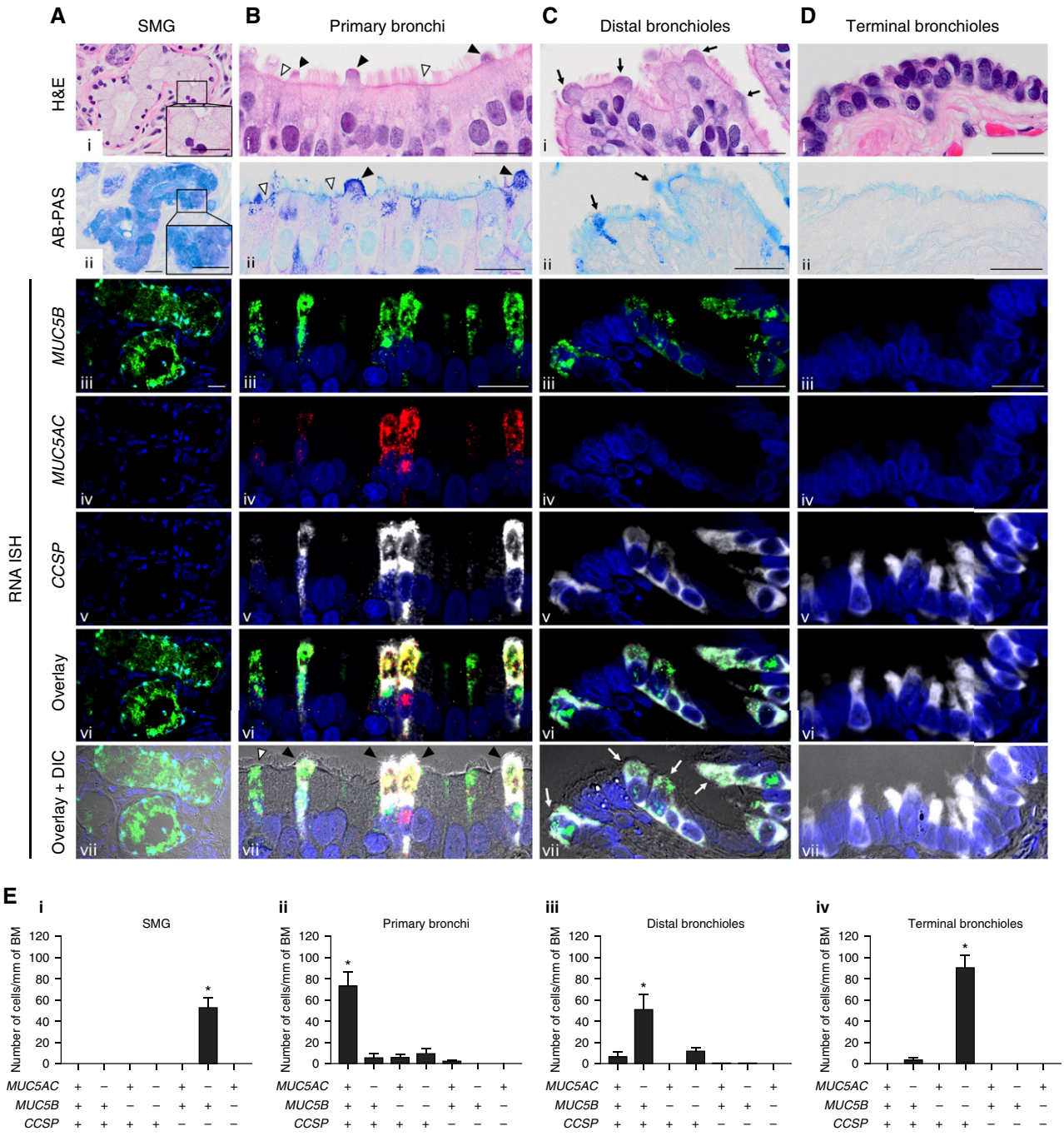


**Figure 6.** *MUC5B*, *MUC5AC*, and *CCSP* mRNA coexpression is region-specific. (*A–D*) Colocalization of *MUC5B* and *MUC5AC* with *CCSP* mRNAs by RNA *in situ* hybridization (ISH) in four different regions of normal/healthy human airways. For cellular localization, *MUC5B* (green), *MUC5AC* (red), and *CCSP* (white) mRNAs were visualized by fluorescent RNA ISH. Single-color images were merged (*Avi–Dvi*, "overlay") and the overlaid images superimposed on differential interference contrast (*Avii–Dvii*). In submucosal glands (*A*), mucus cells exhibited large mucin granules definable by Alcian Blue and periodic acid–Schiff (AB-PAS) staining (insets in *Ai* and *Aii*). In primary bronchial superficial epithelium (*B*), two nonciliated epithelial cell types were identified: *1*) a nonciliated epithelial cell with an AB-PAS–stained apical bulge (*Bi*, *Bii*, and *Bvii*, black arrowheads) and *2*) a nonciliated epithelial cell without the apical bulge (*Bi*, *Bii*, and *Bvii*, white arrowheads). In distal bronchioles (*C*), nonciliated epithelial cells with dome-shaped apical bulges (*Ci* and *Cii*, black arrows) corresponded to the $CCSP^{+}/MUC5B^{+}/MUC5AC^{-}$ cells (*Cvii*, white arrows). Nuclei were stained with DAPI (blue). Scale bars = 20 μm. (*E*) Quantification of cell types expressing *MUC5B*, *MUC5AC*, and/or *CCSP* mRNAs in different regions of normal/healthy human airways. Data are expressed as the number of each cell type per millimeter of basement membrane. Solid bars and error bars represent mean ± SD. $n = 5$. $^{*}P < 0.05$ compared with every other cell type by pairwise Wilcoxon rank sum test for *post hoc* analysis, following significant Kruskal-Wallis test. BM = basement membrane; CCSP = club cell secretory protein; DIC = differential interference contrast; H&E = hematoxylin and eosin; SMG = submucosal glands.

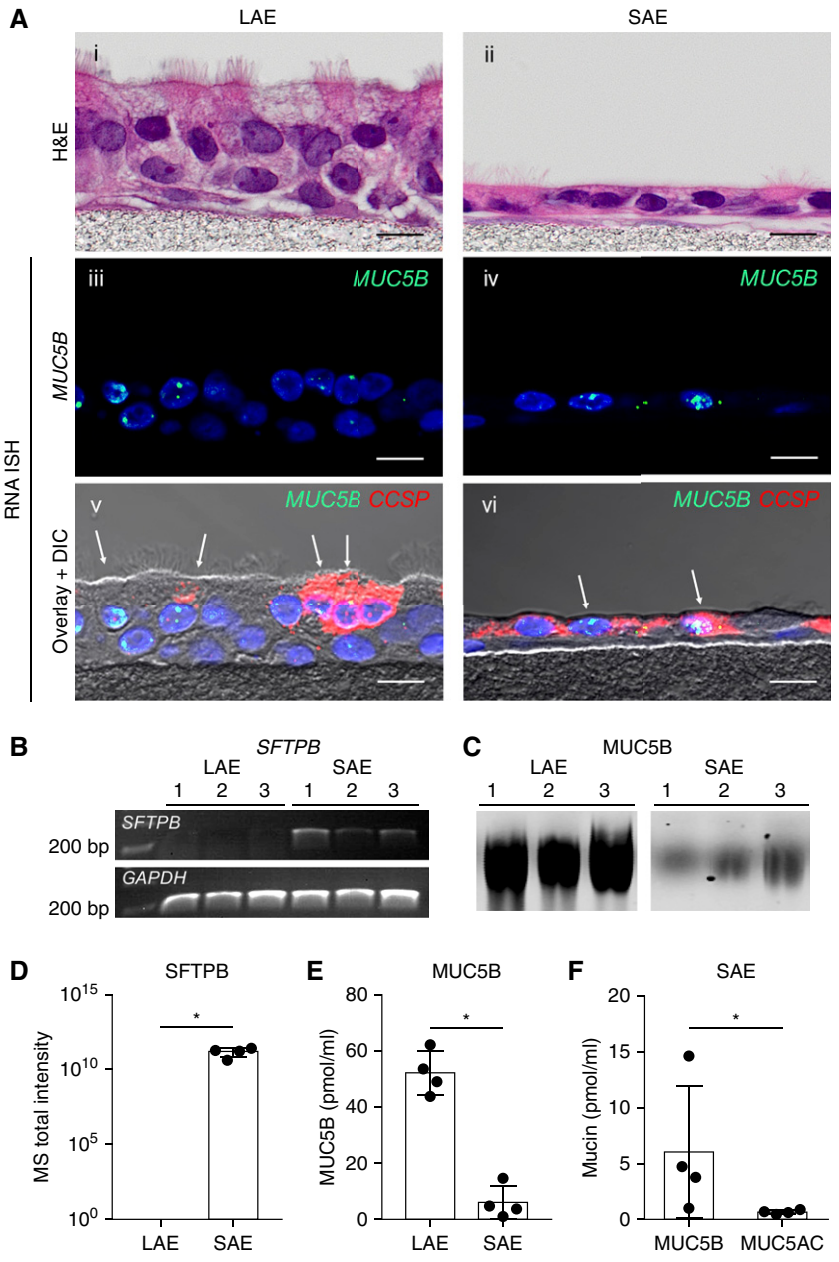


**Figure 7.** MUC5B production in primary human small airway epithelial cell cultures. (*A*) Histologic images of large and small airway epithelial cell cultures. Air–liquid interface cultures of large airway epithelial (LAE) and small airway epithelial (SAE) cells were stained by hematoxylin and eosin (*i* and *ii*) and RNA *in situ* hybridization for *MUC5B* (*iii* and *iv*) and *CCSP* mRNAs (*v* and *vi*). *MUC5B* mRNAs (green) are localized in *CCSP* mRNA (red)–positive nonciliated cells in both LAE and SAE cells (*v* and *vi*, white arrows). Scale bars = 10 μm. (*B*) *SFTPB* transcript expression in LAE and SAE cells. *SFTPB* gene was specifically detected in SAE but not LAE cells by quantitative PCR. *GAPDH* was used as the reference gene. (*C*) Identification of MUC5B protein in apical washes of LAE and SAE cells. Immunoblots of apical washes were probed with antibody to MUC5B. Samples from both LAE and SAE cell cultures were run on the same gel. (*D*) Quantification of SFTPB protein in apical washes of LAE and SAE cells. SFTPB protein was identified by mass spectrometry (MS) using label-free quantification normalized to total precursor intensity. (*E*) Absolute concentrations of MUC5B protein in apical washes of LAE and SAE cells as determined by MS. (*F*) Absolute mucin concentrations for both MUC5B and MUC5AC in apical washes of SAE cells as determined by MS. *B* and *C*: $n = 3$, the order of three biologic replicates were the same in *B* and *C*. *D* and *E*: Histogram bars and error bars represent mean ± SD. $n = 4$, $^*P < 0.05$ by Wilcoxon rank sum test. CCSP = club cell secretory protein; DIC = differential interference contrast; H&E = hematoxylin and eosin; ISH = *in situ* hybridization.

### Region-Specific Superficial Epithelial Mucin Production in Normal/Healthy Human Lungs

To describe the airway regions contributing to total MUC5AC and MUC5B expression in the lung, the numbers, diameters, and total surface areas of airways as reported by Weibel and coworkers (37, 38) were used to calculate: total *MUC5B*, *MUC5AC*, or *CCSP* mRNA–stained volumes for the superficial epithelium of each airway region based on RNA ISH; and the percent of total *MUC5B*, *MUC5AC*, and *CCSP* mRNA–stained volumes for each airway region (Figure 8; *see* Table E4).

*MUC5B* mRNA–stained volume in airway superficial epithelia increased from the proximal to the distal airways, reflecting the exponential increase in distal airway surface area. *MUC5B* mRNA–stained volume peaked in the distal bronchioles with a threefold higher value than observed in the cartilaginous large airways. In contrast, *MUC5AC* mRNA–stained volume peaked in the distal bronchi. Even though the combined surface area of proximal and distal bronchioles is more than 30-fold greater than distal bronchi, the percent of *MUC5AC* mRNA–stained volumes in bronchioles was less than distal bronchi. Importantly, both *MUC5B* and *MUC5AC* mRNA–stained volumes were negligible in the terminal bronchioles despite the fact that terminal bronchiolar surface area is the largest of the airway regions. For reference, *CCSP* mRNA–stained volume increased as a function of more distal regions and peaked in the terminal bronchioles.

## Discussion

A comprehensive regional expression pattern of MUC5B and MUC5AC in normal/healthy human lungs has not been reported. Recent data from mice airways suggest that Muc5b is secreted by superficial epithelial club cells in both larger airways and bronchioles (9–12). Whether this paradigm pertains to normal/healthy human airways was the focus of this study.

It is difficult to obtain intact normal/healthy human lungs for studies of regional mucin expression. We adopted the criteria that a normal/healthy lung region had to be obtained from a lung donor: *1*) without history of lung disease or smoking; *2*) exposed to a mechanical ventilation for less

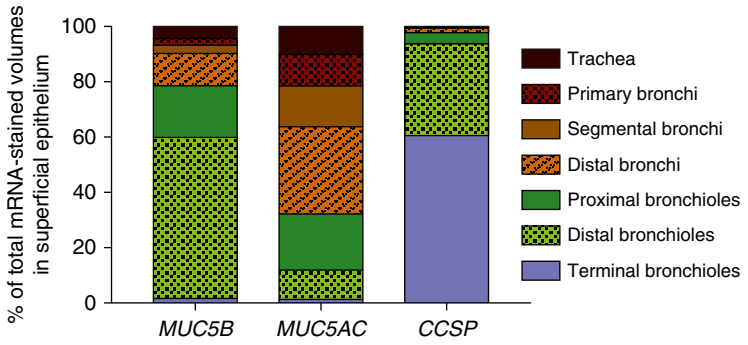


**Figure 8.** Distinct regional distributions of *MUC5AC*, *MUC5B*, and *CCSP* mRNA localization in superficial epithelium of the normal/healthy lung. Data represent the calculated percent of total *MUC5AC*, *MUC5B*, or *CCSP* mRNA–stained volumes for each airway region. Total *MUC5AC*, *MUC5B*, or *CCSP* mRNA–stained volume for each airway region was calculated by multiplying the mean values of the RNA *in situ* hybridization volume densities for each airway region obtained from five normal/healthy lungs by predicted total surface area of corresponding airway regions. CCSP = club cell secretory protein.

than or equal to 7 days; and *3*) did not exhibit GMH as defined by more than 0.005 $mm^3/mm^2$ AB-PAS–stained volume density in a superficial epithelial region, based on the mean value of mucin volume densities in healthy subject group in transbronchial biopsy study (36). Using these criteria, 5 of the 10 lungs from study subjects met the criteria for normal/healthy lungs. We speculate that the five subjects that exhibited airway GMH were more sensitive to the stresses of the mechanical ventilation, which has been shown to rapidly induce GMH in primary human cell culture models, animal models, and preterm infants (27–31). If indeed the excluded lungs were normal/healthy before mechanical ventilation, our data may document how rapidly and substantially large airway GMH can be induced by mechanical stresses (Figure 1B).

Our results demonstrate that MUC5B is extensively expressed in the superficial epithelium, in addition to SMG, of normal/healthy human airways. Importantly, these findings indicate that a major site for MUC5B production is the small airway superficial epithelia where SMG do not exist (Figure 8). These findings are consistent with the results of RNA-seq quantification of the normal human SAE transcriptome (49), and studies describing high expression of MUC5B versus MUC5AC in normal distal airway epithelium (21).

Our studies permitted a semiquantitative description of the relative expression of MUC5B in the SMG versus small airways of the normal/healthy human lung. In the large airways (e.g., from trachea to sixth generation bronchi) SMG occupy a volume of ~0.10 $mm^3/mm^2$ airway surface area in nonsmokers (50), and the mucous cell percentage of total SMG volume is about 40% (51, 52). Based on these numbers and regional airway surface areas (37, 38) (*see* Table E6), the *MUC5B* mRNA–stained volume in distal bronchiolar superficial epithelia exceeded the total mucin volume of the SMG (*see* Figure E11). Although it is difficult to predict secretion rates based on stained volumes, the superficial epithelium of small airways may be considered a source of MUC5B production at least equal to SMG.

Interestingly, the SMG and superficial epithelium provide redundant sources for MUC5B in central airway locations. One unresolved issue is whether the superficial epithelium provides the basal MUC5B secretion in this region, as it likely does in mice (10, 11), with SMG providing intermittent secretion to acutely trap inhaled irritants that provoke cough responses. A second unresolved issue with regard to MUC5B biology in the large airways is whether superficial epithelial MUC5B differs from gland-derived MUC5B. Differences may occur because MUC5B seems to be secreted in large airways from different cell types. A CCSP-positive cell without definable granules secretes MUC5B in the superficial epithelium, whereas MUC5B is secreted in SMG from CCSP-negative cells with large mucin storage granules. MUC5B is known to exist as two glycoforms with different charges, a low and a high charge, and the low-charge MUC5B is elevated in the sputum from the subjects with asthma, cystic fibrosis, and chronic obstructive pulmonary disease (3, 53). Further studies are required to determine whether MUC5B glycan-based charge differs in MUC5B secreted by SMG versus the superficial epithelium.

Our results revealed that neither MUC5B nor MUC5AC is expressed in terminal bronchioles. This finding was also apparent in the group of five subjects with airway GMH (Figure 1B). Interestingly, this finding mimics the absence of Muc5b expression reported in terminal bronchioles of mice (10). Moreover, it is notable that a subtype of $CCSP^+$ cells exists in the terminal bronchioles that do not express *MUC5B* but do express *SFTPB* (*see* Figure E10), consistent with previous RNA ISH findings (54). These data are consistent with reports that SFTPB and mucin gene expression are inversely regulated through NK2 homeobox1 at the transcription level (55–57). The finding that secretory mucins are not expressed in terminal bronchioles suggests that the physiology of terminal bronchioles requires a surfactant-rich, mucin-free zone to protect gas exchange functions in alveoli adjacent to terminal bronchioles. Because MUC5B overexpression in peripheral airways is reported in patients with IPF (21–23), it is possible that the loss of a surfactant-rich terminal bronchiolar mucin-free zone is associated with IPF pathogenesis.

In contrast to MUC5B, which is necessary to sustain mucociliary clearance (14), MUC5AC has been recognized as a "response mucin" with expression regulated by several inflammatory stimuli (9, 19, 29). The highly responsive nature of MUC5AC suggests that it provides critical innate immune functions during airway stresses. Our study revealed that MUC5AC-stained volume density peaked at the level of segmental bronchi. Maximal deposition of particles greater than 1 μm diameter per unit surface area occurs in this region (58), suggesting that MUC5AC is responding to the load of external stimuli deposited in this region. Importantly, it seemed that MUC5AC expression was superimposed on MUC5B/CCSP-expressing cells; and as MUC5AC expression increased, the cell morphology appeared more goblet cell–like.

The RNA ISH approach identified four distinct types of airway secretory cells based on the combination of *MUC5AC*, *MUC5B*, and *CCSP* transcript expression. Each cell-type predominantly exists in different airway regions and SMG versus superficial

epithelium (Figure 6). Airway surface secretory epithelial cells have conventionally been divided into four distinct cell types based on morphology and ultrastructure: *1*) mucous (goblet), *2*) serous, *3*) club (Clara), and *4*) neuroendocrine cells (59). Mucous cells are mainly located in the tracheobronchial tree and rarely in bronchioles (60). Serous cells have been described in surface epithelium in adult human small bronchi and bronchioles, whereas club cells have been considered to be the predominant secretory cell type in the human bronchiole (61). Juxtaposing these data to our results, CCSP was shown to be expressed not only in typical club cells in distal airways but also nonciliated airway secretory cells in large airway superficial epithelia. The *CCSP* mRNA$^+$ cells typically expressed *MUC5B*/*MUC5AC* mRNAs in large airway epithelia and *MUC5B* mRNAs in small airway epithelia. Thus, a subset of CCSP$^+$ cells seems to define a mucin secretory cell type in superficial epithelia.

Consistent with our findings, single-cell transcriptome analysis of human airway epithelial club cells revealed that a subset of CCSP$^+$ cells coexpressed MUC5B/MUC5AC, indicating possible lineage relationship between CCSP$^+$ secretory cells and mucin-secreting cells in human airways (62). In contrast, MUC5B-producing cells in SMG may reflect a different lineage because CCSP expression was absent in SMG mucous cells. Morphologically, *CCSP*$^+$/*MUC5B*$^+$/*MUC5AC*$^-$ cells in distal airways resembled typical club cells, whereas the morphology of *CCSP*$^+$/*MUC5B*$^+$/*MUC5AC*$^+$ cells in proximal larger airways was variable, reflecting club cells and cells that were termed "mucous cells," "serous cells," or "indeterminate cells" (63, 64). Our data suggest that the current nomenclature of airway secretory cells based on morphologic characteristics may need to be revisited.

Our *in vivo* finding that CCSP$^+$ cells in small airways have the capacity to produce MUC5B was supported by *in vitro* LAE and SAE cell culture data. Interestingly, airway epithelial cells isolated from large and small airway regions were differentiated into phenotypes that maintained region-specific characteristics when cultured under identical conditions. This result recapitulates the previous findings that proximal and distal airway basal cells exhibited region-specific gene expression profiles and progenitor properties during lung regeneration in mice (46, 65).

There are limitations to our morphologic studies. First, the study group was small, particularly for the RNA ISH and immunohistochemistry studies, because of the strict inclusion criteria used to select normal/healthy lungs. Second, it is possible that degranulation of the stored mucin protein from superficial epithelial cells occurred during the tissue isolation, which might result in underestimation of AB-PAS- and mucin protein–stained volumes compared with those *in vivo*. However, MUC5B and MUC5AC localization patterns obtained from RNA ISH were consistent with carbohydrate/protein localization data, suggesting degranulation may not have occurred (Figure 3). Moreover, we tested whether expression of forkhead box A3 (FOXA3), a transcription factor typically expressed in goblet cells (66), might be helpful in addressing this question. Immunohistochemistry experiments revealed positive signals for FOXA3 protein in the nuclei of goblet cells in airway epithelium with GMH, whereas FOXA3 staining was absent in normal/healthy airway epithelium (*see* Figure E12). These data, therefore, suggest that the "normal/healthy" airway epithelium with less AB-PAS–stained area did not contain the degranulated goblet cells. Finally, the affinity of probes for RNA ISH differed for *MUC5AC* and *MUC5B*. Direct measurements of relative RNA ISH *MUC5B* versus *MUC5AC* probe affinities in gene-edited A549 cells, however, provided a useful correction factor. Collectively, the RNA ISH quantitation of *MUC5AC* and *MUC5B*, the absolute mucin transcript expression data by ddPCR in freshly isolated airway surface epithelium, and the cell culture data all agreed that MUC5B is the dominant expressed and secreted mucin in distal human superficial airway epithelia.

In conclusion, MUC5B is extensively expressed in human airway superficial epithelia in addition to SMG. The distal airway region is the major site for MUC5B production in the superficial epithelium of the human lung. MUC5AC is normally only produced by the superficial epithelia of the cartilaginous larger airways. Both MUC5B and MUC5AC are colocalized in CCSP$^+$ cells in proximal superficial epithelium, whereas MUC5B is colocalized in CCSP$^+$ cells of distal airway superficial epithelia. Our study suggests an important contribution to mucin secretion by the superficial epithelium of the distal airways that are the initial site of mucus plugging in mucoobstructive and possibly IPF diseases. ■

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

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