Glandular Gene Expression of Sinus Mucosa in Chronic Rhinosinusitis with and without Cystic Fibrosis

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Secretory cells in submucosal glands (SMGs) secrete antibacterial proteins and mucin glycoproteins into the apical lumen of the respiratory tract, and these are critical for innate immune mucosal integrity. Glandular hyperplasia is manifested in diseases with obstructive respiratory pathologies associated with mucous hypersecretion, and is predominant in the sinus mucosa of patients with chronic rhinosinusitis (CRS), cystic fibrosis (CF), and clinical symptoms of CRS. To gain insights into the molecular basis of SMG hyperplasia in CRS, gene expression microarray analyses were performed to identify the differences in global and specific gene expression in the sinus mucosa of control, CRS, and CRS/CF patients. A marked up-regulation of 11 glandular-associated genes in CRS and CRS/CF sinus mucosa was evident. The RNA and protein expressions of the four most highly up-regulated genes (DSG3, KRT14, PTHLH, and OTX2) were evaluated. An increased expression of DSG3, KRT14, and PTHLH was demonstrated at the mRNA and protein levels in both CRS and CRS/CF sinus mucosa, whereas the increased expression of OTX2 was evident only for CRS/CF sinus mucosa, implicating OTX2 as a CF-specific gene. Immunofluorescence analysis localized DSG3, PTHLH, and OTX2 to serous cells, and KRT14 to myoepithelial cells, in SMGs. Because glandular hyperplasia is a central histologic feature of CRS, the identification of overexpressed glandular genes in the sinus mucosa lays the groundwork for future studies of glandular hyperplasia, and may ultimately lead to the development of novel treatments for mucous hypersecretion in patients with CRS.

Keywords: chronic rhinosinusitis; cystic fibrosis; submucosal glands; hyperplasia; microarrays

CLINICAL RELEVANCE

To gain insights into the molecular basis of submucosal gland hyperplasia/hypertrophy in chronic rhinosinusitis, we performed gene expression microarray analyses and identified differences in global and specific gene expression in the sinus mucosa of control, chronic rhinosinusitis (CRS), and CRS/cystic fibrosis (CF) patients, focusing especially on genes related to glandular development. Because glandular hyperplasia is a central histologic feature of CRS, identifying overexpressed glandular genes in the sinus mucosa lays the groundwork for future studies on glandular hyperplasia, and may ultimately lead to the development of novel treatments for mucous hypersecretion in patients with CRS. Submucosal gland hyperplasia in the sinus mucosa is a characteristic of patients with CRS. This study delineated genes expressed in sinus mucosa, and determined the cellular localization of glandular-associated gene products that are markedly differentially expressed in CRS or CRS/CF arrays. These findings, in the context of future studies on glandular hyperplasia, may help lead to the development of novel treatments for mucous hypersecretion in patients with CRS.

The mammalian upper and lower respiratory tracts are covered by a mucosal layer that provides a physiologic barrier and a dynamic interface to protect the underlying epithelium against inhaled pathogens and environmental toxins. Secretory cells, for example, goblet cells in the surface epithelium and mucous and serous cells in the submucosal glands (SMGs), are major contributors to respiratory tract mucus. They secrete mucin glycoproteins and antibacterial proteins, which are key components in the mucosal innate immune responses that maintain normal respiratory tract function and homeostasis. Obstructive respiratory pathologies associated with mucous hypersecretion reflect the histologic changes exemplified by goblet-cell hyperplasia, glandular hyperplasia, and hypertrophy. Hyperplasia of goblet cells in the surface epithelium is evident in the lower respiratory tract of patients with asthma (1) or other chronic obstructive pulmonary diseases (2), but is not a characteristic finding in the sinus mucosa of adult (3) or pediatric (4) patients with chronic rhinosinusitis (CRS). Conversely, SMG hyperplasia or hypertrophy is prevalent in the sinus mucosa of adult (3) and pediatric (5) patients with CRS, and in the lower respiratory tracts of patients with chronic obstructive pulmonary diseases (6). Patients with cystic fibrosis (CF) and clinical symptoms of CRS (designated here as CRS/CF) also exhibit SMG hyperplasia/hypertrophy in their sinus mucosa (7).

Submucosal glands are invaginations of the surface epithelium in the cartilaginous airways, and comprise a series of ducts with interconnecting serous and mucous tubules that terminate in acini. They are present at birth in the trachea and bronchi of the lower respiratory tract and in the oronasal passages of the upper respiratory tract, reflecting the initiation of glandular development during embryogenesis (8, 9). The molecular basis of glandular morphogenesis during development was studied in murine (10) and ferret (11) tracheas. The lymphoid enhancer binding factor 1 (Lef1) and the canonical Wnt/wingless signaling pathway were shown to be involved in the development and formation of the initial buds that develop into SMGs in ferrets (12, 13) and mice (14), whereas the bone morphogenetic protein

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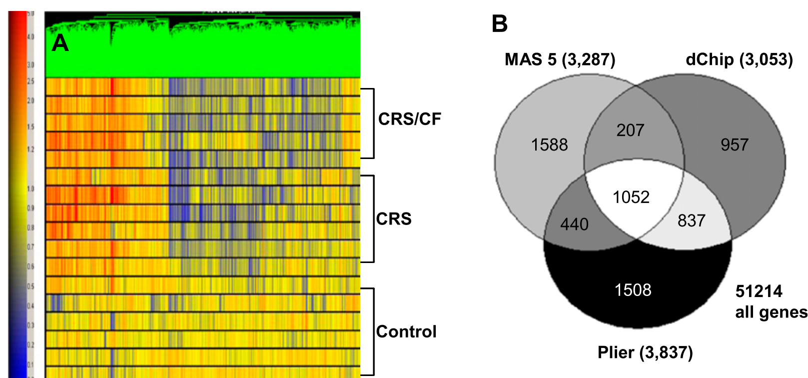

Figure 1. Gene tree derived from PLIER algorithm analyses of 3,837 differentially expressed genes in sinus mucosa from control, chronic rhinosinusitis (CRS), and CRS/cystic fibrosis (CF) patients. *(A)* The color scale for the gene tree is shown at *left*. *Red* and *blue* represent positive and negative changes in expression, respectively, versus the control condition. Color intensity is proportional to the magnitude of differential expression. Each row represents a separate patient in one of the three cohorts. Each vertical line represents an individual gene. *(B)* Venn diagrams show the number of statistically significantly altered probe sets generated by the MAS 0.5, dCHIP, and PLIER algorithms in the CRS and/or CRS/CF sinus mucosal specimens. *White* denotes the common differentially expressed probe sets (1,052) in all three algorithms. $P < 0.05$.

Bmp4 appears to control the development and homeostasis of murine SMGs in the larynx and proximal trachea (15). Studies on the molecular basis of glandular development in the sinus mucosa have not been reported, to the best of our knowledge.

Genes and mechanisms activated during development may likewise be activated during the *de novo* gland development that results in SMG hyperplasia in the sinus mucosa of patients with CRS. Alternatively, because CRS is considered an inflammatory disease, mediators activated during the response of the sinus mucosa to infection and inflammation may induce SMGs, much as the Th2 cytokine IL-13 mediates goblet cell metaplasia in murine models of allergic asthma (16, 17). The genes and pathways that lead to SMG hyperplasia in respiratory mucosa are unknown. To gain insights into SMG hyperplasia in the upper respiratory tract, gene expression microarray analyses were performed to identify differences in global and specific gene expressions in the sinus mucosa of control and CRS patients. Our initial study identified and validated specific inflammatory and innate immune mediator genes that are up-regulated in CRS and control sinus mucosa (18). Here, we identify glandular gene products differentially expressed in the sinus mucosa of control, CRS, and CRS/CF patients, and we evaluate their cellular localization in SMGs.

MATERIALS AND METHODS

Specimens of Sinus Mucosa

Sinus tissues from patients who underwent craniofacial or neurosurgical procedures for pathologies other than CRS served as control samples. Sinus tissue was obtained from patients with CRS who underwent functional endoscopic sinus surgery at Children's National Medical Center (CNMC, Washington, DC) for CRS refractory to medical management. Exclusion criteria for the control population and the criteria for diagnosing CRS were described previously (18). A diagnosis of CRS in the CRS/CF cohort was based on a combination of clinical presentation, endoscopic nasal examinations, and computerized tomography imaging of the paranasal sinuses. All patients with CRS/CF underwent preoperative evaluations by the Pulmonary Division at CNMC. Patients with ciliary dyskinesias or craniofacial abnormalities were excluded. Clinical data from control and CRS patients were recorded as described previously (4). All patients were entered consecutively into the study after we had obtained appropriate surgical and research consents (and assents, when applicable). This study was reviewed and approved by the Institutional Review Board of the CNMC.

Mucosa from the paranasal sinuses that typically included maxillary and ethmoid tissues was collected from CRS and control patients, as described previously (18). When clinically appropriate, mucosa from the frontal and sphenoid sinuses was also collected. No patients with CRS exhibited nasal polyps, whereas two patients with CRS/CF manifested polyps and polypoid changes in their sinonasal mucosa. The nasal polyps were excised from the mucosa, and were not used in these experiments. Specimens were immediately frozen in liquid nitrogen for RNA or protein isolation, or fixed with 10% neutral-buffered formalin (pH 6.8–7.2; Richard-Allan Scientific, Kalamazoo, MI) for paraffin-embedding and subsequent microscopy.

Gene Expression Profiling and Microarray Data Analyses

Sample preparation, expression profiling, and microarray analyses of sinus mucosa samples were performed at the Microarray Core of CNMC, as previously described (18). All data were analyzed using Microarray Analysis Software, version 5.0 (MAS 5.0 algorithm; Affymetrix, Fremont, CA), along with dChip and PLIER algorithms. We

TABLE 1. UP-REGULATED GENES ($P < 0.05$) IN THE SINUS MUCOSA RELATED TO GLANDULAR DEVELOPMENT AND MORPHOGENESIS

Symbol	Probe Set	Gene Bank Identification Number	Gene Name	Fold Change	
				CRS	CRS/CF
KRT14	**209351_at**	**BC002690**	**Keratin 14**	**25.33**	**11.94**
DSG3	**235075_at**	**AI813438**	**Desmoglein 3**	**18.88**	**12.39**
PTHLH	**210355_at**	**J03580**	**Parathyroid hormone–like hormone**	**3.11**	**2.25**
OTX2	**242128_at**	**BE779765**	**Orthodenticle homologue 2**	**3.33**	**7.91**
TNFAIP2	202510_s_at	NM_006291	TNF-α–induced protein 2	1.99	1.78
TNFAIP6	206025_s_at	AW188198	TNF-α–induced protein 6	1.86	1.52
TNFRSF18	224553_s_at	AF117297	TNF receptor superfamily member 18	1.26	1.09
TCF19	223274_at	BC002493	Transcription factor 19 (SC1)	1.55	1.33
BMPR2	238393_at	AL047534	Bone morphogenetic protein receptor, Type II	1.78	2.63
BMP1	205574_x_at	NM_001199	Bone morphogenetic protein 1	1.76	1.30
FGFR3	204380_s_at	M58051	Fibroblast growth factor receptor 3	1.68	1.58

Definition of abbreviations: CF, cystic fibrosis; CRS, chronic rhinosinusitis.
Genes listed in bold were further evaluated in this study.

only used probe sets that were statistically significant according to one-way ANOVA and a P value "cutoff" of less than 0.05 in all three algorithms. The dataset from each algorithm was loaded into the data-mining program Gene Spring GX, version 7.3.1 (Silicon Genetics, Redwood City, CA), and the results were visualized using the gene tree cluster feature of the program, which rearranges the order of the probe sets and groups them based on the similarity (Pearson's correlation) of their expression dynamics (19). Hierarchical clustering analyses of all three algorithm datasets were performed using the Hierarchical Clustering Explorer 3.5 program (20). Similarity and distance measures were assessed according to the Pearson correlation coefficient. Functional clustering analyses of the identified genes were performed via Gene Notes software (http://combio.cs.brandeis.edu/GeneNotes/index.htm), which identifies the gene ontology (GO) biological processes significantly enriched with the differentially expressed genes, with Bonferroni correction (21).

Quantitative RT-PCR Analysis

Please *see* the online supplement for details.

Western Blot Analysis

Please *see* the online supplement for details.

Immunohistochemical Staining

Please *see* the online supplement for details.

Cellular Localization by Multichannel Fluorescence Microscopy

Please *see* the online supplement for details.

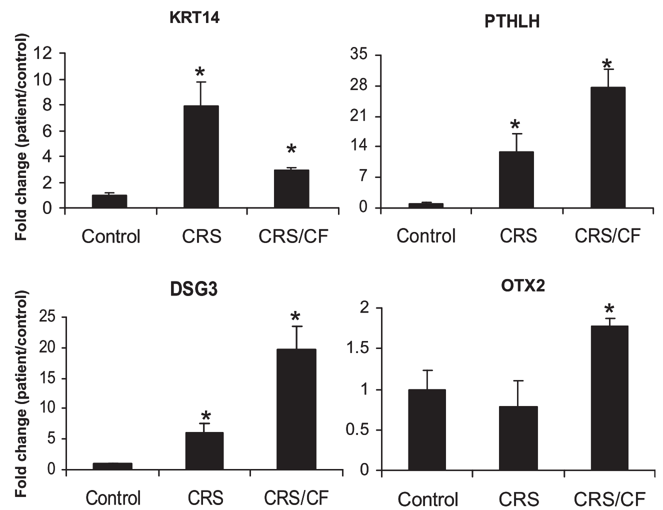

Figure 2. Quantitative RT-PCR analyses of four up-regulated genes identified in our sinus mucosa microarray study. Data show fold-changes (with standard errors) in transcript levels of tissues from CRS ($n = 6$) and CRS/CF ($n = 5$) compared with control samples ($n = 6$), which are normalized to one. *Statistically significant differences ($P < 0.05$) between CRS/CF and CRS cohorts and control subjects. KRT14, keratin 14; PTHLH, parathyroid hormone–like hormone; DSG3, desmoglein 3; OTX2, orthodenticle homologue 2.

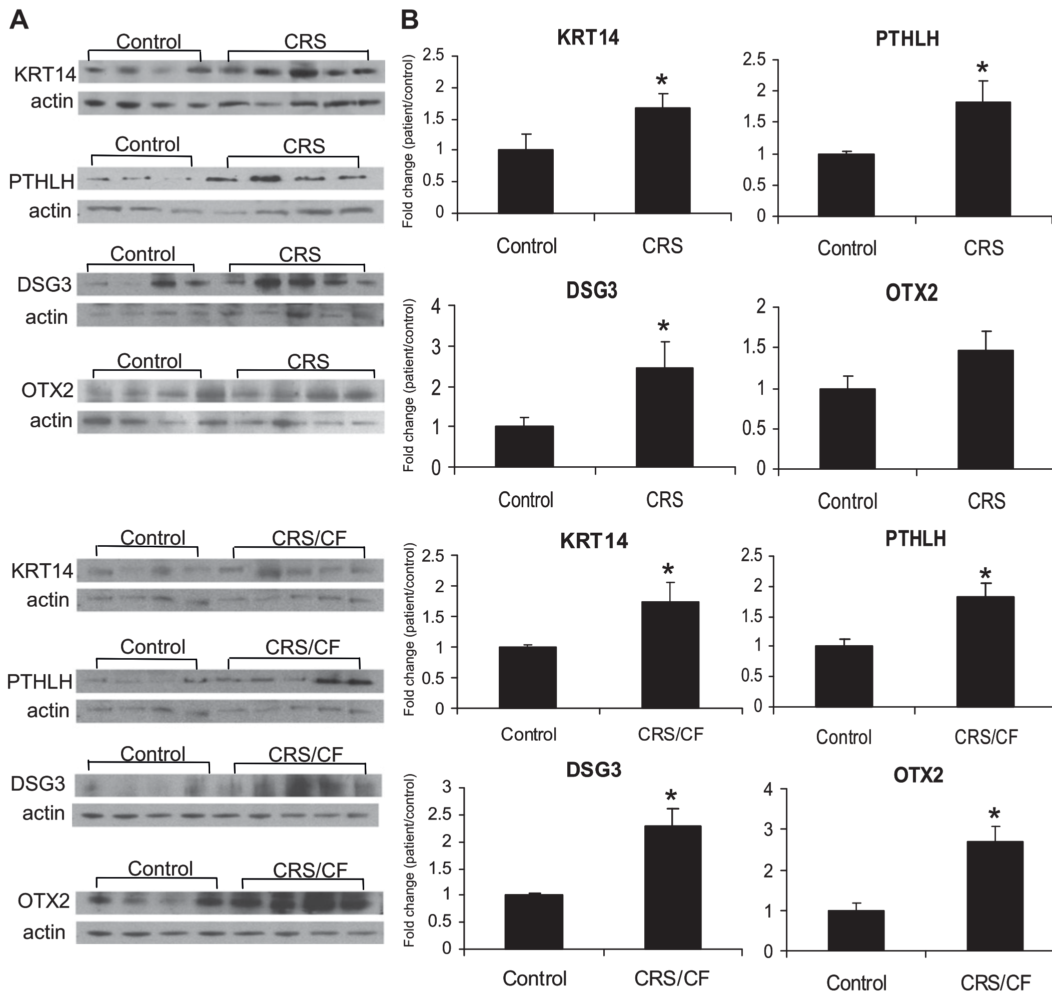

Figure 3. Quantification analysis of protein expression of four glandular gene products by Western blot analysis used sinus mucosa tissue from control, CRS, and CRS/CF patients. *(A)* Representative Western blots depict the protein expression of KRT14, PTHLH, OTX2, and DSG3 in sinus mucosa tissue homogenates from control, CRS, and CRS/CF patients. Actin was immunoblotted as an internal control. *(B)* Quantitation of band intensity from blots (*A*) by densitometry. Results are presented as means ± SEM. The relative expression of each protein was set at 100% for control tissues after normalization against actin and protein measurements, respectively. $n = 4$ for control, $n = 4$ or 5 for CRS or CRS/CF patients. *$P < 0.05$, compared with control subjects.

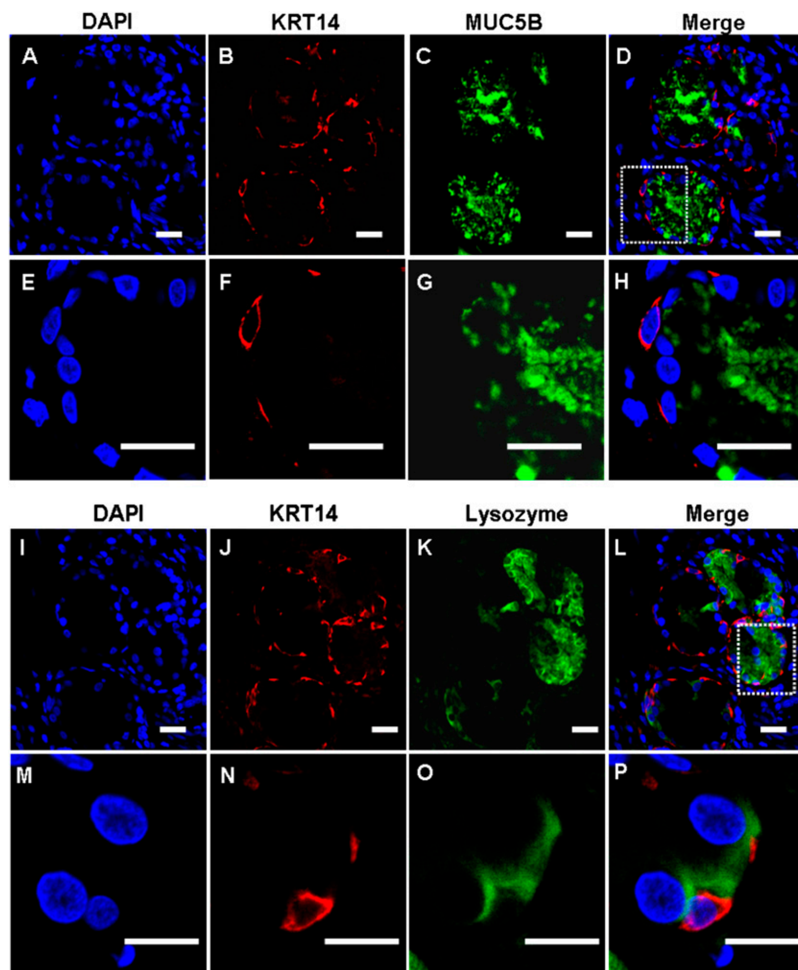

Figure 4. Immunofluorescent double labeling of KRT14 and mucin-5B (MUC5B) or lysozyme in human sinus submucosal glands (SMGs). (*A–H*) Double labeling of KRT14 and MUC5B. (*I–P*) Double labeling of KRT14 and lysozyme. Labeling by the KRT14 antibody was detected with a rhodamine-labeled secondary antibody (*red*). Nulear was stained with 4′,6-diamidino-2-phenylindole (DAPI) (*blue*). Labeling of MUC5B and lysozyme were detected with FITC-labeled secondary antibody (*green*) in the same section. Double labeling revealed no colocalization of KRT14 and MUC5B or lysozyme in SMG cells. Instead, KRT14 was expressed in myoepithelial cells adjacent to the acini. *Scale bars*, 20 μm in *A–L*; 10 μm in *M–P*. The images in the white dotted box in *D* and *L* are magnified and shown in *E–H* and *M–P*, respectively.

RESULTS

Specimens and Patient Demographics

In total, 51 sinus mucosa specimens from three patient cohorts (control, CRS, and CRS/CF) were used for genome-wide microarray, quantitative RT-PCR (qRT-PCR), Western blot, and immunohistochemical/immunofluorescent analyses. Samples were obtained from 17 control patients (8 females) aged 121–222 months, with a mean age of 197 months and a median age of 206 months; from 19 CRS patients (six females) aged 30–214 months, with a mean age of 111 months and a median age of 93 months; and from 15 CRS/CF patients (eight females) ranging in age from 66–355 months, with a mean age of 169 months and a median age of 162 months. Clinical information is provided in the online supplement and in MATERIALS AND METHODS.

Expression Array Analyses of Sinus Mucosa from Control, CRS, and CRS/CF Patients

To evaluate the differential expression of genes in the sinus mucosa between control and CRS patients and/or CRS/CF patients, a genome-wide microarray analysis of sinus mucosa from 17 individuals (six patients with CRS, five patients with CRS/CF, and six control subjects) was performed using Affymetrix Human Genome U133_2.0 Arrays. To obtain the most accurate dataset of differentially expressed genes in the three cohorts, the data for each array were subsequently analyzed separately by the MAS 5.0, dChip, and PLIER algorithms. In total, 3,287 probe sets were obtained from MAS 5.0, 3,053 from dChip, and 3,837 from PLIER. The significant probe sets were analyzed by hierarchical clustering in conjunction with the use of an overall F-measure score (which can range from 0–1), with a higher F-measure score reflecting better clustering results in biological samples (20). The results showed that the PLIER algorithm, at $F = 0.681$, yielded the highest F-measure score and the most consistent grouping of samples in the three cohorts. Thus, the PLIER software program was used for the data presentation of each probe set. The 3,837 probe sets from PLIER were loaded into GeneSpring. The differential expression for each of the 17 patient samples is depicted as a gene tree (Figure 1A). Results showed similarities in expression patterns among patients in each cohort, and a clear-cut stratification of genes in the two CRS cohorts versus the control cohort.

To better assess the differential expression of CRS, CRS/CF, and control genes in sinus mucosa, the 3,000–3,800 statistically significantly altered probe sets ($P < 0.05$) in each of the three algorithms were overlapped in a Venn diagram (Figure 1B). The data yielded a list of 1,052 differentially expressed probe sets common to all algorithms that had changed in a statistically significant way in the CRS and/or CRS/CF cohorts. These probe sets were categorized by biological functions in a GO analysis that focused on genes with terms that were overrepresented more frequently than expected by chance, and that proved significant in sinus mucosa after a Bonferroni correction ($P < 0.0005$) for multiple comparisons. Gene products related to development (GO, 7,275), cell morphogenesis/differentiation (GO, 30,154; GO, 8,283; GO, 9,653; and GO, 902), and inflammatory/immune responses (GO, 6,955; and GO, 6,952) were

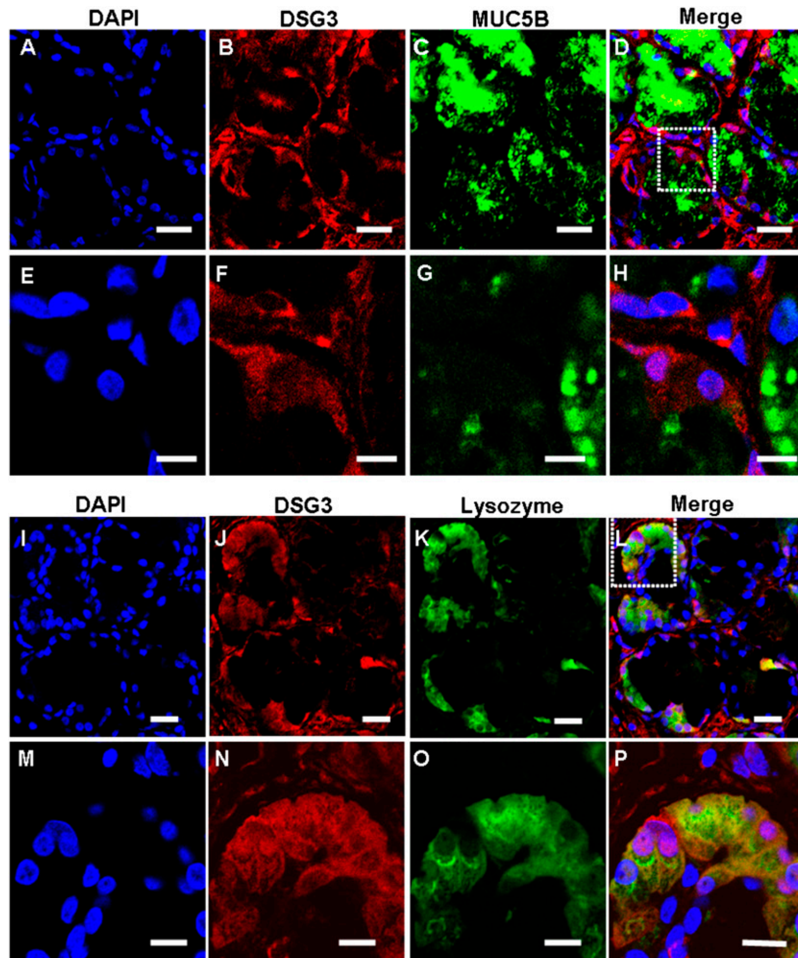

Figure 5. Immunofluorescent double labeling of DSG3 and MUC5B or lysozyme in human sinus SMGs. (*A–H*) Double-labeling of DSG3 and MUC5B. (*I–P*) Double labeling of DSG3 and lysozyme. Labeling by the DSG3 antibody was detected with a rhodamine-labeled secondary antibody. Labeling of MUC5B and lysozyme were detected with FITC-labeled secondary antibody in the same section. The overlap of DSG3 (*red*) and MUC5B or lysozyme (*green*) labeling appeared *orange* (*Merge*). Double labeling revealed colocalization of DSG3 and lysozyme in the same SMG cells, but no colocalization of DSG3 and MUC5B. No staining was evident under negative control conditions. *Scale bars*, 20 μm in *A–D* and *I–L*; 10 μm in *E–H* and *M–P*. The images in the white dotted box in *D* and *L* are magnified and shown in *E–H* and *M–P*, respectively.

expressed in sinus mucosa. Inflammatory/immune mediator genes were previously described as significantly up-regulated in the CRS cohort (18). Here, we focused on evaluating the differentiation and developmental gene products relevant to glandular functions (as indicated by mining the Medline database) that were significantly up-regulated in the CRS and CRS/CF cohorts.

Microarray Analysis of Glandular Genes

Glandular development and morphogenesis genes that were up-regulated with statistical significance ($P < 0.05$) in the CRS and CRS/CF cohorts are listed in Table 1. These genes encoded keratin 14 (KRT14), desmoglein 3 (DSG3), parathyroid hormone-like peptide (PTHLH), transcription factors (OTX2 and TCF19), TNF-associated proteins (TNFAIP2, TNFAIP6, and TNFRSF18), bone morphogenetic-related proteins (BMPR2 and BMP1), and growth factor–related genes (FGFR1). Four of these genes (DSG3, KRT14, PTHLH, and OTX2) were markedly up-regulated (3-fold to 27-fold) in both CRS and CRS/CF sinus mucosa (Table 1) and were selected for further analysis, because their expression and localization in human sinus mucosa were not previously reported.

Evaluation of Glandular Gene mRNA Expression Levels by qRT-PCR

An independent set of tissues (separate from those used in the microarray analyses) from four control, five CRS, and four CRS/CF patients was used to determine the validity of the expression array results. The data indicated that DSG3, KRT14, and PTHLH mRNA were up-regulated in both CRS and CRS/CF sinus mucosa compared with control sinus mucosa, and that the differences were statistically significant (Figure 2). This independent mRNA analysis confirmed the significant increase observed between CRS and CRS/CF patients and controls in the microarray data. However, OTX2, which showed a statistically significant increase in both the CRS and CRS/CF cohorts according to microarray analyses, was only increased in sinus mucosa from CRS/CF patients, according to qRT-PCR analyses in the independent set of sinus mucosa tissues.

Differential Expression of Glandular Proteins in CRS Sinus Mucosa

The four glandular gene products of interest were evaluated by Western blot analysis (Figure 3) in sinus tissues from five control, seven CRS, and five CRS/CF patients, to determine whether differential expression also occurred at the protein level. The data showed a statistically significant increase in DSG3, KRT14, and PTHLH protein levels in both CRS and CRS/CF sinus mucosa compared with control sinus mucosa ($P < 0.05$), in agreement with the qRT-PCR validation data (Figure 2). Likewise, in agreement with the RNA validation studies, OTX2 protein was increased with statistical significance ($P < 0.05$) only in the sinus mucosa of CRS/CF but not CRS patients.

Localization of DSG3, KRT14, PTHLH, and OTX2 in Sinus Mucosa

The localization of the four proteins shown by Western blot analysis (Figure 3) to be expressed in the sinus mucosa was

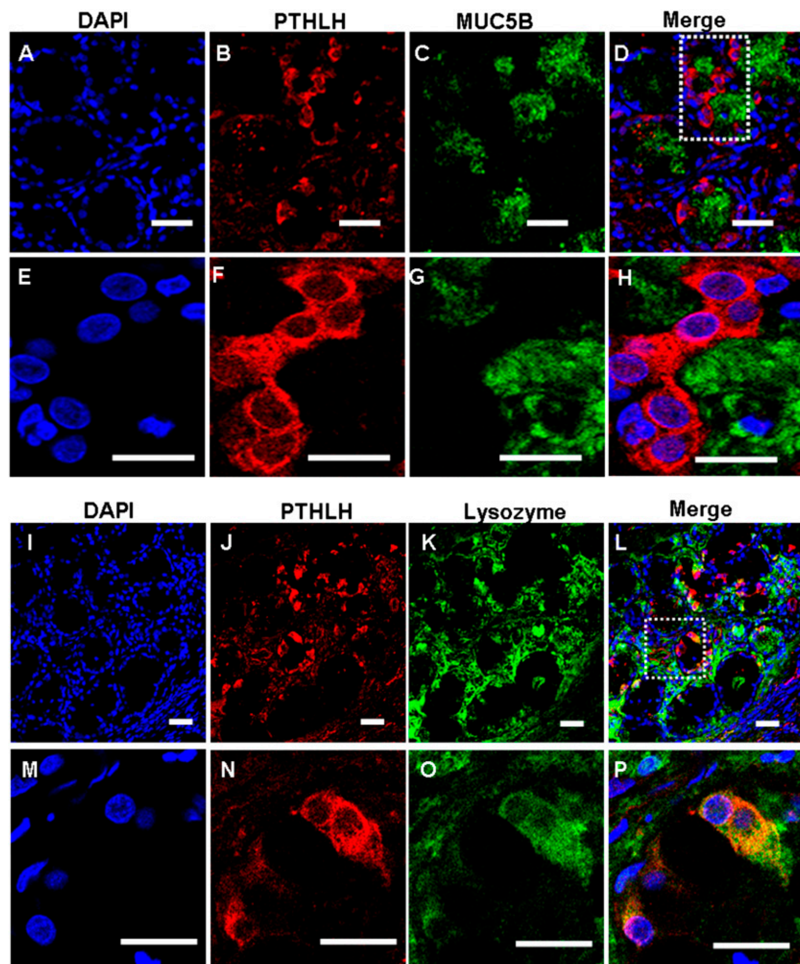

Figure 6. Immunofluorescent double labeling of PTHLH and MUC5B or lysozyme in human sinus SMGs. (*A–H*) Double labeling of PTHLH and MUC5B. (*I–P*) Double labeling of PTHLH and lysozyme. Labeling by the PTHLH antibody was detected with a rhodamine-labeled secondary antibody. Labeling of MUC5B and lysozyme were detected with FITC-labeled secondary antibody on the same section. The overlap of PTHLH (*red*) and MUC5B or lysozyme (*green*) labeling appeared *orange* (*Merge*). Double labeling revealed colocalization of PTHLH and lysozyme in the same SMG cells, but no colocalization of PTHLH and MUC5B. *Scale bars*, 20 μm in *A–D* and *I–L*; 10 μm in *E–H* and *M–P*. The images in the white dotted box in *D* and *L* are magnified and shown in *E–H* and *M–P*, respectively.

determined. Two distinct microanatomic compartments in the sinus mucosa (the surface epithelium and submucosa) were evaluated using sinus tissues of three CRS, four CRS/CF, and four control patients. KRT14, DSG3, PTHLH, and OTX2 were detected in ciliated and basal cells in the surface epithelium (data not shown). All four proteins were strongly expressed in SMGs, as shown in the online supplement (Figure E1). Among control samples, those with detectable glands were selected for analyses. The expression patterns were similar in CRS, CRS/CF, and control sinus mucosa, which typically contained far fewer SMGs than CRS sinus mucosa.

Localization of DSG3, KRT14, PTHLH, and OTX2 in SMG Cells

To determine the cellular localization of the four glandular genes of interest in sinus SMGs, double immunofluorescent staining and multichannel fluorescence microscopy were performed. Colocalization studies of each of the four glandular proteins with mucin-5B (MUC5B) or lysozyme, which are markers for mucous or serous cells, respectively (22, 23), were performed (Figures 4–7). Immunofluorescence showed that KRT14 was not expressed in mucous or serous cells (Figure 4). However, KRT14 was expressed in myoepithelial cells, and the colocalization of KRT14 with the myoepithelial cell marker, α–smooth muscle actin (24), was evident (Figure E2). The double-labeling studies also showed that DSG3 (Figure 5), PTHLH (Figure 6), and OTX2 (Figure 7) colocalized with lysozyme but not MUC5B, indicating the restriction of DSG3, PTHLH, and OTX2 to serous cells in sinus SMGs.

Colocalization of KRT14 and DSG3

Previous studies showed that Dsg3 binds to Krt14 in mouse keratinocytes (25), and increases the expression of Krt14 in murine suprabasal epidermis (26). To determine whether DSG3 might interact with KRT14 in SMG cells in sinus mucosa, their cellular localization was investigated using multichannel fluorescence microscopy. Immunofluorescence data indicated the colocalization of KRT14 and DSG3 in a subset of myoepithelial cells (Figure E3).

DISCUSSION

Mucus hypersecretion, a characteristic phenotype of patients with obstructive respiratory pathologies, to some extent reflects the epithelial tissue remodeling subsequent to infection and inflammation (27). In the sinus mucosa, this hypersecretion is manifested as SMG hyperplasia (3, 5) rather than goblet cell hyperplasia (3, 4). Microarray analyses of the sinus mucosa of control and CRS patients, including CRS/CF patients, were performed to identify differences in global and specific gene expression between healthy and remodeled sinus mucosa, and to gain initial insights into the genes implicated in glandular hyperplasia in CRS. Several inflammatory and immune mediator genes significantly up-regulated in CRS versus control sinus mucosa were previously identified and validated (18).

The present study focused on glandular-associated genes, to identify, validate, and characterize the cellular localization of four gene products (DSG3, KRT14, PTHLH, and OTX2) that exhibited significantly increased mRNA expression in the sinus

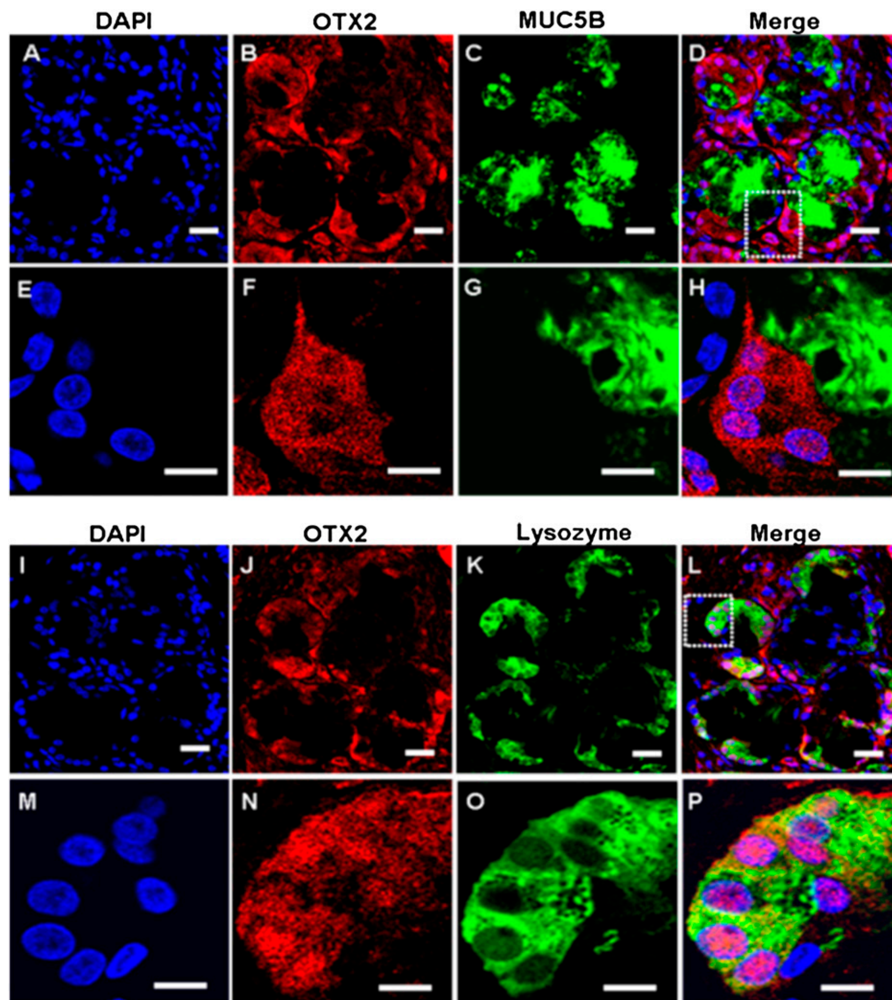

Figure 7. Immunofluorescent double labeling of OTX2 and MUC5B or lysozyme in human sinus SMGs. (*A–H*) Double labeling of KRT14 and MUC5B. (*I–P*) Double labeling of OTX2 and lysozyme. Labeling by the OTX2 antibody was detected with a rhodamine-labeled secondary antibody. Labeling of MUC5B and lysozyme was detected with FITC-labeled secondary antibody in the same section. The overlap of OTX2 (*red*) and MUC5B or lysozyme (*green*) labeling appeared *orange* (*Merge*). Double labeling revealed no colocalization of OTX2 and MUC5B, but colocalization of OTX2 and lysozyme in the same SMG cells. *Scale bar*, 20 μm. The images in the white dotted box in *D* and *L* are magnified and shown in *E–H* and *M–P* respectively.

mucosa of both CRS and CRS/CF patients relative to control patients. The increased expression of DSG3, KRT14, and PTHLH mRNA and protein was validated both in CRS and CRS/CF sinus mucosa, indicating that they may comprise glandular-associated genes up-regulated in CRS and CRS/CF sinus mucosa in response to the chronic inflammation inherent in the sinus mucosa of all patients with CRS. In contrast, the increased mRNA and protein expression of glandular transcription factor OTX2 was validated only in CRS/CF sinus mucosa, suggesting that OTX2 may be a CF-specific gene that contributes to SMG hyperplasia in CF respiratory mucosa.

We reasoned that if these four gene products were relevant to glandular hyperplasia in CRS or CRS/CF patients, they would be well-expressed in glandular cells and perhaps detectable in the surface epithelium, which is thought to be the source of epithelial stem and progenitor cells, at least in the trachea (10). Each of the four proteins was detected in basal or ciliated cells in the surface epithelium, and each was highly expressed in SMGs, with the expression of DSG3, PTHLH, and OTX2 observed in serous but not mucous cells, and KRT14 observed in myoepithelial cells adjacent to glandular acini. Serous cells are thought to be stem cells for the renewal of respiratory tract epithelium (28, 29). The expression of DSG3, PTHLH, and OTX2 in serous cells (but not mucous cells) raises the possibility that progenitor cells in the surface epithelium can differentiate into serous cells that then function as the "leading edge" of gland tubules during invagination of the epithelium. Although neither serous nor stem cells in the human sinus mucosal epithelium have yet been identified, progenitor cells that are serous cell precursors may proliferate after inflammation, which could lead to SMG hyperplasia.

The genes that participate in the morphogenesis and differentiation of SMGs in respiratory tract mucosa are markedly understudied. However, the four proteins that are markedly up-regulated in sinus mucosa (DSG3, KRT14, PTHLH, and OTX2) were implicated in glandular formation in other epithelial systems. In the mammary gland, desmosomal adhesion is important during epithelial morphogenesis. Blocking of the cell adhesion recognition sites in desmosomal cadherins inhibits alveolar morphogenesis by epithelial cells from the mammary lumen, and disrupts the positional sorting of luminal and myoepithelial cells in aggregates formed by the reassociation of isolated cells (30). The desmosomal cadherin superfamily provides mechanical strength to epithelial tissues by forming stable intercellular contacts anchored to KRT intermediate filaments. DSG3, a member of the desmosomal superfamily, is implicated in the intercellular attachment of skin and mucous membranes (25). Dsg3 anchors to Krt14 in murine keratinocytes (25), and the expression of human DSG3 in transgenic mice increases the expression of murine Krt14 in the suprabasal epidermis (26).

KRT14 is a member of the Type I keratin family that forms the cytoskeleton of keratinocytes and other epithelial cells. Specific keratins are considered markers for hair-follicle stem cells (31) and rabbit cornea stem cells (32). In the salivary glands, KRT14 is expressed in myoepithelial cells, which were

proposed to function as luminal cell progenitors (33). In murine tracheae, distinct populations of cells in glandular ducts near the cartilage-intercartilage junction that express high concentrations of Krt14, Krt18, and Krt5 after epithelial damage are considered stem cells (10). Krt14/Krt5 is considered a marker of murine lung epithelial stem cells (34, 35). In human sinus mucosa, we observed the colocalization of KRT14 with KRT5 in SMG ductal cells (data not shown), but also observed that KRT14 was expressed in myoepithelial cells adjacent to glandular acini, as well as in basal cells in the surface epithelium. Although DSG3 was expressed in serous cells and KRT14 was expressed in myoepithelial cells, the colocalization of KRT14 and DSG3 in a subset of myoepithelial cells was evident (Figure E3). Taken together, the information suggests that the increased concentrations of DSG3 observed in CRS sinus mucosa may contribute to the marked up-regulation of KRT14 in CRS sinus mucosa, and may subsequently elicit stem cell self-renewal, leading to glandular hyperplasia. Further study of the interaction between DSG3 and KRT14 in sinus mucosa glandular cells could serve as a future direction for experiments into the pathogenesis of glandular hyperplasia in CRS sinus mucosa.

A potential role in glandular development or hyperplasia is less clear for the other two glandular genes of interest (PTHLH and OTX2) that are up-regulated in CRS or CRS/CF sinus mucosa and expressed in serous SMG cells. PTHLH is expressed in the myoepithelial cells of sweat and parotid glands (36), and participates in the regulation of epithelial–mesenchymal interactions during embryonic mammary development and adolescent ductal morphogenesis (37). OTX2, a homeodomain-containing transcription factor well-expressed in the *Xenopus* cement gland, a mucus-secreting neural organ, has an essential role in activating the ectopic formation of the cement gland (38). The increased expression of OTX2 was validated only in CRS/CF and not CRS sinus mucosa, suggesting that OTX2 may be a CF-specific gene that affects the ontogeny of SMG hyperplasia at least in the upper respiratory tract mucosa of patients with CF.

The expression of three members (TNFAIP2, TNFAIP6, and TNFRSF18) of the TNF-associated protein family was indicated by microarray analyses to be modestly but significantly up-regulated in CRS sinus mucosa. This finding may indicate a possible role for the TNF superfamily in glandular formation in the respiratory tract, if the increased expression of these gene products were to be validated. Interestingly, mutations in specific members of the TNF superfamily (EDA, EDAR, and EDARADD) were shown to result in a lack of mucus-producing glands in the nose, larynx, and bronchi of patients with ectodermal dysplasia (39). However, these genes were not significantly up-regulated in patients with CRS or CRS/CF in our array datasets.

The modest but significantly increased expression of growth factors (BMP1, BMPR2, and FGFR3) was also evident in our CRS sinus mucosa microarrays, suggesting that specific members of the BMP and fibroblast growth factor (FGF) families play a role in the differentiation of glands that results in SMG hyperplasia in CRS. Various FGFs, growth factor receptors, BMPs, and transcription factors are known to mediate interactions between mesenchymal and epithelial components during embryonic glandular development (40). For example, Bmp7 and Pax6 play important roles during the embryonic SMG branching morphogenesis of murine salivary glands (41), whereas Bmp4 (42) and Fgf10 (43) mediate interactions between mesenchymal and epithelial components that are important for the initial budding and subsequent branching morphogenesis of the epithelial placodes in mice.

The pathways leading to the cellular differentiation that results in gland formation in the upper or lower respiratory mucosa are not well understood. The canonical Wnt signaling pathway and Lef1 play a role during embryogenesis in the development and formation of the initial buds that develop into tracheal SMGs in ferrets (12, 13). These genes and pathways may be transiently activated during the differentiation of respiratory mucosa that leads to *de novo* glandular development and hyperplasia in respiratory tract diseases, but may not be up-regulated in chronic conditions. Along this line, Lef1 mRNA was not differentially regulated in CRS sinus mucosa, suggesting either that the Wnt pathway is not active in chronic conditions, or that an alternate differentiation pathway is initiated by chronic inflammation in patients with CRS. Investigations of the processes that lead to the initiation and development of glands in the sinus mucosa and subsequent glandular hyperplasia will likely be facilitated by the recent development of an *in vitro* three-dimensional model for the differentiation of glandular acini from respiratory tract epithelial cells (44).

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