ORIGINAL ARTICLE

Characterization of human upper airway epithelial progenitors

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Background: New epithelial cells are generated through the proliferation and differentiation of resident progenitor cells in the nasal cavity. In several upper airway diseases, such as cystic fibrosis and chronic rhinosinusitis, self-renewing progenitor cells may be functionally defective, or compromised in their ability, to regenerate cells that maintain normal mucociliary clearance. Herein, we describe our early work to define and characterize a rare population of human nasal epithelial putative progenitors.

Methods: Single-cell suspensions of human ethmoid sinus tissues were prepared following endoscopic sinus surgery. Cell surface antibodies were analyzed as candidate markers for detecting progenitor cells. A panel of antibodies, including epithelial cell adhesion molecule (EpCAM, epithelial cells), CD45 (hematopoietic cells), nerve growth factor receptor (NGFR/CD271), intercellular adhesion molecule-1 (ICAM1/CD54), and integrin-$\alpha6$ (ITGA6/CD49f) were used to resolve epithelial progenitor candidates by high-dimensional flow cytometry and the gating technique of fluorescence minus one (FMO) controls.

Results: A rare population of approximately 0.06% of total ethmoid cells was discriminated as EpCAM⁻CD45⁻NGFR⁺ICAM1⁺ by surface markers. Use of ITGA6 was excluded based on FMO control analysis. This lineage-negative population was purified to 99% homogeneity by cell sorting and analyzed by immunofluorescence microscopy. Sorted cells were subsequently confirmed to uniformly express the transcription factor p63. Upon in vitro culture, lineage-negative clonal cells were confirmed to spontaneously differentiate into epithelial lineage-positive cells.

Conclusion: Using the NGFR and ICAM1 cellular coordinates, we have identified a promising population of native human nasal epithelial progenitor cells that require more formal investigation for their role in upper airway regeneration. © 2013 ARS-AAOA, LLC.

Key Words:
stem cells; nasal mucosa; ethmoid sinus; NGFR; ICAM1; EpCAM; ITGA6; FACS; flow cytometry; progenitor cells; epithelial regeneration

New epithelial cells are likely generated through the proliferation and differentiation of resident epithelial stem cells, or multipotent progenitors, in the adult nasal cavity. This process of renewal is felt to result from rapid regeneration of the nasal mucosal surface after environmental and infectious insults, and recovery of a functional mucociliary blanket following nasal procedures. While the relevant cells and processes for epithelial remodeling are well-defined in other organ systems, these elements are essentially uncharacterized for the upper airway and nasal cavity. Moreover, the signals underlying the differentiation of specified mature ciliated and goblet daughter cells from their progenitor cell precursors are poorly understood, in part because of the inherent difficulty in studying the latter cells in their native environments.

In several diseases, nasal mucosal epithelial cells are theorized to be either functionally defective, or possess aberrant capacity for regeneration, in order to support the formation of a robust mucosal surface with mucociliary

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function. One such example is cystic fibrosis (CF), where a genetic defect in the CFTR chloride transport gene leads to defective nasal mucosal epithelial barrier,[1–3] mucostasis, and secondary infections.[4] Other examples include common sinus diseases such as chronic rhinosinusitis (CRS), a recurrent inflammatory/infectious condition of the upper airway.[5] For these reasons, it is valuable to understand the cellular and molecular players responsible for the restoration of upper airway respiratory mucosa, and, moreover, whether these components may be dysregulated in upper airway disorders.

In this work we describe our early studies to identify and characterize human nasal epithelial cells that have the capacity to support regeneration of the upper respiratory airway epithelium at the mucosal surface. Cell surface markers NGFR, ITGA6, and ICAM1 were analyzed independently for use as candidates in cell sorting strategies aimed at lineage-negative (EpCAM−/CD45−) cell populations. NGFR and ITGA6-positive basal cell populations have been identified as epithelial stem cells of the lower airways in human and mouse.[6] Similarly, expression of both ITGA6 and ICAM1 has been identified in horizontal basal cells, the resident stem cells of the neighboring olfactory epithelium.[7,8] Our current analysis determined that both NGFR and ICAM1, but not ITGA6, defined optimal cellular coordinates for the isolation of a pure population of putative human upper airway epithelial progenitor cells. Furthermore, cells purified using these markers were found to express the transcription factor p63, a nuclear protein expressed by progenitor cells from various tissues including the lower airways and olfactory epithelium.[6,7,9–11] Finally, the capacity for differentiation of our candidate cell population was additionally confirmed through use of in vitro culture.

Materials and methods

Human nasal tissue

Human tissue was acquired and used for research with informed consent under the Institutional Review Board of the Stanford University School of Medicine (Protocol ID #18981). Protocol and informed consent procedures were provided according to Declaration of Helsinki guidelines. Ethmoid sinus tissues were harvested at the Stanford University hospital during primary endoscopic sinus surgeries for chronic rhinosinusitis, in which ethmoidectomies were performed as a planned part of the procedure. All tissues were taken from patients with mild to moderate CRS disease based on endoscopy, preoperative imaging, and intraoperative assessment, keeping in mind the need to have appropriate working tissue volume given the relative rarity of the progenitor cell pool under study. Patients with severe sinus disease (such as pansinus opacification on radiography), nasal polyposis, cystic fibrosis, or evidence of frank, purulent infection at surgery were excluded from use this work.

TABLE 1. Antibodies used for flow cytometry

Antigen	Species specificity	Conjugation	Application
CD45	Human	Pacific Orange	Leukocytes
EpCAM	Human	PerCP-Cy5.5	Pan-epithelial cells
NGFR	Human	Pacific Blue	Progenitor cells
ICAM1	Human	PE	Cell adhesion/progenitor cells
ITGA6	Human	FITC	Cell adhesion/progenitor cells

Fluorescence staining

Fluorochrome-conjugated monoclonal antibodies against human EpCAM, NGFR, ICAM1, and ITGA6 were purchased from BD Pharmingen (San Diego, CA). Anti-human CD45 antibody was purchased from Life Technologies (Carlsbad, CA); Cytokeratin 8 antibody from the Developmental Studies Hybridoma Bank (Iowa City, IA), and p63 antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Hoechst nucleic acid stain was purchased from Life Technologies Corporation (Carlsbad, CA). The complete list of antibodies used in flow cytometry and cell sorting is presented in Table 1.

Ethmoid sinus tissues were washed extensively in PBS and minced into fine pieces. The minced tissues were incubated with 10 mL of 1.5 mg/mL pronase E (Sigma-Aldrich, St. Louis, MO) in Ham's F-12 media supplemented with antibiotics for 18 hours at 4°C. After incubation, cells were dissociated by repeated pipetting. Finally, undigested tissues were removed, filtering the cell suspension through 70-μm nylon filters and cells were collected by centrifugation. To eliminate red blood cell contamination, single-cell suspensions were incubated with red cell lysis buffer (150 mM NH_4Cl) for 5 minutes and washed with 10 volumes of PBS.

Single-cell suspensions were stained with cocktails of fluorochrome-conjugated antibodies to cell surface proteins (presented in Table 1) as described.[12] "Fluorescence-minus-one" (FMO) controls were included to determine the level of nonspecific staining and autofluorescence associated with subsets of cells in each channel as detailed in the Results section. Propidium iodide (Life Technologies, Carlsbad, CA) was added to all samples before data collection to identify dead cells. High-dimensional flow cytometry data were collected on a LSRII FACS instrument. FLOWJO (TreeStar, San Carlos, CA) software was used for fluorescence compensation and acquisition analysis. Data were depicted as 5% probability contour plots or histograms displaying fluorescence intensity (FI) plotted against cell numbers/FI interval with a total of 256 intervals per parameter.

Purification of putative nasal progenitor cells

Single-cell suspensions of sinus tissues were stained with combinations of fluorescent conjugated anti-human
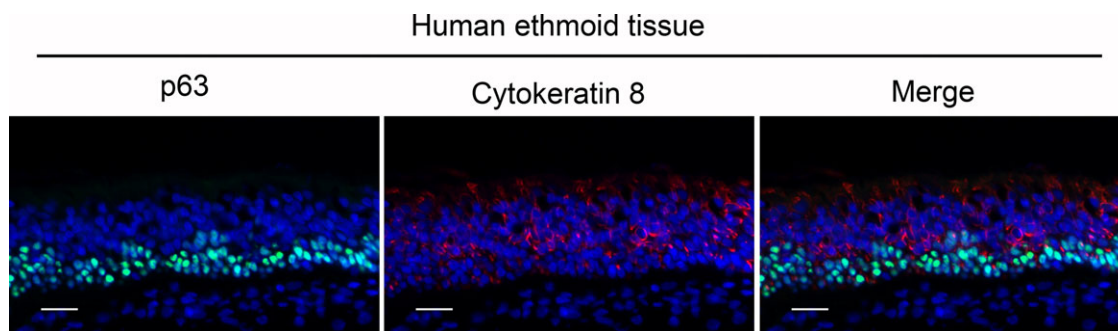
FIGURE 1. Putative basal cell epithelial progenitors in human upper airway mucosal tissues express p63. Cryo-sections of human ethmoid tissues were stained with fluorescent conjugated anti-p63 antibody (FITC, green), cytokeratin 8 (TRITC, red) and Hoechst stain (blue). Note, specific p63 staining in the basal layer of the ethmoid tissue above the basement membrane (right panel) where as luminal/suprabasal cells (marked by cytokeratin 8 staining, middle panel) are devoid of p63 staining. Scale bar, 25 μm.

EpCAM, CD45, ICAM1, ITGA6, and NGFR antibodies as described. To determine the possibility of background, artifactual staining of each antibody into neighboring fluorescence channels, we prepared FMO controls. In each FMO control, 1 antibody against NGFR, ICAM, or ITGA6 was individually omitted from bulk staining cocktails containing the remaining 2 antibodies. These controls allow determination of "spillover" fluorescence signal from the remaining antibodies into the channel of the omitted single antibody. Once the optimized combination of appropriate antibodies was identified with minimal background signal as noted in Results, the cells were stained with the indicated specific antibodies and sorted using a BD Biosciences Aria FACS cytometer (San Jose, CA).

Immunofluorescence microscopy

Cytospin preparations of 1×10^4 sorted cells, and cross-sectioned human tissues, were fixed in 4% paraformaldehyde (PFA). Human tissues were previously cryoprotected by successive incubations in 15%, 20%, and 30% sucrose in PBS and embedded in OCT compound (Sakura Finetek, Torrance, CA), and frozen sectioned at 9-μm thickness using a Leica CM1950 cryostat (Leica, Bannockburn, IL). Samples were blocked and permeabilized (10% normal goat serum, 0.5% Triton-X 100) at room temperature (RT), then incubated overnight at 4°C with primary antibodies as indicated.

For visualization, secondary anti-human antibodies conjugated to Alexa Fluor 488 and 546 (Molecular Probes, Invitrogen Corp., Carlsbad, CA) were used. Nuclei were stained with Hoechst dye in PBS. Cells were then mounted using antifade fluorescent mounting media (DAKO, Carpinteria, CA). Microscopic images were obtained using a Zeiss Axioimager/LSM 5 Exciter fluorescence confocal microscope with AxioVision acquisition software (Zeiss, Germany). Images were processed with Photoshop software (Adobe Systems, San Jose, CA).

Cell culture

Single-cell suspensions of isolated putative progenitor cells were plated onto collagen coated (BD Biosciences, San Jose,

CA) air-liquid interface (ALI) transwell-clear polyester filters, 0.4-mm pore (Corning Corp., Tewksbury, MA) in Bronchial Epithelial Growth Medium (BEGM) (Lonza, Basel, Switzerland). Media was changed the following day and changed every other day afterward. After approximately 1 week in culture, BEGM media was supplemented with retinoic acid at 50 nM (Sigma-Aldrich, St. Louis, MO) to promote cellular differentiation. Spontaneous differentiation was allowed to proceed for 4 weeks following fixation and fluorescence staining as described in Materials and methods.

Results

Human ethmoid basal epithelial cells express p63

Human nasal mucosal tissues were initially analyzed for the presence of the transcription factor p63, known to be widely expressed in many epithelial stem cells.[10,11] Immunofluorescence imaging of primary human ethmoid tissue cryosections demonstrated a punctate nuclear staining pattern for this protein, restricted to the basal cells atop the lamina propria (Fig. 1, left panel). Additionally, the specialized cells of the suprabasal layer of the luminal epithelial lining appropriately stained with cytokeratin 8 (Krt8), a marker expressed by differentiated epithelial cells (Fig. 1, middle panel). These results suggest that the putative epithelial progenitor cells, found in the basal layer of the upper airway epithelium, express intranuclear p63 protein.

Defining optimal cellular markers using FMO

To assess whether the p63$^+$ population of basal cells possess the properties of candidate progenitor cells within human upper airway mucosa, selected cell surface markers were required for use in downstream cell sorting applications (Table 1). Our strategy utilized specific antibodies that were successfully employed to identify progenitors from both upper and lower airway epithelial surfaces.[6,7] This antibody panel initially consisted of epithelial cell markers EpCAM, pan-leukocyte marker CD45, NGFR, ICAM1, and ITGA6. The stepwise isolation of these cells is depicted in Figure 2A. Leukocytes (CD45$^+$EpCAM$^-$) and
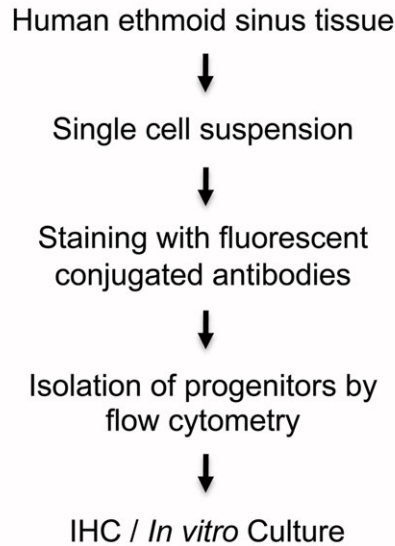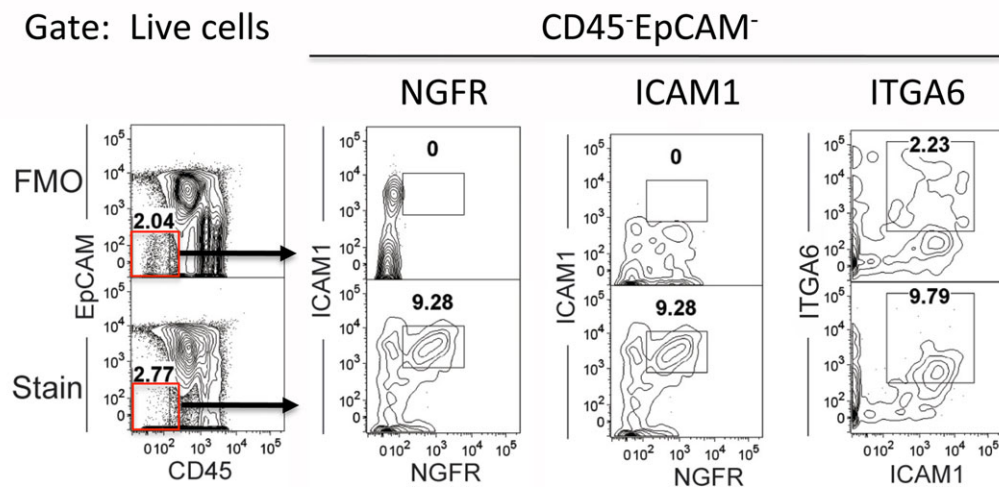
FIGURE 2. Flow cytometry subgating strategy for isolation of nasal progenitor cells. (A) Overview of the experimental strategy for identification of nasal epithelial stem cells. (B) Single-cell preparations of primary ethmoid mucosal tissues stained with fluorescent conjugated anti-human EpCAM, CD45, ICAM1, NGFR, and ITGA6. Leukocytes represent the CD45$^+$EpCAM$^-$ population of cells and epithelial cells represent the CD45$^-$EpCAM$^+$ population of cells (1st column). CD45$^-$EpCAM$^-$ cells were further selected, and analyzed for NGFR, ICAM1, and ITGA6 patterns of staining (boxed areas, 1st column). Top rows indicate the FMO antibody, which was excluded from cell staining in that tube. The respective bottom rows indicate the staining achieved after re-addition of all antibodies. NGFR antibody was omitted from the staining cocktail (FMO, 2nd column) yielding no background interference. ICAM1 antibody was omitted from the staining cocktail (FMO, 3rd column) also yielding no background interfering fluorescence. However, when ITGA6 was omitted from the staining cocktail (FMO, 4th column), background interference was notably observed. NGFR and ICAM1, but not ITGA6, were determined to be the most suitable markers for further purification studies in the upper airway tissues under study.

committed epithelial cells (CD45$^-$EpCAM$^+$) were excluded from the purification process (Fig. 2B). Therefore, lineage-negative (EpCAM$^-$CD45$^-$) cells, representing approximately 2% to 3% of the total cell population, were used in the initial subgating scheme for the isolation of the putative nasal progenitors. Subsequently, FMO cocktails were prepared as noted, omitting each of the putative progenitor cell antibodies (NGFR, ICAM1, and ITGA6) from the total antibody cocktail mixture. This strategy was performed to minimize background interference between antibodies and to obtain optimal purifica-

tion. NGFR (Fig. 2B, 2nd column), ICAM1 (3rd column), and ITGA6 (4th column) were excluded from the complete cocktail, respectively, as indicated in the top row of Figure 2. The signal from these samples was used to subtract background fluorescence from the complete antibody cocktails (Fig. 2B, bottom rows). FMO control staining for NGFR and ICAM1 showed no background interference while FMO control staining for ITGA6 in Figure 2B (top 4th column) had significant background staining. Based on these findings, although other groups have used ITGA6 in their isolation strategies, we felt
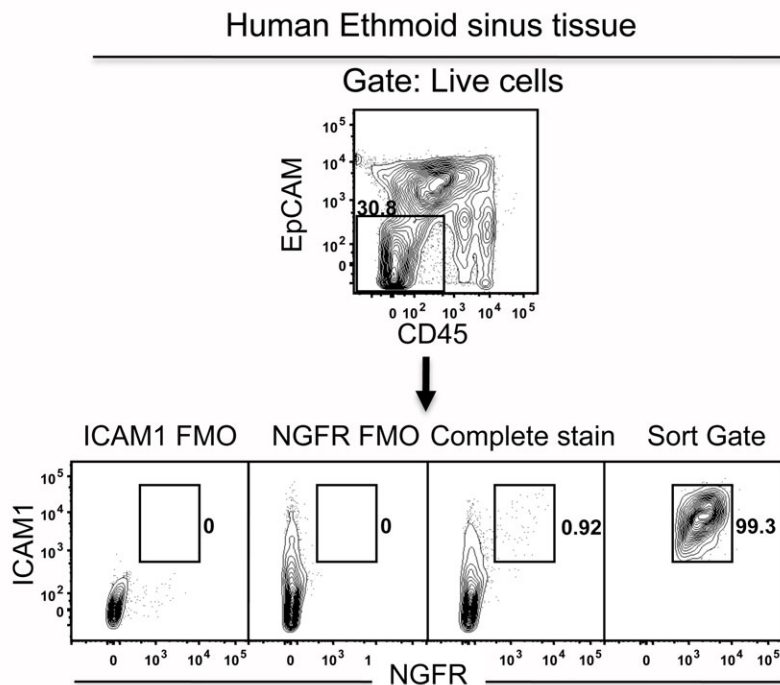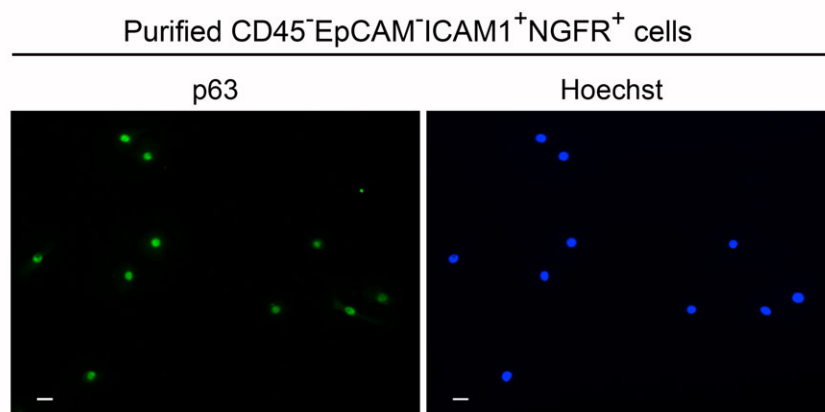
FIGURE 3. FACS purification of CD45⁻EpCAM⁻NGFR⁺ICAM1⁺ cells and p63 staining. (A) Single-cell preparations of ethmoid sinus mucosal tissues were stained with fluorescent conjugated anti-human EpCAM, CD45, ICAM1, and NGFR antibodies. Cells excluding propidium iodide were initially gated as live cells. These cells were then subgated for CD45⁻EpCAM⁻ cells. NGFR⁺ICAM1⁺ coordinates were then resolved based on FMO controls as indicated above the plot. Finally, NGFR⁺ICAM1⁺ cells were sorted to 99% homogeneity (bottom right panel) and used for subsequent studies. (B) Cytospin preparation of purified sorted cells were stained with antibody to p63 (left panel) and Hoechst stain (right panel) for all nucleated cells. Note specific nuclear p63 staining in all purified cells. Scale bar, 20 μm.

compelled to exclude this marker, and proceeded with purification of EpCAM⁻CD45⁻NGFR⁺ICAM1⁺cells in downstream studies.

Purified upper airway candidate progenitors express p63, and show spontaneous differentiation into epithelial cells

Cells were then further tested for their expression of p63 and potential to differentiate into specialized epithelial cells. EpCAM⁻CD45⁻NGFR⁺ICAM1⁺ cells were purified to 99.3% homogeneity (Fig. 3A), and comprised approximately 0.06% of total nasal cell preparations. When sorted cells were cytospun onto glass slides and permeabilized for intracellular staining, we observed that the EpCAM⁻CD45⁻NGFR⁺ICAM1⁺ cells uniformly express p63 (Fig. 3B, left panel), the same transcription factor initially identified in this work. The expression of p63 is localized to the cell nucleus as evident from overlap with Hoechst staining (Fig. 3B, right panel).
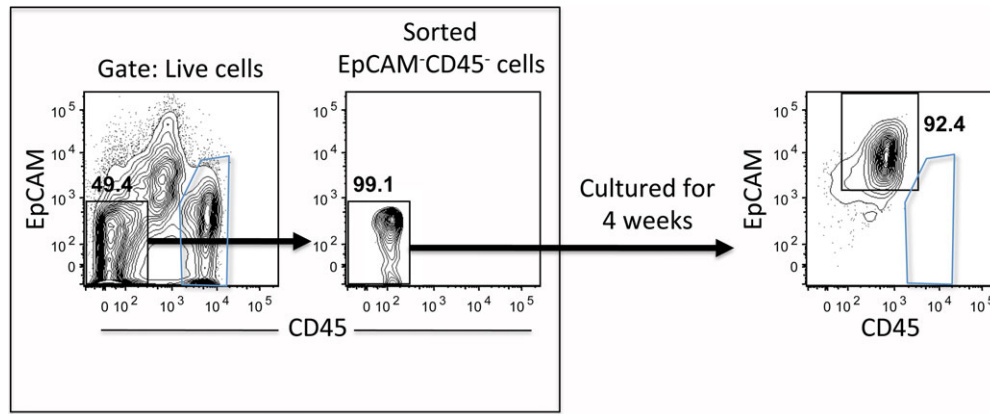
FIGURE 4. Spontaneous differentiation of CD45⁻EpCAM⁻ cells to epithelial lineage. EpCAM⁻CD45⁻ cells were cultured to determine their capacity to differentiate into epithelial lineages. The cells were analyzed by flow cytometry following 4 weeks of culture and found to express the epithelial lineage marker, EpCAM.

However, when single sorted cells were additionally cultured in vitro, they spontaneously differentiated into epithelial lineage cells over a 4-week period (data not shown). Additionally, EpCAM⁻CD45⁻ sorted cells were cultured to determine their capacity to differentiate. After 4 weeks in appropriate culture medium, these EpCAM-negative cells spontaneously differentiated to EpCAM-positive cells, suggesting that the cells under active investigation can become committed to epithelial cell fates (Fig. 4).

Discussion

In this study we attempted to better define reliable phenotypic cell-surface characteristics of a rare population of upper airway epithelial progenitors cells. To achieve this objective we used cellular markers described for lower airway and olfactory epithelial cells, which have only seen limited use in the nasal cavity.[6,7] We used high-dimensional flow cytometry techniques to purify these putative progenitor cells followed by assessing their expression of p63 and differentiation potential using in vitro cultures. We observed that a lineage-negative cell population that expresses NGFR and ICAM1 (Fig. 3A) also expresses the nuclear transcription factor p63 (Fig. 3B). In addition, when these cells were cultured in vitro they spontaneously differentiated into epithelial cell lineages (Fig. 4).

The technique of using FMO has been successfully employed in past studies involving B cells.[13] This technique allows a clear definition of background staining. By using this technique, we found the need to exclude 1 of our 3 potential target molecules, ITGA6, from our original cell sorting strategy given the elevated background/"spillover" fluorescence (Fig. 2B). We were then able to refine our studies to use only NGFR and ICAM1 for purifying putative progenitors.

There is an isolated report of nasal epithelial progenitor cells shown to produce functional ciliated cells in vitro,[14] using whole nasal tissue single-cell preparations to produce ciliated nasal epithelial cells. However, in this study, putative progenitors were not purified based on cell surface markers. In contrast, in our study, we have performed rigorous sorting of candidate progenitor cells to homogeneity based on defined cell-surface markers. Subsequent differentiation analyses are required to fully assign these cells as true progenitor cells for upper airway epithelial tissue. These assays will include assessing the true potential of these progenitor cells to generate mucin-producing cells and ciliated cells. Use of promising progenitor cell pools may be of clinical significance to better understand the cellular contributors involved in regeneration of the epithelial barrier in inflammatory diseases of the upper airway respiratory tract.

Conclusion

Based on cellular coordinates of murine and human lung epithelial cells, we have advanced early investigations into a promising population of putative multipotent primary human upper airway epithelial progenitors within sinonasal tissues. Identification of upper airway epithelial progenitor cells may hold promise for future functional regenerative therapies of the nasal mucosal epithelium.

Acknowledgments

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