Airway Progenitor Clone Formation Is Enhanced by Y-27632–Dependent Changes in the Transcriptome

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Abstract

The application of conditional reprogramming culture (CRC) methods to nasal airway epithelial cells would allow more widespread incorporation of primary airway epithelial culture models into complex lung disease research. In this study, we adapted the CRC method to nasal airway epithelial cells, investigated the growth advantages afforded by this technique over standard culture methods, and determined the cellular and molecular basis of CRC cell culture effects. We found that the CRC method allowed the production of 7.1×10^{10} cells after 4 passages, approximately 379 times more cells than were generated by the standard bronchial epithelial growth media (BEGM) method. These nasal airway epithelial cells expressed normal basal cell markers and could be induced to form a mucociliary epithelium. Progenitor cell frequency was significantly higher using the CRC method in comparison to the

standard culture method, and progenitor cell maintenance was dependent on addition of the Rho-kinase inhibitor Y-27632. Whole-transcriptome sequencing analysis demonstrated widespread gene expression changes in Y-27632–treated basal cells. We found that Y-27632 treatment altered expression of genes fundamental to the formation of the basal cell cytoskeleton, cell–cell junctions, and cell–extracellular matrix (ECM) interactions. Importantly, we found that Y-27632 treatment up-regulated expression of unique basal cell intermediate filament and desmosomal genes. Conversely, Y-27632 down-regulated multiple families of protease/antiprotease genes involved in ECM remodeling. We conclude that Y-27632 fundamentally alters cell–cell and cell–ECM interactions, which preserves basal progenitor cells and allows greater cell amplification.

Keywords: conditionally reprogrammed cells; clone-forming cell frequency; airway stem progenitor; Y-27632

The airway epithelium forms the interface through which the respiratory system first interacts with inhaled environmental agents. Dysfunction of the airway epithelium is associated with lung disorders, including asthma, chronic obstructive pulmonary disease, cystic fibrosis, and idiopathic pulmonary fibrosis (1). Study of these airway diseases and the responses to

exposures have been greatly facilitated by the use of primary lung airway epithelial cultures. Due to a limited supply of lung tissue and the small number of cells recovered from brushing and biopsy samples, isolated airway epithelial cells are typically expanded on collagen-coated plastic in proprietary bronchial epithelial growth media (BEGM) (2). Well

established air–liquid interface (ALI) culture methods can then be used to differentiate these expanded cells into a mucociliary epithelium, which is an excellent model of the normal or diseased airway (3–5).

Although the BEGM method has increased accessibility of primary airway culture methods, its wide-spread adoption

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Clinical Relevance

Studies that are designed to distinguish airway disease endotypes require primary airway cell cultures from a broad cross-section of subjects with clinically defined airway diseases. This goal can be achieved through the marriage of minimally invasive nasal airway epithelial sampling with conditional reprogramming culture (CRC) methods. Furthermore, an understanding of CRC-mediated basal cell amplification may, on its own, delineate the cellular and molecular mechanisms that regulate the epithelial response to severe airway injury. Our analysis of the CRC mechanism reveals an unforeseen ability of basal cells to adapt to their environment and the importance of the extracellular milieu in governing the normal proliferative capacity of these airway progenitor cells. We suggest that such basal cell adaptation may contribute to basal cell dysfunction in response to chronic lung diseases.

has been hindered by an inability to generate a large number of cells over multiple passages. These problems were recently addressed in both bronchial and alveolar epithelial cells using a new culture method called conditional reprogramming culture (CRC) (6–9). The near-limitless number of cells generated from a single specimen using this method has made extensive characterization of bronchial airway epithelial cell function possible in small numbers of donor subjects (7, 8, 10). However, the analysis of bronchial airway epithelium from a broad cross-section of subjects with clinically defined airway diseases is needed to help distinguish airway disease endotypes (11–13). Large-scale collection of bronchial epithelial cells is difficult, given the need to perform an invasive bronchoscopy for specimen collection. Moreover, well controlled studies require airway cells from healthy age-/sex-/race-matched subjects, who would rarely undergo bronchoscopy outside of a dedicated research protocol.

The nasal respiratory epithelium is readily accessible and is an excellent surrogate for lower airway epithelium. The same epithelial cell subtypes (secretory,

ciliated, basal) compose the upper and lower airway epithelium (14, 15). Importantly, we have shown that the nasal transcriptome and its regulation mirror that of the lower airways (16). Finally, we and others have shown dysfunction in the nasal airway epithelium among subjects with lower airway diseases, including asthma, chronic obstructive pulmonary disease, and cystic fibrosis (16–20). Based on this finding, we believe that the marriage of minimally invasive nasal airway epithelium sampling with CRC methods would allow unprecedented population-based analysis of airway dysfunction across multiple chronic lung diseases.

Before application of CRC methods to large subject cohorts, a more complete understanding of the cellular and molecular phenotype of cells produced with this method is needed. Relatedly, it is critical to understand the mechanism that underlies the enhanced expansion and passage of CRC cells. CRC-mediated cell amplification has been previously associated with several pathways, including those involved in cell immortalization (6–8, 21), induced pluripotency (22), and reprogramming to a more stem-like state (8).

In this study, we adapted the CRC method to the growth of respiratory airway epithelial cells recovered from nasal airway brushings. Importantly, we tested the ability of nonproprietary NIH3T3 cells to support nasal airway basal cell growth, while retaining other aspects of the published CRC method (7). We directly compared the growth characteristics and cellular phenotype of nasal airway epithelial cells cultured with the CRC and BEGM methods. Moreover, we explored the mechanism by which the CRC method confers greater cellular amplification and the dependence of this process on Y-27632 supplementation. Finally, we determined if Y-27632 supplementation mediated specific changes in the pattern of gene expression by whole-transcriptome expression profiling.

Materials and Methods

Human Subjects and Nasal Brushing Procedure

Human nasal airway epithelial cells were collected from four healthy subjects after receiving written informed consent, as previously described (16). The Institutional

Review Board at National Jewish Health (Denver, CO) approved this study.

Culture Techniques

Culture techniques were adapted from previously reported methods (8), and are described in detail in the online supplement.

Clone-Forming Cell Frequency

The number of progenitor cells was determined using the limiting dilution assay (23) modified for lung (24–26).

Flow Cytometry

Cell surface phenotype was determined using antibodies and sorting methods as previously described (24, 27). Cell cycle analysis was performed using the method of Krishan (28) and assayed on a Gallios 561 instrument equipped with a Hypercyte 96-well plate reader (Beckman Coulter, Indianapolis, IN).

Air-Liquid Interface Cultures

Cultures were generated and analyzed according to published methods (5, 24, 29), and are described in detail in the online supplement.

Whole-Transcriptome RNA Sequencing and Analysis

Passage (P) 2 nasal airway epithelial cells from three donors were grown using the CRC method, both with and without Y-27632 supplementation, and extracted RNA used for whole-transcriptome sequencing were per the methods listed in the online supplement.

Results

The CRC Method Better Preserves Nasal Airway Basal Progenitor Cells Relative to the BEGM Culture Method

A cytology brush was used to recover nasal respiratory epithelial cells from the posterior surface of the inferior turbinate of four donors. Brushed cells were divided evenly between plates, and P0 cultures were established using the BEGM culture and CRC methods. Cells grown using the BEGM and CRC methods exhibited distinct growth patterns (Figure 1A). Specifically, BEGM-cultured cells (grown on collagen-coated plastic) assumed a “scattered” growth pattern. In contrast, CRC cells (grown on a

Figure 1. The conditional reprogramming culture (CRC) method enhances preservation of nasal airway basal progenitor cells. (A) Phase-contrast microscopy of nasal epithelial cells grown using the bronchial epithelial growth media (BEGM) and CRC techniques reveals “scattered” and “colony” growth patterns, respectively. *Black arrows* indicate individual cells (A) or the colony edge (B). *Red arrows* in A indicate feeder cells. *Scale bars*: 200 μ m. Values represent the mean ($\pm$ SEM). (B) Frequency of cytokeratin-5- and -14-expressing passage (P) 2 cells recovered from BEGM and CRCs. Representative of studies done in three donors. (C) The surface phenotype of P2 cells recovered from BEGM cultures or CRCs was determined by flow cytometry. Bivariate FACS plots of cells stained for the tetraspanin (CD151) and tissue factor or α 6 integrin (CD49f). Representative of three to four donors. (D) Quantification of cell subtype frequency as determined by flow cytometry ($n = 3-4$ donors). Values represent the mean ($\pm$ SEM). (E) The cell cycle distribution of P2 nasal cells recovered from CRC or BEGM cultures was determined by saponin/propidium iodide staining and flow cytometry. G1, gap 1; G2M, gap 2 mitosis; S, DNA synthesis ($n = 3-4$ donors). Values represent the mean ($\pm$ SEM). (F) The effect of culture technique on clone-forming cell frequency (CFCF). Nasal brushings were cultured at P2 using the CRC or BEGM method. At P3, cells were cultured in the same medium or switched to the alternative medium. CFCF was determined by limiting dilution analysis. Values represent the mean ($\pm$ SEM). Representative of studies done in three donors.

fibroblast feeder layer) grew as well defined colonies.

We cultured P2 cells using both BEGM and CRC methods and compared their phenotypes. Cytospin preparations of P2 cells were immunostained with basal airway epithelial cell markers to determine cell type frequency. Cells recovered from BEGM were 95.6 (± 0.9)% cytokeratin (KRT) 5⁺ and from CRC were 95.7 (± 2.2)% KRT5⁺ (Figure 1B). The majority of KRT5⁺ cells coexpressed KRT14⁺ (BEGM, 81.0 $\pm$ 5.2%; CRC, 80.2 $\pm$ 5.4%). The KRT staining pattern indicated that P2 cells were basal epithelial cells, and that the frequency of basal cells did not vary as a function of culture method.

We then evaluated the surface phenotype of P2 cells by flow cytometry using known basal airway epithelial cell markers: tissue factor, integrin $\alpha 6$ (CD49f), and tetraspanin CD151 (Figures 1C–1D). All cells for both culture methods expressed tissue factor. BEGM-cultured cells were 90.8% positive for CD49f, whereas all CRC cells expressed this marker. Costaining studies demonstrated that 11.4 (± 5.1)% of CD49f-positive BEGM-cultured cells were also CD151 positive. In contrast, culture using the CRC method resulted in a significantly higher frequency of CD151/CD49f dual-positive cells (57.1 $\pm$ 10.9%). The cell cycle distribution of P2 cells was determined by saponin/propidium iodide staining and flow cytometry. The frequency of cells in G1, S, and G2M phases did not vary according to culture method (Figure 1E). Viability, as assessed by trypan blue exclusion or 4',6-diamidino-2-phenylindole staining and FACS analysis, did not vary as a function of culture method or passage (data not shown).

The ability of the CRC and BEGM methods to maintain clone-forming progenitor cells was tested by comparing cells that were initially cultured using the CRC or BEGM methods and passaged using either the CRC or BEGM culture method. Progenitor cell number was determined by limiting dilution analysis and reported as the clone-forming cell frequency (CFCF). The CFCF for cells that were passaged from CRC to CRC was significantly greater than that of cells that were passaged from CRC to BEGM (Figure 1F). Serial passage in BEGM resulted in the lowest CFCF, and this value was significantly increased by subculture

with CRC. These data demonstrate that both the CRC and BEGM culture methods result in selection for basal airway epithelial cells, and that these cells exhibit a similar cell cycle frequency. However, the CRC method results in significantly more CD151-positive cells and a significant enrichment of progenitor cells.

Nasal Airway Basal Cell Culture Using the CRC Method Permits More Extensive Cell Amplification than the BEGM Method

We evaluated the amplification metrics across four passages for brushed nasal epithelial cells using both the BEGM culture and CRC methods. P0 nasal cells from four donors were first cultured with CRC, and 4×10^5 cells were then subcultured for four sequential passages using the BEGM and CRC methods. Median cell numbers recovered from the CRCs were 3.4-, 4.9-, 2.7-, and 5.8-fold higher for P1–P4, respectively, as compared with the BEGM-cultured cells for the corresponding passages (Figure 2A). However, the cell recovery data indicate a clear downward trend in mitotic potential with increasing passage for both the BEGM or CRC methods. This increased cell recovery is reflected in a higher burst size for the cells grown by the CRC method versus the BEGM method (Figure 2B). The empirically determined amplification metrics were used to project the total potential amplification of both methods from a starting population of 8×10^5 P1 cells. Based on this projection, BEGM will permit generation of 1.9×10^8 ($\pm 1.4 \times 10^7$) P4 cells (Figure 2C). In contrast, CRC will allow generation of 7.1×10^{10} ($\pm 2.4 \times 10^{10}$) P4 cells. These data demonstrate that the CRC method can generate 379 times as many cells as the BEGM method by P4.

Nasal Airway Basal Cells Amplified with the CRC Method Exhibit Normal Differentiation

The ALI culture method is a well established model system to generate a mucociliary differentiated airway epithelium for cell biology studies (3–5). Therefore, we tested the differentiation capacity of P4 nasal airway basal cells expanded with the CRC method. Differentiation was induced using a variation of the method developed by Wu (5). The cells were cultured for 28 days after establishing a dry apical surface, and

differentiation was assessed by immunostaining. Confocal microscopy was used to determine the cellular organization within ALI cultures derived from CRC basal cells. This study found that the epithelium was pseudostratified (Figure 3A). Examination of the immunostained cultures from four donors revealed secretory cells (expressing mucin 5ac [MUC5AC] and/or MUC5B) and ciliated cells (cilia stained by acetylated α -tubulin) in cultures from all donors (Figure 3B).

We observed mucociliary differentiation in all donors; however, the extent of differentiation varied among donors, as judged by stereological examination of secretory and ciliated cell frequency (Figures 3C–3E; *see also* Figure E1 in the online supplement). Namely, we found that the frequency of MUC5B⁺ cells varied significantly among donors, with donor 1 exhibiting two to three times more cells than observed for donors 3 and 4. Donor 2 generated the fewest MUC5B⁺ cells. Variation was also observed in the frequency of MUC5AC⁺ cells across donors. Similar to secretory cell differentiation, ciliated cell differentiation varied significantly among donors. The frequency of ciliated cells did not correlate with frequency of MUC5B⁺ cells, although donor 1 generated the highest frequency of both ciliated cells and MUC5B⁺ cells. To explore if this variation in differentiation is related to progenitor cell frequency, we determined the CFCF for donors 1–4 at P4. The variation in CFCF values across donors (donor 1, 200; donor 2, 132; donor 3, 140; donor 4, 100) was not related to the extent of mucus or ciliated differentiation ($P > 0.05$).

We also examined differentiation by measuring gene expression of cell type-specific markers. Markers of basal (KRT5), secretory (MUC5AC, MUC5B), and ciliated (forkhead box J1 [FOXJ1]) cells were evaluated by quantitative RT-PCR in cultured cells recovered from the expansion stage and cells from the same two donors after 28 days of ALI differentiation (Figure 3F). Consistent with differentiation, KRT5 expression decreased 3.3-fold from the expansion to differentiated cultures. The decrease in KRT5 expression was accompanied by a 606-, 25,420-, and 658-fold increase in MUC5AC, MUC5B, and FOXJ1 expression, respectively. These data are consistent with differentiation of basal cells into both

Figure 2. CRC permits greater nasal airway basal cell amplification compared with BEGM culture. Comparison of cell amplification metrics for paired cell cultures grown using the CRC and BEGM culture methods. (A) Number of airway basal cells recovered at each of four consecutive passages. Median, interquartile range. (B) Airway basal cell burst size at each passage, calculated as the number of cells recovered after culture divided by the number of cells plated. Values represent the mean ($\pm$ SEM). (C) The total number of airway basal cells that could be generated at each passage based on the empirically determined cell burst size and starting with 8×10^5 P0 cells. Median, interquartile range. Data shown for four donors. Asterisks represent Mann-Whitney test P value < 0.05 .

secretory and ciliated cells. Together, these immunohistochemistry and gene expression results demonstrate that nasal airway basal cells cultured with the CRC method retain the ability to generate a mucociliary epithelium.

The Fibroblast Feeder Layer in the CRC Method Is Required to Maintain the Clone-Forming Basal Cell Population

We first examined whether other fibroblast feeder cell types would support basal progenitor maintenance as previously shown for NIH3T3 mouse embryonic fibroblast line (Figures 1 and 2). We found no difference in CFCF for basal cells grown on adult human primary fibroblast or smooth muscle cells (Figure 4A). We next examined whether progenitor cell maintenance of the CRC method was dependent on the presence of an irradiated fibroblast feeder layer. We found the CFCF for basal cells was reduced from 225 to 160 with omission of the feeder layer (Figure 4B). We also examined the synergy between Y-27632 and the feeder layer in maintaining progenitor cells. We found that the gain in CFCF mediated by the feeder layer was dependent on Y-27632 supplementation, and that feeder layer effects on CFCF were not observed in the absence of Y-27632 (Figure 4B). Relatedly, we found that the unique colony characteristic of CRC (e.g., small, tightly packed cells; Figure 1A) was dependent on the use of a fibroblast feeder layer (compare Figures 4C and 4E to Figures 4D and 4F).

We tested the effect on CFCF of feeder layer removal at various time points after basal cell seeding. We found that the differential trypsinization method employed for mature cultures removed the fibroblast feeder layer, but did not disturb the epithelial cells, allowing us to test this question. We found that high CFCF levels required continuous culture with feeders, as the time with feeder cells was positively correlated with CFCF levels (Figure 4G). We also found that the time with feeder cells was negatively correlated with the cells per colony (Figure 4H). Visual inspection of the colonies revealed that prolonged culture of the basal cells without feeders decreased cellular density of the colonies (Figures 4I–4N). Taken together, these results indicate that the fibroblast feeder layer is a critical determinant of CFCF, and that this effect is related to increased

Figure 3. P4 CRC amplified airway basal cells retain the ability to generate a mucociliary epithelium *in vitro*. (A) Confocal imaging analysis of epithelial structure. Arrows indicate the orientation of the X-, Y-, and Z-planes. Scale bar: 25 μm . (B) Immunohistochemical analysis of air-liquid interface (ALI) membranes for markers of mucociliary differentiation (images from a single donor, representative of four donors). Scale bar: 100 μm . (C–E) Morphometric analysis of mucin (MUC) 5B (C), MUC5AC (D), and acetylated α -tubulin (E) immunopositive cell frequency on ALI membranes. Values represent the mean ($\pm$ SEM). Data from three membranes across four donors. (F) Relative gene expression for markers of mucociliary differentiation during CRC expansion (EXP) and after differentiation (ALI). Lines connect data from a single donor. Marker gene expression is presented relative to β -glucuronidase gene expression. Data are from two donors. Average fold changes of gene expression in the ALI phase relative to the EXP phase are shown. * <0.05, ** <0.01, *** <0.001, **** <0.0001 for Mann–Whitney or *t* test *P* values. DAPI, 4',6-diamidino-2-phenylindole.

cellular density and, possibly, alteration of cell–cell interactions.

Transient Y-27632 Stimulation Drives Nasal Airway Basal Cell Amplification by Increased Clone Formation

Our comparison of the CRC and BEGM culture methods revealed that the burst size and CFCF of nasal airway basal cells is dramatically increased with the CRC method. Prior reports for other epithelial cell types suggested that addition of Y-27632 to the CRC medium enabled indefinite proliferation of these cells (7). Therefore, we examined growth effects of the CRC method with and without Y-27632. We found that addition of Y-27632 increased burst size 29-fold compared with cultures without Y-27632 (Figure 5A, $P = 0.0079$). Moreover, there was a 4-fold increase in the number of clones formed per number of cells plated when Y-27632 was included in the CRC media (Figure 5B). This increase in clone number required Y-27632 during the first 2 days of culture, but not at later time points (Figure 5B). Relatedly, the CFCF was eightfold higher in the cultures growing with Y-27632 relative to those without Y-27632 (Figure 5C, $P < 0.0001$). This effect was preserved when Y-27632 was removed after the first 2 days of culture (Figure 5C). We did observe that continuous culture in Y-27632-containing CRC medium generated slightly higher CFCFs, whereas the number of cells per clone did not vary as a function of Y-27632 addition (Figure 5D). These results suggest that the Y-27632-dependent increase in culture burst size is due to an enhanced capacity of the nasal airway basal cells to form clones. Moreover, the increased clone formation is largely mediated in the first 2 days of culture.

Y-27632 Treatment Modulates Cytoskeletal and Extracellular Matrix–Modifying Pathway Gene Expression

Our results indicate that Y-27632, in the context of the CRC method, enhances nasal epithelial clone formation, which results in dramatically increased cell yields. The molecular mechanism(s) underlying these observations are unclear. Therefore, we performed an agnostic RNA-sequencing study of nasal airway basal cells to determine the effects of Y-27632 on the transcriptome. The cells from three donors used for this experiment were continuously cultured with

Y-27632 stimulation before P2. During P2, cells were cultured for 8 days with or without continuous Y-27632 stimulation. On Culture Day 8, nasal airway basal cells were purified by flow sorting (Figure 1) and extracted RNA was used for RNA sequencing (Table E1). Multidimensional scaling of gene expression profiles demonstrated consistent transcriptome-wide changes in response to Y-27632 stimulation (Figure 6A).

To identify functional gene groupings and pathways affected by Y-27632 treatment, we used the Generally Applicable Gene-set Enrichment analysis method (30), which permits the analysis of all expression data rather than just differentially expressed genes. This method has the advantage of detecting small, but coordinated, changes in pathway expression that are biologically relevant, yet would be missed by single-gene differential expression analyses. We first tested the Gene Ontology cellular component database with our data set. We identified seven gene sets that were significantly perturbed (false discovery rate of 5%) by Y-27632 stimulation: the intermediate filament, keratin filament, intermediate filament cytoskeleton, extracellular matrix (ECM), proteinaceous ECM, anchored component of the membrane, and cell surface categories (Figure 6B, Table E2). We then tested the Gene Ontology molecular function database. We found eight significant groups: olfactory receptor activity, cytokine activity, G protein-coupled amine receptor activity, type I IFN receptor binding, cytokine receptor binding, peptidase inhibitor activity, adrenergic receptor activity, and endopeptidase inhibitor activity (Figure 6C, Table E3).

Y-27632 Treatment Modulates Expression of Genes Involved in Establishing Cell–Cell and Cell–ECM Interactions

To determine the genes that underlie the Y-27632-dependent shift in gene set expression, we performed paired single-gene differential expression analysis (31). We identified 639 differentially expressed genes (false discovery rate of 5%; Tables E4–E7). Among these, 462 were down-regulated and 177 were up-regulated in response to Y-27632. Single-gene differential expression corroborated gene set analysis results implicating Y-27632 in

mediating wide-spread expression changes for genes involved in cytoskeletal structure, ECM modifications, interactions between cells and the ECM, and cytokines, as detailed subsequently here (Figure 6D, Table E5).

Cytoskeleton genes. Intermediate filaments link the airway epithelial cell cytosolic compartment to adjacent cells and the extracellular environment via desmosomes and hemidesmosomes. We found that Y-27632 treatment modulated fundamental genes in this cellular–ECM network. Intermediate filaments are composed of KRT dimers. In basal airway epithelial cells, the intermediate filaments are composed of type I and type II KRTs; specifically, *KRT-5*, *-6C*, *-14*, *-15*, and *-16* (32, 33). All of these KRTs were up-regulated by Y-27632 treatment (Figure 6D, Table E5). In contrast, the type I KRTs, *KRT10* and *KRT23*, not previously reported to mark airway basal cells, were up- and down-regulated by Y-27632 treatment, respectively. Vimentin, an intermediate filament component that is highly expressed in mesenchymal cells (34), was also down-regulated by Y-27632. The intermediate filaments are anchored to cell–cell junctions via desmosomes and to the ECM via hemidesmosomes. An obligate component of the anchoring complex is desmoplakin (35), which was up-regulated in Y-27632-treated cells. Plakophilin-1, an interacting partner of desmoplakin and a component of the desmosome (35), was also up-regulated by Y-27632 treatment.

Cell–cell interaction genes. Airway epithelial cells form intercellular tight junctions via multiprotein complexes. We found that Y-27632 treatment down-regulated expression of four genes that are essential to forming these junctions (36). These included the occludin gene and claudin genes 1, 4, 7, and 8 (Figure 6D, Table E5). Claudins-10 and -11 were down-regulated as well, whereas claudin-19 was up-regulated with Y-27632 treatment. We note that claudin-10 was previously identified as a club cell marker in mice (37). Airway epithelial cells also interact via adherens junctions, which are formed by cadherin proteins (38). P- and E-cadherins (*CDH3*, *CDH1*) are two of the most highly expressed cadherin genes in airway epithelial cells (39, 40). We found that *CDH3* expression was down-regulated by Y-27632 treatment, whereas *CDH1* expression was unaffected. Importantly,

Figure 4. Impact of fibroblasts on nasal airway basal progenitor frequency. (A) CFCF was determined for nasal airway basal cells plated on embryonic mouse (NIH3T3), adult human lung fibroblasts from normal (NHLF), or idiopathic pulmonary fibrosis (IPF) 11 or normal human lung smooth muscle (SM). Mean ($\pm$ SEM) ($n = 3$). Representative of four subjects. (B) CFCF was determined for nasal airway basal cells cultured with or without Y-27632 in the presence or absence of NIH3T3 feeders. Mean ($\pm$ SEM) ($n = 3$). Representative of four subjects. (C–F) Phase-contrast images of Geimsa-stained nasal airway basal cells grown with Y-27632 and NIH3T3 feeders (C), Y-27632 without feeders (D), without Y-27632 with feeders (E), and without Y-27632 or feeders (F). Solid lines in C, D, and E demarcate clones. Arrows in F indicate single cells. Scale bars: 200 μ m. (G) CFCF was determined for nasal airway basal cells that were cultured with NIH3T3 fibroblast feeders for 2, 3, 4, 5, 6, or 8 days and assayed on Culture Day 8. Values represent the mean ($\pm$ SEM). (H) Cells per colony were determined for nasal airway basal cells that were cultured with NIH3T3 fibroblast feeders for 2, 3, 4, 5, 6, or 8 days and assayed on Culture Day 8. Values represent the mean ($\pm$ SEM). (I–N) Phase-contrast images of Geimsa-stained nasal airway basal cells that were cultured with NIH3T3 fibroblast feeders for 2 (I), 3 (J), 4 (K), 5 (L), 6 (M), or 8 (N) days and assayed on Culture Day 8. Scale bars: 200 μ m.

Figure 5. CRC amplification and increase in nasal airway basal progenitor cell clone-forming frequency is dependent on Y-27632 stimulation. (A) Comparison of burst size for airway basal cells grown with the CRC methods with and without Y-27632. (B–D) Effect of Y-27632 supplementation on cell behavior in CRCs: none, Y-27632 was not added; 0–6, 0–4, 0–2 indicate the intervals (in days) during which Y-27632 was present. (B) Effect of Y-27632 supplementation on the number of clones per cell plated. (C) Effect of Y-27632 supplementation on the CFCF. (D) Effect of Y-27632 supplementation on the number of cells per clone. * <0.05, ** <0.01, *** <0.001 for Mann–Whitney or *t* test *P* values.

down-regulation of *CDH1* when paired with up-regulation of N-cadherin (*CDH2*) is a marker of malignant transformation (41). *CDH2* was expressed at extremely low levels, and was not altered by Y-27632 treatment.

Protease/antiprotease genes. We found that the expression of many protease genes was down-regulated by Y-27632 treatment (Figure 6D, Table E5). Specifically, four matrix metalloproteinase (MMP) genes, including *MMP2*, -9, -14, and -28, were down-regulated. Three of these four proteases are secreted (*MMP2*, -9, -28), whereas *MMP14* is membrane associated. We also found a disintegrin and metalloproteinase (ADAM) genes were down-regulated with Y-27632 treatment. Among the ADAM family genes affected were *ADAM8*, -9, and -28. Similarly, four

thrombospondin motif-containing ADAM protease genes, *-TS1*, *-TS6*, *-TSL3*, *-TSL4* (lacks activity), were down-regulated with Y-27632. Finally, multiple genes in the serine protease inhibitor (*SERPIN*) family were down-regulated with Y-27632 treatment. These genes included intracellular *SERPINS* (*B3*, *B6*, *B7*, *B9*, and *B13*), extracellular *SERPINS* (*A3* and *E1*), and *SERPINB2*, which is found in both the intracellular and extracellular compartments. In contrast, the extracellular *SERPINE2* was up-regulated.

Inflammatory cytokines, chemokines, and their receptors. In line with the pathway analysis, we observed that expression of multiple inflammatory cytokines was significantly down-regulated in response to Y-27632 treatment (Figure 6D, Table E5). For example, the most prominent

inflammatory cytokine produced by airway epithelial cells, *IL8*, was down-regulated 2.53-fold with Y-27632 treatment. *IL-15*, -32, and -33 (a driver of type 2 cytokine gene expression) were all down-regulated. Cytokine receptors *IL1R1* and *IL7R* were down-regulated, whereas *IL6R* was up-regulated. Inflammatory epithelial chemokines that were down-regulated by Y-27632 treatment included *CXCL3*, *CXCL5*, *CXCL6*, *CXCL16*, and *CXCL17*. Notably, *CCL20* (macrophage inflammatory protein 3 α), a chemoattractant for lymphocytes and neutrophils, but not monocytes, was down-regulated as well. In contrast, *CXCL14* (macrophage inflammatory protein 2 γ) has chemotactic activity for monocytes, but not for lymphocytes, dendritic cells, neutrophils, or macrophages. This gene

Figure 6. The effects of Y-27632 supplementation of CRC on the airway basal cell transcriptome. (A) Multidimensional scaling plot of whole-transcriptome data from three subjects' cultures grown with and without Y-27632. Dotted lines connect the subject pairs and show similar directional change in transcriptome expression between Y-27632-treated and untreated cultures for all donors. Red, +Y-27632; blue, -Y-27632. (B and C) Differentially expressed (B) cellular component and (C) molecular function gene ontology categories with Y-27632 stimulation are shown. Box plot of fold changes for genes within each set are shown. Individual gene fold changes shown by points. Red points indicate genes, the expression of which is differentially regulated by Y-27632. (D) Fold changes with Y-27632 treatment for genes in select functional groups (median, interquartile range). Genes represented by points are shown in Table E5. (E) *In situ* analysis of matrix metalloproteinase activity. Solid lines in E demarcate clones; arrows point toward areas of matrix metalloproteinase activity. Scale bars: 200 μm . (F) Quantification of cells with matrix metalloproteinase activity. Note: Gene Ontology (GO) term 0005882 intermediate filament was also significant in the cellular component analysis, but greater than 98% of genes within this term were also part of the GO:0045111 term, intermediate filament cytoskeleton, displayed. ADAM, a disintegrin and metalloproteinase ADAM; MMP, matrix metalloproteinase; SERPIN, serine protease inhibitor.

was up-regulated with Y-27632 treatment. Because of the dynamics in chemokine expression, we queried receptors for these proteins, and found that none of these receptors were differentially expressed (*CCR*, *CXCR*, *CX3CR*, and *XCR1*).

Growth factor cytokines. We also note that IL-33 has been reported as a marker of the basal airway stem cell (42), although immunostaining shows expression by all basal cells. Other cytokines implicated in proliferation of basal epithelial cells were down-regulated by Y-27632 treatment, including leukemia-inhibitory factor and Factor 3 (Figure 6D, Table E5). Leukemia-inhibitory factor is structurally related to *IL6*, which was identified as a basal cell growth factor (43). Factor 3 is a member of the clotting cascade, which was previously shown to function in basal cell survival (27).

Y-27632-regulated genes. Y-27632-mediated cell amplification has been associated with several pathways, including those involved in cell immortalization (6–8, 21), although this mechanism has been challenged (44). In keratinocytes, the Y-27632 immortalization pathway was associated with expression of cMyc, up-regulation p53 and telomerase enzymes, and was characterized by decreased expression of differentiation markers (6). Although we observed down-regulation of secretory differentiation genes in Y-27632-treated cells, the molecular mediators that were previously associated with the immortalization process were expressed at similar levels with and without Y-27632, or were not expressed under either condition. Similarly, three of the four genes associated with induced pluripotency (*OCT4*, *NANOG*, and *KLF4*) (22) were not differentially expressed. In contrast, *SRY-box 2* (*SOX2*), which also plays a role in epithelial differentiation, was up-regulated with Y-27632 treatment.

Y-27632 Treatment Modulates ECM Modification Activity

Several of the functional gene groupings and pathways affected by Y-27632 were related to ECM modification and cell interaction with the ECM. Thus, we used *in situ* zymography to determine if Y-27632 altered matrix metalloproteinase activity at the cellular level. *In situ* analysis of gelatinase-type matrix metalloproteinases revealed that Y-27632 treatment decreased activity (Figures 6E and 6F). A similar

analysis of collagenase activity demonstrated that Y-27632 treatment altered the location of cells with collagenase activity (Figure 6E), but did not alter the number of cells with activity (Figure 6F). In cultures treated with Y-27632, cells with collagenase activity were located in central regions. In contrast, collagenase activity was located at the perimeter of clones grown in the absence of Y-27632. These studies indicate that Y-27632 treatment alters the ECM and cell–ECM interactions, as predicted by the RNA-sequencing study.

Discussion

Conditional reprogramming methods have made primary cell culture models accessible to the greater scientific community. In this article, we detail application of the CRC method to upper (nasal) airway epithelial cells. The standard methodology for culture of nasal epithelial cells involves plating on collagen-coated plastic in proprietary BEGM media. By applying the CRC method, we were able to produce approximately 400 times more cells than the BEGM method during the first four culture passages. Moreover, the P4 CRC cells were still able to polarize and generate a mucociliary epithelium using the ALI method, whereas, in our experience, P4 BEGM cultured cells do not generate a mucociliary epithelium.

The ability to produce considerably more cells, which retain differentiation capacity, affords significant advantages in many research settings. For example, gene-editing methods are coming to the forefront of primary cell disease model research. We have shown that our production of edited primary nasal epithelial cells required extensive cell amplification and the ability to repeatedly passage transduced cells (45). Similarly, enhanced proliferation also opens the door to other cell-intensive studies, such as drug screening, environmental agent response studies, and biochemical analyses.

Although our study and others have described increased amplification potential of multiple epithelial cell types mediated by the CRC method, the mechanism responsible for this amplification has remained elusive. For both the BEGM culture and CRC methods, our cell cycle analysis indicated a normal distribution of cells across the cycle stages. This result is in line with previous analyses of CRC cells,

which did not indicate cancerous transformation (6, 7). Several investigators have also suggested that the majority of cells grown with the CRC method are “reprogrammed” to a more stem-like state (8). Although we cannot rule out this possibility, we find that cells generated using both the CRC and BEGM methods exhibit a normal basal airway epithelial phenotype. This is evidenced by expression of the same KRTs (KRT5 and -14) and surface markers (tissue factor and CD49f) using both methods. Finally, we note previous work, which has shown the ability to passage airway epithelial cells with this technique at least 11 times (8). In contrast, a projection of our passage data indicates that, although the mitotic potential of cells grown with this method is increased, it remains finite. This finding further supports our interpretation that the CRC method propagates normal airway basal cells.

Additional studies suggest that the CRC technique promotes clone formation rather than selecting for a tissue stem cell subpopulation (7, 46, 47). We find that nasal airway basal cells exhibit an eightfold increase in CFCF when cultured using the CRC method. We show that this increase in CFCF is dependent on Y-27632 supplementation, and that Y-27632 addition is necessary only during the first 2 days of culture. Similar results were reported for CRC basal cells from skin, prostate, mammary gland, cervix, and trachea (7, 8). Because the Y-27632 effect occurs before the emergence of clones, and Y-27632 addition did not change the number of cells per clone, these results suggest that the CRC method permits more efficient propagation of extant basal progenitor cells within the cell population.

Treatment with Y-27632 inhibits binding of ATP to the rho kinase, and prevents rho-associated protein kinase-dependent phosphorylation of enzymes involved in cell type-dependent actin–myosin cytoskeleton formation, organization, and contraction (48, 49), and regulates focal adhesion formation, maturation, and turnover (reviewed in Ref. 50). We found that Y-27632 treatment also mediates wide-spread gene expression changes. Surprisingly, we observed expression changes to intermediate filament, rather than to actin microfilament, genes. Specifically, the KRTs (e.g., KRT5 and -14) were up-regulated by Y-27632. These genes

are diagnostic of airway basal cells, and are among the most highly expressed genes in this cell type (33, 51). These *KRT* genes are expressed at ~10 times the average expression of the other Y-27632-regulated genes, suggesting that KRT-mediated changes to the cytoskeleton may be fundamental to the effect of Y-27632 on basal cell amplification. Up-regulation of keratins in basal cells may be a direct consequence of a Y-27632-dependent increase in the *FOXN1*, a transcription factor, which is known to activate keratin promoters (52). Because KRT gene expression changes were not observed in Y-27632-treated human alveolar type II cells (9), we suggest that Y-27632-driven KRT up-regulation may be a unique response of airway basal progenitor cells.

In a pseudostratified epithelium, such as that found in the human airway, basal cells adhere tightly to the basement membrane; whereas phenotypically distinct luminal cells adhere primarily through cell–cell interactions (53, 54). Basal cells use desmosomal proteins to attach to the basement membrane, whereas luminal cell interactions are primarily mediated through tight junction proteins. This bias in cell adhesion mechanisms is critical for cell proliferation and wound repair (55). We found that expression of key airway desmosomal genes was up-regulated with Y-27632 treatment, and that expression of critical airway epithelial tight junction genes was down-regulated. This Y-27632-dependent variation in cell–cell and cell–ECM interactions may be related to our finding that genes involved in release of growth factors from the matrix and activation of growth factor receptors were also down-

regulated by Y-27632 treatment. These observations and the known roles for cell–cell and cell–ECM interactions in epithelial cell proliferation suggest a decreased need for exogenous growth factor stimulation in the context of Y-27632. Finally, we found that Y-27632 treatment resulted in down-regulation of an epithelial differentiation transcription factor SAM pointed domain-containing Ets transcription factor (SPDEF) and markers typical of a differentiated epithelium (mucin 1 and secretoglobin family 1A member 1). Together, these data suggest that Y-27632 supports basal progenitor cell maintenance by: (1) promoting the basal cell phenotype; (2) stimulating basal cell-specific cell–cell and cell–ECM interactions; and (3) suppressing differentiation to an airway luminal cell phenotype.

Consistent with the importance of cell–ECM interactions for basal cell function, we found that a large majority of the genes that were differentially expressed $\pm$ Y-27632 are involved in formation of the ECM. Namely, multiple classes of ECM modifying genes and their inhibitors were highly down-regulated in response to Y-27632 treatment. This gene expression pattern suggests that the clone-forming process used by Y-27632-treated cells requires little remodeling of existing extracellular environment. This mechanism is consistent with the previously proposed idea that Y-27632 treatment suppresses anoikis (46), an integrin-mediated cell death mechanism that is activated by cell disadhesion or contact with ECM proteins that are not appropriate to the cell's integrin composition (56). Beyond the positive effects of Y-27632 treatment on clone formation, our study also suggests that clone formation

in the absence of Y-27632 is mediated by nasal airway basal progenitors that acquire or possess an innate ability to actively remodel the ECM. The apparent capacity of basal cells to adapt their milieu may contribute to basal cell dysfunction in response to severe epithelial injury (57) and chronic lung diseases, including idiopathic pulmonary fibrosis (29).

In summary, our analysis of CRC nasal airway basal cells indicates that the greater expansion afforded by this method is mediated by the ability of Y-27632 treatment to enhance basal cell clone formation, and thus preserve the basal airway progenitor cell population. Molecular analysis of these cells suggests that Y-27632 treatment fundamentally alters cell–cell and cell–ECM interactions to a phenotype more consistent with basal cells as opposed to luminal epithelial cells. This highlights the ability of the basal cell to adapt to its environment and the importance of the extracellular milieu in governing the normal proliferative capacity of airway progenitor cells. In total, these results highlight the great potential of airway basal cells that are isolated by nasal brushing and expanded with the CRC method to allow population-based analyses of airway dysfunction across many chronic lung diseases. ■

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