ORIGINAL ARTICLE

Rapid Expansion of Human Epithelial Stem Cells Suitable for Airway Tissue Engineering

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Abstract

Rationale: Stem cell–based tracheal replacement represents an emerging therapeutic option for patients with otherwise untreatable airway diseases including long-segment congenital tracheal stenosis and upper airway tumors. Clinical experience demonstrates that restoration of mucociliary clearance in the lungs after transplantation of tissue-engineered grafts is critical, with preclinical studies showing that seeding scaffolds with autologous mucosa improves regeneration. High epithelial cell–seeding densities are required in regenerative medicine, and existing techniques are inadequate to achieve coverage of clinically suitable grafts.

Objectives: To define a scalable cell culture system to deliver airway epithelium to clinical grafts.

Methods: Human respiratory epithelial cells derived from endobronchial biopsies were cultured using a combination of mitotically inactivated fibroblasts and Rho-associated protein kinase (ROCK) inhibition using Y-27632 (3T3+Y). Cells were analyzed by immunofluorescence, quantitative polymerase chain reaction, and

flow cytometry to assess airway stem cell marker expression. Karyotyping and multiplex ligation-dependent probe amplification were performed to assess cell safety. Differentiation capacity was tested in three-dimensional tracheospheres, organotypic cultures, air–liquid interface cultures, and an *in vivo* tracheal xenograft model. Ciliary function was assessed in air–liquid interface cultures.

Measurements and Main Results: 3T3-J2 feeder cells and ROCK inhibition allowed rapid expansion of airway basal cells. These cells were capable of multipotent differentiation *in vitro*, generating both ciliated and goblet cell lineages. Cilia were functional with normal beat frequency and pattern. Cultured cells repopulated tracheal scaffolds in a heterotopic transplantation xenograft model.

Conclusions: Our method generates large numbers of functional airway basal epithelial cells with the efficiency demanded by clinical transplantation, suggesting its suitability for use in tracheal reconstruction.

Keywords: epithelium; adult stem cells; trachea; respiratory mucosa; tissue engineering

At a Glance Commentary

Scientific Knowledge on the Subject: Cell therapies aiming to apply an autologous epithelium to tracheal transplants are in their infancy. Current airway epithelial cell culture techniques are limited in their scalability to large constructs and produce cells that lack appropriate differentiation potential and functionality at clinically relevant time points.

What This Study Adds to the Field: We define a method for epithelial cell expansion capable of generating sufficient numbers of clinically useful human epithelial cells for airway tissue engineering.

Patients with tracheal disease have a poor quality of life and often prognosis due to the limited reconstruction options available. Organ transplantation has dramatically improved patient mortality and morbidity, but donor organ supply cannot meet demand and life-long immunosuppression is required (1). Tissue engineering aims to bioengineer cell–scaffold technologies as an alternative strategy (2). The first bioengineered tracheal transplantation took place in 2008 and more have followed (3–5), making upper airway reconstruction among the first in the field to see clinical translation of advanced tissue-engineering methods (6). Although the clinical need for these transplants is established, many aspects of this nascent therapy remain to be investigated in detail (7), including the use of decellularized versus synthetic scaffolds (8), the value of graft prevascularization or enhanced angiogenesis (9), and the optimal

combination of growth factors and cultured cells to stimulate regeneration (10, 11).

In healthy airways, a pseudostratified epithelium consisting of three primary cell types allows mucociliary clearance: basal stem cells line the basement membrane, goblet cells produce mucus to trap inhaled particles and pathogens, while ciliated cells produce motile force to remove these from the lungs (12, 13). After tracheal transplantation, compromised mucociliary clearance represents an important challenge as secretions are retained at the distal anastomosis site, promoting infection and airway obstruction (14, 15). Therefore, inclusion of a functional epithelium in tracheal transplants is desirable, and some of the first tracheal transplants have included autologous epithelial cells with a view to expediting mucosal recovery (3, 4). However, there is limited time available to culture cells owing to the emergency nature
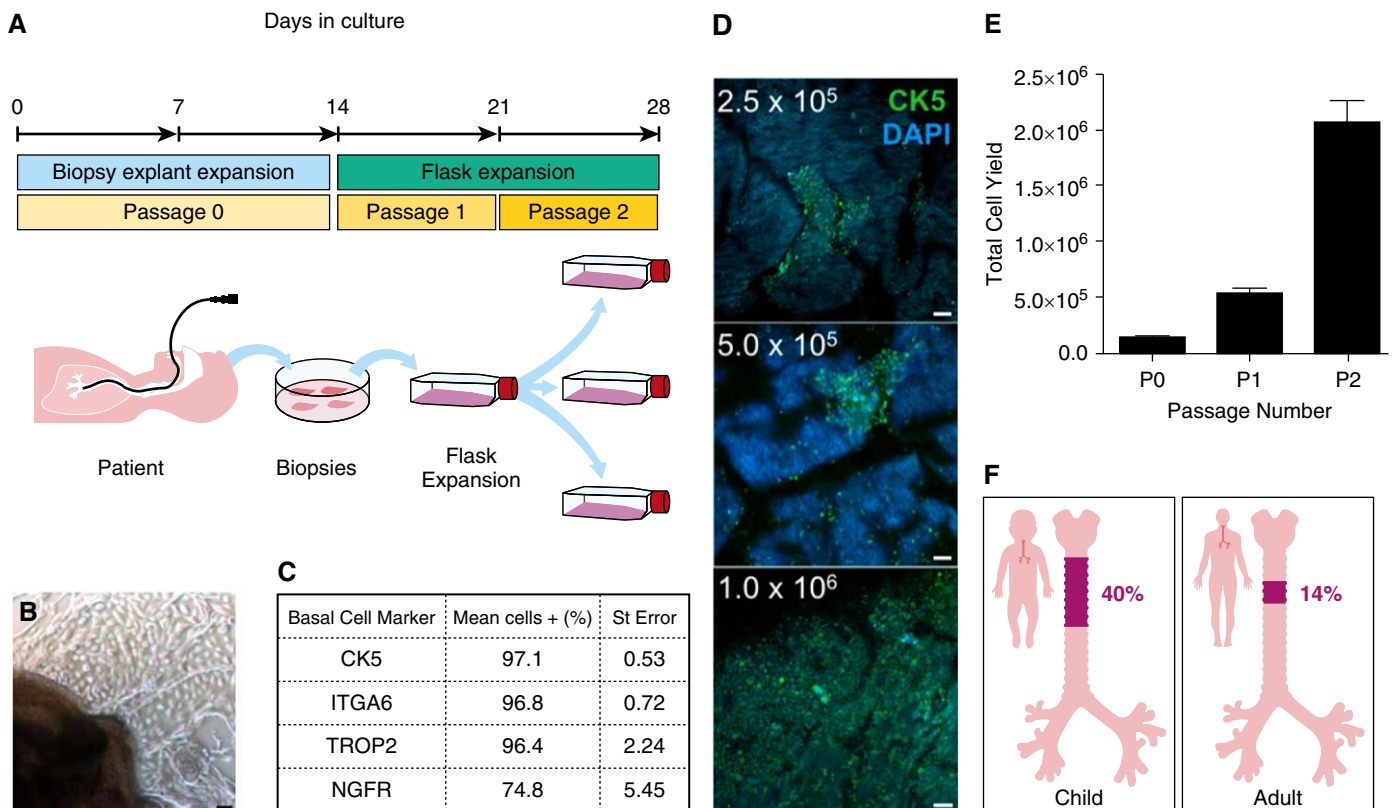
Figure 1. Isolation of autologous human basal epithelial cells from tracheobronchial biopsies. (*A*) Schematic representation of epithelial cell culture pipeline from bronchoscopic biopsy explants. (*B*) Bright-field image of biopsy outgrowth (*scale bar*, 20 μm). (*C*) Table summarizing flow cytometric analysis of the proportion of basal cell marker–expressing cells grown from biopsies in bronchial epithelial growth medium (BEGM) (n = 5 biopsies). (*D*) Decellularized human tracheal scaffolds seeded with expanded CK5$^+$ airway epithelial cells (*green*) show poor coverage at seeding densities of less than 1×10^6 cells/cm^2 (counterstained with 4′,6-diamidino-2-phenylindole [DAPI], *blue*; *scale bar*, 50 μm). (*E*) Cumulative cell numbers generated from biopsies expanded in BEGM (n = 19 donors) over passage. (*F*) Estimation of maximum graft size possible using existing culture protocols (assumes five biopsies can be obtained and 100% culture success) in children (*left*) and adults (*right*). CK5 = cytokeratin 5; St Error = standard error.
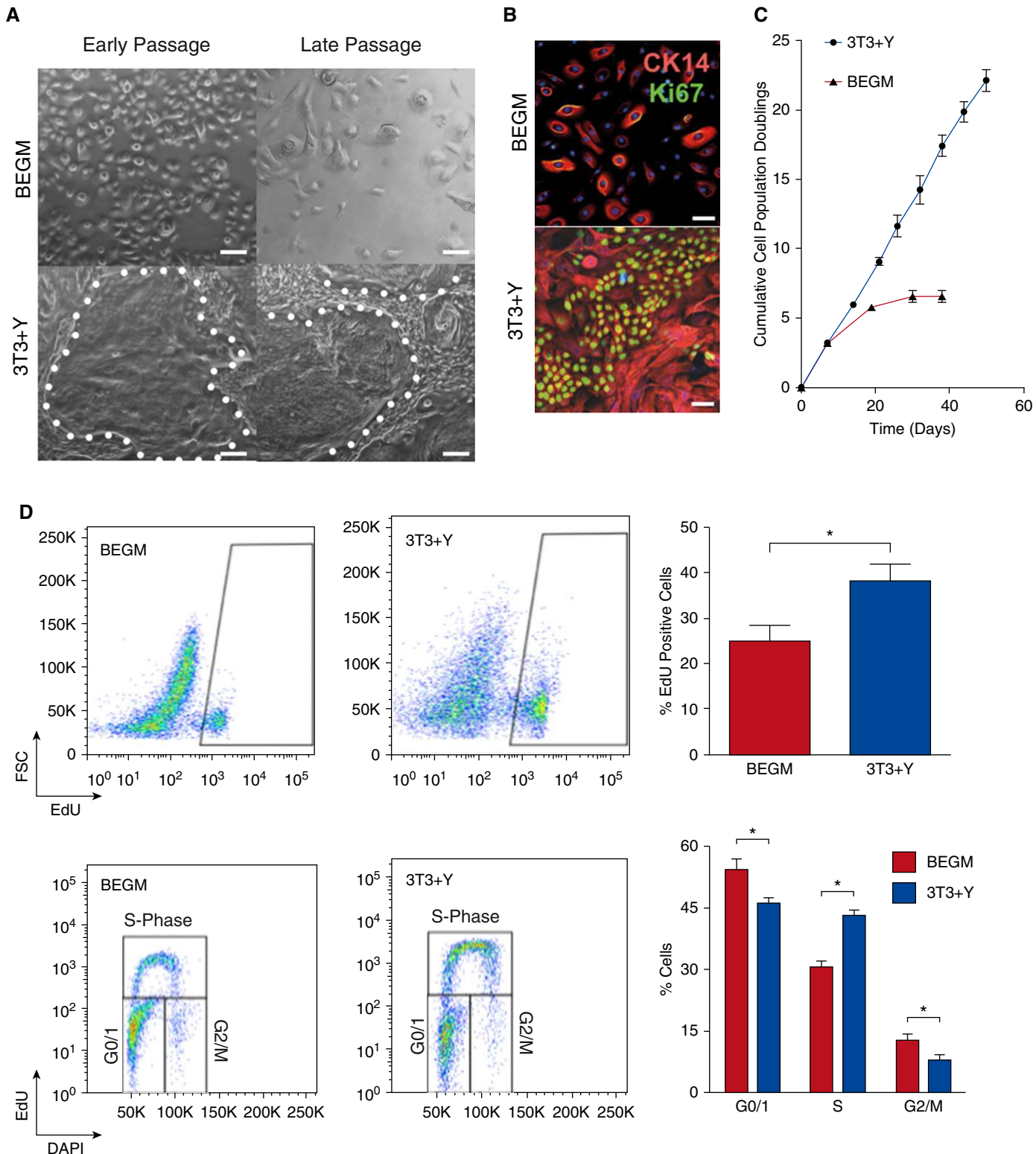
Figure 2. 3T3-J2 coculture and Rho-associated protein kinase inhibition allow rapid expansion of human airway epithelial cells. (*A*) Bright-field images show the morphology of cells in bronchial epithelial growth medium (BEGM) and in 3T3+Y at both early (P1) and late (P4) passage. *White dotted lines* indicate epithelial colonies in 3T3+Y cultures (*scale bar*, 20 μm). (*B*) Airway epithelial cells stained by immunofluorescence with a marker of actively dividing cells (Ki67; *green*), cytokeratin 14 (CK14; *red*), and 4′,6-diamidino-2-phenylindole (DAPI; *blue*) after 4 days of culture in BEGM or in 3T3+Y (*scale bar*, 50 μm). (*C*) Population doublings for human airway epithelial cells grown in BEGM and 3T3+Y plotted over time. (*D*) Representative plots showing

of some interventions, and an inability to study cell fate in humans means that little is known about their contribution. Clinical observations showed that patients were slow to regenerate healthy mucosa (3, 4, 15, 16).

High cell-seeding densities are required for bioengineering applications and, given the large surface area of clinical tracheal grafts, obtaining sufficient numbers of autologous epithelial cells is a challenge for the field. Previously, epithelial cells were obtained from endobronchial biopsies and cultured in serum-free bronchial epithelial growth medium (BEGM) for multiple passages (3). This is a useful tool to generate basal cells for *in vitro* investigations (17), but we find it inefficient for regenerative applications as many cultures fail and those that grow cannot provide sufficient cell numbers for graft coverage. In addition, in BEGM, cells undergo a well-characterized decline in their capacity for multipotent differentiation into a ciliated epithelium over passaging (18–20), suggesting that self-renewal capacity begins to be lost in culture after one or two passages. A method to generate sufficient numbers of airway epithelial cells for use in tissue-engineered tracheal transplants therefore represents a significant and unmet need.

Successful *ex vivo* long-term expansion of human epidermal stem cells is achieved by coculture with mitotically inactive mouse embryonic fibroblast feeder cells (21). Inhibition of Rho-associated protein kinase (ROCK) increases proliferation and conditionally immortalizes cells, allowing indefinite propagation of stem cells with tissue-appropriate differentiation capacity (22–25). Here, we investigate the suitability of this method for expansion of primary human airway epithelial cells. Cells expressing airway basal stem cell markers with multilineage airway differentiation capacity are expanded rapidly and efficiently, suggesting that this technique may generate the quantities of functional epithelial cells demanded by future tissue-engineered constructs. Some of these results have previously been published as abstracts (26, 27).

Methods

Complete methods can be found in the online supplement.

Human Airway Epithelial Cell Culture

Human bronchial epithelial cell cultures were derived from biopsies taken during tracheobronchoscopy procedures with patient consent. Ethics approval was obtained through the National Research Ethics Committee (REC references 06/Q0505/12 and 11/LO/1522). Biopsies were obtained from healthy regions of airways and received on ice in transport medium (αMEM supplemented with penicillin–streptomycin and amphotericin B). Explant cultures were plated directly onto 25-cm^2 flasks and enough bronchial epithelial growth medium (BEGM) was applied to cover the flask. Explants (P0) were cultured for a maximum of 14 days before first passage. Experiments that required a significant number of cells grown in BEGM were performed on cells derived from cadaveric donor airways or from airways removed as part of lobectomy procedures. These cells were isolated according to protocols described by Fulcher and colleagues (17) and frozen at first passage, using standard protocols. For experiments comparing matched donor cells under different culture conditions, cells were thawed in BEGM for one passage and then divided according to experimental culture conditions.

For cocultures, epithelial culture medium consisted of Dulbecco's modified Eagle's medium (cat. no. 41966; Gibco) and F12 (cat. no. 21765; Gibco) at a 3:1 ratio with penicillin–streptomycin (cat. no. 15070; Gibco) and 5% fetal bovine serum (cat. no. 10270; Gibco) supplemented with 5 μM Y-27632 (cat. no. Y1000; Cambridge Bioscience, Cambridge, UK), hydrocortisone (25 ng/ml) (cat. no. H0888; Sigma-Aldrich, St. Louis, MO), epidermal growth factor (0.125 ng/ml) (cat. no. 10605; Sino Biological, Beijing, China), insulin (5 μg/ml) (cat. no. I6634; Sigma-Aldrich), 0.1 nM cholera toxin (cat. no. C8052;

Sigma-Aldrich), amphotericin B (250 ng/ml) (cat. no. 10746254; Fisher Scientific, Loughborough, UK), and gentamicin (10 μg/ml) (cat. no. 15710; Gibco). Epithelial cells were cultured at 37°C and 5% CO_2 with three changes of medium per week. For experiments requiring isolation of a pure epithelial cell population from cocultures, we performed differential trypsinization, taking advantage of the greater trypsin sensitivity of feeder cells in comparison with strongly adherent epithelial cells. All trypsinization was performed with TrypLE (Life Technologies, Carlsbad, CA), a recombinant enzyme, avoiding the use of porcine trypsin. Population doublings (PD) were calculated as PD = 3.32 × [log(cells harvested/cells seeded)].

Results

Requirement for Epithelial Cell Expansion in Airway Tissue Engineering

To include expanded autologous epithelial cells in clinical transplants requires the ability to derive cells several weeks in advance of surgery. To date, this has been achieved through procurement of endobronchial biopsies (3) that are expanded in culture (Figure 1A; and *see* Figure E1A in the online supplement). We confirmed that we could expand airway epithelial cells in this way using bronchial epithelial growth medium (BEGM; Figure 1B). Basal epithelial cells grew from biopsies as assessed by flow cytometric analysis of their expression of basal cell markers cytokeratin 5 (CK5), integrin α_6 (ITGA6), tumor-associated calcium signal transducer 2 (TROP2), and nerve growth factor receptor (NGFR) (Figure 1C; and Figures E1B and E1C).

However, large numbers of epithelial cells are required to achieve coverage of tracheal scaffolds *in vitro*. Data from our laboratory showed that epithelial cell–seeding densities used to prepare previous grafts were suboptimal for complete coverage of synthetic scaffolds

Figure 2. (Continued). 5-ethynyl-2′-deoxyuridine (EdU) uptake in P2 cells grown in BEGM (*top left*) or in 3T3+Y (*top center*) for 3 days. Summary data are shown for six donors (*top right*; mean ± SEM; experiment performed in technical triplicate for each donor and averaged). Cells were costained with DAPI to analyze cell cycle progression. Representative plots for cells grown in BEGM (*bottom left*) or in 3T3+Y (*bottom center*) are shown. Summary data are shown for six donors (*bottom right*; mean ± SEM; experiment performed in technical triplicate for each donor and averaged). Differences between conditions were assessed using a Wilcoxon matched-pairs signed-rank test (*$P < 0.05$). FSC = forward scatter.
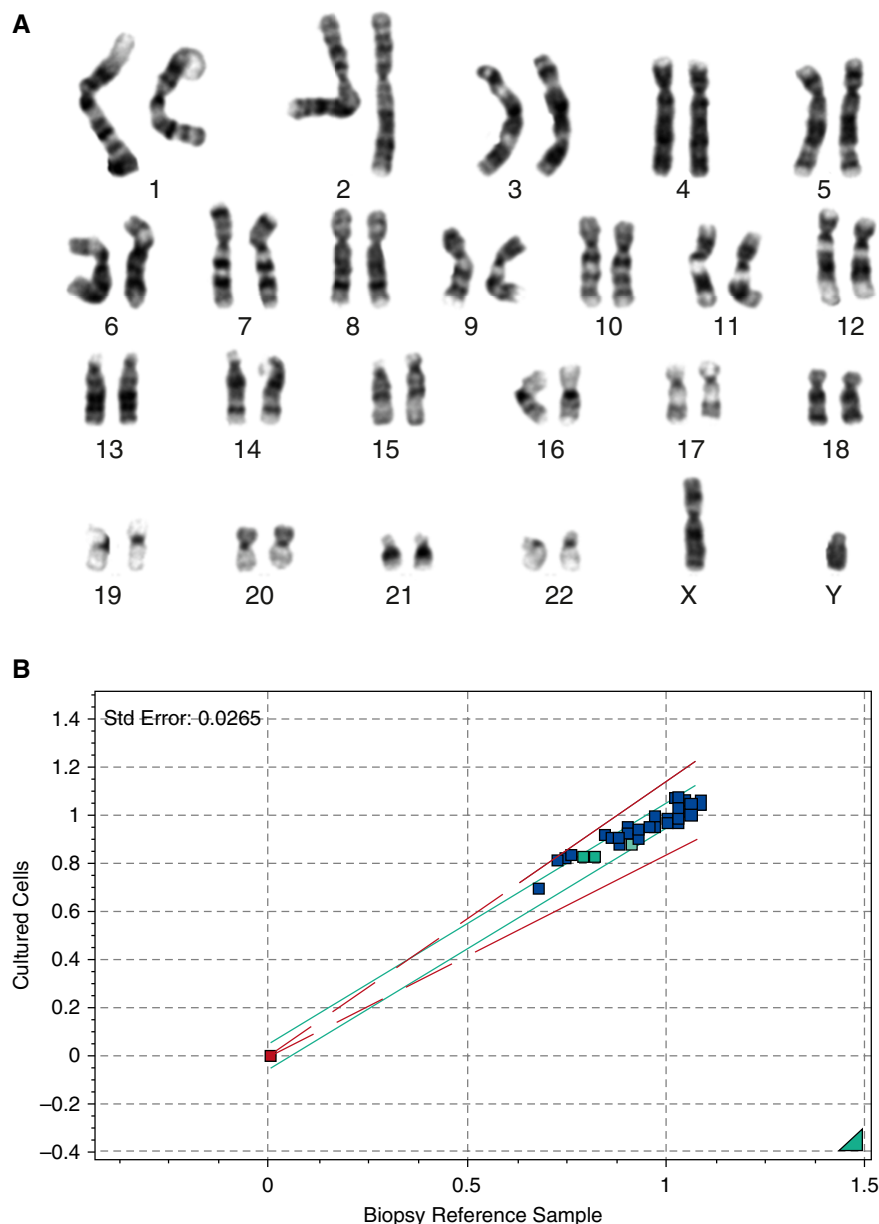
A

B

Figure 3. Airway epithelial cells are karyotypically normal after clinically relevant periods in culture. (A) Representative karyotyping image for airway epithelial cells grown in 3T3+Y for more than 6 weeks. Normal karyotype was found in all three donor cell cultures tested. (B) Multiplex ligation-dependent probe amplification analysis was performed in GeneMarker version 2.4.0 to compare the normalized peak height ratio of a reference biopsy sample and donor matched cells grown in 3T3+Y for 6 weeks. A clear correlation is demonstrated; no subtelomeric copy number changes were detected. Std Error = standard error.

senesce and lose the capacity for multiciliated differentiation (18–20). These data suggest that current strategies for epithelial cell expansion produce only enough cells for the replacement of approximately 14% of the trachea in adults and approximately 40% in a child aged 6–8 years (Figure 1F and Figure E2) (30). These estimations assume that five biopsies can be obtained and a 100% success rate of biopsy culture; in fact, we found only 49% (26 of 53; 19 donors) of biopsies yielded epithelial cells. Clinically, short-length airway defects will be treated by resection with end-to-end anastomosis whereas for larger defects, for which tracheal replacement may be indicated, our data show that a BEGM-based culture system is unlikely to succeed.

Expansion of Human Basal Epithelial Stem Cells in Coculture

To improve the suitability of human airway epithelial cell culture for tissue-engineering applications, we adopted an alternative protocol (22, 23). After cell outgrowth from biopsies in BEGM, we compared growth of matched donor epithelial cells in BEGM and in 3T3+Y, where we combined a murine embryonic feeder layer (mitotically inactivated 3T3-J2 fibroblasts) with pharmacological inhibition of the ROCK pathway (here using 5 μM Y-27632; 3T3+Y). In BEGM alone, cells with cuboidal morphology were expanded with minimal cell–cell contact at early passages (P1; Figure 2A, top left). Cells became larger and flatter at later passages (P4; Figure 2A, top right). By contrast, serum-containing epithelial growth medium in combination with 3T3-J2 feeder cells and Y-27632 led to the formation of colonies of smaller epithelial cells that retained cell–cell contact and whose morphology did not change with passage (Figure 2A, bottom). These conditions also supported the growth of cells in colony-forming assays, which BEGM did not (Figure E3). Consistent with studies in other epithelia (22, 23), we observed rapid proliferation of cells in 3T3+Y compared with those grown in BEGM after 4 days of culture (Figure 2B). This growth advantage was sustained at the same rate over serial passage, whereas cells in BEGM underwent a well-characterized senescence process (Figure 2C). We verified that 3T3+Y increased the number of cells in S phase compared with BEGM by analyzing 5-ethynyl-2′-deoxyuridine (EdU)

in vitro (28). Instead, a higher seeding density exceeding 1×10^6 cells/cm^2 was suggested, a figure close to the number of basal cells that line healthy airways (29). Similar to results obtained with synthetic scaffolds, airway epithelial cell seeding onto decellularized human trachea showed that at low seeding densities of 2.5×10^5 and

5.0×10^5 cells/cm^2 coverage was sparse, whereas a higher density of 1×10^6 cells/cm^2 achieved more uniform coverage (Figure 1D). On average, we obtained approximately 2×10^6 cells from a single endobronchial biopsy after two passages (Figure 1E), a time point beyond which cells grown under these conditions begin to

A

B

C

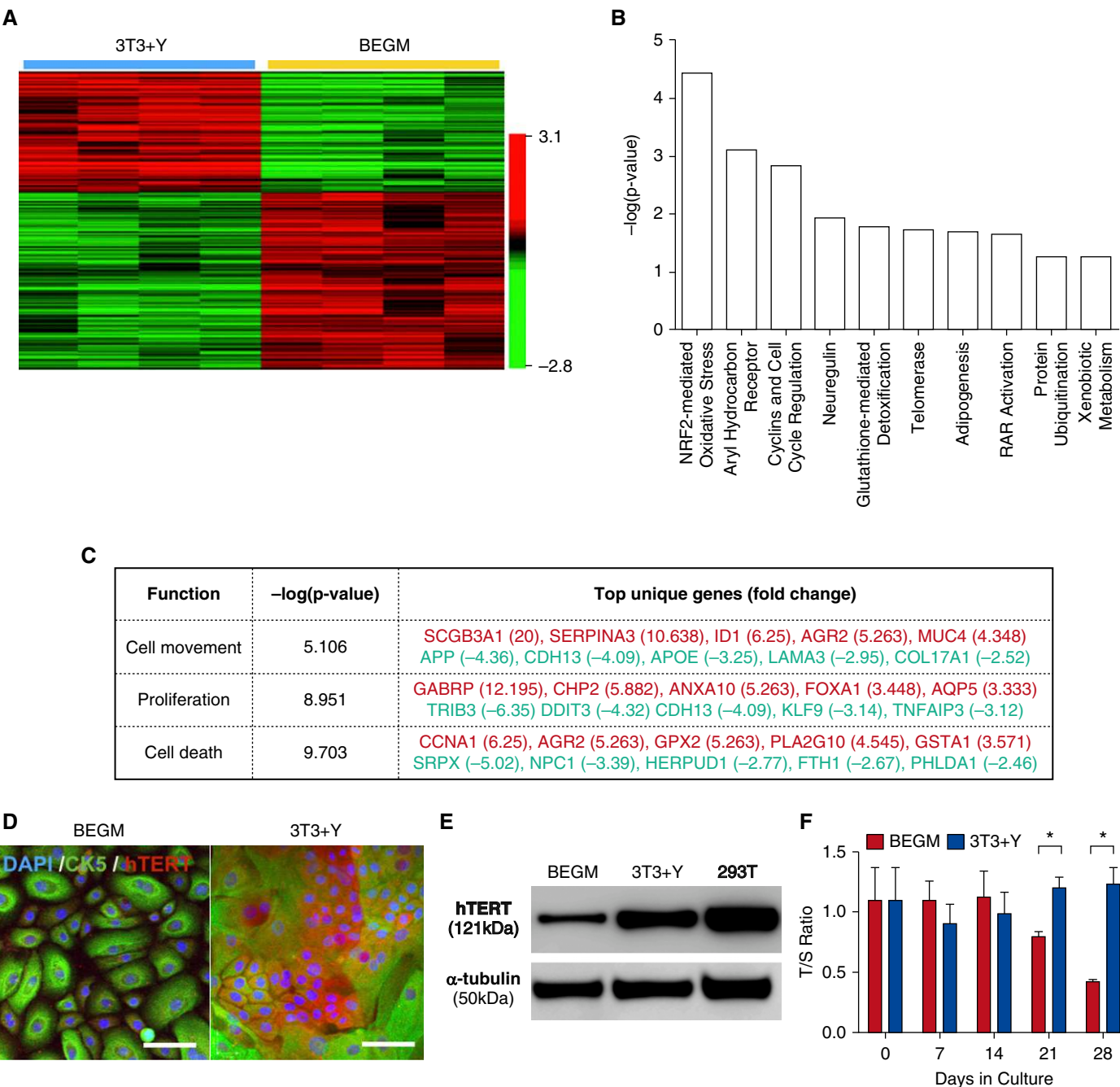| Function	−log(p-value)	Top unique genes (fold change)
Cell movement	5.106	SCGB3A1 (20), SERPINA3 (10.638), ID1 (6.25), AGR2 (5.263), MUC4 (4.348) APP (−4.36), CDH13 (−4.09), APOE (−3.25), LAMA3 (−2.95), COL17A1 (−2.52)
Proliferation	8.951	GABRP (12.195), CHP2 (5.882), ANXA10 (5.263), FOXA1 (3.448), AQP5 (3.333) TRIB3 (−6.35) DDIT3 (−4.32) CDH13 (−4.09) KLF9 (−3.14) TNFAIP3 (−3.12)
Cell death	9.703	CCNA1 (6.25), AGR2 (5.263), GPX2 (5.263), PLA2G10 (4.545), GSTA1 (3.571) SRPX (−5.02), NPC1 (−3.39), HERPUD1 (−2.77), FTH1 (−2.67), PHLDA1 (−2.46)

D

E

F

Figure 4. Transcriptomic profile of human airway epithelial cells expanded in 3T3-J2 coculture and Rho-associated protein kinase inhibition. (*A*) Cluster diagram plotting significantly differentially expressed genes between cells grown for one passage in either bronchial epithelial growth medium (BEGM) or 3T3+Y. (*B*) Ingenuity Pathway Analysis (IPA) was applied to investigate cell signaling pathways containing significantly differentially expressed genes. The IPA −log(P value) is plotted on the *y-axis* versus biological processes on the *x-axis*. RAR = retinoic acid receptor. (*C*) Functional analysis of differentially expressed genes was performed by IPA. The major functions altered by culture in 3T3+Y are shown, along with the relevant −log(P value) and the top 10 relevant genes (5 up-regulated and 5 down-regulated). Genes already displayed in a function were subsequently ignored to avoid overlap. (*D*) Airway epithelial cells (P2) stained by immunofluorescence for human telomerase reverse transcriptase (hTERT; *red*), cytokeratin 5 (CK5; *green*), and 4′,6-diamidino-2-phenylindole (DAPI; *blue*) after 5 days of culture in BEGM or in 3T3+Y (*scale bar*, 100 μm). (*E*) Western blot analysis of hTERT in lysates from airway epithelial cells (P2) grown in either BEGM or in 3T3+Y. HEK293T cells were included as a positive control. (*F*) Quantitative polymerase chain reaction analysis of telomere length in matched BEGM and 3T3+Y cultures. T/S ratio = telomere-to–single-copy gene ratio. Differences between conditions were assessed using the Mann–Whitney test ($n \geqslant 3$; *$P < 0.05$).
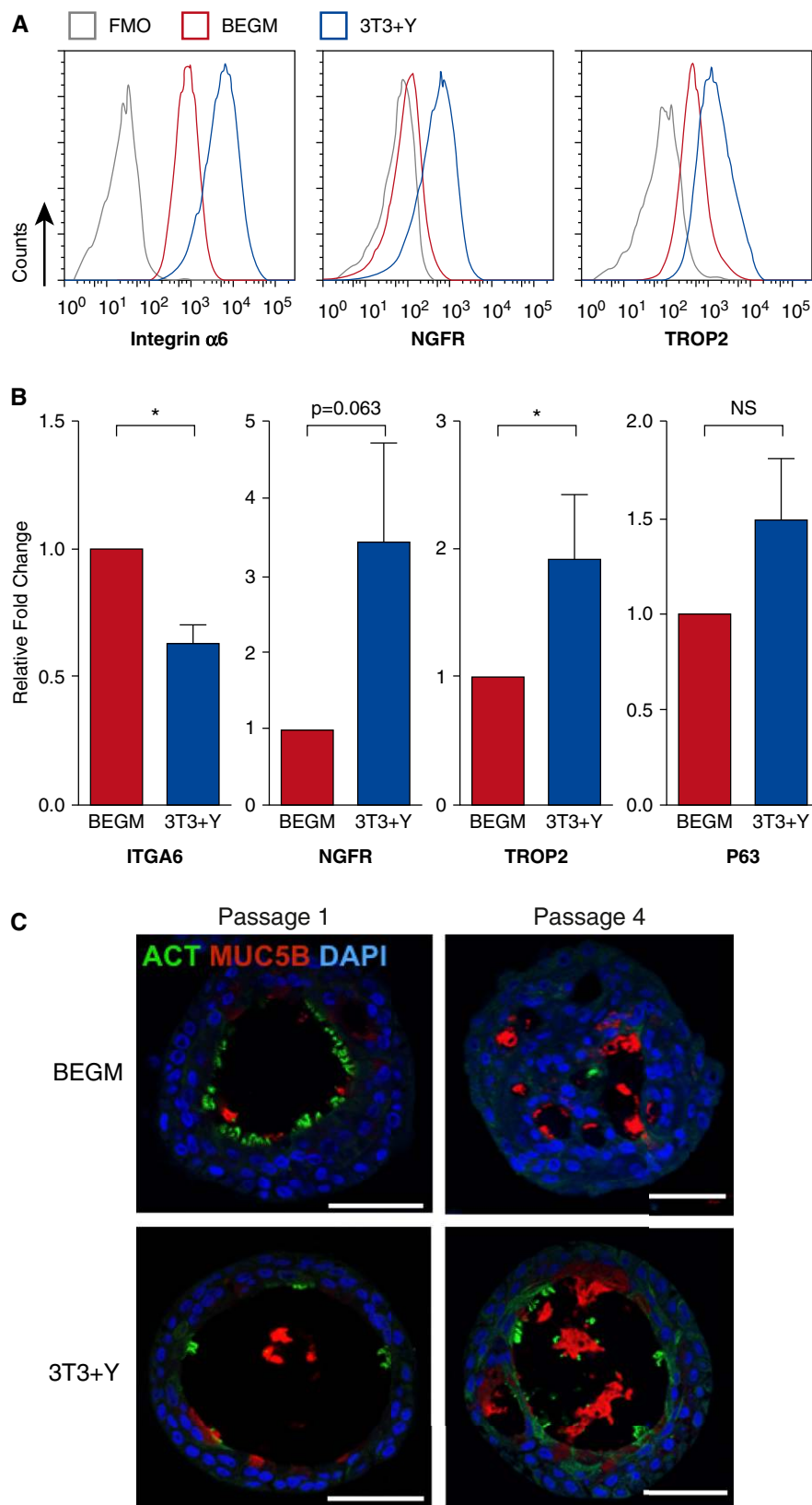
Figure 5. The combination of 3T3-J2 coculture and Rho-associated protein kinase inhibition expands basal epithelial stem cells. (*A*) Flow cytometric analysis of airway basal stem cell marker expression on the surface of cells grown in bronchial epithelial growth medium (BEGM; *red*) or in

uptake and the DNA content of cells (Figure 2D). We have passaged cells continuously in 3T3+Y at a 1:5 split ratio for more than 3 months, and cultures were easily reinitiated after cryopreservation. Epithelial cells were reliably separated from 3T3-J2 feeder cells by differential trypsinization (Figure E4).

Expanded Epithelial Cell Karyotype and Copy Number Are Stable after Coculture and ROCK Inhibition

To generate transplantable epithelium it will be important to avoid the generation of genetic abnormalities during the culture period, which for tracheal tissue-engineering applications is likely to be 3–6 weeks. Importantly, airway epithelial cells grown in 3T3+Y displayed a normal 46,XX or 46,XY karyotype after more than 6 weeks in culture (Figure 3A). However, deletions below approximately 5 megabases are not reliably detected by conventional karyotyping, and therefore we obtained two biopsies from a single donor to compare the tissue of origin with matched cells grown in 3T3+Y. We investigated copy number change in these cells by multiplex ligation-dependent probe amplification (31), as gene-rich subtelomeric regions share significant homology between chromosomes, making them vulnerable to inappropriate recombination during meiosis (32). Our analysis revealed that both samples were normal with no evidence of subtelomeric copy number alterations after 6 weeks in culture (Figure 3B and Figure E5). In addition, cells grown in 3T3+Y retain the capacity for contact inhibition on confluence (Figure E6A) and do not form tumors in a subcutaneous tumorigenesis assay (Figure E6B).

Gene Expression Pathway Analysis Reveals 3T3+Y Leads to Increased Cell Cycle Progression, Increased Telomerase Protein Expression, and Maintenance of Telomere Length

To understand the effects of 3T3+Y culture conditions, we performed genome-wide transcriptional profiling, using microarrays to explore the major pathways altered by 3T3+Y culture. After initial expansion in BEGM, four matched donor cell lines grown in either BEGM or 3T3+Y for 7 days were compared. We analyzed data using the significance analysis of microarrays method with a false discovery

rate of 5% and found 507 significantly differentially expressed transcripts, with 297 down-regulated in 3T3+Y and 210 up-regulated. Significant differences were visualized in a cluster diagram (Figure 4A) that clearly showed clustering of expression according to culture condition rather than donor. This remained the case when selected airway epithelial genes were analyzed independently of their significance value (Figure E7). In all samples, we confirmed the expression of six differentially expressed genes by qPCR; these showed a high correlation with the microarrays ($r = 0.96$). To categorize these genes, we used Ingenuity Pathway Analysis (QIAGEN, Redwood City, CA) and observed alterations in several pathways including cell cycle regulation (Figure 4B), which correlated with the increased proliferation of cells in 3T3+Y. Function analysis revealed significant up-regulation of genes associated with cell movement and proliferation and decreased expression of genes associated with cell death in 3T3+Y (Figure 4C).

We were particularly interested in the changes observed in telomerase signaling in 3T3+Y (Figure 4B) and investigated the expression of human telomerase reverse transcriptase (hTERT) under the two culture conditions, reasoning that telomere shortening must be inhibited to allow sustained expansion in 3T3+Y. Both immunofluorescence (Figure 4D) and Western blot analysis (Figure 4E) showed higher protein expression of hTERT in 3T3+Y, suggesting this is the case. qPCR analysis (33) revealed that telomere length shortened over time in cells grown in BEGM but was maintained in matched donor 3T3+Y cultures (Figure 4F).

3T3+Y Augments an Airway Stem Cell Phenotype

As there is clear correlation between epithelial stem cell maintenance and engraftment success in epidermal allografts (34), we hypothesized that the maintenance of airway stem cells in culture would be similarly important to the clinical success of airway epithelial transplantation. In human airways, basal cells are abundant stem cells during homeostasis and contribute to repair after injury (35, 36). Using flow cytometry and qPCR, we validated changes in specific airway basal cell gene and protein expression between cells grown in BEGM and 3T3+Y based on changes seen in our microarrays. Higher levels of stem cell markers integrin-α_6 (37), NGFR (35), and TROP2 (38) were present on the surface of cells expanded in 3T3+Y (Figure 5A and Figure E8). Expression of these genes varied between donors, but consistent patterns of change did occur between culture conditions. Gene expression of TROP2 was significantly up-regulated, and there was a trend toward NGFR up-regulation (Figure 5B). However, integrin-α_6 was down-regulated at the level of gene expression (Figure 5B), suggesting that posttranscriptional regulation may underlie the increased surface expression observed in cells cultured in 3T3+Y (Figure 5A and Figure E8). Expression of the ΔN P63 isoforms expressed by basal epithelial stem cells (39, 40) was not significantly altered by culture conditions (Figure 5B). In contrast to the tissue-resident airway epithelial cell markers investigated, 3T3+Y did not induce expression of pluripotent stem cell markers (Figure E9).

As adult stem cells are capable of recapitulating organ-specific differentiation programs in three-dimensional culture (41), we compared cells grown in BEGM with matched donor cells grown in 3T3+Y in a three-dimensional tracheosphere assay (35, 42). Tracheospheres derived from passage 4 BEGM cells were smaller than those from passage 1 BEGM cells, whereas the size of those derived from 3T3+Y basal cells was not affected by passage (Figure E10). Further, a single lumen was evident in the majority of tracheospheres in early-passage cultures, but at late passage this occurred only in 3T3+Y cultures; late-passage BEGM tracheospheres failed to form a lumen and rarely contained ciliated cells (Figure 5C).

Epithelial Cells Expanded in 3T3+Y Are Capable of Functional Ciliary Differentiation

A functional tissue-engineered graft will require the incorporation of a differentiated epithelium with effective ciliary beat. To further examine whether basal cells expanded in 3T3+Y retained multipotent airway differentiation potential, we devised an organotypic raft assay. Epithelial cells were cultured to confluence on a collagen I/Matrigel raft containing primary human lung fibroblasts before being lifted to air–liquid interface for 3 weeks. Cells cultured for more than 6 weeks in 3T3+Y before seeding formed a pseudostratified epithelium containing both ciliated (ACT; Figure 6A) and mucosecretory goblet cells (MUC5AC; Figure 6A). Next, we investigated whether the cilia produced by these cells were functional. Using conventional air–liquid interface cultures (17), we found that cells from 3T3+Y cultures formed ciliated epithelia (see Video E1 in the online supplement) with high transepithelial electrical resistance values (43), indicative of high epithelial integrity, and cilia with normal beat pattern (44) and frequency and ultrastructure (Figures 6B and 6C) (45). Air–liquid interface cultures again indicated the maintenance of ciliated differentiation potential at late passage in 3T3+Y but not in BEGM cultures (Figure E11).

Repopulation of Tracheal Scaffolds in a Xenograft Model

To test the downstream application of 3T3+Y-expanded airway epithelium *in vivo*, we used a xenogeneic engraftment model (46, 47) to repopulate denuded tracheal grafts (Figure E12). We processed rat tracheal scaffolds using repeated freeze–thaw cycles (48) and generated a matrix depleted of endogenous epithelium (Figure 7A, *left*). Human epithelial cells that had been rapidly expanded in 3T3+Y successfully engrafted onto this scaffold under *ex vivo* conditions. Cells in direct contact with the tracheal matrix were p63$^+$ and all cells retained expression of the basal

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Figure 5. (Continued). 3T3+Y (*blue*) for 4 days. Fluorescence minus one (FMO) controls are shown for comparison. This experiment was repeated three times with different donor cell lines, and representative plots for one donor cell line are shown. NGFR = nerve growth factor receptor; TROP2 = tumor-associated calcium signal transducer 2. (B) Quantitative polymerase chain reaction analysis of airway basal stem cell marker gene expression in airway epithelial cells grown in BEGM or in 3T3+Y for 7 days. Differences between groups were assessed using the Wilcoxon matched-pairs signed-rank test (n ≥ 6 donors; *$P < 0.05$). ITGA6 = integrin-α_6; NS = not significant. (C) Immunofluorescence staining of tracheospheres generated from cells grown in either BEGM (P1 and P4) or 3T3+Y (P1 and P4) for acetylated tubulin (ACT; *green*), mucin 5B (MUC5B; *red*), and 4′,6-diamidino-2-phenylindole (DAPI; *blue*). Scale bars, 50 μm.
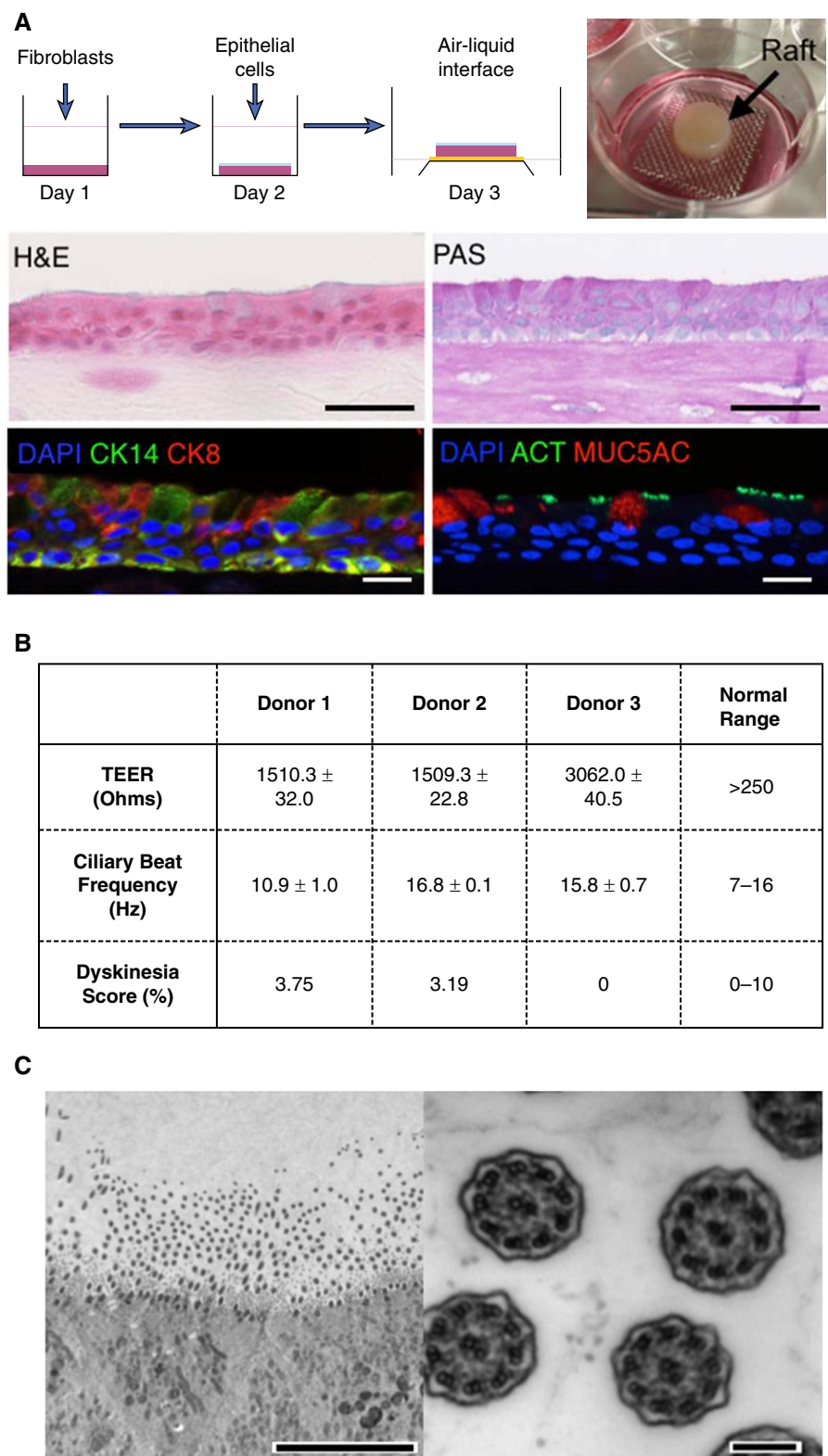
A

Fibroblasts → Epithelial cells → Air-liquid interface

Day 1 → Day 2 → Day 3

Raft

H&E

PAS

DAPI CK14 CK8

DAPI ACT MUC5AC

B

	Donor 1	Donor 2	Donor 3	Normal Range
TEER (Ohms)	1510.3 ± 32.0	1509.3 ± 22.8	3062.0 ± 40.5	>250
Ciliary Beat Frequency (Hz)	10.9 ± 1.0	16.8 ± 0.1	15.8 ± 0.7	7–16
Dyskinesia Score (%)	3.75	3.19	0	0–10

C

Figure 6. 3T3-J2 coculture and Rho-associated protein kinase inhibition preserve the multipotent airway differentiation capacity of human airway epithelial cells. (*A*) Schematic representation of the technique used to create human airway epithelial cell rafts (*top*). A collagen I/Matrigel raft containing human lung fibroblasts is created on Day 1. Epithelial cells are seeded at high density the next day before being lifted to air–liquid interface on Day 3. Hematoxylin and eosin (H&E; *second row*, *left*)

cell marker CK5 after 5 days (Figure 7A, *right*). Scanning electron microscopy revealed good coverage of the scaffold (Figure 7B, *top right*), including regions of intact cobblestone epithelium (Figure 7B, *bottom*). Importantly, to achieve these results we delivered cells at high seeding densities of 1×10^7 cells/ml and achieved a near-confluent epithelial sheet 24 hours after seeding.

Confident that cells grown in 3T3+Y adhered to the denuded scaffold, we next verified the engraftment capacity of cells in an *in vivo* xenogeneic engraftment model. Scaffolds were assembled into tubing and cells were allowed to engraft for 6 hours before subcutaneous implantation in NOD *scid* gamma (NSG) mice (Figure 8A). After 5 weeks, restoration of an epithelial barrier around the full circumference of tracheal grafts was evident. By immunofluorescence, we confirmed that regenerated epithelial cells were derived from transplanted human cells using the human-specific antibody STEM121. Further, we found that the epithelium was organized normally with $CK5^+p63^+$ basal cells and luminal $MUC5B^+$ goblet cells and ACT^+ ciliated cells (Figure 8B).

Discussion

For clinical transplantation, there are three criteria that the ideal epithelial expansion system must meet: (*1*) the cells must not cause an adverse immune response (e.g., autologous origin); (*2*) they must be rapidly expandable to respond to challenging clinical scenarios; and (*3*) they must be of high quality in terms of their karyotype, their expression of tissue-specific markers, their differentiation, and their functional capacity.

Although efforts are underway to obtain autologous airway epithelial cells through the step-wise differentiation of induced pluripotent stem (iPS) cells (49), it has not been defined how safe these cells will be for use in regenerative medicine because of doubts about their genetic stability during culture (50) and the added time burden of iPS-based therapy; current techniques would require several months between cell isolation and delivery (51), unless "off-the-shelf" allogeneic applications proved successful. We argue, therefore, that the ability to culture
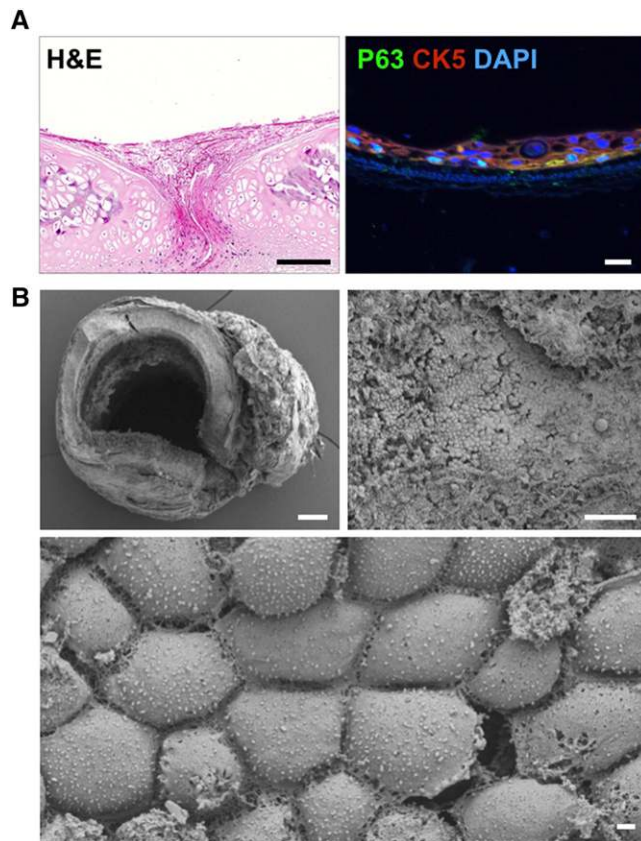
Figure 7. Expanded human airway epithelial cells repopulate a tracheal matrix *ex vivo*. (*A*) Hematoxylin and eosin (H&E) staining of rat tracheal xenografts demonstrated a denuded epithelium before human cell engraftment (*left*; scale bar, 100 μm). Immunofluorescence staining for p63 (*green*), cytokeratin 5 (CK5; *red*), and 4′,6-diamidino-2-phenylindole (DAPI; *blue*) 5 days after seeding of 1×10^7 cells/ml (*right*; scale bar, 20 μm). (*B*) Scanning electron microscopy of the xenograft in cross-section 5 days after seeding at 1×10^7 cells/ml (*top left*; scale bar, 250 μm), the luminal surface shown top down (*top right*; scale bar, 50 μm), and a higher magnification view of a region with cobblestone epithelial appearance (*bottom*; scale bar, 1 μm).

suitable for biopsy. Our estimation is that more than 7×10^7 cells are required for graft coverage in a full-length adult tracheal transplant. More than 35 patient biopsies would be required to grow this number of low-passage cells from BEGM cultures, whereas 3T3+Y could provide appropriate numbers of multipotent cells from a single biopsy and reduce the time required for epithelial cell expansion to less than 4 weeks.

Existing protocols that allow immortalization of airway epithelial cells using plasmid or viral delivery of oncogenes and/or exogenous expression of the catalytic subunit of hTERT are clearly unsuitable for cell therapy due to the uncontrolled proliferation of the resulting cell lines. Importantly, using the system we describe here, we do not find evidence of genetic aberrations in chromosomal analyses after clinically relevant culture periods.

Using microarray and pathway analysis we identify key pathways that are changed in cells grown in 3T3+Y. These include cell cycle regulators, consistent with the increased proliferation of cells in 3T3+Y; oxidative stress pathway genes, which may be of significance given that reactive oxygen species modulate human basal cell behavior through NRF2 (55); and neuregulin signaling, consistent with a previous report that feeder cells cause epidermal growth factor receptor (EGFR) and HER2 phosphorylation in epidermal keratinocytes (54). In addition, we found that telomerase signaling genes were affected by coculture. Although hTERT is not typically expressed in somatic cells, low expression levels are thought to slow telomere shortening in adult stem cells (56) and exogenous addition of hTERT mRNA extends telomeres and the life span of epidermal keratinocytes *in vitro* (57). The apparent stabilization of telomere length during 3T3+Y culture supports our view that basal stem cells are better preserved.

Epithelial cells from other organs expanded in 3T3+Y also retain the features of tissue-specific stem cells (23, 24) and, in

sufficient numbers of autologous patient epithelial cells in a timely and efficient manner would be of more immediate clinical application. As a first step toward the development of scalable, high-quality autologous airway basal cell cultures for bioengineering applications, we characterized a protocol using 3T3-J2 feeder cells and a ROCK inhibitor, Y-27632.

An important translational advantage of this system is the rate of cell expansion.

Whereas ROCK inhibition alone increases the rate of proliferation in airway epithelial cells (52), secreted factors from 3T3-J2 cells have a synergistic effect (53, 54). The ability to derive adequate numbers of autologous cells from small endobronchial biopsies, typically just 1 mm³ in size, is significant as this is the route of tissue acquisition in patients. Tissue availability is particularly limiting in the target population, in whom extensive airway disease restricts regions

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Figure 6. (Continued). and periodic acid–Schiff (PAS; *second row*, *right*) staining of paraffin-embedded sections reveal a multilayered epithelium in which apical cells contain abundant mucosubstances after 3 weeks (scale bar, 50 μm). Immunofluorescence staining was done for cytokeratin 14 (CK14; *green*), a marker of basal cells; cytokeratin 8 (CK8; *red*), a marker of differentiated nonbasal cells (*bottom row*, *left*); acetylated tubulin (ACT; *green*); and the airway mucin MUC5AC (*red*; *bottom row*, *right*; scale bar, 10 μm). *Blue*, 4′,6-diamidino-2-phenylindole (DAPI). (*B*) In traditional air–liquid interface cultures we characterized the transepithelial electrical resistance (TEER), ciliary beat frequency, and ciliary beat pattern. Results are shown as means ± SEM. (*C*) Transmission electron microscopy showed a healthy, well-ciliated strip of respiratory epithelium from air–liquid interface cultures. Normal columnar cells and microvilli are seen (scale bar, 10 μm). The electron micrograph on the *right* shows cilia in cross-section. A normal ciliary ultrastructure is seen with the typical 9 + 2 arrangement of microtubules and inner and outer dynein arms (scale bar, 1 μm).
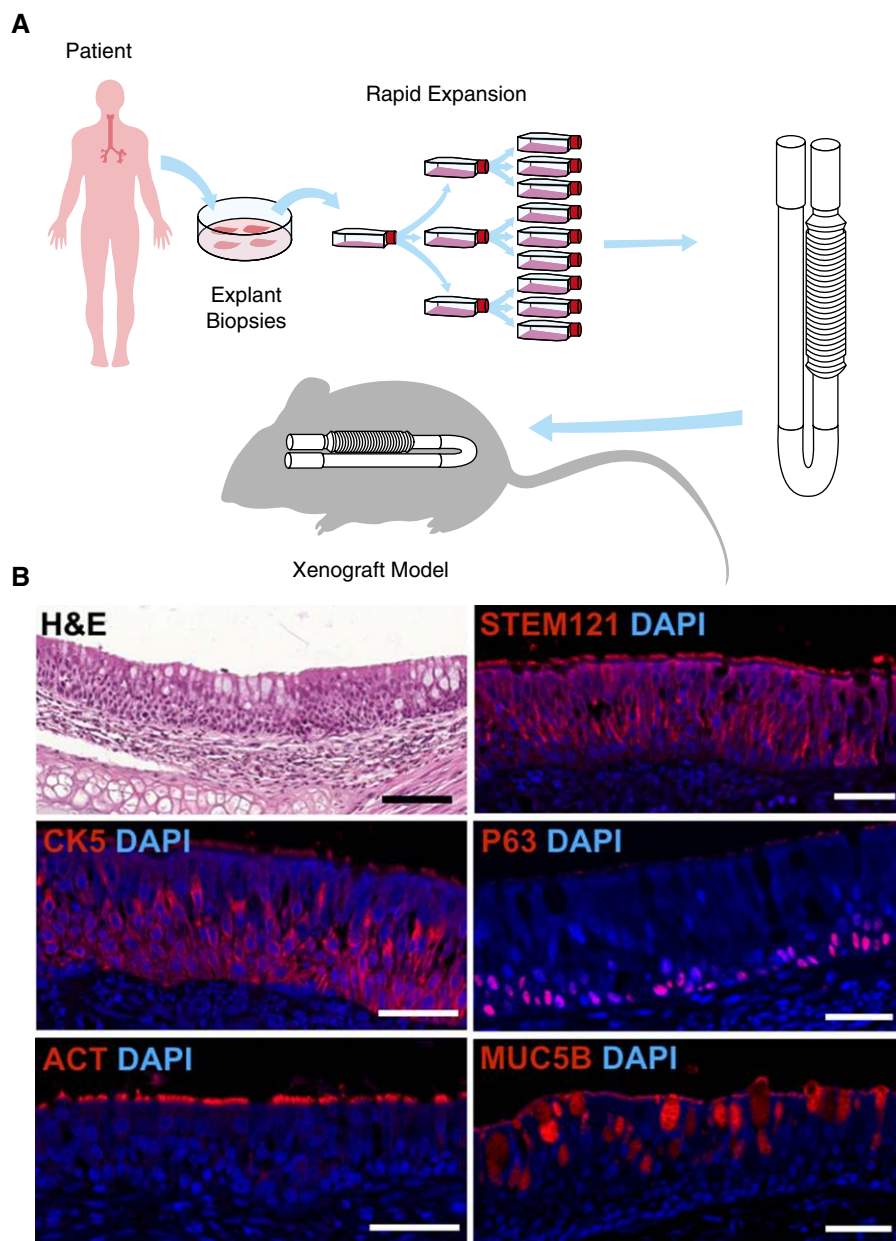
The ability of epithelial cells grown in 3T3+Y to differentiate down mucosecretory or ciliated lineages is not compromised by time in culture, as it is by culture in BEGM (18–20): we produced multiciliated epithelia from cells passaged for more than 6 weeks in tracheosphere and air–liquid interface assays. The normal ciliary ultrastructure, beat pattern, and frequency of these cultures suggest that inclusion of epithelial cells on tissue-engineered grafts may help to restore mucociliary function after transplantation if the appropriate delivery methods are found for decellularized and/or synthetic human-sized scaffolds.

Overall, the human basal epithelial cell culture protocol reported here largely fulfills the criteria demanded by transplantation. Although culturing cells in direct contact with mitotically inactivated murine embryonic fibroblasts leads to their classification as a xenograft, it remains an acceptable method for clinical translation. Although progress is being made toward understanding the factors produced by 3T3-J2 cells that support epithelial cell expansion (54), no effective replacement has yet been reported. Epithelial cells expanded on 3T3-J2 feeder layers form part of clinically approved products in other settings, including the treatment of serious epidermal burns (59, 60) and limbal stem cell deficiency following chemical burns of the eyes (61, 62). It has been suggested that human mesenchymal stromal cells (hMSCs) may provide an alternative feeder layer to avoid the use of xenogeneic cells (63), but we find that neither hMSCs nor human lung fibroblasts support the growth of airway epithelial cells to the same extent as 3T3-J2 fibroblasts (Figure E13).

In addition to providing a means to expand large numbers of human airway basal stem cells for cell culture experiments, our data indicate that 3T3-J2 coculture along with ROCK inhibition allows sufficient cell numbers to be generated for future application in airway cell therapies. The protocol not only substantially reduces the time required for epithelial cell culture but also improves the quality of cells produced after clinically relevant periods in culture. ∎

Figure 8. 3T3+Y-expanded human airway basal cells contribute to the regeneration in a tracheal xenograft model. (*A*) Schematic representation of epithelial preparation and xenograft implantation. Human airway mucosal biopsies were expanded in 3T3+Y, engrafted for 6 hours, and implanted subcutaneously in NOD *scid* gamma mice for 5 weeks. (*B*) Hematoxylin and eosin (H&E) staining of the restored epithelial layer found throughout the graft (*top left*). STEM121, a human-specific antibody, revealed regenerated epithelium that was derived from transplanted cells (*top right*). Immunofluorescence staining for the basal cell markers cytokeratin 5 (CK5) and p63 (*middle row*), the ciliated cell marker acetylated tubulin (ACT; *bottom left*), and the goblet cell marker MUC5B (*bottom right*) revealed a normal organization in regenerated epithelium. All immunofluorescence was costained with 4′,6-diamidino-2-phenylindole (DAPI; *blue*). Scale bars, 50 μm.

airway epithelial cells, we show that markers of basal stem cells, such as integrin-α_6 (37) and NGFR (35), are up-regulated compared with cells grown in BEGM. We further demonstrate that TROP2, a marker of murine basal and submucosal gland epithelial cells (38), is up-regulated in our human cultures, suggesting it may identify basal stem cells in human airways as it does in prostate epithelium (58).

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