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Derivation of Airway Basal Stem Cells from Human Pluripotent Stem Cells

Graphical Abstract



Highlights

- Directed differentiation of human iPSCs generates airway basal cells ("iBCs")
- iBCs self-renew and display multipotent differentiation *in vitro* and *in vivo*
- By single-cell RNA-seq, iBCs are highly similar to adult primary airway basal cells
- iBCs enable modeling of acquired and genetic airway diseases

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In Brief

Hawkins and colleagues report a directed differentiation protocol enabling the derivation of airway basal cells ("iBCs") from human iPSCs. iBCs recapitulate hallmark stem cell properties of primary basal cells, including self-renewal and multi-lineage differentiation, thus enabling modeling of airway diseases *in vitro* and repopulation of tracheal xenografts *in vivo*.



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Derivation of Airway Basal Stem Cells from Human Pluripotent Stem Cells

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SUMMARY

The derivation of tissue-specific stem cells from human induced pluripotent stem cells (iPSCs) would have broad reaching implications for regenerative medicine. Here, we report the directed differentiation of human iPSCs into airway basal cells ("iBCs"), a population resembling the stem cell of the airway epithelium. Using a dual fluorescent reporter system (NKX2-1^{GFP};TP63^{tdTomato}), we track and purify these cells as they first emerge as developmentally immature NKX2-1^{GFP+} lung progenitors and subsequently augment a TP63 program during proximal airway epithelial patterning. In response to primary basal cells, including the capacity to self-renew or undergo multi-lineage differentiation *in vitro* and in tracheal xenografts *in vivo*. iBCs and their differentiated progeny model perturbations that characterize acquired and genetic airway diseases, including the mucus metaplasia of asthma, chloride channel dysfunction of cystic fibrosis, and ciliary defects of primary ciliary dyskinesia.

INTRODUCTION

Basal cells (BCs) of the adult mouse and human airways are capable of self-renewal and multi-lineage differentiation *in vivo* and after culture expansion *ex vivo*, thereby fulfilling the definition of a tissue-specific adult stem cell (Rock et al., 2009). An extensive literature has established that BCs can regenerate the airway epithelium by serving as precursors for essential specialized epithelial cell types, including secretory cells (SCs) and multiciliated cells (MCCs) (Montoro et al., 2018; Plasschaert

et al., 2018; Rock et al., 2009). These stem cell properties make BCs a highly desirable cell type to generate *ex vivo* for modeling airway diseases and a leading candidate for cell-based therapies designed to reconstitute the airway epithelium.

In human airways, BCs are highly abundant in the pseudostratified epithelium extending from the trachea to the terminal bronchioles (Rock et al., 2010). Airway BCs can be identified based on their classic anatomic location along the basal lamina and by the expression of several markers, including tumor protein 63 (TP63), cytoskeletal protein keratin 5 (KRT5), and nerve

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growth factor receptor (NGFR) (Rock et al., 2010). TP63, a member of the p53 family of transcription factors, is essential to the BC program in the airway but also other organs (Yang et al., 1999). Although airway BCs in adult lungs have been extensively studied, only recently has their developmental origin been examined. For example, lineage-tracing experiments in mice (Yang et al., 2018) reveal that a Tp63 program is already present early in lung development at the time of initial lung bud formation (embryonic day 9.5 [E9.5]) within a subset of lung epithelial progenitors expressing the transcriptional regulator that marks all developing lung epithelial cells, NK2 homeobox 1 (Nkx2-1). Early Nkx2-1+/Tp63+ co-expressing cells are not BCs because they lack the BC morphology and molecular program. Rather, these fetal cells function as multipotent progenitors of subsequent alveolar and airway epithelia (Yang et al., 2018). Tp63 expression is then gradually restricted to the developing airways, where it is initially broadly expressed in immature airway progenitors and later restricted to a subset of tracheal cells that localize to the basement membrane and upregulate markers of adult BCs, including Krt5 and Ngfr. The signaling pathways that control BC specification and maturation in the lung are not precisely known; however, in-bred mouse models suggest a temporal role for FGF10/FGFR2b (Volckaert et al., 2013) and recent single-cell RNA sequencing (scRNA-seq) of human fetal airways identified fetal BCs and a role for transient activation of SMAD signaling in BC specification (Miller et al., 2020). Although limited data are available regarding the developmental origins of BCs in humans, a similar pattern to that observed in mice has also been described (Nikolić et al., 2017).

Given the stem cell properties of airway BCs, including their established proliferative capacity, well-established protocols have been developed to expand primary human BCs *in vitro* (Fulcher and Randell, 2013; Fulcher et al., 2005; Mou et al., 2016; Suprynowicz et al., 2017). These BCs, conventionally referred to as human bronchial epithelial cells (HBECs), differentiate into a pseudostratified airway epithelium in air-liquid interface (ALI) culture that recapitulates aspects of *in vivo* airway biology. The understanding of acquired and genetic human airway diseases, including the mucus metaplasia of asthma, the chloride transport defects of cystic fibrosis (CF), and the ciliary dysfunction of primary ciliary dyskinesia (PCD), has advanced through this model (Clancy et al., 2019; Horani et al., 2016; Seibold, 2018).

Several recent reports have demonstrated the successful directed differentiation of human induced pluripotent stem cells (iPSCs) into airway epithelial cell types, including those that express the canonical BC marker TP63 (Dye et al., 2015; Hawkins et al., 2017; Konishi et al., 2016; McCauley et al., 2017). These cultures contain cells with some markers found in BCs; however, the successful generation of bona fide BCs with detailed characterization and demonstration of stem cell properties that are comparable to adult BCs has yet to be reported.

Here, we successfully differentiate iPSCs in vitro into putative BCs that share transcriptional and functional similarities to their in vivo counterparts. The resulting approach recapitulates the sequence of key developmental milestones observed in mouse and human fetal lungs. Initially primordial lung progenitors identified by NKX2-1 expression are produced with only low levels of TP63 expression detectable in a minority of cells. Subsequently, a developing airway program is induced characterized by coexpression of NKX2-1 and TP63, with subsequent maturation into cells expressing the functional and molecular phenotype of BCs. As is observed in mature primary BCs, iPSC-derived BCs ("iBCs") express the cell surface marker NGFR that enables their purification by flow cytometry. The resulting sorted cells display long-term, clonal self-renewal capacity, multi-lineage differentiation in ALI cultures in vitro and in tracheal xenografts. iBCs can be applied for disease-modeling studies, exemplified here by recapitulating essential features of asthma, CF, and PCD. The ability to derive tissue-specific stem cells, such as airway BCs, from iPSCs, including capabilities to purify, expand, cryopreserve, and differentiate these cells, overcomes many of the key hurdles that currently limit the more widespread application of iPSC technology and should accelerate the study of human lung disease.

RESULTS

A TP63 Fluorescent Reporter Allows Visualization, Purification, and Interrogation of iPSC-Derived Airway Progenitors

Because NKX2-1 is the earliest transcriptional regulator expressed in all developing lung epithelial cells and TP63 is a canonical transcription factor required for expression of the BC program, we sought to generate a tool that would allow visualization and purification of NKX2-1+/TP63+ putative lung BCs engineered in vitro from iPSCs while excluding any non-lung BCs (NKX2-1-/TP63+: Figures 1A and 1B). Using our published. normal iPSC line (BU3) that carries a GFP reporter targeted to the endogenous NKX2-1 locus (Hawkins et al., 2017), we additionally targeted a tdTomato reporter coding sequence into one allele of the endogenous TP63 locus at exon 4 to ensure reporter expression reflects expression of the predominant known forms of TP63, DeltaN-type, or TA-type at the N terminus while avoiding bias to C-terminal splice forms, such as α -, β -, and γ type (Figure 1B; Levrero et al., 2000). Karyotypically normal, pluripotent clones with monoallelic targeting of the TP63 locus and biallelic targeting of the NKX2-1 locus were identified (Figures S1A-S1E), thus establishing a bifluorescent reporter iPSC line, BU3 NKX2-1^{GFP};P63^{tdTomato}, hereafter "BU3 NGPT." To test faithfulness and specificity of the reporters, we differentiated this iPSC line into lung epithelium, employing our recently published airway-directed differentiation protocol, in which we previously identified a population of basal-like cells based on the



⁽F) Quantification of the percentage of cells (mean \pm SD), calculated by flow cytometry, co-expressing NKX2-1^{GFP} and TP63^{tdTomato} between days 15 and 16 and 40–42 of differentiation (n = 6).

⁽G) qRT-PCR quantification of *TP63* mRNA levels ($2^{-\Delta\Delta Ct}$) in GFP+/TOM+, GFP-/TOM-, and GFP+/TOM- populations sorted by fluorescence-activated cell sorting (FACS) compared to presort levels on day 36 of directed differentiation (*p < 0.05).

⁽H) Immunolabeling of BU3 NGPT with antibodies against TP63 and RFP on day 30 of directed differentiation. The cells remained in 2D culture from day 15 to facilitate antibody labeling (DNA stained with DAPI; scale bar represents 50 μm).

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expression of NKX2-1, TP63, and KRT5 (Figure 1C; McCauley et al., 2017). As expected, NKX2-1^{GFP+} (hereafter "GFP+") cells, a small fraction of which also expressed tdTomato (hereafter "TOM+"), emerged by day 15 of differentiation, as detected by flow cytometry and fluorescence microscopy (18.1% \pm 19% GFP+; 0.4% \pm 0.8% TOM+; mean \pm SD). In the experiment shown in Figure 1E, 45.4% of cells were GFP+ and 2.5% of cells were GFP+/TOM+ (Figures 1D and 1E). Almost all TOM+ cells identified at this time point expressed the lung epithelial lineage GFP reporter (Figure 1E). We previously determined that GFP+ cells at this stage of the differentiation are similar to primordial lung progenitors and do not express canonical markers of extra-pulmonary NKX2-1 domains, which include the forebrain and thyroid (Hawkins et al., 2017; Serra et al., 2017).

To induce proximal airway differentiation of lung progenitors, GFP+ cells (regardless of TOM expression) were sorted on day 15 of the protocol and suspended in droplets of Matrigel in our previously published "airway" serum-free medium, hereafter "FGF2+10+DCI+Y" (Figure 1C, schematic; McCauley et al., 2017). Between days 28-36, monolayered epithelial spheres emerged and 43.7% ± 8.6% (mean ± SD; 46.6% GFP+, 24.6% GFP+/TOM+ in the experiment shown in Figure 1E) of cells coexpressed NKX2-1^{GFP} and TP63^{TOM}, hereafter "GFP+/TOM+" (Figures 1E and 1F). A single-input GFP+ cell on day 15 yielded 15.7 ± 8.25 GFP+/TOM+ cells by days 30-35. After single-cell dissociation of epithelial spheres and expansion in 3D culture conditions, the percentage of cells GFP+/TOM+ increased further to 73.7% ± 4.7% by day 42 (Figure 1F). Flow cytometry sorting on day 36 demonstrated enrichment of TP63 mRNA in GFP+/TOM+ cells and depletion in TOM- cells (Figure 1G). TP63 protein was detected in TOM+ cells; however, TOM- cells lacked TP63 protein (Figures 1H and S1F). These findings confirmed the specificity of the tdTomato reporter. In summary, we determined that NKX2-1^{GFP+}/TP63^{TOM+} cells emerge and can be expanded in FGF2+10+DCI+Y medium. The early emergence of tdTomato expression in some cells beginning around day 15 is in keeping with our prior observations in vitro (Hawkins et al., 2017; McCauley et al., 2017, 2018a) and in vivo (Yang et al., 2018; Nikolić et al., 2017) that Tp63 mRNA and nuclear protein are initially detected at this time point in a subset of early NKX2-1+ primordial lung epithelial progenitors soon after specification of the respiratory lineage, even prior to airway differentiation (see schematic in Figure 1C).

iPSC-Derived Airway Progenitors Adopt a Molecular Phenotype Similar to Primary Adult Basal Cells

We next sought to characterize NKX2-1^{GFP+}/TP63^{TOM+} cells produced by the airway differentiation protocol in terms of capacity for self-renewal, multi-lineage differentiation, and expression of the canonical BC marker NGFR (Figures S2A–S2E). We sorted day 40–42 iPSC-derived GFP+/TOM+ cells and placed them in ALI 2D cultures for at least 2 weeks and observed differentiation into cells expressing markers of MCCs and SCs, although this differentiation appeared to be patchy (Figure S2A) and cells failed to maintain barrier function based on transepithelial electrical resistance (TEER) measurements (Figures S2A and S2B) in contrast to ALI-differentiated primary HBEC controls (Figures S2A and S2B). In keeping with these results, NGFR was expressed on only a small fraction, $1.9\% \pm 1.8\%$, of GFP+/TOM+ cells on day 40–42

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of differentiation, suggesting cells at this time point were unlikely to be mature BCs. Even after 3 additional months of serial sphere passaging of GFP+/TOM+ cells in 3D culture, NGFR expression did not significantly increase, although GFP and TOM co-expression was maintained in the majority of cells (Figures S2C–S2E), suggesting that increased culture time alone would not induce BC maturation of iPSC-derived NKX2-1+/TP63+ cells.

We reasoned that one possible explanation for the low expression of NGFR and inconsistent multi-lineage differentiation of iPSC-derived cells was that GFP+/TOM+ cells were more similar airway progenitors to immature TP63+/NKX2-1+/KRT5observed in vivo during early airway patterning and were not yet mature BCs (Yang et al., 2018). Thus, we next sought to identify culture conditions that would further differentiate GFP+/TOM+ cells into a more basal-like state, using NGFR expression as a readout. Recently, inhibition of SMAD and Rho-associated protein kinase (ROCK) signaling was found to induce a proliferative state in primary BCs and significantly increase overall yield from serially passaged cultures while maintaining, to an extent, their differentiation capacity (Mou et al., 2016; Zhang et al., 2018). We tested whether GFP+/TOM+ iPSC-derived airway progenitors would selectively proliferate and possibly mature in media optimized for primary human BC culture. On days 30-32 of differentiation, GFP+/TOM+ cells were sorted, replated in 3D Matrigel, and exposed to a commercially available BC medium (Pneuma-Cult-Ex Plus) supplemented with small-molecule inhibitors of SMAD signaling (transforming growth factor β [TGF- β] and bone morphogenetic protein (BMP) inhibition with A 83-01 and DMH1, respectively) and Y-27632 (hereafter "BC medium"; Figures 2A-2D). In response to BC medium, GFP+/TOM+ spheroids exhibited less distinct lumens (Figure 2A) with rapid and robust induction of NGFR in GFP+/TOM+ cells within 4 days of exposure to BC medium (Figures 2B-2D, S2F, and S2G). For example, between days 1 and 3, NGFR expression increased in frequency from \sim 1% to 35% in response to BC medium. By day 6, 71% ± 5% (mean ± SD) of cells expressed NGFR (Figure 2B), suggesting that the change in medium formulation, rather than selective expansion of rare NGFR+ cells, was inducing NGFR expression in previously NGFR- cells. In contrast, parallel aliquots of cells that continued in FGF2+10+DCI+Y (Figures 2B, 2D, and S2F) expressed little to no NGFR.

Next, to determine whether the increase in NGFR expression in response to BC medium was coincident with a broader augmentation of a mature BC program, we performed scRNAseq. We differentiated BU3 NGPT iPSCs in our new protocol (Figure 2A), purifying GFP+/TOM+ cells on days 30-32 followed by replating for 3D culture in either BC medium versus continuing in FGF2+10+DCI+Y medium until scRNA-seq analysis on day 46. After quality control, a total of 7,331 cells were analyzed (Louvain clustering; Figure 2E) and six populations were identified based on the expression of canonical markers, cell cycle, and differentially expressed genes (DEGs) (1-6, in descending order of size): (1) "basal"; (2) "proliferative TP63+"; (3) "secretory"; (4) "TP63+/ NGFR-"; (5) "SCGB3A2+"; and (6) "non-lung endoderm" (Figures 2E, 2F, and S3A-S3C). Clusters 1, 2, and 4, with basallike features, expressed key BC markers as well as TP63 and tdTomato, thus supporting the reporter specificity (Figures S3A-S3C). As expected, NGFR was only expressed in the basal-like population cultured in BC medium (cluster 1;

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Figure 2F). Notably, we have previously demonstrated that, during lung-directed differentiation, cells can revert to non-lung endodermal lineages (Hurley et al., 2020; McCauley et al., 2018a); however, less than 0.05% of all cells were characterized as non-lung endoderm in this analysis (Figure S3B).

We next sought to quantify the transcriptomic similarities and differences of these iPSC-derived clusters compared to their human airway counterparts. We included freshly isolated adult and fetal airway epithelia to determine the transcriptomic profile of endogenous airway epithelial cells and also cultured human airway epithelium, the gold standard in vitro platform of airway biology. First, we generated gene signatures composed of the top 30 DEGs in BCs, SCs, and MCCs from scRNA-seq of cultured HBECs ("P0"; minimal culture) before and after ALI differentiation (Fulcher and Randell, 2013; Table S1; Figures S4A-S4D). The validity of these signatures was confirmed by quantifying their expression in freshly isolated human airway epithelium from 6 adult individuals (Carraro et al., 2020; Figure S3D). The expression of each signature was then visualized on a Uniform Manifold Approximation and Projection (UMAP) of cells from BC medium and FGF2+10+DCI+Y and the enrichment score for BCs, SCs, and MCCs measured in each of clusters 1-6. Cluster 1 (BC medium) had the highest BC enrichment score for the BC signature and expressed canonical BC markers, including S100A2, CAV1, and COL17A1 in addition to TP63, KRT5, KRT17, and ITGB4 (Figures 2G and S3C; Rock et al., 2009; Plasschaert et al., 2018).

In order to compare our iPSC-derived cells with adult and fetal lung cell types and exclude the potential confounding effects of in vitro culture on gene expression, we first integrated the dataset of freshly isolated adult airway epithelial cells with that of fetal airways at different developmental time points (Carraro et al., 2020b; Miller et al., 2020). After identifying DEGs between adult airway cells and their iPSC-derived counterparts (Table S2), we selected primary cell comparators consisting of 7 fetal and 3 adult airway cell types and identified 366 unique markers to quantify the transcriptomic similarities between iPSC-derived cells from FGF2+10+DCI+Y or BC medium and each of these primary cell types using Pearson's correlation coefficients (PCCs) (Figure 2H; Miller et al., 2020). For each iPSC-derived single cell, the primary epithelial cell type exhibiting the maximum PCC was determined and indicated as "best fit." Cells from FGF2+10+DCI+Y were best fit with either "fetal basal" or "adult basal." On the other hand, the majority of cells in BC medium were most transcriptionally similar to adult basal cells. Consistent with this assignment, NGFR expression was infrequent in fetal BCs (1.4%) compared to adult BCs (40%), suggesting it marks more mature BCs. Furthermore, primary HBECs analyzed in the same manner also showed a best fit to adult basal cells, verifying the approach (Figure S3E). In view of their more basal-like transcriptional profile, we designated GFP+/TOM+ cells in BC medium as iBCs.

Next, to interrogate possible biological differences between iPSC-derived cells in BC medium and both fetal and adult primary BCs, we used gene set enrichment analysis (GSEA) (Figure S3F). Based on the observation that KRAS signaling, known to be downstream of fibroblast growth factor (FGF), was identified in both comparisons and data suggesting a role for FGF10 signaling in primary BC development in the airways (Ornitz and Itoh, 2015; Volckaert et al., 2013), we modulated FGF signaling between days 15 and 35 of iPSC differentiation and assessed changes in the yield of GFP+/TOM+ cells per input cell. GFP+/TOM+ cells were rare in the absence of FGFs, however, increased in a FGF dose-dependent manner (Figure S3G), suggesting that FGF plays a role in the differentiation of immature lung progenitors into NKX2-1+/TP63+ cells, findings in keeping with our GSEA results.

iBCs Exhibit Stem Cell Properties: Long-Term Self-Renewal and Multi-lineage Differentiation

We next asked to what extent iBCs share the key stem cell properties of primary BCs: long-term self-renewal and multi-lineage differentiation (Figures 3A-3G). In BC medium, iBCs were propagated for up to 10 passages (170 days) in 3D culture, while retaining GFP/TOM expression (Figures 3B and S5A), NGFR expression, and a normal karyotype (Figure S5B). One input iBC plated on day 45 yielded $6.9 \pm 1.7 \times 10^6$ cells (mean \pm SD) 78 days later with a doubling time of 1.1 days (Figure 3B). First, to assess the differentiation and self-renewal of individual cells, we adapted the 3D tracheosphere assay previously published for primary BCs (Rock et al., 2009). We established a seeding density at which >95% of spheres were clonally derived from a single iBC (Figure S5C). At this clonal density, NGFR+ sorted iBCs were cultured in either BC maintenance or differentiation "ALI" media. In both conditions, spheres formed and were composed of a stratifiedappearing layer of predominantly NKX2-1+ cells (Figure 3C). In BC medium, NKX2-1+/TP63+ cells were readily identified in the outermost cell layer of all spheres. In the differentiation medium,

Figure 2. NKX2-1^{GFP+}/TP63^{tdTomato+} Cells Adopt a Molecular Signature Similar to Primary Basal Cells

⁽A) Schematic of experiment: GFP+/TOM+-sorted cells were suspended in 3D Matrigel between days 30–35 of differentiation in FGF2+FGF10+DCI+Y or primary BC media. After 12–14 days, morphology and tdTomato fluorescence were assessed (left panel) and the expression of NKX2-1^{GFP}, TP63^{tdTomato}, and NGFR quantified by flow cytometry (middle and right panel, representative plots). See Figure S2 for additional details.

⁽B) Kinetics of NGFR induction, quantified by flow cytometry in response to primary BC medium compared to continued FGF2+FGF10+DCI+Y over 8 days between days 33–41 (n = 3).

⁽C) NGFR protein immunostaining (cyan) of BU3 NGPT cells in primary BC medium compared to TP63^{tdTomato} fluorescence (scale bar represents 20 µm).

⁽D) Percentage of NGFR+ cells (mean ± SD) quantified by flow cytometry between days 40–50 (n = 12) (****p < 0.0001).

⁽E) scRNA-seq of day 46 cells from BC or FGF2+FGF10+DCI+Y media. UMAP (left panel) displays the distribution based on culture conditions used to treat cells. Louvain clustering (res = 0.25) identifies 6 clusters (1–6).

⁽F) UMAP of canonical BC (TP63, NGFR, and KRT5) and SC (SCGB3A2) markers.

⁽G) UMAP of the expression of BC signature (left) and graph of BC gene signature enrichment score ("basal" = 0.84 ± 0.24 ; "proliferative *TP*63+" = 0.52 ± 0.24 ; "secretory" = 0.51 ± 0.18 "*TP*63+/*NGFR*-" = 0.51 ± 0.15 ; "SCGB3A2+" = 0.32 ± 0.15 ; mean enrichment score ± SD). See also Figures S3 and S4.

⁽H) Heatmap showing all calculated pairwise PCCs between freshly isolated primary human airway cells (without culturing) and all iPSC-derived cells on day 46 grown in either FGF2+FGF10+DCI+Y or BC medium. Ten fetal and adult primary airway epithelial cell types are shown. Colors of the best fit annotation represent the epithelial cell type with the highest PCCs for each iBC in each cultured sample.

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BCs, MCCs, and SCs were identified in all spheres, suggesting self-renewal and clonal multi-lineage differentiation of iBCs (Figures 3C and S5D). In extended 3D culture in BC medium without passaging, some spontaneous differentiation of iBCs into secretory and MCCs was observed (Figure S5E). Next, to further study differentiation, we generated 2D ALI cultures from iBCs. On day 46, NGFR+ iBCs were sorted and plated on Transwell inserts in differentiation ALI medium and compared to presorted cells (schematic in Figure 3A). Without NGFR sorting, we observed patchy differentiation in Transwell cultures, evident as frequent areas devoid of staining for the above lung markers and some areas devoid of TOM expression (Figure 3D). In contrast, NGFR+ sorted iBCs initially formed a confluent TOM+ epithelial layer in submerged culture, and after 16 days in air-phase culture, SCs (SCGB1A1+ and MUC5AC+) and beating MCCs were distributed homogeneously across the entire Transwell (Figure 3E; Video S1). Confocal microscopy and transverse sections confirmed that NGFR+ sorted cells had formed pseudostratified epithelia composed of MCCs (Acetylated tubulin [ACT]+ and FOXJ1+) and SCs (SCGB1A1+ and MUC5AC+; Figures 3E-3G). Importantly, after ALI differentiation, iBCs had also formed TP63+/ KRT5+/NGFR+ cells with the expected basal location of bona fide BCs, along the basal membrane (Figure 3G). When iBCs and primary HBECs were differentiated in identical ALI conditions, trilineage differentiation from both starting cell types was easily detected by immunostaining (TP63+ cells: 13.3% ± 2.7% versus 27.9% ± 6.1%; FOXJ1+ cells: 42.4% ± 3.3% versus 19.6% ± 2%; MUC5AC+ cells: 9.4% ± 1.7% versus 16.3% ± 4.5%; SCGB1A1+ cells: 8.2% ± 0.4% versus 18.8% ± 2.1% [mean cell frequency ± SD; iBC versus primary HBEC]). Both NGFR sorted and GFP+/TOM+ iBCs formed epithelial layers with similar TEER to primary controls and significantly higher TEER than ALI differentiations prepared in parallel from GFP+/TOM+ cells that had been maintained in FGF2+10+DCI+Y without BC medium maturation (Figure 3H). Next, we tested the extent to which the multi-lineage differentiation capacity of iBCs was maintained after extended in vitro expansion in BC medium. After 10 passages of expansion, iBCs retained their capacity to form trilineage, pseudostratified airway epithelium when transferred to ALI culture (Figure S5F). After cryopreservation and subsequent thaw, iBCs retained their expression of BC markers, proliferated in BC

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medium, and retained TEER and trilineage differentiation potential in ALI conditions (Figures 3I and S6A–S6E). Taken together, these results indicated successful derivation from iPSCs of iBCs that exhibit the airway stem cell properties of self-renewal and multipotent differentiation.

scRNA-Seq of iBC-Derived Airway Epithelium

To more completely assess the molecular phenotypes of the differentiated cell types generated from iBCs, we performed scRNAseq of ALI cultures from NGFR-sorted cells (Figure 4A). Louvain clustering identified five clusters (1-5) with gene expression profiles reminiscent of airway epithelial cell types and one proliferative cluster (6; Figures 4A-4E, S7A, and S7B). We found no discrete cluster of non-lung endoderm cells (low to undetectable CDX2, AFP, and ALB). We compared the top 20 DEGs in each cluster to the scRNA-seq dataset of differential gene expression in primary airway epithelial cell types (Figures 4B and S4; Table S1) and published datasets (Plasschaert et al., 2018) and annotated the cell types as follows: secretory (cluster 2, DEGs: SCGB1A1, SCGB3A2, MUC5B, C3, XBP1, CEACAM6, and CXCL1); basal (cluster 6, DEGs: KRT5, KRT17, and S100A2); "intermediate" (cluster 1, DEGs: KRT4, KRT12, KRT13, and KRT15); "immature MCC" (cluster 3, DEGs: CCNO, CDC20b, and NEK2); and "MCC" (cluster 4, DEGs: TUBB4B, TUB1A1, TPP3, and CAPS; Figures 4A–4E, S7A, and S7B). Enrichment scores for gene signatures of primary BCs, SCs, and MCCs correlated with these annotated clusters with enrichment scores of 0.77 ± 0.16 , 0.54 ± 0.26 , and 1.54 ± 0.27 (mean enrichment score ± SD), respectively (Figures 4D and S7A). FOXJ1, a key transcription factor expressed in MCCs, was found enriched in both ciliated cell clusters (Figure 4C); however, FOXN4, recently identified as a marker of immature primary human airway MCCs, was found only in the immature iPSC-derived MCCs (Figure S7A; Plasschaert et al., 2018). There was no evidence to suggest the presence of ionocytes or neuroendocrine or alveolar epithelial cells among the 3,500 iPSC-derived cells analyzed (data not shown). To quantify the transcriptomic similarities of iPSC-derived SCs and MCCs to fetal and adult MCCs and SCs, we used PCC and the 10 fetal and adult airway cell types as shown in 2H (Figure S7C). Similar to HBEC-derived ALI (Figure S3E), we detected cells with gene-expression patterns consistent with BCs, SCs, and MCCs. iPSC-derived MCCs were

Figure 3. iBCs Undergo Self-Renewal and Multi-lineage Differentiation

(A) Schematic of experiment: GFP+/TOM+ cells sorted on approximately day 40 of differentiation expanded in BC medium and then characterized in terms of selfrenewal and multi-lineage differentiation in ALI.

⁽B) Representative flow cytometry plots of NKX2-1^{GFP} versus TP63^{tdTomato} expression in cells at passage 7, following expansion in 3D culture with BC medium (left panel). Per input sorted GFP+/Tom+ cell on day 45, we calculated the yield up to 123 days of directed differentiation (right panel).

⁽C) Immunolabeling of representative spheroid on day 83 of differentiation in either BC medium (left and middle panel) or after 10 days in ALI differentiation medium (right panel) with antibodies indicated (DNA stained with Hoechst; scale bar represents 50 μm). See also Figure S5D.

⁽D and E) GFP+/TOM+ cells were expanded in BC medium until day 46 and plated on Transwells with (E) or without (D) GFP/TOM/NGFR sorting. Representative images of the endogenous TP63^{tdTomato} fluorescence during culture of BU3 NGPT-derived ALI are shown. Stitched image of whole Transwell insert (\emptyset = 6.5 mm; 1st column) and zoom in (2nd column) is shown. Immunolabeling with antibodies indicated after 16 days of ALI culture is shown (3rd and 4th columns; DNA stained with DRAQ5; scale bar represents 100 µm).

⁽F) Confocal microscopy of BU3 NGPT-derived ALI cultures shown in (E) and immunolabeled with antibodies indicated (DNA stained with DRAQ5; scale bar represents 100 µm).

⁽G) Transverse section of BU3 NGPT-derived ALI cultures shown in (E) and stained with hematoxylin and eosin or antibodies indicated (DNA stained with DAPI; scale bar represents 100 μm).

⁽H) TEER measurements (mean \pm SD) of Transwell ALI cultures, comparing GFP+/TOM+ from FGF2+10+DCI+Y (n = 5), BC medium with (n = 4) or without (n = 5) NGFR sorting, and primary HBEC controls (n = 21).

⁽I) TP63^{tdTomato} fluorescence in BU3 NGPT iBCs after cryopreservation and thaw (scale bar represents 200 µm; left panel). Immunolabeling of ALI cultures generated from cryopreserved iBCs with antibodies indicated is shown (scale bar represents 100 µm).

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transcriptionally distinct and best fit with "fetal MCC" or "fetal MCC precursor" cell types. The remaining cells were predominantly best fit with fetal basal or "fetal club-like" cell types. The expression levels of key airway epithelial markers in iBC-derived ALI cultures were confirmed using gRT-PCR and directly compared to primary controls cultured in identical conditions (Figure 4F). In both primary and iPSC-derived samples, there was robust upregulation of FOXJ1, MUC5AC, and SCGB1A1. FOXJ1 expression was significantly higher and MUC5AC expression lower in iPSC-derived ALI cultures (Figure 4F). SCGB3A2 was expressed in iPSC-derived ALI cultures, but not detected at significant levels in primary HBEC-derived ALI cultures, whereas SCGB1A1 expression was similar between both samples (Figure 4F). We note that iBCs yielded pseudostratified airway epithelium composed of BCs, MCCs, and SCs, irrespective of whether PneumaCult-ALI or UNC-ALI media were utilized in ALI cultures (Figures S7D and S7E).

iBCs Form a Differentiated Airway Epithelium in a Tracheal Xenograft

Engraftment and regeneration in vivo after transplantation is a critical measure of stem cell function that has been successfully tested for several non-lung tissues, such as the hematopoietic system, but remains a major challenge for lung epithelia, with only limited success reported to date in the airways (Ghosh et al., 2017; Miller et al., 2018; Nichane et al., 2017). We sought to determine whether iBCs could establish an airway epithelium in vivo using the tracheal xenograft model (Everitt et al., 1989). Rat tracheas were completely decellularized by repeated freeze thaws, seeded with NGPT-derived iBCs, implanted subcutaneously into the flanks of Foxn1^{nu} immune-compromised mice, and then followed for 3 weeks after transplantation (Figure 5A). Air exposure through the trachea was maintained in vivo using open-ended tubing. After 3 weeks, a stratified or pseudostratified epithelial layer of cells had formed (Figure 5B). Using antibodies against canonical markers, we identified BCs (KRT5+) occupying the expected positions along the basement membrane and SCs (SCGB1A1+, MUC5AC+, and MUC5B+) and MCCs (ACT+) oriented toward the air-filled tracheal lumen (Figure 5C). NKX2-1^{GFP} and TP63^{tdTomato} expression were detected throughout the sections of epithelium analyzed, confirming its origin from transplanted rather than endogenous cells (Figure 5C). In summary, in the tracheal xenograft model, iBCs form an airway epithelium similar in structure and composition to in vivo airways.

Selection of iBCs from Non-reporter iPSCs Lines Using Antibodies Directed against Surface Markers

To expand the application of iBCs for disease modeling, we first sought to adapt our approach so that a diversity of normal or pa-

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tient-specific iPSC lines could be differentiated into iBCs without requiring the use of knockin fluorochrome reporters. We found through repeating our protocol with the BU3 NGPT line, we could replace GFP+/TOM+ sorting with antibody-based sorting of NGFR+ cells to prospectively isolate putative iBCs (Figure 6). For example, cells grown until days 40-42 with our protocol (Figures 6A–6C) were 73.7% \pm 5.9% (mean \pm SD) GFP/TOM double positive, and gating on NGFR+/EpCAM+ cells yielded a population of cells highly enriched in GFP/TOM co-expressing cells (94.3% ± 1.1%; mean ± SD; Figure 6B). A representative experiment is shown in Figure 6B: 73.6% of cells were GFP+/TOM+; 55.3% were NGFR+/EPCAM+; and 94.5% of sorted NGFR+/ EPCAM+ cells were GFP+/TOM+. Sorting solely on NGFR+ or NGFR+/EpCAM+ and replating cells in ALI cultures resulted in successful derivation of a TOM+ pseudostratified airway epithelium, similar to sorting GFP+/TOM+/NGFR+ cells and in contrast to unsorted controls (Figure 6C). Four additional iPSC lines (DD001m, PCD1, 1566, and 1567) and an embryonic stem cell (ESC) line (RUES2) were also differentiated, recapitulating our BC differentiation protocol while completely replacing the need for fluorescent reporters through the use of CD47^{hi}/CD26^{neg} sorting on day 15 to purify NKX2-1+ progenitors as we have published (Hawkins et al., 2017), followed by NGFR+ sorting after day 40 to purify candidate iBCs (see STAR Methods and schematic in Figure 6A). NGFR+ cells from all five iPSC/ESC lines differentiated into pseudostratified epithelia composed of MCCs, SCs, and BCs (Figures 6D-6G).

iBCs Model Biological Features of the Airway Diseases: Asthma, CF, and PCD

Human airway epithelium has diverse biologic and physiologic roles, some of which are uniquely affected by disease. We tested whether iBC-derived ALI cultures could recapitulate features of the three airway disorders with distinct epithelial characteristics: (1) mucus cell metaplasia in asthma; (2) abnormal ion-flux seen in CF; and (3) defective ciliary beating seen in PCD. First, we tested whether iBC-derived ALI cultures could recapitulate the mucus cell metaplasia seen in asthma. Interleukin-13 (IL-13) is an inflammatory cytokine in asthma and can induce mucus metaplasia. A number of studies, in mice and primary human airway epithelial cell cultures, have found IL-13 leads to increased MUC5AC+ cell numbers via the activation of STAT6 and SPDEF, at the expense of MCCs and MUC5B+ cell frequencies (Kondo et al., 2002; Seibold, 2018; Woodruff et al., 2009). In response to IL-13 added on day 10 of ALI differentiation of iBCs, we found a significant 1.75-fold increase in numbers of MUC5AC+ cells (p = 0.002), increased expression of the goblet cell transcriptional regulator SPDEF, and a decrease in MUC5B expression (Figures 7A-7C). Next, we applied our antibody-based iBC

Figure 4. scRNA-Seq Profiling of iBCs and Their Differentiated Progeny

⁽A) Schematic of scRNA-seq experiment and UMAP with Louvain clustering (clusters 1–6) of iBC-derived ALI. See also Figures S4 and S7.

⁽B) Top 20 differentially expressed genes (DEGs) per cluster.

⁽C) UMAPs of primary BC, SC, and MCC gene signatures applied to iBC-derived ALI (upper row). Violin plots of the enrichment score of clusters 1–6 for BC, SC, and MCC gene signatures (lower panel).

⁽D) UMAPs of the canonical BC, SC, and MCC markers across clusters 1-6.

⁽E) Violin gene expression plots of same panel of markers shown in (D).

⁽F) qRT-PCR validation of key airway markers in iBC-derived ALI (iBC-ALI) compared to primary HBEC-derived ALI (HBEC-ALI) in PneumaCult ALI medium. Fold change is $2^{-\Delta\Delta Ct}$ normalized to undifferentiated iPSCs (*p < 0.05; **p < 0.01, error bars represent mean ± SD).

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Figure 5. iBCs Establish Pseudostratified, Well-Differentiated Airway Epithelium *In Vivo* in Tracheal Xenografts

(A) Schematic of experimental procedure used to generate tracheal xenografts.

(B) Magnified image (left panel; scale bar represents 50 μm) of xenograft epithelium established from BU3 NGPT iBCs stained with hematoxylin and eosin; the location of this region within the transverse section of the xenograft is shown in the right panel. White arrows indicate examples of MCCs.

(C) Immunolabeling of the xenograft epithelium with the indicated antibodies. Anti-GFP and anti-RFP antibodies were used to detect the expression of each fluorochrome reporter that had been targeted to the donor human cell loci, NKX2-1^{GFP}, and TP63^{tdTomato}, respectively. DAPI staining indicates DNA (scale bar represents 50 μ m).

ACT KRT5 DNA SCGB1A1 MUC5AC DNA MUC5B MUC5AC DNA MUC5B MUC5AC DNA MUC5B MUC5AC DNA

purification protocol for modeling the genetic airway diseases CF and PCD (Figure 7D). We identified a CF patient homozygous for the most common CFTR mutation (c.1521_1523delCTT, p.Phe508del) and a PCD patient homozygous for one of the most common mutations in DNAH5 (c.12617G>A, p.Trp4206Ter) and generated iPSCs from these individuals (see STAR Methods and Figure S1). For CF modeling, we first used gene editing to correct the F508del CFTR mutation. Preand post-gene corrected paired syngeneic iPSC clones (hereafter CF and Corr CF) were differentiated into iBCs in our protocol, sorted using our antibody-based-method and generated ALI cultures composed of BCs, SCs, and MCCs (Figures 6A and S7F). CFTR-dependent current, representing ion flux regulated by apically localized CFTR, was measured using the gold-standard Ussing chamber approach. As expected, Ussing chamber analysis indicated minimal CFTR-dependent current in CF ALI cultures (forskolin Δ Isc = 0.8 μ A/cm²; CFTRInh-172 Δ Isc = -0.8 µA/cm²). In marked contrast, correction of the F508del mutation led to restoration of CFTR-dependent current (forskolin Δ Isc = 35.1 ± 1.8 μ A/cm²; CFTRInh-172 Δ Isc = -43.2 ± 2.5 μA/cm²; Figures 7E and 7F). Similar results were obtained from 2 additional CF iPSC lines following CFTR gene correction (data not shown). A limitation of primary HBECs in CF studies is the decrease in CFTR-dependent current with extended passage of primary HBECs (Gentzsch et al., 2017). To address this possibility in iPSC-derived airway epithelium, we performed Ussing chamber analysis on ALI cultures generated from the NGPT line before and after cryopreservation of iBCs and after in vitro expansion of iBCs up to day 87 and found CFTR-dependent current was retained (Figure S6F).

To develop a model for PCD, we differentiated our PCD iPSC line carrying the DNAH5 mutation and generated ALI cul-

tures containing MCCs. We compared these cells to control ALI cultures from BU3 NGPT iBCs, primary nasal epithelium from the PCD donor, and normal primary HBECs. Cilia motility was not detected in the MCCs generated from either the *DNAH5* mutant iPSC line or from *DNAH5* mutant primary cells, in contrast to normal controls (Video S2). The possibility that *DNAH5* mutant iPSCs had failed to produce cilia was excluded by immunofluorescent staining with antibodies against ACT, which confirmed the widespread presence of normal-appearing cilia in all samples (Figures 7G, S7F, and S7G). In addition, increased tethering of MUC5AC+ mucus strands was observed on the apical surface of the differentiated epithelium generated from the mutant iPSCs (Figure S7F).

In MCCs generated from normal iPSCs and normal HBECs, DNAH5 protein was present and colocalized with ACT along the length of cilia although DNAH5 protein was not detected in either PCD iPSC-derived or primary nasal-derived MCC cells (Figure 7G). In contrast, DNALI1, an inner dynein arm (IDA) protein, was detectable by immunostaining, suggesting an intact IDA complex (Figure S7G). Transmission electron microscopy

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of *DNAH5* mutant iPSC-derived MCCs cells showed lack of the outer dynein arm (ODA), identical to the defect in cilia of nasal cells obtained from the patient and in contrast to MCCs obtained from control iPSC and normal HBEC (Figures 7G and 7H). Ciliary beat frequency (CBF) was not detected in *DNAH5* mutant MCCs from the patient or from mutant iPSCs, whereas CBF was similar between normal HBECs and BU3 NGPT-derived MCCs (Figure 7I). Taken together, these results indicate that iPSC-derived airway epithelium shares key physiologic and biologic features with human airway epithelium. In addition, patient-specific iBCs purified without using fluorescent protein reporters can be successfully applied to model a variety of airway epithelial diseases, including CF and PCD.

DISCUSSION

Here, we report the differentiation of iPSCs into iBCs, cells that are molecularly and functionally similar to the predominant stem cell of human airways, the basal cell. We demonstrate the potential of these iBCs to model human development and disease and provide evidence of their capacity to regenerate airway epithelium *in vivo* in a tracheal xenograft model.

The derivation of a tissue-resident stem cell from human iPSCs has considerable implications for regenerative medicine research as it overcomes several important hurdles currently limiting progress in this field. For example, despite significant progress in recent years, directed differentiation protocols are often lengthy, complex, and yield immature and heterogeneous cells (Dye et al., 2015; Firth et al., 2014; Gotoh et al., 2014; Hawkins et al., 2017; Huang et al., 2014; Jacob et al., 2017; McCauley et al., 2017; Miller et al., 2018). Frequently, these issues are due to lack of precise knowledge regarding developmental roadmaps associated with cellular embryonic origin. TP63+ populations have previously been observed stochastically in iPSC-directed differentiation protocols, and their characterization was based only on the expression of a handful of canonical markers, limiting any conclusions as to whether basal-like cells had been produced (Hawkins et al., 2017; McCauley et al., 2017; Chen et al., 2017; Konishi et al., 2016; Dye et al., 2015). Without the ability to purify these cells from the heterogeneous mix of other iPSCderived lineages, little progress has been made in phenotyping or testing their functional potential. Here, we report several advances that culminate in the efficient derivation and purification of BCs from iPSCs. Using a dual-fluorescence BC reporter iPSC line, we recapitulated in vitro the milestones of in vivo airway development and BC specification (Yang et al., 2018). First, during the emergence of the earliest detectable lung epithelial pro-



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From a practical perspective, insight and methodologies developed herein should expand the application of iPSC technology. The shared properties of primary BCs and iBCs for expansion and differentiation in ALI culture makes them attractive for a variety of *in vitro* or *in vivo* applications. Specifically, the ability to purify iBCs based on the surface marker NGFR, expand the resulting cells in 3D culture, and cryopreserve cells should minimize variability associated with directed differentiation and can potentially offset the cost and time required for these protocols.

In terms of the utility of this platform for disease modeling, we selected diseases with unique research challenges. In asthma, the epithelium plays a sentinel role in pathogenesis, and there is significant interest in understanding epithelial dysfunction and the contribution of genetic variants to asthma susceptibility (Loxham et al., 2014). Here, we demonstrate that a mucus metaplasia phenotype is induced in iPSC-derived airway epithelium in response to stimulation with the Th-2 cytokine IL-13. In CF, where many mutations in one gene necessitate individual models of disease for predicting personalized therapeutics, we demonstrated that patient-specific iPSCs or their gene-edited progeny can be differentiated into iBCs and give rise to airway epithelia exhibiting quantifiable CFTR-dependent currents of sufficient magnitude for disease modeling using the gold-standard Ussing chamber assay of CFTR function. Although we did not detect airway ionocytes in our scRNA-seq profiles of iPSCderived cells, given the known rarity of this cell type in vivo or in cultured HBEC preparations, further work is required to determine whether ionocytes, which are known to be rich in CFTR (Plasschaert et al., 2018), occur at low frequencies in our cultures or contribute in any way to the currents we measured. For PCD, where many mutations in many genes controlling ciliogenesis can contribute to the disease, we determined that iBC-derived MCCs model both the functional and ultrastructural defects observed in DNAH5 mutant primary-donor-derived cells. These proof-of-concept experiments suggest that the iPSC platform may be helpful in determining mechanisms of pathogenicity for

Figure 6. A Surface Marker Strategy for Purifying iBCs Using NGFR Replaces the Need for Fluorescent Reporters

(A) Schematic of NKX2-1^{GFP}/TP63^{tdTomato} reporter versus surface marker iBC protocols.

⁽B) Representative flow cytometry plots of BU3 NGPT iBCs on day 40 of differentiation and labeled with antibodies against NGFR and EpCAM. Red arrow indicates sorted NGFR+/EpCAM+ cells are 94.5% GFP+/TOM+. Enrichment is quantified (mean ± SD) in the right panel (n = 3).

⁽C) Representative images of the endogenous TP63^{tdTomato} fluorescence in whole Transwell filters ($\emptyset = 6.5$ mm) seeded with sorted (NGFR+/EpCAM+) or unsorted cells during submerged culture and after ALI culture.

⁽D) Representative flow cytometry plot of a non-reporter iPSC line (DD001m) stained for NGFR is shown.

⁽E) Representative image of sorted NGFR+ cells plated on Transwell filters and after 7 days immunolabeled with an anti-TP63 antibody (DNA stained with Hoechst; scale bar represents 100 µm).

⁽F) Confocal microscopy of DD001m iPSC-derived ALI cultures immunolabeled with antibodies indicated (scale bar represents 200 µm).

⁽G) Representative image of RUES2 ESC-derived ALI immunolabeled with an anti-ACT antibody (DNA stained with DRAQ5; scale bar represents 200 µm; upper left) and confocal microscopy of it immunolabeled with antibodies indicated (DNA stained with DRAQ5; scale bar represents 100 µm).



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genetic airway diseases and may serve as a platform to develop novel therapeutics.

Limitations of Study

Although we conclude that iBCs share essential features with their endogenous counterparts, several questions remain, including the maturity status, regionality, genetic stability, and *in vivo* competence of iBCs. Typically, iPSC-derived specialized cell types are at fetal stages of development (Studer et al., 2015). Although the transcriptomes of iBCs were most similar to adult BCs in our analysis, their differentiated progeny (SCs and MCCs) were more similar to fetal equivalent cell types than to freshly isolated adult cells. This may indicate differences in maturity or reflect the limited duration of *in vitro* differentiation from the BC state or regional differences not represented in scRNA-seq adult airway samples.

We note differences in the pattern of expression of two secretoglobins, *SCGB3A1* and *SCGB3A2*, in iPSC-derived cells compared to primary cells. *SCGB3A1*, predominantly expressed in the larger airway, is the most highly expressed gene in primary BC-derived SCs although *SCGB3A2* is expressed at very low levels. iBC-derived SCs express high *SCGB3A2* and low/absent *SCGB3A1*. These differences further raise the question of immaturity and/or proximal/distal patterning (Reynolds et al., 2002) of iBCs.

Finally, we provide evidence of the potential of iBCs to regenerate an airway epithelium in vivo in a tracheal xenograft. This approach does not recapitulate the ultimate clinical goal of orthotopic transplantation of cells for in vivo airway reconstitution. Limited progress has been made to date in engrafting cells in the airways (Ghosh et al., 2017; Miller et al., 2018; Nichane et al., 2017). As this field advances, determining the extent to which transplanted iBCs might engraft and integrate with endogenous airway epithelium will be required as a first step toward future therapies, whereby autologous gene-edited iBCs may eventually be employed therapeutically for in vivo reconstitution of airway epithelial function for individuals with genetic airway disease (Berical et al., 2019). That said, efficient derivation of an identifiable, tissue-specific stem cell capable of long-term selfrenewal, cryopreservation capacity, and retained multi-lineage differentiation overcomes many of the hurdles that face iPSC technology for studies of respiratory mucosa and diseases of pulmonary epithelium.

STAR*METHODS

Detailed methods are provided in the online version of this paper and include the following:



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Supplemental Information can be found online at https://doi.org/10.1016/j. stem.2020.09.017.

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Figure 7. iBCs Enable In Vitro Modeling of Asthma, Cystic Fibrosis, and Primary Ciliary Dyskinesia

(A) Representative images of BU3 NGPT iBC-derived ALI cultures, with or without IL-13 treatment, immunolabeled with antibodies indicated (DNA stained with Hoechst; scale bar represents 200 μm).

(D) Schematic of CF and PCD disease-modeling experiments, relying on surface markers. See also Figures S7F and S7G.

(G) Immunolabeling of MCCs in ALI cultures generated from *DNAH5* mutant nasal epithelial cells, iPSCs, non-diseased primary HBECs, and BU3 NGPT iPSCs with antibodies indicated (DNA stained with DAPI; scale bar represents 10 µm). Transmission electron microscopy of cilia is shown (right column).

(H and I) The number of outer dynein arms detected in cross sections of cilia (H, mean ± SD) or ciliary beat frequency (I, mean ± SEM) from the samples detailed in (G) (***p < 0.001).

⁽B) Quantification of the number of MUC5AC+ cells (mean ± SD) per high power field for IL-13-treated versus untreated wells (n = 3) (**p < 0.01).

⁽C) qRT-PCR quantification of mRNA expression levels of *MUC5AC*, *SPDEF*, and *MUC5B* in IL-13 treated (+IL-13) versus untreated cells (no IL-13; n = 3). Data are preselected as fold change over untreated iBC-derived ALI cultures (*p < 0.05; **p < 0.01; ***p < 0.001). Error bars represent mean \pm SD.

⁽E) Representative electrophysiological traces from Ussing chamber analysis of ALI cultures generated from CF iPSCs and corrected CF iPSCs (corr CF). (F) Mean and SD of electrophysiological values from (E) (n = 3).

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AUTHOR CONTRIBUTIONS

F.J.H., S.S., B.R.D., and D.N.K. conceived the work, designed the experiments, and wrote the manuscript. S.S., A.M.C., and B.R.D. designed the targeting strategy and generated the TP63^{tdTomato} iPSCs. F.J.H., S.S., B.R.D., and D.N.K. developed the BC differentiation protocol. F.J.H., S.S., C.B., A.B., M.L.B., R.W., and J.L.S. performed differentiation experiments. C.V.-M. performed bioinformatic analysis of scRNA-seq. Y.T. and T.M.S. generated CF iPSCs and performed gene correction. R.W. generated ALIs from CF iPSCs. S.S. and N.M. performed *in vivo* experiments. A.R. and E.J.S. performed Ussing chamber analysis. S.X.L.H. provided critical input. S.H.R. provided primary HBECs and critical input. J.W., J.R.S., G.C., and B.R.S. provided scRNA-seq datasets of fetal and adult airway epithelium. A.H. and S.L.B. performed the experiments characterizing cilia in iPSC-derived MCCs, including TEM and CBF.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR*METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-FOXJ1 (2A5)	Invitrogen	Cat.# 14996580; RRID: AB_1548836
Mouse monoclonal anti-Acetylated Tubulin (ACT) (6-11B-1)	Millipore-Sigma	Cat.# T7451; RRID: AB_609894
Rabbit monoclonal anti-Acetyl-α-Tubulin (Lys40) (D20G3)	Cell Signaling Technologies	Cat.# 5335S: RRID: AB_10544694
Mouse monoclonal anti-TP63 (4A4)	Biocare	Cat.# CM163A: RRID: AB_10582730
Rabbit monoclonal anti-MUC5AC (E309I)	Cell Signaling Technologies	Cat.# 61193: RRID: AB_2799603
Mouse monoclonal anti-MUC5AC (45M1)	Invitrogen	Cat.# MA5-12178: RRID: AB_10978001
Mouse monoclonal anti-MUC5B (A-3)	Santa Cruz	Cat.# Sc-393952:
Rabbit monoclonal anti-KRT5 (D4U8Q)	Cell Signaling Technologies	Cat.# 25807: RRID: AB_2798912
Chicken polyclonal anti-KRT5 (Poly9059)	BioLegend	Cat.# 905901: RRID: AB_2565054
Mouse monoclonal anti-NGFR (NGFR5)	Invitrogen	Cat.# MA5-13314: RRID: AB_10982037
Rabbit monoclonal anti-p75NTR/NGFR (D4B3)	Cell Signaling Technologies	Cat.# 8238: RRID: AB_10839265
Mouse monoclonal anti-CC10 (E-11)	Santa Cruz	Cat.# Sc-365992; RRID: AB_10915481
Rabbit polyclonal anti-RFP	Rockland Immunochemicals	Cat.# 600-401-379; RRID: AB_2209751
Chicken polyclonal anti-GFP	Invitrogen	Cat.# A10262; RRID: AB_2534023
Rabbit monoclonal anti-NKX2-1 (EP1584Y)	Abcam	Cat.# ab76013; RRID: AB_1310784
Rabbit monoclonal anti-ZO-3 (D57G7)	Cell Signaling Technologies	Cat.# 3704S; RRID: AB_2203606
APC-mouse monoclonal anti-CD47 (CC2C6)	Biolegend	Cat.# 323123; RRID: AB_2716202
PE-mouse monoclonal anti-CD26 (BA5b)	Biolegend	Cat.# 302705; RRID: AB_314289
APC/Fire750-mouse monoclonal anti-EpCAM (9C4)	Biolegend	Cat.# 324234: RRID: AB_2629703
APC-mouse monoclonal anti-human CD271/NGFR (ME20.4)	Biolegend	Cat.# 345108; RRID: AB_10645515
Mouse monoclonal anti-EpCAM (AUA1)	Abcam	Cat.#ab20160: RRID: AB_445379
Goat polyclonal anti-SOX17	R&D system	Cat.# AF1924; RRID: AB_355060
Mouse monoclonal anti-NESTIN (Clone 25)	BD	Cat.# 611658; RRID: AB_399176
Mouse monoclonal anti- Tubulin β 3 (TUJ1)	Biolegend	Cat.# 801201; RRID: AB_2313773
Goat polyclonal anti-Brachyury	R&D system	Cat.# AF2085; RRID: AB_2200235
Mouse IgG1kappa isotype control, APC-conjugated	Biolegend	Cat.# 400122; RRID: AB_326443
Mouse IgG1 isotype control, PE-conjugated	Biolegend	Cat.# 400113; RRID: AB_326435
Mouse IgG1 isotype control, PerCP/Cy5.5-conjugated	Biolegend	Cat.# 400149; RRID: AB_893680
Rabbit anti-DNAH5	Millipore-Sigma	Cat.#HPA037470; RRID: AB_10672348
Rabbit anti-DNALI1	Millipore-Sigma	Cat.# HPA028305; RRID: AB_10601807
Bacterial and Virus Strains		
pHAGE-EF1alphaL-eGFP-W	Addgene	Plasmid# 126686; RRID: RRID:Addgene_ 126686
pHAGE- EF1alphaL-TagBFP-W	Addgene	Plasmid# 126687; RRID: RRID:Addgene_ 126687
pHAGE-CMV-DsRED-W	This paper.	N/A
Biological Samples		
Paraffin-embedded section of human trachea	The MLI Tissue Procurement and Cell Culture Core, University of Chapel Hill, North Carolina.	N/A
Chemicals, Peptides, and Recombinant Proteins		
SB431542	Tocris	Cat.# 1614
Dorsomorphin	Stemgent	Cat.# 04-0024
CHIR99021	Tocris	Cat.# 4423

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant human BMP4	R&D Systems	Cat.# 314-BP
Retinoic acid	Sigma	Cat.# R2625
Y-27632 dihydrochloride	Tocris	Cat.# 1254
Recombinant human FGF10	R&D Systems	Cat.# 345-FG-025
Recombinant human FGF2	R&D Systems	Cat.# 233-FB
Dexamethasone	Sigma	Cat.# D4902
8-bromoadenosine 30,50-cyclic monophosphate sodium salt (cAMP)	Sigma	Cat.# B7880
3-Isobutyl-1-methylxanthine (IBMX)	Sigma	Cat.# I5879
A83-01	ThermoFisher Scientific	Cat.# 293910
DMH1	ThermoFisher Scientific	Cat.# 412610
Critical Commercial Assays		
RNeasy Mini Kit	QIAGEN	Cat. #74104
QIAzol Lysis Reagent	QIAGEN	Cat.#79306
TaqMan Fast Universal PCR Master Mix (2XX), no AmpErase UNG	ThermoFisher Scientific	Cat.#4364103
Chromium Next GEM Single Cell 3' Kit v3.1	10C Genomics	1000269
Deposited Data		
Sc-RNA-Seq data of iBCs and iBC-derived ALI and primary HBECs and HBEC-ALI	Kotton/Hawkins Labs	GEO: GSE142246
Experimental Models: Cell Lines		
Human: Normal donor iPSC line targeted with NKX2-1GFP and P63tdTomato (BU3-NGPT)	Kotton Lab (this paper)	http://www.bu.edu/dbin/stemcells/
DD001 iPSC	Hawkins Lab (this paper)	N/A
Human: Normal donor iPSC line targeted with NKX2-1GFP (BU3-NG)	Kotton Lab (Hawkins et al., 2017)	http://www.bu.edu/dbin/stemcells/
Human: Primary ciliary dyskinesia donor iPSC line	Generated in the CReM, BU.	N/A
Human: Cystic fibrosis donor iPSC line	Generated at Boston Children's Hospital.	N/A
Human: Corrected cystic fibrosis donor iPSC line	Generated at Boston Children's Hospital.	N/A
Human: RUES2 embryonic stem cell line	Gift from Dr. Ali H. Brivanlou, Rockefeller University	N/A
Experimental Models: Organisms/Strains		
Rat: Crl:NIH-Foxn1rnu	Charles river	Strain Code: 118
Mouse: Nu/Nu:Foxn1nu	The Jackson Laboratory	JAX stock #002019
Oligonucleotides		
TaqMan Gene Expression Assay Primer/Probe Sets (Table S3)	ThermoFisher Scientific	See Table S3 per gene
Primers for characterization of the integrated reporter, see Table S4	This paper	N/A
Recombinant DNA		
P63-tdTomato donor	GenScript, This paper	N/A
P63gRNA-CAG-Cas9-2A-GFP plasmid	DNA2.0, ATUM This paper	See target sequence in the method
Software and Algorithms		
ImageJ	National Institutes of Health	https://Imagej.nih.gov/ij/
Prism Software, version 7	Graphpad Software	https://www.Graphpad.com
FlowJo software	Becton, Dickinson & Company	https://www.Flowjo.com
Cell Ranger version 3.0.2	10X Genomics	N/A
Seurat version 3.1.0	Satija Lab, NYU	https://satijalab.org/seurat/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
mTeSR1	StemCell Technologies	Cat.# 05850
StemDiff Definitive Endoderm Kit	StemCell Technologies	Cat.# 05110
PneumaCult ALI Medium	StemCell Technologies	Cat.# 05001
PneumaCult ExPlus Medium	StemCell Technologies	Cat.# 05040
StemFlex medium	ThermoFisher Scientific	Cat.# A3349401
Matrigel Growth Factor Reduced	Corning	Cat.# 356230
Matrigel hESC-Qualified Matrix	Corning	Cat.# 354277
UNC-ALI	The MLI Tissue Procurement and Cell Culture Core, University of Chapel Hill, North Carolina	N/A
BEGM	The MLI Tissue Procurement and Cell Culture Core, University of Chapel Hill, North Carolina	N/A

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the Lead Contact, Darrell Kotton (dkotton@bu.edu).

Materials Availability

Induced pluripotent stem cell lines generated in this study are available from the CReM Biobank at Boston University and Boston Medical Center and can be found at http://www.bu.edu/dbin/stemcells/. The embryonic stem cell line RUES2 was received from Dr. Ali Brivanlou, Rockefeller University, NY.

Data and Code Availability

The accession number for the data reported in this paper is deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE142246 as well on the Kotton Lab's Bioinformatics Portal at http://www.kottonlab.com. The scRNA-Seq data of primary fetal airway (Miller et al., 2020) and primary adult airway Carraro et al., 2020 were previously deposited in NCBI's Gene Expression Omnibus.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

The Institutional Review Board of Boston University approved the generation and differentiation of human iPSCs with documented informed consent obtained from participants. The generation of CF iPSCs was approved by Boston Children's Hospital Institutional Review Board. Human airway tissue and primary HBECs were received from the CF Center Tissue Procurement and Cell Culture Core (Dr. Scott Randell) at the University of North Carolina. Human lung tissue was procured under the University of North Carolina Office of Research Ethics Biomedical Institutional Review Board Protocol No. 03-1396. For PCD studies, human protocols were approved by the institutional review board at Washington University in St. Louis. Subjects with known mutations causative of PCD were recruited from the PCD and Rare Airway Disease clinic at St. Louis Children's Hospital and Washington University. Informed consent was obtained from individuals (or their legal guardians). All human samples were de-identified. Details of procurement and isolation of airway epithelial cells from fetal and adult human lungs were previously described (Miller et al., 2020).

Human iPSC reprogramming and iPSC/ESC maintenance

All iPSC and ESC lines were maintained in feeder-free conditions on hESC-qualified Matrigel (Corning) in StemFlex Medium (Thermo Fisher Scientific, Waltham, MA) or mTeSR1 medium (StemCell Technologies, Inc., Vancouver, Canada) and passaged with Gentle Cell Dissociation Reagent (StemCell Technologies, Inc.) or ReLeSR (StemCell Technologies, Inc.). All human ESC/iPSC lines used were characterized for pluripotency and were found to be karyotypically normal. The reprogramming and gene-editing methods to derive BU3 iPSCs and target the NKX2-1 locus to generate BU3 NKX2-1^{GFP} iPSCs were previously described (Hawkins et al., 2017). PCD1 iPSC line (*DNAH5* mutation) was generated by reprogramming peripheral blood mononuclear cells with the human EF1a-STEMCCA-loxp lentiviral vector followed by Cre-mediated vector excision, according to our detailed protocol (Sommer et al., 2012). DD001m iPSCs were generated from "DD001m" P0 primary HBECs by modifying a Sendai virus based reprogramming

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protocol to substitute the base medium for bronchial epithelial growth medium (Park and Mostoslavsky, 2018). Homozygous F508del (p.Phe508del) iPSCs were generated from peripheral blood mononuclear cells at Boston Children's Hospital Stem Cell Program by Sendai virus using the Cytotune 2 kit (Thermo Fisher), yielding clones 791 and 792 ("CF"). Clone 792 was selected for correction of the p.Phe508del deletion and the experiments in Figure 7. The RUES2 human embryonic stem cell line was a generous gift from Dr. Ali H. Brivanlou of The Rockefeller University, New York City, NY. Standard immunolabeling for pluripotency markers and G-band karyotyping was performed to ensure pluripotency and normal karyotype in clones selected for these experiments (as shown in Figure S1). For BU3 NGPT iPSCs spontaneous differentiation into mesoderm, endoderm and ectodermal lineages was demonstrated by generating embryoid bodies with iPSC medium in ultra-low attachment plate (Corning) and then replacing with base medium supplemented with 10% fetal bovine serum in adherent culture. The cell types from each lineage were identified with standard immunolabeling (as shown in Figure S1) using anti-bodies listed in Key Resources Table. Primocin (Invivogen, San Diego, CA) was routinely added to prevent mycoplasma contamination and all iPSC and ESC lines screened negative for mycoplasma contamination and were routinely tested and remained negative.

Basal cell reporter iPSC

The dual reporter, NKX2-1^{GFP} and P63^{tdTomato}, iPSC lines (BU3 NGPT) were derived from the published single reporter, NKX2-1^{GFP}, iPSC line (BU3 NG), a normal donor iPSC carrying homozygous NKX2-1^{GFP} reporters (Hawkins et al., 2017). The BU3 NG line was targeted and integrated with a P63^{tdTomato} fluorescent reporter using CRISPR/Cas9 technology. A gRNA was designed to target exon 4 of the endogenous *TP63* (target sequence TGCGCGTGGTCTGTGTTATA) and a donor template was constructed to contain the tdTomato sequence and removable antibiotic selection cassette via Cre recombination flanked by arms of homology. In this report we used the clone BU3 NGPT c5-cre22, where P63^{tdTomato} fluorescent reporter-integrated clone 5 was further cre-excised to remove selection cassette (sub-clone 22).

Gene corrected CF-iPSCs

Monoallelic correction of homozygous F508del CF iPSC, clone 792, was performed by nucleofecting 1M TrypLE-Select dissociated cells with 5µg Cas9-GFP plasmid (Addgene, 44719), 5 µg of a U6 promoter driven sgRNA plasmid (target sequence ACCATTAAA-GAAAATATCAT), 10µg of a plasmid containing a 1.4kb piece of the *WT CFTR* that includes the exon encoding F508. Repaired clones were identified by ddPCR and Sanger sequencing of long-range PCR products. Clones 1566 (CFTR WT/ F508del) and 1567 (CFTR F508del / F508del) were confirmed by DNA STR fingerprint (match with donor) and karyotype analysis (no abnormalities).

Primary HBECs and human airway tissue

De-identified, cryopreserved human bronchial epithelial cells (HBECs) were received from the CF Center Tissue Procurement and Cell Culture Core, Marsico Lung Institute, University of North Carolina where they were harvested and cultured as previously described in detail. Paraffin-embedded sections of de-identified human airway tissue were also received. These samples are exempt from regulation by HHS regulation 45 CFR Part 46. Freshly isolated P0 HBECs were expanded in culture on collagen type I/III-coated tissue culture plates in non-proprietary bronchial epithelial growth medium (BEGM). At 70%–90% confluence HBECs were dissociated with Accutase (Sigma), counted and transferred to human placental type IV collagen-coated membranes (Transwell, Corning Inc., Corning, NY) at a density of 100,000-150,000 cells per 6.5mm insert and differentiated using air-liquid interface conditions in either a non-proprietary medium, "UNC-ALI" medium, or PneumaCult-ALI for 2-3 weeks or longer before analysis. Frequency of each epithelial cell-type was assessed 14 days after establishing ALI culture for both iBC and HBEC samples previously expanded in Basal Cell medium described below. Figure 4F analysis was performed 21 days after establishing ALI culture for both iBC and HBEC samples. Figure S7D analysis was performed 30 days after establishing ALI culture.

Animals

All animal procedures were performed at an AAALAC accredited facility and in accordance with National Institutes of Health's Guide for the Care and Use of Laboratory Animals and were approved by the University of Texas Health Science Center at Houston (UTHealth) Institutional Animal Care and Use Committee.

Adult mice (Foxn1^{nu}, The Jackson Laboratory) and rats (NIH-Foxn1^{rnu}, Charles River) used for these experiments were housed in the barrier facility of the Brown Institute of Molecular Medicine at the UTHealth. Animals were group housed in autoclaved, individually ventilated cages (IVC), and provided irradiated corncob bedding with cotton square nestlets for enrichment. Animals were fed *ad libitum* a commercially available, irradiated, balanced mouse diet (no. 5058, LabDiet, St Louis, MO) and had free access to acidified water. A mouse received xenografts was single housed. Rooms were maintained at 21-23°C and under a 12:12-h light:dark cycle. All animals were maintained specific pathogen free (the list of tested pathogens is available upon a request).

METHOD DETAILS

Differentiation of hPSCs into airway organoids

This protocol is based on previously described approaches to derive airway organoid from hPSCs (Hawkins et al., 2017; McCauley et al., 2017) including a detailed step-by-step protocol prior to basal cell medium (McCauley et al., 2018b). NKX2-1+ lung progenitors were generated from hPSCs first by inducing definitive endoderm with STEMdiff Definitive Endoderm Kit (StemCell Technologies,

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Inc.) for 60-72 hours. Endoderm-stage cells were dissociated and passaged in small clumps to hES-qualified Matrigel-coated (Corning) tissue culture plates (Corning) in a complete serum free differentiation medium (cSFDM) consisting of a base medium of IMDM (Thermo Fisher, Waltham, MA) and Ham's F12 (Thermo Fisher) with B27 Supplement with retinoic acid (Invitrogen, Waltham, MA), N2 Supplement (Invitrogen), 0.1% bovine serum albumin Fraction V (Invitrogen), monothioglycerol (Sigma, St. Louis, MO), Glutamax (Thermo Fisher), ascorbic acid (Sigma), and antibiotics. To pattern endoderm into anterior foregut the cSFMD base medium was supplemented with 10 μM SB431542 (Tocris, Bristol, United Kingdom) and 2 μM Dorsomorphin (Stemgent, Lexington, MA) for 72 hours (Green et al., 2011). Cells were then cultured for 9-11 additional days (typically, 144 hr - day 15) in cSFDM containing 3 μM CHIR99021 (Tocris), 10 ng/mL recombinant human BMP4 (rhBMP4, R&D Systems), and 50-100 nM retinoid acid (Millipore-Sigma) to induce NKX2-1 primordial lung progenitors (Serra et al., 2017). NKX2-1+ lung progenitors were sorted by NKX2-1^{GFP} expression for BU3 NGPT or enriched based on the expression of cell surface markers CD47hi/CD26- for non-reporter iPSC (see below) and then were resuspended in three-dimensional growth factor reduced Matrigel (Corning) at a density of 400 cells per μl and pipetted in 25-50 μL droplets onto the base of tissue culture plates (Hawkins et al., 2017). After 10-15 minutes at 37°C to allow gelling of Matrigel airway medium composed of cSFDM containing 250ng/ml FGF2 (rhFGFbasic; R&D Systems), 100ng/ml FGF10, 50nM dexamethasone, 100nM 8-Bromoadenosine 3',5'-cyclic monophosphate sodium salt (cAMP; Millipore-Sigma), 100 μM 3-Isobutyl-1-methylxanthine (IBMX; Millipore-Sigma), and 10 μM Y-27632 (Y; Tocris) (FGF2+10+DCl+Y) was added.

Sorting of PSC-derived lung and airway progenitors

To facilitate directed differentiation from hPSCs, NKX2-1+ lung progenitors and airway progenitors of iBCs were purified by cell sorting. This sorting protocol is further detailed in previous publications (Hawkins et al., 2017; McCauley et al., 2018b). On ~day 15, cells were harvested by incubation with 0.05% Trypsin-EDTA for 15-20 min at 37°C. Trypsin was neutralized and cells were washed with medium containing 10% fetal bovine serum (FBS, Thermo Fisher). Harvested cells were spun at 300 RCF for 5 min and resuspended in the buffer containing Hank's Balanced Salt Solution (Thermo Fisher), 2% FBS, 25mM HEPES 2mM EDTA (FACS buffer) supplemented with 10 µM Y-27632 and stained with propidium iodide (PI, ThermoFisher) or calcein blue AM (Thermo Fisher) for live cell selection during flow cytometry. Live cells were sorted based on NKX2-1^{GFP} expression for BU3 NGPT or by staining for CD47 (Biolegend) and CD26 (Biolegend) for non-reporter PSCs and gating for CD47hi/CD26-. Airway progenitors were sorted on ~day 30 of directed differentiation. Organoids cultured in 3D Matrigel were harvested by incubation with 1 U/mL Dispase (Stemcell technologies) or 2mg/ml Dispase (Thermo Fisher) for ~60 min at 37C until Matrigel was fully dissolved. Dissociated organoids were collected with a 1000 µL pipette, transferred to a 15ml conical and and centrifuged at 200 RCF for 3 min. The cells were resuspended and incubated in 0.05% trypsin at 37°C for approximately 10min until a single cell suspension was achieved. Trypsin was inactivated by adding 10% fetal bovine serum (Hyclone) in DMEM (GIBCO). Cells were then centrifuged at 300 RCF for 5 min at 4°C, counted and resuspended in FACS buffer. 10 µM of the cell viability dye Calcein blue (ThermoFisher) was added and Calcein Blue+/NKX2-1^{GFP+}/TP63^{tdTomato+} or PI⁻/NKX2.1^{GFP+}/TP63^{tdTomato+} cells were sorted and replated at 400 cells per µl of density in Matrigel (Corning) and cultured in FGF2+10+DCI+Y medium.

Generation, purification and maintenance of iBCs

The iPSC-derived basal cells (iBCs) were generated from airway organoids in FGF2+10+DCI+Y described above. For BU3 NGPT, one day after (unless indicated) sorting and replating NKX2-1^{GFP+}/TP63^{tdTomato+} on ~day 30 of directed differentiation, culture medium was switched to Pneumacult ExPlus (StemCell Technologies, Inc.) supplemented with 1 µM A83-01 (Tocris), 1 μM DMH1 (Tocris), and 10 μM Y-27632 (Basal cell medium). In some cases, Pneumacult Ex (Stemcell technologies) supplemented with 1 μM A83-01, 1 μM DMH1, and 10 μM Y-27632 was used as indicated in figures. For non-reporter iPSCs, single cells dissociated from airway organoids in FGF2+10+DCI+Y were replated without a sorting step and changed to basal cell medium as above. Viable iBCs at ~Day 40 of directed differentiation or later were sorted based by dissociating organoids to a single-cell suspensions as above and flow sorting based NKX2-1GFP+/TP63tdTomato+/NGFR+ cells for BU3 NGPT, or by labeling with mouse monoclonal anti-NGFR (Cat.# 345108, Biolegend) and mouse monoclonal anti-EpCAM (Cat.# 24234, Biolegend) with isotype controls (mouse monoclonal IgG1k APC-conjugated, Cat.#400122, Biolegend). Both anti-NGFR and anti-EpCAM antibodies were used at a dilution of 1:100 in a suspension of 1x10⁶ cells per 50 or 100 μL on ice for 30 min. For these sorting experiments a MoFlo Astrios Cell sorter (Beckman Coulter, Indianapolis, IN) was used at Boston University Medical Center Flow Cytometry Core Facility or BD FACSMelody (BD biosciences) was used at the UTHealth Flow Cytometry Service Center. Sorted iBCs were either replated in ALI culture (see below) or resuspended in Matrigel (Corning) droplets at 400 cells/ul, plated and cultured in basal cell medium as above for expansion of iBCs. iBCs in 3D Matrigel droplets were fed with basal cell medium every 2-3 days and required passaging every ~10-14 days. iBCs were cryopreserved by first dissociating organoids to single-cells as above and resuspending in basal cell medium supplemented with 10% dimethylsulfoxide (ThermoFisher) and transferred to cryovials. Cryovials were placed in cryostorage containers in a -80°C freezer to cool at -1°C /min for 24 hours and then transferred to -150°C freezer or into liquid nitrogen the following day for long-term storage. iBCs were thawed by warming to 37°C in a water or bead bath, diluting in 5-10ml of basal cell medium (37°C) added dropwise and centrifuged at 300 RCF for 5min. The cell pellet was then resuspended in Matrigel at 1000cells/µl, plated in tissue culture plates and after 10-15 min at 37°C basal medium was added to the well. Expanded or cryopreserved cells-derived organoids in 3D Matrigel were dissociated and sorted for iBC makers prior to replating in ALI culture.

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ALI culture of iBCs or other iPSCs-derived AECs

To differentiate iBCs in Air Liquid Interface (ALI) culture we adapted existing, detailed protocols for primary HBECs (Fulcher and Randell, 2013). iPSCs-derived airway epithelial cells (AECs) including iBCs were seeded on 6.5 mm Transwells with 0.4 µm pore polyester membrane inserts (Corning Inc., Corning, NY), coated with Matrigel diluted in DMEM/F12 (GIBCO) according to the recommended dilution factor from the lot-specific certificate of analysis, at 150,000-200,000 cells per insert with a medium used to culture each cell culture (e.g., iBCs cultured in Basal cell medium were plated on Transwells and cultured in Basal cell medium) for 3 to 7 days. Once visual inspection confirmed the Transwell membranes were covered by a confluent sheet of cells the culture medium was switched to PneumaCult-ALI medium (StemCell Technologies, Inc.) or non-proprietary "UNC-ALI medium (Fulcher and Randell, 2013) in both apical and basal chambers. The following day, the medium from top chamber was removed and the cells were further differentiated in air-liquid interface conditions for 2-3 weeks or longer before analysis. ScRNA-Seq (Figures 4A–4E), qRT-PCR (Figures 4F and S7E) were analyzed 21 days after establishing ALI culture.

Flow cytometry analysis

Flow cytometry analysis was performed as described above. NKX2-1^{GFP}, TP63^{tdTomato} expression or fluorochrome-conjugated primary antibodies (listed in the Key Resources Table) were detected using a FACS Calibur (BD Biosciences), Stratedigm S1000 EXi (Stratedigm Inc, San Jose, CA) or MoFlo Astrios (Beckman Coulter) at Boston University Medical Center or with BD FACSMelody and BD FACSChorus software (BD Biosciences) at the UTHealth Flow Cytometry Service Center. Calcein blue or PI was used to identify viable cells depending on the flow cytometer used. FlowJo software (BD biosciences) was used for further analysis.

Histological analysis

Organoids in 3D Matrigel were harvested by incubation in Dispase as described above, and then fixed in 4% paraformaldehyde (PFA) (ThermoFisher or Electron Microscopy Sciences, Hatfield, PA) for four hours at room temperature, paraffin-embedded and immunostained as previously described (McCauley et al., 2017, McCauley et al., 2018b). ALI cultures were fixed by placing Transwell filters in 4% PFA at 4°C overnight. Either whole mount immunostaining of Transwell filters or paraffin-embedding was performed. Tracheal xenografts were also fixed with 4% PFA at 4°C overnight and embedded in the paraffin. For paraffin sections, fixed samples were dehydrated with a series of increasing concentrations of ethanol, cleared with xylene and infiltrated with paraffin before embedding in wax. Sections of 5 µm thickness were transversely cut using an HM 325 Rotary Microtome (Thermo Fisher Scientific). Sectioned samples were stained with hematoxylin and eosin. For immunofluorescence analysis, antigen retrieval was performed on sectioned samples using Antigen retrieval reagent-Basic (R&D Systems) or Antigen Unmasking Solution, Citric Acid Based (Vector Labs, Burlingame CA) following manufacturer's instructions. Whole inserts and sectioned samples were stained with primary and secondary antibodies (antibody information and sources are detailed in the Key Resources Table). Briefly, samples were permeabilized with with 0.1% or 0.3% Triton X-100 (Sigma) in PBS for 15-30 min and blocked with 2% bovine serum albumin (BSA) with or without 4% Normal Donkey Serum and 0.1% Triton X-100 or 0.1% Tween20 for 1 hour at room temperature. Samples were then incubated with primary antibodies overnight at 4°C, followed by the incubation with the respective secondary antibodies at room temperature for one to two hours. Prolong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) was added to counter-stain and coverslipped. Images were acquired using a Leica DMi8 microscope (Leica Microsystems, Wetzlar, Germany) and Leica Application Suite Software (Leica Microsystems) or a Nikon Eclipse Ni-E microscope (Melville, NY). In order to generate an image of whole transwell or of large field with AECs in Xenograft model, the automated tile-scanning and stitching functions were used through Leica Application Suite Software. Confocal microscopy was performed for whole mount staining samples stained with antibodies as described above and counterstained with DRAQ5 (Cell Signaling Technology, Beverely, MA) using Leica Application Suite Software. Quantification of each epithelial cell-type was performed manually by counting the number of cells stained for specific markers in transverse sections. Three inserts for each condition were independently prepared and approximately 1,000 cells in random 40x magnification fields were analyzed for each insert. The frequency of each cell-type was calculated relative to the total number of cells as determined by DAPI-stained nuclei.

To characterize the ciliary defect in *DNAH5* mutant cells (Figure 7; Video S2), airway cells were fixed and immunostained as previously described (Pan et al., 2007; You et al., 2002). Primary and secondary antibodies used to detect dynein arms included anti-DNAH5 (1:100, HPA037470, Millipore-Sigma, St. Louis, MO), ACT (1:4000, clone 6-11-B1, Millipore-Sigma), DNAL11 (1:100, HPA028305, Millipore-Sigma). Primary antibodies were detected using fluorescently labeled secondary antibodies (Alexa Fluor, Life Technologies, Grand Island, NY, USA). Nuclei were stained using DAPI (Vector Laboratories, Burlingame, CA, USA). Images were acquired using an epiflourescent microscope interfaced with imaging software (LAS X, Leica, Buffalo Grove, IL) and adjusted globally for brightness and contrast using Affinity Photo (Serif Ltd, Nottingham, UK).

Single-cell RNA-sequencing

Cells were prepared for sc-RNA-Seq by dissociating organoids or ALI cultures using the methods described above. Live cells were sorted on a MoFlo Astrios Cell sorter (Beckman Coulter, Indianapolis, IN) at Boston University Medical Center Flow Cytometry Core and scRNA-Seq was performed using the Chromium Single Cell 3' system (10X Genomics) at the Single Cell Sequencing Core at Boston University Medical Center according to the manufacturer's instructions (10X Genomics). Single cell reads were aligned to the reference genome GRCh38 to obtain a gene-to-cell count matrix with Cell Ranger version 3.0.2 (10x Genomics). The average sample had a mean of 40,154 reads per cell (ranging from 18,415 to 50,219 depending of the library). The median number of genes

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detected per cell on the average sample was 3,303 genes. This matrix was pre-processed using Seurat version 3.1.0 (Butler et al., 2018), and filtered to remove stressed or dead cells (those with a high percentage of reads mapping to mitochondrial genes, that is beyond 10%-20%, depending on the shape of the distribution of mitochondrial reads per cell) and potential doublets (where the number of genes detected is above a percentile set as threshold based on the expected proportion of doublets at any given cell density (100 - (number of cells/1000) / 100). After normalizing, scaling and regressing out unwanted sources of variation (like cell degradation), we identified highly variable genes and used them for linear dimensionality reduction (PCA). The first 20 principal components were then used for clustering based on the Louvain method for community detection at different resolutions (from 0.25 to 1.5 at intervals of 0.25). Further non-linear dimensionality reduction was performed using Uniform Manifold Approximation and Projection (UMAP) for visualization purposes. Clusters identified with the Louvain method were annotated based on their differentially expressed genes (computed using hurdle models for sparse single cell data from the MAST package) (Finak et al., 2015). Gene set enrichment analysis for molecular signatures was done using the method described by Tirosh et al. (2016), as implemented in Seurat. In order to obtain a molecular signature of airway epithelial cell types that could be used to characterize iPSC-derived lung cells, we first analyzed a combination of two datasets of primary airway epithelial cells: (1) P0 HBECs in BEGM medium and (2) ALI cultures subsequently derived from these cells. We used the single cell pipeline above with one variation during the preprocessing: in addition to regressing the mitochondrial content, we also regressed out the "library" batch effect. Once we had annotated (based on the expression of known markers of basal, secretory and ciliated cells) the clusters obtained by the Louvain method (Figure S4B), we proceeded to compare each cell type against all others. In the cases where the cell type of interest was represented in more than one cluster, we combined such clusters before performing the comparison to ensure the purity of both query and background populations. This rationale is to ensure that the fold changes were not diluted by the presence of the same cell type in both the query and the background of each comparison, for example, when we grouped basal cell populations that were originally in different clusters belonging to HBEC samples and ALI samples, and to avoid noise by excluding clusters with poorly characterized or intermediate phenotypes. All single cell visualizations were made with Seurat (heatmaps, UMAPs, violin plots).

Quantification of the similarity between AECs

In order to quantify the similarity between the iPSC-derived airway epithelial cells and the freshly isolated primary airway epithelial cells of both fetal and adult tissues, we used a similar procedure as Miller et al. (2020). As a positive control, we included correlations between primary cultured HBECs and HBECs differentiated in ALI. Briefly this assessment consists of two steps: first, selecting a list of informative markers whose profile can discriminate between cell types and developmental stages; and second, calculating the correlation between cells in the in-vitro organoid cultures and the primary fresh cell types using the markers selected. For the first step (marker selection), we analyzed separately the single-cell primary fetal airway(Miller et al., 2020) and the single-cell primary adult airway(Carraro et al., 2020). Each dataset was normalized using regularized-negative binomial transformation (Hafemeister and Satija, 2019), and its percentage of mitochondrial reads was regressed out. Then we performed differential expression test (Finak et al., 2015) and selected the top 50 markers for each cell type. This resulted in 353 unique markers for fetal cell types and 149 unique markers for adult cell types. These two lists were combined into one and confounding genes (cell cycle markers) were excluded, resulting in a total of 366 unique markers. For the second step (computing the correlations), we averaged the normalized expression for each cell type (for both adult and fetal cell types), and then iteratively computed the Pearson correlation of the features selected in the previous step between in-vitro cells (normalized with the same method) and the in-vivo averaged cell types. A heatmap with the resulting coefficients for the epithelial cell types to relation.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number:GSE142246 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142246).

Tracheal xenografts

The tracheal xenograft model was utilized to assay the ability of iBCs to establish well-differentiated airway epithelium in an *in vivo* setting. Open-ended xenografts were prepared as previously described (Filali et al., 2002). Briefly, following multiple freeze and thaw cycles to decellularize rat tracheas, tubing was attached, and xenografts were seeded with 10⁶ iBCs (NKX2-1^{GFP+}/TP63^{tdTomato+}/NGFR⁺/EpCAM⁺) per trachea. Tracheas (two per mouse) were then implanted subcutaneously into the flanks of immune-compromised Nu/Nu mice, maintaining air exposure through the open-ended tubing. Three weeks after transplantation, tracheas were removed and examined via H&E staining and immunostaining for the establishment of pseudostratified, well-differentiated airway epithelium.

Epithelial cell electrophysiological analysis

Barrier function of the Air Liquid Interface Cultures (ALI) was measured using the Millicell ERS-2 Volt-ohm Meter (Millipore cat# MERS00002). The transepithelial electrical resistance (TEER) was measured at varying time points after the medium was removed from the apical chambers. All Transwells, including blanks (no cells), received 0.6ml of fresh PneumaCult-ALI medium in the baso-lateral chamber and 0.2ml in the apical chamber. Ohms (Ω) were measured by inserting the longer portion of the electrode into the lower chamber of the Transwell while the shorter end was kept in the apical chamber, and after readings were stabilized for at least six seconds. All measurements were performed in triplicate. The resistance across each cell layer (R_{tissue}) was obtained by calculating the average resistance of the blank wells (R_{blank}) and subtracting them from the average of an individual Transwell's 3 readings (R_{total}): (R_{tissue} = R_{total}- R_{blank}). TEER (Ω . cm²) = (R_{tissue})* surface area (cm²) of the Transwell.

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Ussing chamber experiments were performed on EasyMount Ussing Chamber Systems at voltage clamp mode, and Acquire & Analyze software (Physiologic Instruments) was employed to record and analyze data. Briefly, Transwell inserts were mounted into chambers and bathed in low chloride Ringer's solution (1.2 mM NaCl, 140 mM Na-gluconate, 25 mM NaHCO₃, 3.33 mM KH₂PO₄, 0.83 mM K₂HPO₄, 1.2mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose) at apical and Ringer's solution (120 mM NaCl, 25 mM NaHCO₃, 3.33 mM KH₂PO₄, 0.83 mM K₂HPO₄, 0.83 mM K₂HPO₄, 1.2mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose) at basolateral side of monolayer. After base line is stabilized, 100 μ M amiloride (Sigma) applied to both sides of the chamber to carry out complete inhibition of ENaC (Epithelial so-dium channel). Subsequently, 10 μ M forskolin (Sigma) were administered to stimulate chloride current. At the end of experiments, 10 μ M CFTR inhibitor-172 (Sigma) employed at the apical side to specifically inhibit CFTR function followed by 100 μ M UTP at the apical side to assess the integrity of developed epithelium. For BU3 NGPT derived ALI, 5 μ M VX-770, CFTR modulator, was treated subsequent to forskolin. The resulting change in short circuit current was calculated as Δ Isc. The data were expressed in mean \pm SD.

Ussing chamber analysis was also used to measure TEER at the beginning of each recording, prior to compound administration, in some cases. Briefly, a blank insert was mounted into the Ussing chamber and each side was incubated with Ringer solution as above, followed by compensation for voltage electrode asymmetry and fluid resistance. The blank insert was now replaced with the insert with cells, which was then voltage clamped and monitored for short-circuit current to calculate TEER based on Ohm's law.

Limiting dilution and tracheosphere assays

On day 10 of directed differentiation, cells were treated with 5 μ g/ml polybrene and then separate wells were infected with pHAGE-EF1alphaL-eGFP-W, pHAGE-Ef1alphaL-TagBFP-W, or pHAGE-CMV-DsRED-W at an MOI = 1 for 24 hr. On Day 20 of differentiation, cells were dissociated and GFP+, BFP+ and DsRed+ cells were sorted according to the same protocol described above. Equal numbers of GFP+, BFP+ and dsRED+ cells were combined and plated at 1000 cell/ μ L, 400 cells/ μ L and 200 cells/ μ L in 50ul droplets of 3D Matrigel in triplicate. On day 34, the Matrigel droplets were imaged and the number of GFP, BFP, dsRed or mixed colonies were manually counted. For the tracheosphere assay GFP+/TOM+/NGFR+ BU3 NGPT cells were sorted as above and plated at a density of 200cells/ μ L in basal cell medium. Once organoids had formed, ~10 days, triplicate wells were either continued in basal cell medium or changed to PneumaCult-ALI for an additional 10 days. For the scRNA-Seq experiment in Figure S5E, GFP+/TOM+/NGFR+ BU3 NGPT cells were sorted as above and plated at a density of 400cells/ μ L in basal cell medium and cultured for 29 days.

Mucus cell metaplasia model

BU3 NGPT iBSC-derived ALI culture was prepared as described above. Ten days after air-lifted, cells were treated with 10ng/ml IL-13 and cultured for 10 days prior to the assessments. Mucus cell metaplasia was assessed by immunofluorescence staining for the increment of MUC5AC expressing cells or gene expression changes via qRT-PCR. Quantification of MUC5AC+ cells was performed using ImageJ software by manually counting MUC5AC+ cells in 3 randomly selected fields per well (n = 3 wells per condition).

Quantitative RT-PCR

RNA was extracted by first lysing cells in QIAzol (QIAGEN) and subsequently using the RNeasy Mini kit (QIAGEN). Taqman Fast Universal PCR mastermix (Applied Biosystems) were used to reverse transcribe RNA into cDNA and analyzed during 40 cycles of real time PCR using Taqman probes (Applied Biosystems). Relative gene expression, normalized to 18S control, was calculated as fold change in 18S-normalized gene expression, over baseline, using the $2^{(-\Delta\Delta CT)}$ method. Baseline, defined as fold change = 1, was set to undifferentiated iPSCs. If undetected, a cycle number of 40 was assigned to allow fold change calculations.

Transmission electron microscopy

Electron microscopy was performed as previously described (Horani et al., 2012, 2013). In short, cultured airway epithelial cells collected from subjects with PCD, or iPSC derived cells were fixed using 2.5% glutaraldehyde + 2% paraformaldehyde in 0.15M cacodylate buffer at 37°C. Secondary fixation was performed in 1% OsO4 / 1.5% potassium ferrocyanide, and then in 2% uranyl acetate. Post-staining with 0.1% lead citrate and 0.2% tannic acid was done for additional contrast enhancement. At least 10 cilia cross sections were evaluated per group.

High speed video-microscopy

Cilia beat frequency was analyzed live in at least 5 fields obtained from each preparation, using a high-speed video camera and processed with the Sisson-Ammons Video Analysis system (Amons Engineering, Mt Morris, MI) as described (Horani et al., 2012; Sisson et al., 2003).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical methods relevant to each figure are outlined in the accompanying figure legend or described in Results section. Unless otherwise indicated unpaired, two-tailed Student's t tests were applied to two groups of n = 3 or more samples, where each replicate ("n") represents either entirely separate differentiations from the pluripotent stem cell stage or replicates differentiated simultaneously and sorted into separate wells. A p value < 0.05 was considered to indicate a significant difference between groups.