Lentiviral vectors transduce lung stem cells without disrupting plasticity

Ashley L. Cooney,1,2,4 Andrew L. Thurman,2,3,4 Paul B. McCray, Jr.,1,2 Alejandro A. Pezzulo,2,3 and Patrick L. Sinn1,2

1Stead Family Department of Pediatrics, The University of Iowa, Department of Pediatrics, 169 Newton RD, 6320 PBDB, Iowa City, IA 52242, USA; 2Pappajohn Biomedical Institute and the Center for Gene Therapy, The University of Iowa, Iowa City, IA 52242, USA; 3Department of Internal Medicine, The University of Iowa, Iowa City, IA 52242, USA

Life-long expression of a gene therapy agent likely requires targeting stem cells. Here we ask the question: does viral vector transduction or ectopic expression of a therapeutic transgene preclude airway stem cell function? We used a lentiviral vector containing a GFP or cystic fibrosis transmembrane conductance regulator (CFTR) transgene to transduce primary airway basal cells from human cystic fibrosis (CF) or non-CF lung donors and monitored expression and function after differentiation. Using chamber measurements confirmed CFTR-dependent chloride channel activity in CF donor cells. Immunostaining, quantitative real-time PCR, and single-cell sequencing analysis of cell-type markers indicated that vector transduction or CFTR expression does not alter the formation of pseudostratified, fully differentiated epithelial cell cultures or cell type distribution. These results have important implications for use of gene addition or gene editing strategies as lifelong curative approaches for lung genetic diseases.

INTRODUCTION

Cystic fibrosis (CF) is caused by mutations in cystic fibrosis transmembrane conductance regulator (CFTR), which encodes an anion channel that contributes to regulation of airway surface liquid volume and composition. Without functional CFTR protein at the cell surface, dysregulated chloride and bicarbonate permeability ultimately leads to reduced innate immune defenses, bacterial colonization, inflammation, and mucus plugging that gradually and irreversibly destroy the lungs. Complementing or repairing CFTR in the appropriate pulmonary cell types early in life would prevent CF-related lung complications.

Many cell types in the conducting airways express CFTR, including ciliated, non-ciliated, and secretory cells and ionocytes at the airway surface, as well as serous cells within the acini of the submucosal glands.1,2 These cell types are obvious targets for CF gene therapy; however, they are often terminally differentiated, and corrected CFTR expression would be lost as cells turn over. To achieve lifelong correction from a gene therapy intervention, permanent genomic modification of self-renewing cells is likely required. Unlike many organs, the respiratory epithelium has multiple progenitor cell populations. Different progenitor cell types are responsible for maintaining discrete niches from the trachea to the alveoli,3 and their roles may vary in health and disease. The heterogeneous population of basal cells, which comprise the progenitor cells of the trachea and mainstem bronchi,4 have remarkable plasticity to respond to injury and repair the surrounding epithelia (reviewed in Tata and Rajagopal5).

Primary cultures of airway basal cells grown at an air-liquid interface (ALI) mimic human airways and will reconstitute a pseudostratified epithelial sheet with multiple CFTR-expressing cell types;6 however, basal cells do not typically express CFTR protein.7 The ramifications of exogenous expression of CFTR in basal cells are unknown and have important implications for gene therapy. Early evidence of CFTR expression in non-epithelial cells suggested altered metabolism, growth abnormalities, and potential consequences of exogenous transgene expression.8,9 Since then, no significant progress has confirmed this, and no studies have been performed with human primary basal cells. Here, we transduce a population of primary human basal cells with a lentiviral vector expressing either a GFP reporter gene or CFTR to address fundamental unanswered questions, including (1) will lentiviral transduction or CFTR expression in basal cells alter their multipotency and differentiated cell type distribution? and (2) do CF cells complemented with CFTR have a global mRNA transcript expression profile that more closely resembles CF or non-CF patterns?

RESULTS

Basal cell collection, transduction, differentiation, and phenotypic correction

Human lungs from multiple CF and non-CF donors were used for these studies. Primary basal cells were isolated from the trachea and bronchi as previously described11 (Figure 1A). Cells isolated from both CF and non-CF airways expressed high levels of the known CFTR transmembrane regulator (CFTR) gene or

Received 22 December 2020; accepted 9 June 2021;
https://doi.org/10.1016/j.omtn.2021.06.010.

1These authors contributed equally

Correspondence: Ashley L. Cooney, Stead Family Department of Pediatrics, The University of Iowa, Department of Pediatrics, 169 Newton RD, 6320 PBDB, Iowa City, IA 52242, USA.
E-mail: ashley-peterson@uiowa.edu
transduced at the time of seeding with either HIV-GFP or HIV-CFTR (shown schematically, Figure S2) (MOI = 5) overnight, left submerged for 2–3 days, and then grown at an ALI until well differentiated (>4 weeks). Untransduced control cells were cultured in parallel. We quantified GFP in well-differentiated epithelial cells by flow cytometry to confirm that transgene expression was retained after differentiation. We observed 75%–80% GFP-positive cells in both CF and non-CF cultures (Figure 1C).

To examine the morphology of cultured epithelia, immunostained cultures were imaged with confocal microscopy. Polarized airway epithelial cells had a pseudostratified columnar morphology with actin belts (phalloidin, white) and cilia ([α-tubulin, red] (Figures 2A–2C). XZ images (lower panels) revealed pseudostatification. As expected, GFP expression (green) was restricted to HIV-GFP-treated cultures (Figure 2B). We next measured the anion transport properties of each condition. We hypothesized that complementing CFTR in CF basal cells using a lentiviral vector would achieve phenotypic correction after differentiation of transduced basal cells. As shown in Figure 2D, CF cells transduced with HIV-CFTR exhibited a greater change in short-circuit current (ΔIsc) in response to the cAMP agonists forskolin and 3-isobutyl-1-methylxanthine (IBMX) (F&I) and the CFTR inhibitor GlyH-101 (GlyH) compared to untreated or HIV-GFP-treated cells. These data demonstrate that complementing CFTR in CF cultures restores Cl− currents to levels indistinguishable from non-CF cultures. Moreover, supplemental expression of CFTR by a lentiviral vector in non-CF cultures did not result in supraphysiological changes in CFTR-dependent current.

Single-cell transcriptome profiles of transduced primary basal cells after differentiation

To further analyze how basal cells respond to lentiviral transduction and CFTR expression, we used single-cell RNA (scRNA) sequencing (scRNA-seq) to assess transcript levels by cell type. We compared multiple CF (n = 4) and non-CF (n = 3) donors. For each donor, we prepared a scRNA library and performed single-cell sequencing following 3 conditions (untreated, HIV-GFP, and HIV-CFTR). In total, 21 individual scRNA libraries were sequenced. We first confirmed that the appropriate cell types arose from basal cell differentiation, including secretory, basal, and ciliated cells, ionocytes, and pulmonary neuroendocrine cells (PNECs)2,4,12 (Figure 3A). Approximately 50% of the cells were secretory, 25% were basal, 25% were ciliated, and <1% were ionocytes or PNECs (Figure 3B). The major cell types (secretory, basal, and ciliated) appeared in similar ratios regardless of treatment (untreated, HIV-GFP, or HIV-CFTR) or disease (CF or non-CF). However, there were fewer ciliated cells in CF (~15%) than in non-CF (30%) cells. The 10 most highly expressed transcripts in each cell type are listed in a heatmap (Figure 3C) where color intensity represents expression levels (yellow = high, black = low). The expression levels of canonical cell type markers were also visualized with a violin plot for secretory cells (BPIFA1), basal cells (KRT5), ciliated cells (FOXJ1), ionocytes (ASCL3), and PNECs (ASCL1) (Figure 3D). Of note, subpopulations within these common cell types have been previously described.13,14 Indeed, not all cells fit perfectly into common cell populations; for example, cell populations that are dual positive for basal (TP63+) and ciliated (DNAAF1+) markers can be identified (Figure S3).

As further confirmation of appropriate expression levels of cell type markers, we used quantitative real-time PCR to determine the relative abundance of p63 (basal cells), FOXJ1 (ciliated cells), and MUC5AC and MUC5B (secretory cells) markers compared to undifferentiated basal cells in the cultures (Figures S4A–S4D). Relative to the initial expression in basal cells, we observed similar levels of Pan-ΔNp63, a marker for basal cells, and high levels of FOXJ1, MUC5AC, and MUC5B, confirming well-differentiated airway epithelia.15 These levels were unchanged between untreated, GFP, and CFTR conditions. Moreover, the minimal change in pattern observed with exogenous CFTR expression suggests that the genotype-associated difference may not be determined by CFTR function.
Changes to the transcriptome following lentiviral vector integration or exogenous CFTR expression could reveal the impact of gene therapy on cellular gene expression. Here, we analyzed genes differentially expressed in both CF and non-CF cells after treatment with a lentiviral vector expressing either GFP or CFTR relative to their untreated controls. We observed few differentially expressed genes. In each case, we analyzed differentially expressed transcripts among all cell types. As expected, we observed GFP expression in the HIV-GFP- or HIV-CFTR-treated samples. We observed GFP expression in all cell types untreated non-CF cells. We next compared treated cells than the HIV-GFP or untreated conditions (Figure 6C). We measured total CFTR mRNA expression by quantitative real-time PCR and observed a trend toward higher levels in the HIV-CFTR-treated cells than the HIV-GFP or untreated conditions (Figure 6D). We observed an increase in total (endogenous and CFTR transgene) CFTR transcripts in ciliated CF cells treated with HIV-CFTR compared to untreated (Figure 6F). This is consistent with the differential gene expression data in Figure S5 (CF, ciliated cells). Also, PNECs seemed to show less GFP reads were predominately detected in the HIV-GFP- and transcripts supplied by the vector. Representative alignment coordinates shows the number of reads at each nucleotide position within GFP (Figure 6B). This indicates that the GFP reads were predominately detected in the HIV-GFP-treated samples. We observed GFP expression in all cell types including secretory, basal, and ciliated cells, ionocytes, and PNECs by scRNA-seq (Figure 6E). These data indicate that all cell types appropriately differentiated from a population of basal cells transduced with a lentiviral vector expressing GFP and that using a lentiviral vector to transduce airway progenitor cells does not preclude stem cell differentiation.

We next confirmed that the delivered transgenes were expressed in all cell types. As expected, we observed GFP expression in the HIV-GFP-transduced cells by quantitative real-time PCR (Figure 6A). An example of the scRNA-seq alignment coordinates shows the number of reads at each nucleotide position within GFP (Figure 6B). This indicates that the GFP reads were predominately detected in the HIV-GFP-treated samples. We observed GFP expression in all cell types including secretory, basal, and ciliated cells, ionocytes, and PNECs by scRNA-seq (Figure 6E). These data indicate that all cell types appropriately differentiated from a population of basal cells transduced with a lentiviral vector expressing GFP and that using a lentiviral vector to transduce airway progenitor cells does not preclude stem cell differentiation.

We measured total CFTR mRNA expression by quantitative real-time PCR and observed a trend toward higher levels in the HIV-CFTR-treated cells than the HIV-GFP or untreated conditions (Figure 6C). We next sought to differentiate between endogenous CFTR mRNA and transcripts supplied by the vector. Representative alignment coordinates show the number of reads that match the input sequence (Figure 6D). We observed an increase in total (endogenous and CFTR transgene) CFTR transcripts in ciliated CF cells treated with HIV-CFTR compared to untreated (Figure 6F). This is consistent with the differential gene expression data in Figure S5 (CF, ciliated cells). Also, PNECs seemed to show less CFTR in untreated CF cells than untreated non-CF cells. We next compared CFTR transgene levels from GFP- or CFTR-treated cells relative to untreated CF cells.
and observed that CFTR transcripts were increased in secretory, basal, and ciliated cells in HIV-CFTR-transduced cells only (Figure 6G). In CF cells treated with HIV-CFTR, CFTR transcript levels were restored to non-CF levels in ciliated cells. Interestingly, ionocytes expressed the highest CFTR levels, but expression was not increased after the delivery of HIV-CFTR (Figure 6G). In summary, modest detectable increases in CFTR transcripts were sufficient to restore functional correction to CF epithelia. Additionally, CFTR expressed in basal cells is retained in basal cells and expressed in all major cell types after differentiation.

DISCUSSION

For a gene therapy treatment to correct CF airway disease for the life of a person, stable expression of a functional CFTR protein in surface airway epithelial cells is likely necessary. Of the many proposed gene therapy approaches that may satisfy this benchmark, lentiviral delivery of a constitutively expressed CFTR cDNA to airway progenitor cells is among the products with near-term translational potential. Basal cells are the progenitor cells of the large airways; however, prior to this study, important questions about the consequences of CFTR expression in basal cells were unaddressed. Although mouse studies suggest that lentiviral vectors can persistently express CFTR for the life of the animal,\textsuperscript{16} evaluating a gene therapy treatment specifically in human airway progenitor cells is crucial. Here, we analyzed primary basal cells from CF and non-CF donors and their responses to lentiviral vectors expressing GFP or CFTR. Our results suggest that neither a lentiviral vector nor CFTR expression significantly altered the differentiation potential of primary basal cells acquired from human donors.

Little is known about how restoring CFTR in airway epithelia affects the transcriptome. Small-molecule correctors and potentiators, such as ivacaftor and Trikafta, are beginning to inform us of the long-term effects of restoring CFTR activity.\textsuperscript{18,19} Transcriptome profiling, including scRNA-seq, is a robust approach that can address fundamental questions about cellular responses to CFTR complementation and provide clues to underappreciated CF defects. Here we evaluated well-differentiated airway epithelia derived from primary human basal cells using single-cell sequencing. We found that lentiviral vector transduction of basal cells was associated with very few differentially expressed genes after differentiation. Supplemental CFTR expression in either CF or non-CF cells resulted in only minor transcriptomic changes, regardless of cell type.

A potential hurdle for either gene addition or gene editing strategies is access to lung progenitor cells. Basal cells line the basement membrane of the conducting airways and repopulate the surface epithelium after cell turnover or injury.\textsuperscript{B} Topical aerosol delivery deposits
gene therapy vectors on the luminal surface of the airways, where access to basal cells is limited by tight junctions. However, we and others have shown that agents such as the natural airway surfactant lysophosphatidylcholine (LPC)\textsuperscript{20–22} and the calcium chelator EGTA\textsuperscript{23} transiently increased epithelial permeability and allow access to the basolateral surface of columnar epithelial cells as well as basal cells. Additionally, some basal cell extensions may reach the luminal surface\textsuperscript{24}.

The percentage of basal cells is highest in the trachea (\textasciitilde30\%) and gradually decreases throughout the airway tree, reaching <6\% in the proximal small airways.\textsuperscript{25} Progenitor cells of the mouse small airways lie at the bifurcation of the bronchiolar and respiratory epithelium\textsuperscript{26} and include club cells and alveolar type II cells, respectively. A major difference between progenitor cells in the large versus small airway is accessibility from the lumen. The small airways are an important target for CF gene therapy, and progenitor cells of this region are accessible without the need to disrupt tight junctions. We have previously shown that an aerosol delivery of vectors transduces both proximal and distal airways of a large animal model.\textsuperscript{22}

Delivering \textit{CFTR} by a lentiviral vector to CF cells complements the anion channel defect.\textsuperscript{16,17,27,28} Supraphysiological current changes were previously observed with \textit{CFTR} delivered to fully differentiated primary cultures with an adenoviral-based vector. In that setting, mislocalization of \textit{CFTR} to the basement membrane resulted in a net loss of anion transport.\textsuperscript{29} In our studies, supplemental \textit{CFTR} expression by a lentiviral vector showed no evidence of aberrant expression based on bioelectric properties. Transcriptome analysis revealed no consequential differences between untreated, GFP-treated, and CFTR-treated non-CF cells.

One observation consistent among all conditions was the ratio of cell types that arose from basal cells. These data suggest an intrinsic ability for progenitor cells to maintain an appropriate proportion of cell types that comprise the pseudostratified epithelium. Representing one of the smallest populations of cells in our dataset, ionocytes expressed the highest levels of \textit{CFTR} expression, consistent with previous reports.\textsuperscript{2,20} Importantly, although expression of \textit{CFTR} in non-CF secretory, basal, and ciliated cells increased mRNA transcript levels, this did not result in an increase in \textit{CFTR}-dependent Cl\textsuperscript{−} current.
This could suggest a mechanism to regulate CFTR levels among various cell types. Secretory, basal, and ciliated cells from CFTR-transduced cells expressing CFTR indicate that basal cells can indeed differentiate into other cell types while retaining CFTR expression, further suggesting that CFTR expression does not disrupt stem cell plasticity.

We hypothesized that restoring CFTR would modify the transcriptome to mirror a non-CF cell-like state but instead found that transcripts from CF cells complemented with HIV-CFTR more closely resembled CF cells than non-CF. These findings were consistent in the scRNA-seq data as well as quantitative real-time PCR. Through these studies, we compiled a list of non-vector-encoded genes that were upregulated or downregulated in response to restoring CFTR. Understanding the impact that the expression of these genes have will require further investigation. Another interesting observation was that restoring CFTR to CF or non-CF cells increased transcript levels of CFTR in all cell types except ionocytes. We postulate that there may be an upper physiological limit of expressing CFTR in ionocytes, but this has yet to be investigated.

A primary objective of this study was to quantify the effects of CFTR expression on the multipotency of airway progenitor basal cells. We transduced pure populations of human basal cells collected from multiple CF and non-CF human donors. This is a relevant and representative in vitro model system; however, this study has limitations. Because this is an in vitro model of epithelial cells derived from the trachea and mainstem bronchi, we did not evaluate immune responses to the vector or vector components, basal cell transduction efficiencies in an animal model, or progenitor cell populations of the submucosal glands, small airways, or alveoli. We did not measure persistence through basal cell proliferation for the following reasons: (1) passaging basal cells leads to senescence after 6–8 passages, (2) cell turnover in vitro is not representative of an in vivo setting, (3) immune responses in vivo may play a role in selective cell-mediated clearance, and (4) efforts to passage basal cells resulted in the inability to form an ALI after passage 4 (~3–4 population doublings per passage).

Here we addressed the differentiation potential of CFTR-expressing basal cells compared to untreated or GFP-expressing cells in CF and non-CF primary cells. Based on quantitative real-time PCR, morphology, physiological assays, and scRNA-seq analyses, we provide evidence that lentiviral delivery of CFTR to basal cells does not preclude formation of a well-differentiated airway epithelium with complementation of CFTR anion channel activity.

MATERIALS AND METHODS

Ethics statement
Basal cells from human CF and non-CF donors were isolated from discarded tissue, autopsy, or surgical specimens. Cells were provided by The University of Iowa In Vitro Models and Cell Culture Repository. We were not provided with any information that could be used to identify a subject. All studies involving human subjects received University of Iowa Institutional Review Board approval.

Viral vector production
VSV-G-pseudotyped HIV viral vectors with a PGK promoter driving either GFP (cloned by Laura Marquez Loza) or CFTR expression were produced at the University of Iowa Viral Vector Core (https://medicine.uiowa.edu/vectorcore) by a four-plasmid transfection method as previously described. The lentiviral vector was previously described and provided by Stefano Rivella. Lentiviral vectors were
titered by droplet digital PCR\(^3\) and/or by flow cytometry as part of the Vector Core service.

**Cell culture**

Basal cells were cultured in Lifeline BronchiaLife (Lifeline Cell Technology, Carlsbad, CA, USA) medium for 3–5 days after lung harvest. When the cells reached 80% confluency they were washed in phosphate-buffered saline (PBS), lifted in TrypLE (Gibco, Gaithersburg, MD, USA), and counted. 1 \times 10^5 cells were seeded on each 0.33 cm\(^2\) collagen IV-coated polycarbonate Transwell insert (Corning Costar, Cambridge, MA, USA), and counted. Cultures were left untransduced or transduced with either VSVG-HIV-PGK-GFP or VSVG-HIV-PGK-CFTR overnight at a MOI of 5 at the time of seeding. Transwells remained submerged in USG for 2–3 days post-seeding, apical medium was removed, and cells were grown at an ALI until well differentiated (>28 days).

**Flow cytometry**

After differentiation of the cultures, GFP was quantified by flow cytometry. Briefly, cells were stained with a LIVE/DEAD Fixable Stain (Thermo Fisher Scientific, Waltham, MA, USA), lifted in Accutase at 37°C for 15 min, and run through an Attune NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA), and percentage of GFP-positive cells was calculated. Basal cells were assayed for basal cell markers Krt5 (ab193894; 1:600, Abcam, Cambridge, MA, USA), p63 (ab246728; 1:600, Abcam, Cambridge, MA, USA), NGFR (345110; 1:600, BioLegend, San Diego, CA, USA), and \(\alpha\)-tubulin (NB100-69AF405, 1:300, Novus, Centennial, CO, USA). Cells were treated with the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer’s recommendations and stained for 1 h at 4°C with the antibodies listed above. An entire 10 cm\(^2\) dish was assayed per donor. Cells were run on the Attune NxT Flow Cytometer, and expression was gated on live cells.

**RNA isolation and quantitative real-time PCR**

Basal cells or well-differentiated epithelia were treated with TRIzol, and RNA was isolated with the Zymo Direct-zol Miniprep isolation kit (Zymo Research, Irvine, CA, USA) according to the manufacturer’s instructions. cDNA was generated with the Applied
biosystems high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR was performed with Power SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Primer sets are listed in Table S2. Fold change was calculated as change in gene expression of well-differentiated epithelia over untreated basal cells.

**Electrophysiology**

Isc was measured in well-differentiated airway epithelia derived from primary basal cells from CF and non-CF donors. Conditions included untreated, HIV-GFP-treated, or HIV-CFTR-treated cells. Airway cultures were mounted in the Ussing chamber, and their bioelectric properties were quantified as previously reported.27 Cells were pretreated with F&I overnight before Ussing chamber analysis. Assay protocol is as follows: amiloride, 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid (DIDS), F&I, and GlyH. Results are reported as ΔIsc in response to F&I or GlyH.

**Single-cell RNA sequencing**

CFTR genotypes used in these studies are 621+1G > T/2184insA, df508/3659delC, W1282X/CFTR-del2,3, and 711+1G > T/A455E. Libraries were generated for scRNA-seq according to the Chromium Single Cell Gene Expression v3 Kit 10X Genomics protocol (10X Genomics, Pleasanton, CA, USA). Approximately 5,000 cells were combined with Gel Beads, Master Mix, and Partitioning Oil and loaded into a Chromium Next GEM Chip. Single cells were partitioned in oil to generate gel beads in emulsion (GEMs). GEMs were then dissolved and barcoded with Illumina TruSeq sequencing primer, barcode, and unique molecular identifier (UMI). A reverse transcription step next generated full-length cDNA. After an additional round of cDNA amplification, cDNA underwent enzymatic fragmentation to select the appropriate ampiclon size and then labeled via End Repair, A-tailing, Adaptor Ligation, and PCR to construct final single-cell libraries for sequencing. Sequencing was performed on the HiSeq or NovaSeq 6000 platform.

**Bioinformatic analyses**

Raw sequencing reads were processed with Cell Ranger version 3.0.2 with alignment to a hybrid genome consisting of human genome reference GRCh38.p13 and the GFP and CFTR transgenes. Gene-by-cell count matrices were processed with the R package Seurat version 3.1.1.28 Counts for each cell were normalized by total UMIs and log transformed to quantify gene expression for each cell. To reduce the data dimensionality for clustering and visualization, centered and scaled gene expression for the 2,000 mostly highly variable genes were further reduced to the first 20 principal component scores for input to a shared nearest neighbor clustering algorithm. Cell type identities were associated with each cluster by identifying upregulated genes in each cluster with a Wilcoxon rank-sum test and comparing upregulated genes to a list of known airway epithelial markers.

**Statistics**

Statistical comparisons were performed with cultures as the units of analysis. For each culture, cells were stratified by cell type, and gene counts were summed across cells, resulting in a gene-by-culture count matrix for each cell type. Comparisons of different cell proportions between CF and non-CF cultures and comparisons of gene expression levels for individual genes were performed with a t test. Genome-wide differential gene expression analyses for the effect of VSVG-HIV-PGK-GFP and VSVG-HIV-PGK-CFTR transductions were performed with the R package DESeq2 version 1.22.27 (R Core Team [2020]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/). Differentially expressed genes were defined as those having an FDR < 0.05 and at least a 2-fold difference in expression.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.06.010.

**ACKNOWLEDGMENTS**

We thank Bo Ram Kim, Guillermo Romano Ibarra, Laura Marquez Loza, Ian Thornell, Chris Wohlford-Lenane, and Brajesh Singh for technical assistance. We thank Phil Karp and Ping Tan in the In Vitro Models and Cell Culture Core for providing the basal cells from the many lung donors used in these studies. We thank Laura Marquez Loza, Amber Vu, Christian Brommel, Cami Hippee, and Miguel Ortiz for their critical review of this manuscript. This work was supported by the NIH (P01 HL51670, P01 HL091842, P01 HL152960, R01 HL133089), the Cystic Fibrosis Foundation (COO-NEY18F00, SINN19XX0), the University of Iowa Center for Gene Therapy (DK54759), and the Roy J. Carver Chair in Pulmonary Research (P.B.M.).

**AUTHOR CONTRIBUTIONS**

A.L.C.: designing experiments, data collection, analyzing and interpreting results, writing the manuscript; A.L.T.: data collection, analyzing and interpreting results, writing the manuscript; P.B.M.: designing experiments, analyzing and interpreting results; A.A.P.: designing experiments, analyzing and interpreting results; P.L.S.: designing experiments, analyzing and interpreting results, writing the manuscript.

**DECLARATION OF INTERESTS**

P.B.M. is on the SAB consults and performs sponsored research for Spirovant Sciences.

**REFERENCES**

1. Thelin, W.R., Kesimer, M., Tarran, R., Kredo, S.M., Grubb, B.R., Sheehan, J.K., Stutts, M.J., and Milgram, S.L. (2005). The cystic fibrosis transmembrane conductance...
1. Hisert, K.B., Heltshe, S.L., Pope, C., Jorth, P., Wu, X., Edwards, R.M., Radey, M., Accursio, F.I., Wolter, D.I., Cooke, G., et al. (2017). Restoring Cystic Fibrosis Transmembrane Conductance Regulator Function Reduces Airway Bacteria and Inflammation in People with Cystic Fibrosis and Chronic Lung Infections. Am. J. Respir. Crit. Care Med. 195, 1617–1628.

2. Plasschaert, L.W., Žilioniš, R., Choo-Wing, R., Savova, V., Knehr, J., Roma, G., Klein, A.M., and Jaffe, A.B. (2018). A single-cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte. Nature 560, 377–381.

3. Rock, J.R., Randell, S.H., and Hogan, B.L. (2010). Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. Dis. Model. Mech. 3, 545–556.

4. Rock, J.R., Onatii, M.W., Rawlins, E.L., Lu, Y., Clark, C.P., Xue, Y., Randell, S.H., and Hogan, B.L. (2009). Basal cells as stem cells of the mouse trachea and human airway epithelium. Proc. Natl. Acad. Sci. USA 106, 12771–12775.

5. Tata, P.R., and Rajagopal, J. (2017). Plasticity in the lung: making and breaking cell identity. Development 144, 755–766.

6. Karp, P.H., Moninger, T.O., Weber, S.P., Nesselhauf, T.S., Launspach, J.L., Zabner, J., and Welsh, M.J. (2002). An in vitro model of differentiated human airway epithelia. Methods for establishing primary cultures. Methods Mol. Biol. 188, 115–137.

7. Carvalho-Oliveira, I., Efthymiadou, A., Malhó, R., Nogueira, P., Tzetis, M., Kanavakis, E., Amaral, M.D., and Penque, D. (2004). CFTR localization in native airway cells and cell lines expressing wild-type or F508del-CFTR by a panel of different antibodies. J. Histochem. Cytochem. 52, 193–203.

8. Jiang, Q., and Engelhardt, J.F. (1998). Cellular heterogeneity of CFTR expression and function in the lung: implications for gene therapy of cystic fibrosis. Eur. J. Hum. Genet. 6, 12–31.

9. Stutts, M.J., Gabriel, S.E., Olsen, J.C., Gatzy, J.T., O., 4th, and Cheng, S.H. (1996). Biosynthetic and growth abnormalities are associated with high-level expression of CFTR in heterologous cells. Proc. Natl. Acad. Sci. USA 93, 4677–4681.

10. Carver, J. (2001). Transcriptional Responses to Ivacaftor and Prediction of Ivacaftor Clinical Responsiveness. Am. J. Respir. Cell Mol. Biol.

11. Accurso, F.J., Wolter, D.I., Cooke, G., et al. (2017). Restoring Cystic Fibrosis Transmembrane Conductance Regulator Function Reduces Airway Bacteria and Inflammation in People with Cystic Fibrosis and Chronic Lung Infections. Am. J. Respir. Crit. Care Med. 195, 1617–1628.

12. Cmielewski, P., Anson, D.S., and Parsons, D.W. (2010). Lyso phosphatidylcholine as an adjuvant for lentiviral vector mediated gene transfer to airway epithelium: effect of acyl chain length. Respir. Res. 11, 84.

13. Koehler, D.R., Fndrova, H., Leung, K., Louca, E., Palmer, D., Ng, P., McKerlie, C., Cox, P., Coates, A.L., and Hu, J. (2005). Aerosol delivery of an enhanced helper-dependent adenovirus formulation to rabbit lung using an intratracheal catheter. J. Gene Med. 7, 1409–1420.

14. Cooney, A.L., Singh, B.K., Loza, L.M., Thornell, I.M., Hipper, C.E., Powers, L.S., Ostegaard, L.S., Meyerholz, D.K., Wohlford-Lenane, C., Stoltz, D.A., et al. (2018). Widespread airway distribution and short-term phenotypic correction of cystic fibrosis pigs following aerosol delivery of piggyBac/adenovirus. Nucleic Acids Res. 46, 9591–9600.

15. Wang, G., Zabner, J., Deering, C., Launsbach, J., Shao, J., Bodner, M., Jolly, D.J., Davidson, B.L., and McCray, P.B., Jr. (2000). Increasing epithelial junction permeability enhances gene transfer to airway epithelia In vivo. Am. J. Respir. Cell Mol. Biol. 22, 129–138.

16. Shum, W.W., Da Silva, N., McKee, M., Smith, P.J., Brown, D., and Breton, S. (2008). Transpositional projections from basal cells are luminal sensors in pseudostratified epithelia. Cell 135, 1108–1117.

17. Boers, J.E., Amberg, A.W., and Thunnissen, F.B. (1998). Number and proliferation of basal and parabasal cells in normal human airway epithelium. Am. J. Respir. Crit. Care Med. 157, 2000–2006.

18. Giangreco, A., Reynolds, S.D., and Stripp, B.R. (2002). Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. Am. J. Pathol. 161, 173–182.

19. Cooney, A.L., Abou Aliawi, M.H., Shah, V.S., Bouzek, D.C., Struk, M.R., Powers, L.S., Gansemaker, N.D., Meyerholz, D.K., Welsh, M.J., Stoltz, D.A., et al. (2016). Lentiviral-mediated phenotypic correction of cystic fibrosis pigs. JCI Insight 1, 88370.

20. Wang, G., Slepushkin, V., Zabner, J., Keshavey, S., Johnston, J.C., Sauter, S.L., Jolly, D.J., Dubensky, T.W., Jr., Davidson, B.L., and McCray, P.B., Jr. (1999). Feline immunodeficiency virus vectors persistently transduce nondividing airway epithelia and correct the cystic fibrosis defect. J. Clin. Invest. 104, R55–R62.

21. Farmen, S.L., Karp, P.H., Ng, P., Palmer, D.I., Koehler, D.R., Hu, J., Beaudet, A.L., Zabner, J., and Welsh, M.J. (2005). Gene transfer of CFTR to airway epithelia: levels of expression are sufficient to correct Cl− transport and overexpression can generate basalotateral CFTR. Am. J. Physiol. Lung Cell. Mol. Physiol. 289 L1123–L1130.

22. Montoro, D.T., Haber, A.L., Biton, M., Vinarsky, V., Lin, B., Birken, S.R., Yuan, F., Chen, S., Leung, H.M., Villoria, I., et al. (2018). A revised airway epithelial hierarchy includes CFTR-expressing ionocytes. Nature 560, 319–324.

23. Levardon, H., Yonker, L.M., Hurley, B.P., and Mou, H. (2018). Expansion of Airway Basal Cells and Generation of Polarized Epithelium. Bio Protoc. 8, e2877.

24. Hogan, B.L., Barksaaukas, C.E., Chapman, H.A., Epstein, J.A., Jain, R., Hsia, C.C., Niklog, L., Calle, E., Le, A., Randell, S.H., et al. (2014). Repair and regeneration of the respiratory system: complexity, plasticity, and mechanisms of lung stem cell function. Cell Stem Cell 15, 123–138.

25. Sinn, P.L., Coffin, J.E., Ayithan, N., Holt, K.H., and Maury, W. (2017). Lentiviral Vectors Pseudotyped with Feline Glycoproteins. Methods Mol. Biol. 1628, 65–78.

26. Breda, L., Cau, C., Gardenghi, S., Bianchi, N., Cartegni, L., Narla, M., Yazdanbakhsh, K., Musso, M., Manwani, D., Little, J., et al. (2012). Therapeutic hemoglobin levels after gene transfer in β-thalassemia mice and in hematopoietic cells of β-thalassemia and sickle cells disease patients. PLoS ONE 7, e32345.

27. Wang, Y., Bergelson, S., and Feschenko, M. (2018). Determination of Lentiviral Infectious Titers by a Novel Droplet Digital PCR Method. Hum. Gene Ther. Methods 29, 96–103.

28. Pratt, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., 3rd, Hao, Y., Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive Integration of Single-Cell Data. Cell 177, 1888–1902.e21.

29. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.