A functional genomics approach to investigate the differentiation of iPSCs into lung epithelium at air-liquid interface

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Abstract
The availability of robust protocols to differentiate induced pluripotent stem cells (iPSCs) into many human cell lineages has transformed research into the origins of human disease. The efficacy of differentiating iPSCs into specific cellular models is influenced by many factors including both intrinsic and extrinsic features. Among the most challenging models is the generation of human bronchial epithelium at air-liquid interface (HBE-ALI), which is the gold standard for many studies of respiratory diseases including cystic fibrosis. Here, we perform open chromatin mapping by ATAC-seq and transcriptomics by RNA-seq in parallel, to define the functional genomics of key stages of the iPSC to HBE-ALI differentiation. Within open chromatin peaks, the overrepresented motifs include the architectural protein CTCF at all stages, while motifs for the FOXA pioneer and GATA factor families are seen more often at early stages, and those regulating key airway epithelial functions, such as EHF, are limited to later stages. The RNA-seq data illustrate dynamic pathways during the iPSC to HBE-ALI differentiation, and also the marked functional divergence of different iPSC lines at the ALI stages of differentiation. Moreover, a comparison of iPSC-derived and lung donor-derived HBE-ALI cultures reveals substantial differences between these models.

KEYWORDS
functional genomics, iPSC to HBE-ALI, open chromatin, transcriptional network, transcriptome

1 | INTRODUCTION
The technological advances that initiated the field of induced pluripotent stem cell (iPSC) biology1,2 provided a new toolbox for examining the establishment of cell identity and the organization of tissues. The availability of stem cells, which upon activation with defined stimuli can produce one of many different human tissue types, has facilitated many aspects of medical research (reviewed
in 3). Here, we focus on the epithelium of the human airway that is intimately involved in the aetiology of many common respiratory diseases including chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis. Among the best resources for investigating human bronchial (HBE) and tracheal (HTE) epithelial cells are primary cultures derived from lung tissue and grown on plastic or differentiated on permeable supports.6-8 However, these are always in limited supply as they are dependent on the availability of suitable donor organs and tissue. More recently, robust protocols were developed to differentiate iPSCs into lung epithelial cells at air-liquid interface (ALI), thus modelling the HBE cells on permeable supports.7-14 Extensive characterization of the iPSC-to-ALI cell cultures has largely focused on their relevance to disease states (particularly cystic fibrosis 10,17) and also generated elegant data on aspects of stem cell biology and the detailed lineages of the cell types.18-24 However, complete maturation of iPSC-derived ALI cultures into functional HBE-ALI cells is often challenging. This obstacle may depend not only on the differentiation capacity of individual iPSC lines but also on the absence of appropriate biochemical and/or physical cues.25-27 Our goal was to develop a robust and unbiased functional genomics pipeline to benchmark the differentiation of iPSC lines from different sources cultured according to diverse protocols in individual laboratories. Here, we utilize genome-wide methods to examine the gene regulatory networks that drive the pathways of differentiation from iPSCs to ALI cultures and compare the model ALI cultures with HBE-ALI cells. By intersecting open chromatin data (generated by Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq))28 with the transcriptome (generated by RNA-seq) of defined stages of differentiation of the iPSC to ALI pathway, we reveal the dynamic changes in the transcriptional networks that drive these alterations. We also show the divergence in the molecular signatures of iPSC-derived ALI and lung donor-derived HBE-ALI cells, which may provide insights into the factors that are required to overcome the hurdles of complete maturation of the model ALI cells into mature HBE cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture

iPSC lines CR0000008 (Cell line ID: ND2.0), CR0000011 (Cell line ID: ND1.4) were purchased from RUCDR Infinite Biologics (https://www.nimhgenetics.org/stem_cells/crm_lines.php (www.rucdr.org)). CWRU205 was donated by Dr Paul J. Tesar (Dept. of Genetics and Genome Sciences, Case Western Reserve University). All three lines were from male donors. Differentiation of iPSC to ALI culture was based on the method by Wong et al.10 with minor modifications in coating of the membrane inserts (Corning 346012 mm Transwell inserts) for the differentiation protocol. After testing different coating methods described by others,10,11 we found Matrigel (Corning 35623, 1/60 dilution) with collagen IV (60 μg/mL) supported the most robust and consistent ALI cultures. The stages of differentiation were confirmed by 1) RT-qPCR assays specific for NKX2.1, FOXA2, SOX2, SOX9, SOX17, MUC5AC, MUC16, FOXJ1, TP63 and KRT5 transcripts, using primers shown in Table S1; and specific antibodies to CXCRR (CD184, BD Pharmigen 5559740), SOX17 (BD Pharmigen 562594), NKX2.1 (TTF1; Abcam ab76013), SOX2 (GeneTex GTX101507), FOXA2 (Abcam ab60721) for FACS at DE and AFE stages; and immunofluorescence for NKX2.1, FOXA2, SCGB1A1 (CC10, Santa Cruz Biotechnology (SCB) sc-365992) and MUC5B (SCB sc-21768) at AFE, LP3a and ALIw5 stages.

Donor-derived HBE cells were obtained from the Marsico Lung Institute CF Center Tissue Procurement and Cell Culture Core (University of North Carolina (UNC), Chapel Hill, NC) and cultured according to the published protocols5,6 in accordance with relevant guidelines. The cells were obtained under protocol #03-1396 approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board. All donors or their authorized representatives provided informed consent for research use of explanted lungs. This work was also approved by the Case Western Reserve University Institutional Review Board.

2.2 | RNA preparation, reverse transcription quantitative PCR (RT-qPCR) and RNA-seq

Total RNA was extracted from iPSCs at different stages of differentiation using TRizol (Invitrogen) using their protocol. RT-qPCR was done using our standard protocols29 with primers shown in Table S1. RNA-seq (SR 50bp) was performed as described previously.30

2.3 | ATAC-seq

ATAC-seq was performed on 50 000 cells as previously described28 with the following minor modifications. The initial cell pellet was washed 1-2 times in 100 μL cold PBS and centrifuged for 5 minutes at 500 x g at 4°C. The washed cell pellet was resuspended in 100 μL cold lysis buffer (100 mmol/L Tris-Cl, pH 7.4; 10 mmol/L NaCl; 3mol/L MgCl2; 0.1% (v/v) IGEPAL CA-630, freshly prepared), incubated on ice for 10 minutes and centrifuged for 10 minutes at 500 x g at 4°C. The nuclear pellet was washed once in 50 μL home-made TD buffer22 (20 mmol/L Tris(hydroxymethyl)ammonium methanethiol; 10 mmol/L MgCl2; 20% (v/v) dimethylformamide) and centrifuged for 5 minutes at 500 x g at 4°C, before proceeding with tagmentation using Nextera Tn5 Transposase and commercial 2X TD buffer (Illumina). Following preparation, the final ATAC-seq libraries were purified with Agencourt AMPure XP magnetic beads (Beckman-Coulter) at a sample to bead ratio of 1:1.2, following the manufacturer’s protocol, and eluted into 20 μL Buffer EB (Qiagen). ATAC-seq library size distributions were visualized by TapeStation (Agilent) and quantified using the KAPA Library Quantification Kit (Roche). Six to seven libraries were pooled, per lane, and sequenced on a HiSeq4000 (Illumina) using 100 bp paired-end sequencing.
2.4 | RNA-seq analysis

Raw reads were aligned with STAR 2.6 (https://github.com/alexbobin/STAR).32 Aligned reads were then assigned to genomic features with featureCounts version 1.6.3 in the Subread package (http://subread.sourceforge.net/)33 and differential gene expression was analysed using DESeq2 version 1.22.1 (https://www.bioconductor.org/packages/release/bioc/html/DESeq2.html).34

Raw reads of six untreated healthy donor-derived HBE-ALI samples were acquired from the GEO database Series GSE97036.35 Data sets were processed using the same pipeline. The cells used in this series were as described above for donor-derived HBE cells.

2.5 | Gene ontology analysis

Differentially expressed genes were filtered to enrich for those with a fold change ≥1.5 and Benjamini-Hochberg adjusted P-value ≤ 0.01. Gene lists were read into the PANTHER gene ontology database (http://pantherdb.org/)26 using the biological processes term. Results were further validated using gProfiler. Statistically significant results were filtered for categories passing a P-value of 0.001 with the Bonferroni correction for multiple testing.

2.6 | ATAC-seq data processing

Raw reads from the HiSeq4000 were processed using the ENCODE ATAC-seq pipeline (Dec 2018)(https://github.com/kundajelab/atac-seq-pipeline). Replicates were used to generate peaks with an IDR threshold of 0.5. Analysis of enriched motifs within the IDR peaks was performed with HOMER (4.7.2q) (http://homer.ucsd.edu/homer/index.html).37 PCA and heatmaps were generated by DiffBind (2.10.0) (http://bioconductor.org/packages/release/bioc/vignettes/DiffBind/inst/doc/DiffBind.pdf).38

FIGURE 1  Schematic to show the protocol used to differentiate iPSC into lung epithelial cells at air-liquid interface (ALI) and the experimental pipeline. A, Summary of protocol and timeline for iPSC to ALI differentiation. The same colour-coding is used throughout the figures to denote each stage of differentiation. The red * at day 6 denotes when cells are transitioned to permeable supports. B, Workflow of experimental procedures and bioinformatics analysis of data for each experiment.
2.7 | Binding and expression target analysis BETA

Differential gene expression for STAR aligned reads was analysed using the Cufflinks suite v2.2.1. (http://cole-trapnell-lab.github.io/cufflinks/). Cuffdiff output for pairwise comparisons was processed against ATAC-seq peak files for each stage in the pair independently using the BETA software version 1.0.7 (http://cistrome.org/BETA/) to determine peak distribution at differentially expressed genes (DEGs). Peaks were chosen within 20 kb from the gene body, and function prediction was determined by the rank product of the estimated regulatory potential of factor binding and change in expression of the gene target.

2.8 | Immunofluorescence

iPSC ALI cultures (week 5) on membrane filters were harvested and fixed in 4% paraformaldehyde (in PBS), embedded in paraffin and cut into 5-μm sections. After deparaffinization and rehydration, antigen retrieval was performed by heating with citric acid (10 mmol/L, pH 6.0) in a 98°C water bath for 45 minutes. The tissue sections were subsequently washed 3 times in blocking solution (1% BSA and 0.05% Saponin in PBS). The sections were incubated with the primary antibodies (diluted in PBS containing 0.1% BSA) overnight at 4°C. The sections were then washed 3 times in PBS containing 0.05% Tween 20, followed by incubation with secondary antibodies (diluted in PBS with 0.1% BSA) for 1 hour at room temperature. After 3 washes and counterstaining with 4,6-diamidino-2-phenylindole (DAPI), the samples were mounted with ProLong Gold Antifade Mountant (Invitrogen). The specimens were imaged on a fluorescence microscope (Leica DM 6000). Primary antibodies used were as follows: NKX2.1 (TTF1; Abcam ab76013), FOXA2 (Abcam ab60721) and CC10 (SCB sc-365992). Secondary antibodies used were as follows: Rhodamine Red™-X (RRX) AffiniPure Donkey Anti-Mouse IgG (H + L) (Jackson ImmunoResearch 715-295-151) and Alexa Fluor 488 (AF488) AffiniPure Donkey Anti-Rabbit IgG (H + L) (Jackson ImmunoResearch 711-545-152).

3 | RESULTS

Our experimental and analysis pipelines are summarized in Figure 1. In initial experiments, we differentiated three iPSC lines, ND2.0, ND1.4 and CWRU205, which came from 2 different sources. We followed the standard protocols as described in the methods section to culture the iPSCs and differentiate them into airway epithelial cells at ALI (Figure 1A). Cells were collected for ATAC-seq and RNA-seq at 6 time-points: definitive endoderm (DE), anterior foregut endoderm (AFE) and lung progenitor cells 3a, 3b (LP3a, LP3b), at air-liquid interface after weeks 3 and weeks 5 (ALIw3 and ALIw5). The ND1.4 iPSC line repeatedly failed to differentiate fully to the ALI stage (either through not adhering to membrane supports at the transition to AFE cells, or due to reduced cell proliferation starting at LP3a), so this line was not analysed further. Open chromatin and RNA-seq data were derived from cells in multiple differentiation processes of the ND2.0 and CWRU205 lines. However, data presented here are from one differentiation process where these 2 lines were grown in parallel using the same batches of culture media, supplements and culture inserts, to minimize variation that was not inherent to the cells. The RNA-seq and ATAC-seq pipeline are summarized in Figure 1B.

3.1 | Open chromatin mapping through differentiation from iPSC-derived definitive endoderm through to air-liquid interface lung epithelial cell cultures

We first used ATAC-seq to identify the regions of open/active chromatin at each developmental stage and combined data from the ND2.0 and CWRU205 lines to generate a reproducible peak set by irreproducible discovery rate (IDR) analysis. Principal component analysis (PCA) (Figure 2A) showed close concordance between the open chromatin profiles of the two donors at each time-point from DE to LP3b. In contrast, at the ALIw3 and ALIw5 stages the within donor similarities were greater than time-point–associated features. Of note, the open chromatin profiles of the DE stage were most divergent from all other stages of differentiation. We also examined the IDR peak distributions across the different genomic regions (intergenic, intronic, promoter [within 2 kb 5′ of the transcriptional start site] or other) and found these to be largely consistent between differentiation stages (Figure 2B). Next, we performed transcription factor (TF) motif enrichment analysis on the IDR peaks at each stage of differentiation using HOMER (Figure 2C, Figure S1). Focusing on known motifs, many factors are significantly enriched in peaks of open chromatin including binding sites for architectural proteins CCCTC-binding factor (CTCF)23 and Brother of the Regulator of Imprinted Sites (BORIS),24 also known as CCCTC-binding factor-like (CTCFL). CTCF is the most overrepresented motif at DE, AFE and ALIw3 stages (Figure 2C i, ii, iv). Consistent with its pivotal role in organizing higher order chromatin structure genome-wide, the CTCF motif is among the top 7 motifs at all stages of differentiation (Figure 2C, Figure S1). Among TFs with pioneer activity (opening chromatin to increase accessibility for other TFs) and also with

FIGURE 2 | Analysis of open chromatin through iPSC to ALI differentiation. A, Principal component analysis (PCA) plot showing relationships between open chromatin at different stages of differentiation from the ND2.0 (ND) and CWRU205 (CW) iPSC lines. Each developmental stage is colour coded and numbered according to the inset panel. B, Genomic distribution of open chromatin peaks at each stage of differentiation from an IDR analysis of the ND2.0 and CWRU205 iPSC lines. Peaks called as: intergenic, intronic, promoter (defined as 2 kb upstream of the transcription start site) or other. C, HOMER analysis showing overrepresented binding motifs for known transcription factors under open chromatin peaks at 4 developmental stages i) DE, ii) AFE, iii) LP3b, iv) ALIw3
3.2 | The transcriptome through differentiation from iPSC-derived definitive endoderm through to air-liquid interface lung epithelial cell cultures

To compare temporal changes in gene expression, RNA-seq was performed at each stage of differentiation in the ND2.0 and CWRU205 iPSC lines. Gene expression values were quantified using the featureCounts software in the Subread package (r3.1) and normalized for sequencing depth and DNA composition with DESeq2. The PCA plot in Figure 3A shows a good correlation between the samples from the two lines at each stage until the ALI cultures. Normalized count distributions for the two iPSC lines consistently clustered together across all principal components. Once the cells were exposed to ALI conditions, each line remained more similar to itself between 3 and 5 weeks at ALI than to the other line at the same time-point. To accommodate the variance between the two lines at the ALI stages, results for each time-point were used as biological replicates. Following adjustment for sequencing depth between samples, we identified 8940 differentially expressed genes (P < 0.005) at one or more stage in the full developmental pathway from DE to ALIw5. A heatmap of the expression profile matrix for the top 1000 differentially expressed genes (DEGs) revealed several of these trajectories including genes most up-regulated in the early stages or those that are ALI-specific (Figure 3B). Among the most significant DEGs, passing a P-value threshold of 0.005, in DE compared to all other stages are Cerberus 1, DAN family BMP agonist (CER1), a cytokine that binds directly to bone morphogenetic proteins and inhibits their activity and Left-Right Determination Factor 1 (LEFTY1), which encodes a transforming growth factor-beta (TGFβ) family member (Table 1). LEFTY1 regulates LEFTY2 and NODAL and is required for left-right axis formation during development.59

Expression of both CER1 and LEFTY1 at later stages of differentiation has a log2 fold difference of ~1.93 compared to DE. In contrast, among the most significant DEGs in the combined ALIw3 and ALIw5 samples compared to other stages of differentiation are the two subunits of sodium/potassium Na+/K+ ATPase (ATP1A1 and ATP1B1), which together are essential for the active transport of Na+ and K+ across cell membranes (Table 2). Also more highly expressed in ALI cultures is the long non-coding RNA Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1) and two members of the Serpin family of protease inhibitors SERPINA1 and SERPINA3 (Table 2), which have a log2 fold change of 1.87, 7.57 and 9.08, respectively, compared to the earlier stages. A key target of SERPINA1 (alpha 1 antitrypsin) is elastase, and defects in the protein are associated emphysema.50

To confirm the differentiation protocol was as expected based on previous publications,9,10 we examined the expression of a number of known markers by both RT-qPCR and RNA-seq, including the key TFs SOX17 and FOXJ1. As expected, SOX17, an important marker of endoderm differentiation,9,10 is >3-fold higher in DE cells compared to AFE, and levels decline further as cells enter lung progenitor stages (Figure 3Ci). Expression of FOXJ1, which marks ciliated epithelial cells in the proximal airway,65 is maintained through ALIw5 (Figure 3Ci). Other markers for airway epithelial cells that are expressed through ALIw5 include the cell surface-associated mucin MUC4, the gel-forming mucin MUC5B, and the basal cell progenitor marker tumour protein p63 (TP63) (see GEO GSE136859, CompletePath_Counts.csv). Also shown in Figure 3D are immunofluorescence images for the lung and thyroid lineage marker NK2 homeobox 1 (NKK2.1), the FOXA2 transcription factor and the club cell marker secretoglobin family 1A member 1 (SCGB1A1/CC10) in iPSC ND2.0-derived ALIw5 cells. These
data suggest that the cells are undergoing successful differentiation to the multiple cell types found within the proximal airway epithelium, though do not exclude the persistence of sub-populations of non-lung fated cells.

Next, to identify key biological processes that alter during the course of differentiation from DE to ALIw5 we performed clustering with an infinite Gaussian process mixture model. Data in Figure 4A show the GO biological process enrichment analysis with time in the ND2.0 iPSC line. Only 20 of the 29 clusters of genes with terms passing statistical significance (P-value < 0.05) across a wide spectrum of biological functions are shown. Among processes of particular relevance to the lung epithelium are 'epithelial tube branching involved in lung morphogenesis' and several involved in immune and antimicrobial responses. Also of note are examples of the different gene expression profiles shown by individual gene clusters (Figure 4B and Figure S2). Cluster 2 genes show a peak of expression between AFE and LP3 with no transcripts at the start and end of the differentiation pipeline. Cluster 5 genes show no expression at the early stages of differentiation with a gradual increase through AFE and the LP stages, while reaching a plateau at ALI.

### Expression of genes involved in key pathways of lung development shows substantial changes between stages of differentiation

To further delineate the biological functions that are significantly altered across individual stages of differentiation, we filtered the DEG data sets for each linear pairwise RNA-seq comparison. Here, we focused on transitions between DE to AFE (Figure 5A) and LP3b to ALIw3 (Figure 5B) due to their relevance to early lung tissue development and airway epithelial cell identity, respectively. DEGs passing a 0.01 adjusted P-value threshold were stratified by fold change of ±2 and then analysed using the PANTHER database (r5.1) and gProfiler (r5.2) to identify biological process enrichment gene ontology terms.

In the transition from DE to AFE, 633 genes were down-regulated (Figure 5Ai) and 1107 genes were up-regulated (Figure 5Aii), illustrating the occurrence of both substantial repression and activation of genes during this key developmental modification. A
significant enrichment of genes associated with general pathways of cell and organ development was seen in DE cells (Figure 5Ai, in blue and Table S3). Among the DE-specific DEGs are the NANOG homeobox gene (\textit{NANOG}) and the Nodal Growth Differentiation Factor (\textit{NODAL}), which both have pivotal roles in early development. Also overexpressed in DE cells are fibroblast growth factor 8 (\textit{FGF8}), which has broad ranging functions in regulating embryonic development and cell proliferation, migration and differentiation, and Ripply 1 transcriptional repressor 1 (\textit{RIPPLY1}), which has a critical role in somitogenesis. In contrast, genes more highly expressed in AFE cells are involved in anatomical structure development and, more specifically, tube development (Figure 5Aii, in pink and Table S4). Notably, a profound enrichment of genes involved in epithelial processes such extracellular matrix production and cell adhesion was evident in AFE-specific DEGs. These include Transforming Growth Factor Beta 1 and 2 (\textit{TGFB1}, \textit{TGFB2}) and the Laminin Subunits Alpha 1, Alpha 2, Gamma 1 and Gamma 2 (\textit{LAMA1}, \textit{LAMA2}, \textit{LAMC1}, \textit{LAMC2}). These findings are consistent with the specialization of anterior foregut endoderm and loss of stem cell pluripotency.

Fewer genes showed significant alterations in expression levels between LP3b and ALIw3 (Figure 5B) where 344 are down-regulated (Figure 5Bi) and 445 up-regulated (Figure 5Bii). Genes up-regulated in the transition from LP3b to ALIw3 were enriched for many processes integral to epithelial cell function (Figure 5Bii, in green and Table S5). These include defence responses, including innate immunity, inflammatory response and several metabolic pathways. Among the ALIw3-specific genes contributing to these processes are multiple cytokines such as C-C motif Chemokines ligand 2 and 7 (\textit{CCL2} and \textit{CCL7}), interleukin 1 beta (\textit{IL1B}), interleukin receptor like 1 (\textit{IL1RL1}) and interleukin 1 receptor agonist (\textit{IL1RN}), which inhibits \textit{IL1B} activity, and the antimicrobial peptide defensin beta 4A (\textit{DEFB4A}). Similarly, multiple membrane-associated mucins including \textit{MUC1}, \textit{MUC3A}, \textit{MUC13}, \textit{MUC17} and \textit{MUC21} are significantly up-regulated between LP3b and ALIw3. Genes involved in these pathways are essential for the proper airway epithelial integrity and response to the environment. Many of the pathways up-regulated in the differentiated ALI cultures are impaired by lung disease.

### 3.4 Intersecting open chromatin with differential gene expression through differentiation from DE to ALI cultures

Next, we investigated the correlation between open chromatin within 20 kb of gene bodies and differential gene expression from

| Gene       | log2 (FoldChange) | log2 (FC) std. error | Wald statistic | P-value  | P.adjusted |
|------------|-------------------|-----------------------|----------------|----------|------------|
| ATP1A1     | 2.80              | 0.36                  | 7.84           | 4.35E-15 | 8.99E-14   |
| MALAT1     | 1.87              | 0.40                  | 4.70           | 2.58E-06 | 1.56E-05   |
| COL1A2     | 4.05              | 0.75                  | 5.39           | 7.14E-08 | 5.64E-07   |
| SERPINA1   | 7.57              | 0.55                  | 13.66          | 1.67E-42 | 3.01E-40   |
| ITGB1      | 1.95              | 0.36                  | 5.36           | 8.29E-08 | 6.50E-07   |
| CEACAM6    | 8.09              | 0.95                  | 8.54           | 1.38E-17 | 3.72E-16   |
| B2M        | 4.12              | 0.56                  | 7.36           | 1.77E-13 | 3.03E-12   |
| H19        | 2.65              | 0.82                  | 3.23           | 1.26E-03 | 4.13E-03   |
| SPP1       | 7.52              | 0.75                  | 10.02          | 1.23E-23 | 5.72E-22   |
| SERPINA3   | 9.08              | 0.96                  | 9.43           | 4.19E-21 | 1.55E-19   |
| CDH1       | 2.86              | 0.32                  | 8.88           | 6.58E-19 | 2.03E-17   |
| NEAT1      | 1.81              | 0.44                  | 4.07           | 4.60E-05 | 2.14E-04   |
| KRT8       | 1.35              | 0.26                  | 5.17           | 2.32E-07 | 1.69E-06   |
| CFTR       | 4.44              | 0.38                  | 11.55          | 7.62E-31 | 6.46E-29   |
| C3         | 7.81              | 0.95                  | 8.22           | 2.03E-16 | 4.89E-15   |
| ATP1B1     | 3.29              | 0.37                  | 8.79           | 1.51E-18 | 4.49E-17   |
| TNFAIP2    | 6.63              | 0.55                  | 12.10          | 9.95E-34 | 1.04E-31   |
| SPTBN1     | 1.96              | 0.28                  | 6.94           | 3.99E-12 | 5.67E-11   |
| ALDH1A3    | 4.83              | 0.57                  | 8.44           | 3.22E-17 | 8.35E-16   |

aDEseq2 normalized counts were filtered for those genes with a standard deviation of \( \leq 1000 \) for all ALI samples (ND2.0 ALIw3, ND2.0 ALIw5, CWRU205 ALIw3 and CWRU205 ALIw5). Using the filtered list, genes are presented in order of high to low by subtracting the average DEseq2 normalized counts from all other stages from the average DEseq2 normalized counts for ALI samples.

bAdjusted P-value is calculated with the Benjamini-Hochberg procedure.
Gene ontology (GO) biological process enrichment analysis identifies key cellular, developmental and immune pathways. A. Dot plot of GO biological process terms enriched in gene expression clusters identified in the differentiation of ND2.0. Clustering was performed using an infinite Gaussian process mixture model. Only trajectories with statistically significant enrichment for a biological process category (20/29) are shown. B. Gene expression trajectories from iPSC to ALIw5 for Clusters 2, 5 and 17. The expression profile for individual genes across the developmental pathway (Table S2) is stratified into cluster models with the aggregate mean and standard deviation plotted for each.
**FIGURE 5** Pairwise transition gene ontology process enrichment analysis identifies key developmental and innate immune response processes. Volcano plot of weighted fold change as a function of \(-\log_{10}\) adjusted \(P\)-values for genes during the transitions from A) DE to AFE and B) LP3b to ALIw3. DEGs passing a \(-\log_{10}\) adjusted \(P\)-value of 2 and log₂ fold change of 2 or −2 determined to be stage specific were processed using gene ontology process enrichment analysis. Biological process terms were chosen by passing a \(-\log_{10}\) adjusted \(P\)-value with Bonferroni correction of 4 (Ai), 20 (Aii), 3 (Bi) or 6 (Bii). Notable categories are highlighted in colour for each analysis.
FIGURE 6  Differential gene expression correlates with presence of open chromatin. A, Distributions of stage-specific DEGs correlated with ATAC-seq peaks unique to either stage. Genes passing a 0.01 adjusted P-value threshold with a peak within 20 kb of the gene body were binned by fold change for each linear pairwise comparison. Comparisons shown are i) DE to AFE, ii) AFE to LP3a, iii) LP3a to LP3b, iv) LP3b to ALIw3. B, RNA-seq DEseq normalized counts for TFs with stage-specific overrepresented motifs (by HOMER) in ATAC peaks i) DE-specific TFs, CRX, EOMES and OTX2; ii) LP3b-ALIw3/5 epithelial selective transcription factors, ETS1, KLF5 and EHF. C, Integrated Genomics Viewer (IGV) browser view of ATAC-seq tracks and RNA-seq read alignments at i) EOMES and ii) EHF loci comparing DE and AFE, or LP3b and ALIw3 stages, respectively. Arrows indicate stage-dependent changes in chromatin accessibility at gene promoters.
the RNA-seq data. DEG results for each linear transition through the differentiation were combined with annotation data for ATAC-seq peaks specific to each gene. DEGs with at least one peak passing the IDR threshold for each stage were binned by log2 fold change in the pairwise RNA-seq comparison (Figure 6A). While the overlap analysis shows consistent gene activation and repression around regions of open chromatin, substantially more genes are involved in the early DE to AFE transition (Figure 6Ai), somewhat lower but equivalent numbers are seen at the AFE to LP3a and LP3b to ALIw3 transitions (Figure 6Aii, iv), while many fewer are evident between LP3a and LP3b (Figure 6Aiii). These data are consistent with the major shift in cellular identity from DE to AFE and the more gradual changes through later differentiation and upon transition to ALI.

To reveal more about the TFs driving these changes in cellular identity and differentiation, we examined the expression profiles of specific TFs with important roles in early-stage or late-stage differentiation, for which binding motifs are enriched at peaks of open chromatin (Figure 6B). Among the top enriched motifs in DE cells, 12 are observed only in DE open chromatin (Figure 2C, Figure S1); moreover, 6 are also DE DEGs, including genes encoding the important differentiation factors EOMES and OTX2. Figure 6Bi shows the DEseq2 normalized counts at each state of differentiation for Cone-Rod Homeobox (CRX), EOMES and OTX2. While CRX expression is only seen in DE cells, EOMES and OTX2 show gradual reduction in activity through LP3b and AFE, respectively. Expression of KLF5, for which the binding motif is significantly enriched in peaks of open chromatin in AFE cells and all later stages, gradually increases through post-AFE differentiation, in line with its role in airway epithelial cell differentiation (Figure 6Bii). In contrast to the abundance of ETS1, which is constant through differentiation, expression of EHF, for which the binding motif is only enriched in ALIw3 and ALIw5 cells, reaches maximal expression levels in ALI cells. This is consistent with previous reports of the role of EHF on lung epithelial function and modifying cystic fibrosis lung disease severity.63-65 To highlight the concordance between the ATAC-seq signal and gene expression levels, we surveyed the open chromatin and RNA-seq profiles at the EOMES and EHF loci. Both genes show some positive correlation of ATAC-seq peak intensity and RNA-seq reads (Figure 6C). Most notable are alterations in ATAC-seq signal adjacent to the gene promoters that correspond to changes in gene expression level, evident from total aligned reads (Figure 6C). For example, loss of the ATAC-seq signal at the EOMES promoter in the transition of DE to AFE correlates with reduced aligned RNA-seq reads in AFE cells (Figure 6Ci). In contrast, gain of open chromatin at the EHF promoter correlates with increased aligned reads at the EHF locus in ALIw3 compared to LP3b (Figure 6Cii).

### 3.5 Comparison of iPSC-derived ALI open chromatin and transcriptome with donor-derived HBE-ALI cultures

A critical feature of the iPSC ALI cultures is how well they mirror the transcriptome and functions of ALI cultures generated from adult human bronchial epithelial cells derived from donor lung tissue. We observed significant differences in both open chromatin (Figure 7A) and gene expression profiles (Figure 7B) between the ALI cultures of different origin. The PCA plots of ATAC-seq peaks show that while biological replicates of iPSC and donor-derived ALI cultures cluster well by origin, they are far from each other, and there is more similarity between the two iPSC lines than the two donors (Figure 7A).

With respect to gene expression profiles (Figure 7B), the six replicates of donor-derived HBE-ALI cells cluster well. Though the 2 iPSC-derived lines are quite different from the donor-derived HBE-ALI cells there is only a 6% variance between the biological replicates. Technical replicates of each line also cluster well, reflecting robust and consistent culture protocols. Despite the divergence between iPSC- and donor-derived ALI cultures, the top 24 known TF binding motifs overrepresented in the IDR open chromatin peaks of HBE-ALI cells (Figure 7C) show some overlap with iPSC-derived ALI cells (Figure 2Civ and Figure S1C). Key factors regulating normal airway epithelial function including EHF, KLF5, and BTB domain and CNC homolog 2 (BACH2) are among these TFs in common. Unique to donor-derived ALI cells are overrepresented motifs for CCAAT enhancer binding protein (CEBP), BACH1 and E47-like ETS transcription factor 5 (ELFS). The matrix of differential gene expression (Figure 7D) also shows significant differences between the HBE-ALI cultures of different origins. A total of 3394 genes had a ≥2-fold higher expression in donor-derived HBE-ALI, and 3478 genes were more highly expressed in the iPSC-derived ALI cells. A gene ontology process enrichment analysis on these DEGs (Table S6) showed that processes related to cilia and microtubules were the most markedly up-regulated in donor-derived HBE cultures, consistent with failure of the iPSC-derived cells to generate a robust ciliated cell compartment. In contrast, iPSC-derived ALI cultures showed an increase in processes associated with cell surface components, extracellular matrix and cell adhesion. The DEG matrix also illustrates the high degree variability between the two iPSC-derived ALI cultures consistent with data shown in Figure 3A and B. We then examined the relationship between open chromatin profiles and gene expression levels for two genes, Filamin-binding LIM Protein 1 (FBLIM1), which is more highly expressed in iPSC-derived ALI cultures, and Sodium Channel Epithelial 1 Beta Subunit (SCNN1B) (encoding the beta subunit of ENaC), which is more abundant in donor-derived HBE-ALI cells (Figure 7E). Sites of open chromatin at each locus correlated well with differences in gene expression, with more pronounced accessibility at the promoters, introns and downstream elements in the cultures showing higher expression (Figure 7F). CD47 provided a negative control for this analysis, as neither the normalized counts from DESeq2 nor the ATAC-seq profiles (Figure 7E and Figure S5) vary between the cultures.

### 4 DISCUSSION

The goal of the experiments described here was to generate unbiased genome-wide data on open chromatin and gene expression
during the differentiation of iPSCs into lung epithelial cells at ALI, as this model is frequently used for the study of key events in lung disease such as CF. Though careful characterization of the expression of multiple specific marker genes along the differentiation pathway has been performed by several groups, these studies of individual transcripts or their encoded proteins are by definition limited in scope. The definition of key markers has generally relied on extensive data from other mammalian species, most notably the mouse, which is exceptionally well studied (reviewed in 6). However, the mouse lung has a number of important anatomical and functional differences from the human lung, suggesting that an unbiased review of gene expression profiles in the iPSC-to-ALI differentiation could be valuable. Hence, the genome-wide data reported here are important contributions to understanding the functional genomics of this differentiation pipeline. Several aspects of the differentiation warrant further discussion; although iPSC-derived differentiated cellular models are widely used in medical research, and have facilitated numerous important advances, some pathways of differentiation are more robust than others. Indeed, there are ongoing efforts to optimize the protocols for generating well-differentiated bronchial epithelial cells at ALI from iPSCs. At the time of these experiments, we followed one well-documented protocol of this differentiation pipeline (Figure 1) but are aware that more recent optimizations may enhance the outcome. Two key questions warrant further discussion: first, how robust is the differentiation pipeline in different iPSC lines and second, how similar are iPSC-derived HBE-ALI cultures to organ donor-derived HBE-ALI cells.

To address the first question, we investigated the reproducibility of differentiation in terms of variable efficiency and cell heterogeneity in three iPSC cell lines, from two sources. One line repeatedly failed to reproducibly differentiate to the ALI stage and was excluded for further experimentation and analysis. While the other two lines reproducibly differentiated to ALI cultures, one line apparently differentiated much more efficiently than the other. This phenomenon is well illustrated by the divergence between the ALIw3 and ALIw5 transcription profiles of the two donor lines. After 5 weeks of ALI culture, each line was more similar to itself after 3 weeks than to the other culture at 5 weeks. Though ND2.0 ALI cells expressed many markers for proximal lung progenitors (TP63 and KRT5 (basal cells), FOXJ1 (ciliated cells), ARG2 (goblet cells)), others were expressed at lower levels compared to CWRU205 ALI cells. The latter appeared to provide a more robust model airway epithelium at ALI including expressing markers for secretory cells (SCGB3A2) and goblet cells (MUC5AC), in addition to the cell types seen in ND2.0 ALI cultures. In the context of the divergent differentiation of the CWRU205 and ND2.0 cultures, it is relevant that differentiation of definitive endoderm into airway cells is often associated with the production of non-lung endoderm-derived cell types. These include predominately hepatic-like cells in iPSC-derived proximal airway, and gastric-like cells in iPSC-derived distal airway and other endoderm-derived tissue lineages such as pancreas. The differentiated ALI cultures we generated from iPSC cells similarly expressed some markers for non-lung endoderm cells. These include the hepatic markers fibrinogen beta chain (FGB), apolipoprotein A2 (APOA2) and hepatocyte nuclear factor 4A (HNF4A) and to a lesser extent, pancreas as shown by pancreatic and duodenal homeobox (PDX1) expression. The different abundance of non-lung cell types likely contributes to the deviation of expression and open chromatin profiles between the two iPSC-derived ALI cultures.

Directly addressing the second question, there are clear differences between open chromatin and expression profiles of human donor-derived HBE-ALI and iPSC derived from iPSCs (Figure 7). Many transcription factors that are expressed in murine lung epithelial cells, and play a role in their differentiation, are highly expressed in both groups of ALI cells in addition to enrichment of their binding motifs in both cell types (Figure 2Civ, Figure 5C, Figure 7C). These TFs include JUN, KLF5 and EHF. However, the binding motifs of other TFs with an important role in the lung epithelium are not enriched in open chromatin from iPSC-derived ALI, despite being similarly expressed in iPSC- and donor-derived ALI cultures. These factors include members of the CEBP family, (formerly NRF2) and ELF5. The fact that these TFs are present in the cells but are apparently not being selectively recruited to cis-regulatory elements (open chromatin peaks) suggests that critical co-factors required for DNA-binding might be absent or mis-expressed. Alternatively, these TFs may not be appropriately activated by external stimuli in the iPSC-derived ALI cultures, including from other cell types, and so cannot bind DNA. It is also likely that some of the cell types in the donor-derived HBE-ALI cultures that contribute to the motif enrichment signal are either absent or at lower abundance in the iPSC-derived ALI cells.

In conclusion, we provide a detailed molecular characterization of the gene expression repertoire and the activating and repressive TFs that direct the differentiation of iPSCs into lung epithelial cells at ALI. Our data show that this model to study lung epithelial
function in health and disease has many advantages, according to the protocols we used it currently falls short of producing a fully differentiated functional airway epithelium. This may reflect contamination with non-lung fated cells together with a lack of appropriate environmental cues, which may be biochemical (such as appropriate growth factors) or physical (lack of suitable matrix or substrate and stretch forces), which could result in failure to produce rare but critical cell types. Further higher resolution molecular analysis by single cell RNA-seq and single cell ATAC-seq may reveal the absent components. The application of our analysis pipeline to other protocols for the differentiation of iPSC into lung epithelial cells, would likely be highly informative.

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CONFLICTS OF INTEREST
The authors confirm that there are no conflicts of interest. The funders had no role in the design of the study, in the collection, analyses or interpretation of the data, in the writing of the manuscript, or in the decision to publish the results.

AUTHOR CONTRIBUTION
Jenny L Kerschner: Conceptualization (supporting); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing-original draft (supporting); Writing-review & editing (supporting). Alekh Paranjapye: Conceptualization (supporting); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing-original draft (supporting); Writing-review & editing (supporting). Shiyi Yin: Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (equal). Gurkan Bebek: Data curation (supporting); Formal analysis (equal); Investigation (supporting); Methodology (equal). Danielle L Skander: Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (supporting). Ann Harris: Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Investigation (supporting); Methodology (supporting); Project administration (lead); Writing-original draft (lead); Writing-review & editing (lead).

DATA AVAILABILITY STATEMENT
Genome-wide data are deposited at GEO:GSE136859.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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