Prospective isolation of NKX2-1–expressing human lung progenitors derived from pluripotent stem cells

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It has been postulated that during human fetal development, all cells of the lung epithelium derive from embryonic, endodermal, NK2 homeobox 1–expressing (NKX2-1+) precursor cells. However, this hypothesis has not been formally tested owing to an inability to purify or track these progenitors for detailed characterization. Here we have engineered and developmentally differentiated NKX2-1GFP reporter pluripotent stem cells (PSCs) in vitro to generate and isolate human primordial lung progenitors that express NKX2-1 but are initially devoid of differentiated lung lineage markers. After sorting to purity, these primordial lung progenitors exhibited lung epithelial maturation. In the absence of mesenchymal coculture support, this NKX2-1+ population was able to generate epithelial-only spheroids in defined 3D cultures. Alternatively, when recombined with fetal mouse lung mesenchyme, the cells recapitulated epithelial-mesenchymal developing lung interactions. We imaged these progenitors in real time and performed time-series global transcriptomic profiling and single-cell RNA sequencing as they moved through the earliest moments of lung lineage specification. The profiles indicated that evolutionarily conserved, stage-dependent gene signatures of early lung development are expressed in primordial human lung progenitors and revealed a CD47+CD26+ cell surface phenotype that allows their prospective isolation from untargeted, patient-specific PSCs for further in vitro differentiation and future applications in regenerative medicine.

Introduction

Little is known about the early stages of human lung development, preventing an understanding of whether successful healing from adult lung injury involves recapitulation of embryonic mechanisms and limiting approaches for generating lung progenitors from pluripotent cells in vitro. Inbred mouse models have begun to define the mechanisms regulating lung specification and patterning, but how this breadth of work applies to human lung development is unknown. Current claims suggest that all cells of the postnatal mammalian lung epithelium derive from embryonic NKX2-1+ progenitors; however, scant literature exists formally testing this hypothesis either in mice or humans. Support for this paradigm derives mainly from the observation that Nkx2-1 is the first gene locus known to be activated in cells of the endodermal lung primordium (1, 2). NKX2-1–null mutant mice have hypoplastic lungs that fail to mature, and human children with NKX2-1 mutations develop respiratory insufficiency, hypothyroidism, and neurological impairment (3), but these observations do not necessarily indicate that all lung epithelial cells derive via NKX2-1+ progenitor intermediates. Since lung lineage specification is thought to occur in relatively few endodermal cells during a narrow developmental time period in vivo, it has been difficult to gain access to these cells in human embryos or to follow their cell fate decisions in real time. Hence, we sought to interrogate the earliest moments of human lung lineage specification by engineering an in vitro system that would allow the isolation and differentiation of pure populations of NKX2-1+ putative human lung progenitors. Given the known capacity of mouse pluripotent stem cells (PSCs) to form all cell types, including lung lineages, after transfer into mouse blastocyst embryos and the known broad differentiation repertoire of human PSCs in vitro, we based this system on the in vitro differentiation of PSCs.

Initial published attempts at deriving lung epithelium from PSCs relied on the presence of drug-resistance genes or used incompletely defined media (4–6), resulting in inefficient induction of selected lung markers. Subsequently, a number of groups, including our own, had more success by broadly attempting to recapitulate the key milestones of embryonic lung development in vitro through the exogenous addition of sequential combina-

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Figure 1. Purification of human NKX2-1+ lineages derived from ESCs/iPSCs using NKX2-1GFP reporters. (A) Gene editing strategy based on TALENs or CRISPR technology to target a GFP reporter to the human NKX2-1 locus to engineer NKX2-1GFP iPSC/ESC lines. See also Supplemental Figure 1. (B) Schematic overview of in vitro directed differentiation of ESCs/iPSCs into NKX2-1+ lineages: endodermal lung or thyroid epithelia versus ectodermal forebrain. (C) Representative timeline of GFP expression measured by flow cytometry during lung-directed differentiation (C17). (D) Immunostaining of day 15 lung-directed differentiation for NKX2-1, GFP (anti-GFP), and EPCAM. Scale bar (left panels): 100 μm. Right panel is a zoom in (white dashed-line box). Scale bar: 25 μm (C17). (E) Phase contrast and fluorescence microscopy of C17 iPSC-derived cells generated in the lung (day 15), thyroid (day 27), and forebrain (day 10) protocols. See also Supplemental Figure 2. (F) Flow cytometric analysis of lung (day 15), thyroid (day 17), and forebrain (day 14) protocols (C17). (G) Fold change, compared with day 0, of mRNA expression of sorted NKX2-1GFP- and NKX2-1GFP+ cells from those time points by RT-qPCR; quantified as 2ΔΔCt, n = 3 (C17).

Results

An NKX2-1GFP reporter enables purification of human lung, thyroid, and forebrain lineages. In order to generate a tool for the identification and purification of candidate human lung progenitors, we used gene editing technologies to target an enhanced green fluorescence reporter (GFP) gene to the endogenous human NKX2-1 locus in multiple human PSC lines. Prior reports of targeting GFP to the NKX2-1 locus in human PSCs for the derivation of forebrain lineages resulted in NKX2-1 haploinsufficiency (17). However, pursuing a strategy designed to retain intact expression of targeted loci without haploinsufficiency (Figure 1A and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI89950DS1), we targeted an exon3-2A-GFP cassette to the second intron of NKX2-1 using either transcription activator-like effector nucleases (TALENs) or CRISPR-Cas9 tools deployed in ESCs (H9) or in our previously published iPSC lines: cystic fibrosis patient-specific C17 iPSCs (18) and normal BU3 iPSCs (Figure 1 and Supplemental Figure 1). The resulting NKX2-1GFP reporter PSC clones (hereafter H9NKX2-1GFP, C17NKX2-1GFP, and BU3NKX2-1GFP) demonstrated successful mono- and bi-allelic integration of the donor template by PCR (Supplemental Figure 1B). For further profiling we selected 1 homozygous targeted clone for each of the TALENs-targeted lines (H9 and C17) as well as 1 homozygous CRISPR-Cas9–targeted BU3 clone (Supplemental Figure 1, B and C).

To differentiate each targeted PSC line, we tested protocols previously developed by us and others for the in vitro directed differentiation of human PSCs into the 3 lineages known to express NKX2-1: neural/forebrain (20, 21), lung (7–9), or thyroid (19) (Figure 1B). Protocols for lung and thyroid both required generating anterior foregut–like endoderm followed by the addition of Chir99021 (CHIR), BMP4, KGF, FGF10, and Rara for lung versus BMP4 and FGF2 for thyroid. Consistent with prior publications (9), the percentage of NKX2-1+ cells on day 15 of the lung-directed differentiation was typically 41% ± 21% (mean ± SD) when using the ESC line RUES2 (data not shown). However, when other cells lines including H9 and C17 were differentiated the percentage of NKX2-1+ expressing cells was initially less than 1% (data not shown). Thus, we developed a methodology for the optimization of lung differentiation for each cell line by altering the duration of endoderm induction, the cell density of replated endoderm, and the duration of TGF-β1/BMP inhibition in order to augment the percentage of NKX2-1+ cells emerging by day 15 (Supplemental Figure 1H and Supplemental Figure 2, A and B).

Employing the new lung differentiation protocol optimized for each clone, GFP expression was first detected between days 8 and 10 of differentiation and the percentage of GFP+ cells peaked between days 12 and 16, with an efficiency of 25.6% ± 9.5% for C17NKX2-1GFP (Figure 1, C and F), 29.5% ± 4.4% for BU3NKX2-1GFP, and 19.8%
A
Sorted NKX2-1+ progenitors form lung epithelial organoids

Day 6  Day 15  Matrigel  Day 36
CHIR, BMP4, FGF10, KGF, Ra  CHIR, FGF10, KGF  +DCI

GFP+ outgrowth
NKX2-1GFP-
NKX2-1GFP+

Phase contrast  Fluorescence

NKX2-1
EPCAM
DAPI

B
Day 19 time-lapse

C
Comparison of NKX2-1GFP+ vs. NKX2-1GFP- outgrowth organoids

Sorted on Day 15
FACS analysis on Day 36

% NKX2-1-GFP-

100

GFP outgrowth  GFP outgrowth

D
Day 36 GFP+ outgrowth

MUC1
SFTPBP

DAPI
NKX2-1

MUC1/DAPI
SFTPBP/DAPI

TP63
NKX2-1/TP63
Figure 2. Sorted iPSC-derived NKX2-1
GFP cells exhibit lung progenitor potential and ability to form epithelial spheroids in 3D culture. (A) Schematic overview of organoid generation with representative phase contrast and GFP fluorescence microscopy images (day 25–28) as well as immunostaining (day 36) for NKX2-1 and EPCAM proteins. Cell nuclei are counterstained with DAPI. Each panel shows outgrowth in 3D culture of structures arising from iPSC-derived cells that were either unsorted or sorted on day 15 as GFP+ versus GFP- populations (C17). Scale bars: 100 μm (left and center columns) and 20 μm (right column). (B) Time-lapse microscopy (merged GFP fluorescence and phase contrast) of unsorted organoids over 25 hours. Arrows indicate epithelial organoids undergoing induction of the GFP reporter in real time (C17). See also Supplemental Video file. (C) FACS quantification on day 36 of the percentage of cells expressing GFP in the outgrowth wells shown in A (C17). Data indicate individual biological replicates (squares and triangles) with mean ± SD, n = 6 biological replicates. ***P ≤ 0.001 by Student’s t test. Fold change [RT-qPCR; 2(−ΔΔCt)] in mRNA expression on day 36 compared with day 0 for each GFP+ versus GFP- outgrowth compared with fetal lung control tissue (n = 3 biological replicates) (C17). (D) Immunostaining of GFP+ outgrowth organoids on day 36 for MUC1, SFTPB, and TP63 (C17). Nuclei are counterstained with DAPI. Scale bars: 10 μm.

Employing our human lung–directed differentiation protocol and the C17NKX2-1-GFP iPSC line, we tested whether differentiated lung epithelial cells derive directly from NKX2-1 progenitors. We sorted NKX2-1-GFP versus NKX2-1-GFP cells on day 15 and plated each population (vs. unsorted controls) in 3D Matrigel in serum-free media supplemented with factors we and others have previously shown to support lung epithelial differentiation (8, 9); CHIR, KGF, and FGFI10 for 7 days followed by the addition of dexamethasone, cAMP, and IBMX until day 36 (Figure 2A). We observed the outgrowth of proliferating cell aggregates over the next 2 to 3 weeks (hereafter referred to as organoids) (Figure 2A). Unsorted day 15 cells plated as clumps gave rise to lobular organoids with GFP+ and GFP- areas on day 36 (Figure 2A), and GFP+ cells could be followed in real time in these unsorted cultures by time-lapse photography (Figure 2B and Supplemental Video 1). In contrast, when unsorted cells were plated as single-cell suspensions, simpler spherical organoids formed (data not shown). Immunostaining of the unsorted organoids demonstrated areas of monolayered NKX2-1/EPCAM+ epithelium surrounding an inner lumen but also areas and organoids that were NKX2-1- (Figure 2A). Sorted day 15 GFP+ cells gave rise to GFP+ aggregates in 3D culture, with 50.1% ± 17.6% (mean ± SD; n = 6 runs) of the progeny remaining GFP+ by flow cytometry on day 36 (GFP+ outgrowth) (Figure 2C). Sorted day 15 GFP+ cells remained GFP+ on day 36 (99.8% ± 0.2%) (GFP+ outgrowth) (Figure 2C). The GFP+ outgrowth formed predominantly NKX2-1-EPCAM+ spheroids, whereas the GFP-outgrowth formed EPCAM+ and EPCAM- organoids that were uniformly NKX2-1- (Figure 2A). The sorted NKX2-1-GFP progenitors on day 15 comprised the entirety of cells competent at subsequently expressing the lung-specific marker SFTPC by day 36 (Figure 2C and data not shown), suggesting this population contained lung progenitors. The GFP- outgrowth was also highly enriched for cells competent at expressing lung markers SFTPB and MUC1, although these markers are known to have less lung specificity than SFTPC (Figure 2C). Immunostaining confirmed discrete populations of cells expressing SFTPB and MUC1 proteins in the GFP+ outgrowth (Figure 2D). In contrast, TP63+ cells were present in both GFP+ and GFP- outgrowths, but were more prevalent in GFP+ outgrowths, suggesting TP63 is not a lung-specific marker in this system. Consistent with this interpretation, TP63+ cells in the GFP+ outgrowth coexpressed NKX2-1, whereas TP63+ cells in the GFP outgrowth did not express NKX2-1 (Figure 2D and Supplemental Figure 3, A and B). Reverse transcription quantitative PCR (RT-qPCR) confirmed significantly higher levels of TP63 and the esophageal marker, PITX1, in the GFP outgrowth, raising the possibility that the NKX2-1-TP63+ cells might represent alternative foregut derivatives, such as developing esophageal epithelium (Supplemental Figure 3A) (22).

Fetal LgM augments distal lung differentiation in iPSC-derived lung organoids. Having established that NKX2-1+ primordial progenitors could be induced to upregulate markers of lung epithelial lineages without mesenchymal coculture support, we next sought to determine whether these progenitors might also respond to developmental cues provided by primary embryonic LgM. Lung epithelial-mesenchymal interactions are essential for lung epithelial growth, branching, and differentiation (23–25). For example, separating and recombining rat embryonic lung epithelium and mesenchyme (recombimants) has previously revealed both the importance of LgM and the stage-specific plasticity of the developing lung epithelium in response to mesenchymal signals (23). Hence, we asked whether iPSC-derived NKX2-1+ cells are competent at responding to developing mouse LgM and if distal lung epithelial gene expression might be induced by distal LgM compared with bronchial mesenchyme or standard directed-differentiation conditions without mesenchyme (Figure 3A). Lung organoids generated from unsorted day 15 human iPSCs were cultured in 3D conditions until day 21. Either NKX2-1-GFP or NKX2-1-GFP areas were micromanipulated and recombined with E12 mouse lung
distal or bronchial mesenchyme (Figure 3A). After 5 to 7 further days in culture as recombinants, we observed continued growth of the human GFP+ aggregates in response to mouse distal LgM, continued robust expression of nuclear human NKX2-1 protein, expression of proliferation marker Ki67, and no detectable expression of thyroid markers (Figure 3, B–D and Supplemental Figure 3C). Importantly, we noted induction of cytoplasmic human pro-SFTPC and LPCAT1 protein expression in the majority of the human iPSC-derived cells by immunostaining, with validation of human SFTP C mRNA expression by both in situ hybridization as well as RT-qPCR (Figure 3, B–D and Supplemental Figure 3, D and E). Levels of SFTP C induction were higher in organoids recombined with distal LgM than in those continued solely through directed differentiation without recombination (Supplemental Figure 3D). Since E12 mouse LgM presumably lacks the signals needed for full maturation of the alveolar epithelium, which normally begins in the mouse at E18.5, human cells in these recombinants did not exhibit robust induction of transcripts associated with mature lamellar body biogenesis, such as LAMP3 (data not shown), and did not appreciably display lamellar body-shaped inclusions by microscopy, as expected. Bronchial mesenchyme did not induce SFTP C expression or proximal lung epithelial markers (SOX2, TPE63, or SCGB3A1) in NKX2-1-GFP+ cells (Supplemental Figure 3D and data not shown). In addition, control recombinants generated using GFP- organoids were not competent at inducing either human NKX2-1 or SFTP C expression (Figure 3C and Supplemental Figure 3D). These results suggest that human iPSC-derived NKX2-1+ lung progenitors respond to developing distal lung mesenchymal cues, findings in keeping with our prior observations that recombining rat distal LgM with isolated early distal primary lung epithelium induces distal alveolar marker gene expression (25).

The expression of SFTP C in response to LgM in the majority of epithelial cells analyzed raised the question of whether SFTP C+ cells were being derived by the selective outgrowth of rare distal lung–competent day 15 precursors versus the possibility that distal lung–competent progenitors might be common within the NKX2-1+ day 15 population. To distinguish these possibilities, we purified day 15 NKX2-1-GFP+ cells and replated increasingly dilute numbers of cells (15,000 cells down to 240 cells per well of a 96-well plate) for further directed differentiation from day 15 to day 32 in 3D cultures without mesenchymal coculture support. This limiting-dilution assay should result in declining SFTP C competence with dilution if only rare distal progenitors are present within the day 15 NKX2-1+ population (Figure 3, E and F). On day 32 we observed that lower cell numbers, plated at limiting dilution, resulted in stable to increased SFTP C mRNA expression, consistent with the existence of common rather than rare distal lung–competent progenitors within the NKX2-1+ day 15 population and suggesting inhibition of distal differentiation in increasingly dense replating conditions in epithelial–only sphere outgrowths.

Global gene expression kinetics of early human lung development modeled by directed differentiation of human iPSCs. We next sought to define the fundamental programs of early human NKX2-1+ lung progenitors and their global gene expression kinetics during the course of in vitro directed differentiation. We prepared time series microarray expression profiles representing the following 5 key stages of iPSC lung-directed differentiation (Figure 4A): undifferentiated iPSCs (day 0), definitive endoderm (day 3), anterior foregut–like endoderm (day 6), sorted NKX2-1-GFP+ and NKX2-1-GFP- primordial progenitors (day 15), and sorted NKX2-1-GFP+ and NKX2-1-GFP- differentiated cells (day 28). For positive and negative controls we included primary distal fetal lung epithelial cells (21 weeks of human gestation) and forebrain-like iPSC-derived neural NKX2-1-GFP- cells (as shown in Figure 1B), respectively (Supplemental Table 1).

Principal component analysis (PCA) indicated that the global transcriptome of NKX2-1-GFP+ lung cells was easily distinguished from NKX2-1-GFP- neural cells (Figure 4B). Unsupervised hierarchical clustering of all 27 samples based on the top ~1,000 transcripts differentially expressed as determined by ANOVA (1,032 genes at \( P < 5 \times 10^{-5} \)) revealed that day 15 and 28 cells prepared in the lung-directed differentiation protocol clustered closer to distal fetal lung epithelial controls than to endoderm or neural NKX2-1- cells (Figure 4C). The transcriptional profile of neural NKX2-1-GFP+ cells compared with lung NKX2-1-GFP- cells (day 15) included 4,329 differentially expressed transcripts (FDR-adjusted \( P < 0.01 \)) and a distinct set of transcription factors including SIX3, DBX1, OTX1, OTX2, FOXD1, PAX6, and LHX5 (ranked by fold change [FC], filtered by FC > 5, FDR < 0.01, and gene ontology [GO] classification GO:0003700: “transcription factor activity, sequence-specific DNA binding”; Figure 4D), further emphasizing the marked differences between these early NKX2-1- forebrain and lung progenitors. Directed-differentiation protocols have previously used FOXA2 expression as a marker to define an NKX2-1- population as endodermal and TUI1 to indicate neuroectodermal fate, but we found that FOXA2 is expressed in both neuronal and lung populations (Figure 4F), and that TUI1 is not highly expressed in the neuronal NKX2-1- population, suggesting neither marker is useful in distinguishing NKX2-1- neural from lung lineages. In contrast, the top 10 transcription factors differentially expressed in day 15 lung NKX2-1-GFP versus neuronal NKX2-1-GFP cells include 6 genes expressed in the developing lung in vivo (GRHL2, ELF3, GATA6,
Figure 4. Time series global transcriptomic profiling of human iPSCs undergoing lung-directed differentiation. (A) Schematic overview of the lung-directed differentiation with time points and populations analyzed by microarrays. (B) Principal component analysis (PCA) of global transcriptomes of the biological triplicates from time points shown in A. (C) Unsupervised hierarchical clustering by dendrogram of the samples shown in A, based on the top ~1,000 transcripts differentially expressed as determined by ANOVA across all 27 samples. (D) Heatmap of the top 20 transcription factors differentially expressed in each direction (10 up and 10 down) between neural NKX2-1GFP+ and day 15 lung NKX2-1GFP+ (15+) populations (ranked by fold change; filtered by FDR < 0.01). (E) Heatmap of the top 10 genes differentially expressed between day 15 lung NKX2-1GFP+ (15+) and day 15 lung NKX2-1GFP- (15−; ranked by fold change; filtered by FDR < 0.01). (F) Heatmap of the expression of known markers of neuroectoderm, endoderm, and lung epithelium (separated into progenitor stage and distal/alveolar vs. proximal/airway epithelium). Scale = row-normalized log2 expression. All differentiation samples were from C17 iPSCs.
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The constellation of transcripts better able to distinguish these 2 iPSC-derived populations.

To interrogate the differences between day 15 NKX2-1\(^+\) and NKX2-1\(^{–}\) cells at the primordial progenitor stage, we ranked the top 10 differentially expressed genes by FC (Figure 4E). We found that NKX2-1 as well as neighboring long noncoding RNAs (lncRNAs) SFTA3 and NKX2-1AS were highly upregulated in the GFP\(^+\) population.

Next we sought to identify unbiased gene signatures of primordial (day 15) and maturing (day 28) NKX2-1\(^{–}\) cells. We generated lists of the top 100 differentially expressed genes (ranked by FC, filtered by FDR < 0.01) of each sample across multiple comparisons and identified a common gene set for each sample (Supplemental Figure 4 and Supplemental Table 2). The day 28 GFP\(^+\) population was enriched for diverse but predominantly early endodermal and forebrain markers, while day 15 GFP\(^–\) population was enriched for diverse but predominantly endodermal markers, such as GATA4, GATA6, SOX17, NODAL, and FOXA2 were upregulated early during endodermal differentiation, with retained expression of GATA6 and FOXA2 in day 15 and day 28 NKX2-1\(^{–}\) cells. In contrast, the transcripts SFTPC, SFTPB, and TP63, and FOXP family members were low or absent prior to day 6, and their clear emergence in the day 15 GFP\(^+\) population is consistent with their published expression during the early lung progenitor period in mouse lung development (35, 36). These findings, together with the lack of mature lung marker gene expression in day 15 GFP\(^–\) cells (low SCGB1A1, SCGB3A2, TP63, SFTPB, and SFTPC), further suggest the day 15 GFP\(^+\) population represents a relatively undifferentiated or primordial lung progenitor population, as has been observed in early NKX2-1\(^+\) progenitors in developing mouse cells in vivo (28). In contrast to day 15, by day 28 the GFP\(^+\) population had begun to express markers known to be enriched in maturing alveolar epithelial cells (ETV5, CLDN18, LPCAT1, MUCI, SFTPB, and SFTPC) or in airway epithelia, such as basal (TP63), secretory (SCGB3A2, MUC5B, MUC5AC, and AGR2), and neuroendocrine (ASCL1) cells. PDPN, which has occasionally been referred to as a PSC-derived type 1 pneumocyte marker (8, 9), was actually expressed in day 15 GFP\(^–\) cells, consistent with its expression in prior publications (8, 9), was actually expressed in day 15 and day 28 time points were derived from C17 iPSCs.

**Figure 5.** Transcriptomic signatures of iPSC-derived anterior foregut endoderm and lung progenitors, focused on genes associated with transcription factor activity. Heatmap of the top transcription factors or genes with transcription factor activity GO terms that are differentially expressed (filtered by fold change > 4) between successive stages of lung differentiation starting from day 6 and compared with human fetal lung. Vertical text box and heatmap with black outline identify the signature of each stage being analyzed. *Indicates the gene was previously described in foregut and/or lung development (see Supplemental Table 3 for further publication details). See also Supplemental Figure 4. Day 6, day 15, and day 28 time points were derived from C17 iPSCs.

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To interrogate the differences between day 15 NKX2-1\(^{–}\) and NKX2-1\(^{–}\) cells at the primordial progenitor stage, we ranked the top 10 differentially expressed genes by FC (Figure 4E). We found that NKX2-1 as well as neighboring long noncoding RNAs (lncRNAs) SFTA3 and NKX2-1AS were highly upregulated in the GFP\(^+\) population. Also in this top 10 list were genes (BMP3, CRH, and SPOCK3) previously described in lung development (Figure 4E, with validation by RT-qPCR in S4B) (28–34). The finding that SFTA3 (aka NKX2-1–associated non-coding intergenic RNA [NANCI]) is the top differentially expressed transcript in the genome distinguishing day 15 NKX2-1\(^{–}\) cells is in keeping with recent reports that in developing mouse lungs this transcript is coexpressed with Nkx2-1 and shares the same regional and temporal expression pattern (35). To the best of our knowledge, 4 genes in this list (PALMD, FAM189A2, GRM8, and WDR49) have not been previously identified in the lung epithelium.

From our microarray datasets we selected known markers of definitive endoderm, forebrain, and 24 genes of known importance in the developing lung epithelium and profiled their expression patterns over the course of directed differentiation in comparison with human primary fetal epithelial lung control cells (HEF) (Figure 4F). Endodermal markers, such as GATA4, GATA6, SOX17, NODAL, and FOXA2 were upregulated early during endodermal differentiation, with retained expression of GATA6 and FOXA2 in day 15 and day 28 NKX2-1\(^{–}\) cells. In contrast, the transcripts SFTPC, SFTPB, and TP63, and FOXP family members were low or absent prior to day 6, and their clear emergence in the day 15 GFP\(^+\) population is consistent with their published expression during the early lung progenitor period in mouse lung development (35, 36). These findings, together with the lack of mature lung marker gene expression in day 15 GFP\(^–\) cells (low SCGB1A1, SCGB3A2, TP63, SFTPB, and SFTPC), further suggest the day 15 GFP\(^+\) population represents a relatively undifferentiated or primordial lung progenitor population, as has been observed in early NKX2-1\(^+\) progenitors in developing mouse cells in vivo (28). In contrast to day 15, by day 28 the GFP\(^+\) population had begun to express markers known to be enriched in maturing alveolar epithelial cells (ETV5, CLDN18, LPCAT1, MUC1, SFTPB, and SFTPC) or in airway epithelia, such as basal (TP63), secretory (SCGB3A2, MUC5B, MUC5AC, and AGR2), and neuroendocrine (ASCL1) cells. PDPN, which has occasionally been referred to as a PSC-derived type 1 pneumocyte marker (8, 9), was actually expressed in day 15 GFP\(^–\) cells, consistent with its expression in prior publications (8, 9), was actually expressed in day 0 as well as day 15 GFP\(^+\) cells, consistent with its expression patterns in developing mice where it is robustly expressed in both the foregut endoderm and the developing pseudoglandular lung epithelium prior to the emergence of type 1 cells (37).

Next we sought to identify unbiased gene signatures of primordial (day 15) and maturing (day 28) NKX2-1\(^{–}\) cells. We generated lists of the top 100 differentially expressed genes (ranked by FC, filtered by FDR < 0.01) of each sample across multiple comparisons and identified a common gene set for each sample (Supplemental Figure 4 and Supplemental Table 2). The day 28 GFP\(^+\) population was enriched for diverse but predominantly liver (APOA2, FGB, AFP, CDH17, and TF) and intestinal (CDX2, CDH17, and GIF) markers (Supplemental Table 2). In addition to
Figure 6. Single-cell RNA sequencing of sorted and unsorted iPSC-derived cells reveals NKX2-1+ lung and NKX2-1− non-lung lineages and suggests markers for their identification. (A) Schematic of the single-cell capture and global RNA sequencing of sorted C17 NKX2-1GFP− and unsorted BU3 iPSCs on day 15 of lung-directed differentiation. (B) PCA of the top 150 most variant genes of all sequenced cells reveals 4 cell clusters, color coded to match the clusters shown in panel C. (C) Heatmap of gene expression (global z score) with unsupervised hierarchical clustering of all 153 cells (x axis) and the top 150 most variant genes (y axis). Dendrograms as well as colored boxes indicate 4 cell clusters (CC1−4; matching colors shown in panel B). y-axis dendrograms and thick black lines indicate 3 gene clusters (GC1−3). Key genes indicated on right. CC1 = orange, CC2 = blue, CC3 = red, CC4 = green. (D) Top 10 genes correlated with NKX2-1 expression. (E) Unsupervised cell clustering using Monocle’s pseudotime spanning tree analysis reveals 7 cell states. Individual cells are labeled in subsequent panels by NKX2-1 level, genetic background (iPSC clone), or cell cycle (mitosis), respectively. (F) Pseudotime plots of expression levels of NKX2-1, CD47, SOX9, NFIB, FGB, and APDA2 with cells colored based on the 7 states determined in E. (G) Immunostaining of RUES2-derived day 15 cells for NKX2-1 (red) and SOX9 (green) nuclear proteins. Nuclei counterstained with DAPI. Scale bars: 25 μm.

NKX2-1, SFTA3, CPM, NFIB, and CRH, which are all expressed in primordial lung progenitors, the maturing lung cells (day 28 GFP−) expressed higher levels of SCGB3A2, SFTPB, TP63, ICAMI, IL8, and ITGB6 (Supplemental Figure 4 and Supplemental Table 2). SCGB3A2 was the most differentially expressed transcript of 23,786 probesets ranked by FC (day 28 GFP− vs. GFP+ groups; FC = 76.6; FDR-adjusted P = 1.6 × 10−5). SFTPC was upregulated by day 28 (GFP−), but not yet at levels equivalent to HFL, findings in keeping with our recombinant experiments, which suggested that current differentiation protocols without the use of primary mesenchyme have not yet been optimized for efficient and full distal alveolar maturation (Figure 3, C and D, Supplemental Figure 3D, Figure 4F, and Supplemental Table 2).

Transcription factors play critical roles in organogenesis including in lung development (27). To identify candidate genes that control human lung specification and development, we screened for enrichment of transcription factors or regulators of transcription prior to lung specification (day 6) and at different stages of lung maturity (day 15 NKX2-1GFP+, day 28 NKX2-1GFP+, and HFL; Figure 5). To identify genes of interest, we ranked the significantly differentially expressed genes by FC (FC > 4; FDR < 0.01) and filtered genes based on GO classification for transcription factor activity (GO:0003700, “transcription factor activity”). The majority of the most highly differentially expressed genes in day 6 anterior foregut–like endoderm were known transcription factors of the foregut endoderm previously described in Xenopus and mouse model systems: HHEX, GATA3, GATA4, FOXC1, EOMES, OTX1, OTX2, ISL1, and PITX2 (28, 38, 39) (Figure 5, Supplemental Figure 4C, and Supplemental Table 3). In comparison with day 6, the day 15 NKX2-1GFP+ population was enriched for many transcription factors known to be present in the developing mouse lung (JUN, MECOM, SOX2, HES1, HOXA1, NKX2-1, FOXA1, ELF3, ELF5, NFIB, and FOXP2) (26–28, 38–44) (Figure 5, Supplemental Figure 4D, and Supplemental Table 3). In addition to HOXA1, a number of other HOX genes were upregulated in day 15 NKX2-1GFP+ samples (HOXA4 and HOXC4). SHH, essential for normal lung development in mice (27), was the most highly expressed gene in the day 15 samples in our analysis (FC = 60, FDR = 4.8 × 10−10). Day 28 NKX2-1GFP+ cells expressed higher levels of transcription factors associated with basal cells (TP63) and neuroendocrine cells (ASCL1). Taken together, these time series data provide unbiased stage-dependent signatures of the putative transcriptional programs of human lung progenitors and their differentiated progeny as they emerge during development-directed differentiation. Moreover, these signatures reveal that many evolutionarily conserved transcription factors, previously observed in developing Xenopus and mouse lung endoderm in vivo, also are differentially expressed in the iPSC human lung development model system.

Single-cell RNA sequencing and surface marker profiling of day 15 iPSC-derived lung progenitors. While transcriptomic profiles of purified groups of cells allows a deep understanding of the genetic program of the NKX2-1+ progenitor population, it does not allow interrogation of the heterogeneity of these programs at the individual cell level. Hence, we next sought to profile the transcriptomic programs of individual iPSC-derived cells at the day 15 stage of lung differentiation employing the C17 NKX2-1GFP+ targeted line as well as the untargeted iPSC line, BU3 (19). We performed RNA sequencing (RNA-Seq) of 84 BU3 iPSC-derived cells without any cell sorting and 69 C17 iPSC-derived cells, sorted based on NKX2-1GFP+ expression (Figure 6A).

PCA (Figure 6B) as well as unsupervised hierarchical clustering analysis (Figure 6C) both suggested that 4 broad clusters of cells (hereafter CC1−4) were present on day 15, and these cell clusters could be distinguished based on 3 broad gene clusters (GC1−3). Notably, 3 of the 4 clusters (CC1, CC2, and CC4) expressed high levels of NKX2-1 (Figure 6C), whereas cluster CC3 exhibited an absence of transcripts encoded by the NKX2-1 locus or its neighboring locus, SFTA3. NKX2-1-expressing clusters were most robustly distinguished by whether they exhibited mitotic (CC1 and CC2) or noncycling (CC4) gene signatures. For example, CC1 and CC2 were highly enriched for the expression of genes associated with mitosis or cytokinesis (e.g., KIF11, KIF14, KIF22, KIF23, CDC20, and AURKB). Thus, CC1 was labeled mitotic and CC2 was labeled premitotic based on slightly lower expression levels of these markers in the latter cluster, whereas CC3 and CC4 did not appear to be in active cycle at the time of cell capture for analysis. Importantly, NKX2-1+ cells clustered together (CC4) regardless of whether they were sorted GFP+ C17 iPSCs or unsorted BU3 iPSCs. Furthermore, only 1 GFP− sorted cell could be found misclustering among the 26 cells that comprised the NKX2-1-negative cluster (CC3), and as expected 25 out of 26 cells found in this NKX2-1-negative cluster derived from the unsorted BU3 iPSCs (Figure 6C).

We undertook 3 approaches to interrogate the gene expression differences that distinguished each cell cluster. First, we used unsupervised hierarchical clustering of the top 150 most variant genes (Figure 6C; y-axis dendrograms; hereafter gene clusters GC1−3). We found that GC1 was highly enriched for cell-cycle regulation genes (including AURKB, BIRC5, BUB1, CCNB1, CCNB2, CENPE, CENPF, KIF11, KIF14, KIF22, KIF23, KIF22c, MELK, and TOP2A; Figure 6C), further supporting the interpretation that changes in genes of cytokinesis and cell cycle dominate the first level of clustering of day 15 cells. However, 2 additional distinct gene clusters were also apparent, most notably GC2, including NKX2-1, SFTA3, NFIB,
CD47, WNT5A, CPM, and LAMA3; and GC3 which was associated with the NKX2-1-negative/SFTA3-negative cells of CC3 (Figure 6C). This analysis suggested GC2 genes as potential markers associated with NKX2-1+ lung cells, and GC3 genes as potential markers associated with non-lung (NKX2-1-negative) cells. Consistent with this observation, the top 10 genes most highly correlated with NKX2-1 expression across individual cells were GC2 genes, including CD47, SFTA3, CPM, and LAMA3 (Figure 6D). In contrast, GC3 was enriched in liver-lineage genes (APOA2 and FGB) as well as nonspecific mesenchymal genes (COL19A1 and S100A10). We have previously reported that in iPSC-derived hepatic cells, FGB represents the most upregulated transcript in the genome during hepatic-directed differentiation (45, 46). Furthermore, in postnatal human tissues both APOA2 and FGB are transcripts specifically enriched in liver cells (gtexportal.org). Significantly, all NKX2-1-negative cells of putative hepatic lineage (20 out of 20 APOA2+ cells; Figure 6C) were solely composed of unsorted BU3 iPSCs, indicating that sorting on the NKX2-1(GAP) marker successfully depleted any contaminating hepatic cells in this protocol. The majority of cells (23 of 37) in the mitotic and premitotic groups clustered with NKX2-1+CD47+ cells when hierarchical clustering was run after cell cycle genes were removed (Supplemental Figure 5A).

Second, we applied the Monocle computational algorithm (47) to our single-cell dataset in an effort to identify, in an unbiased manner and irrespective of cell cycle, cell subtypes, or intermediate states. Because a particular challenge in single-cell RNA-Seq experiments is the high cell-to-cell variation observed in most genes, including key developmental regulators, during differentiation (48–51), Monocle was developed to improve the resolution of individual transcriptomes and allow the ordering of cells by potential progress through a biological process without relying on known lineage markers (47). Hence, we used Monocle to order all day 15 cells in pseudotime: an abstract, semiquantitative measure of progress through a biological process (Supplemental Figure 5B). This predominance of the distal progenitor marker, SOX9, in day 15 NKX2-1+ cells is consistent with the efficient distal alveolar differentiation competence of the NKX2-1+ progenitor population observed in our recombiant cultures in Figure 3. Importantly, mature distal or proximal markers (e.g., SFTPC and SCGB1A1) were not detected in any cell on day 15 (Supplemental Figure 6A).

Finally, we performed repeat ANOVA with hierarchical cell and gene clustering of the 97 cells that were not in active mitosis (focusing solely on CC3 and CC4; Figure 6C and Supplemental Figure 6B). This analysis identified 4 cell subgroups (SG1–4;
Supplemental Figure 6B). The largest subgroup of cells, SG2, expressed key genes of the early developing lung (SFTA3, NFIB, and WNT5A) and, in keeping with our population-based transcriptomic profiles, they lacked detectable expression of markers of lung maturation (SFTPC, SFTPB, SCGB3A2, ASCL1, FOXJ1, or SCGB1A1) (Supplemental Figure 6B). In the 3 remaining minor subgroups (SG1, 3, and 4; comprising predominantly NKX2-1-negative BU3 cells), we found differentially expressed genes suggestive of non-lung endoderm or undetermined identity. Gene sets most significantly correlated with SG1 and SG3 were consistent with hematopoietic lineages (Supplemental Figure 6B). Significance testing of SG2 versus SG3 demonstrated that CD47 was the most highly differentially expressed gene in SG2 (ranked by either P value or correlation with NKX2-1 expression; Supplemental Figure 6C), followed by IGBP5, SFTA3, EIF1AY, LAMA3, CPM, SOX9, and LMO7 (Supplemental Figure 6C). LMO7 is a known target of FGFI10 that is upregulated in early developing mouse lung epithelium (52), and NKX2-1, SFTA3, CPM, and SOX9 are all known to be enriched in developing mouse and human lung epithelia.

To determine whether key markers identified in our single-cell RNA-Seq or microarray analyses are expressed in the developing human lung epithelium in vivo, we analyzed available microarray data of human fetal lungs ranging from 53 to 154 days of gestation (53). Consistent with our PSC in vitro model system, we observed increasing in vivo expression with time of known lung differentiation markers (SFTPC, SFTP, and LAMP3), absence at any time point of non-lung markers (APOA2 and CDX2), and, early, unchanging expression of NKX2-1, CD47, NFIB, HOXA3, and JUN (Supplemental Figure 6D). In addition, we confirmed early developmental CD47 protein expression in NKX2-1+ epithelial cells in vivo by immunostaining week 10 human fetal lung (Supplemental Figure 6E). Taken together, our results provided an improved understanding of the heterogeneity of iPSC-derived cells emerging with lung-directed differentiation, supporting the utility of NKX2-1-GFP sorting to deplete non-lung endodermal lineages that contribute to this heterogeneity, and suggested transcripts associated with NKX2-1+ cells in this in vitro model system.

Prospective isolation of iPSC-derived NKX2-1+ primordial lung progenitors by CD47+ cell sorting. Because our single-cell RNA-Seq profiles revealed that CD47 was the transcript in the genome most highly correlated with NKX2-1 (Figure 6D), we sought to determine whether NKX2-1+ primordial progenitor cells might be prospectively isolated based on cell surface protein expression of CD47 without the need for a GFP knockin reporter. Using both immunofluorescence microscopy as well as FACS of day 13–15 unsorted PSCs (C17, BU3, and RUES2 lines), we observed that the brightest CD47+ cells selectively coexpressed NKX2-1 nuclear protein as well as the NKX2-1-GFP reporter (Figure 7, A and B and Supplemental Figure 7).

In independent experiments we screened day 15 iPSC-derived NKX2-1-GFP+ progenitors by FACS using a panel of 243 antibodies, validating that CD47 was in the top 4 cell surface markers most associated with GFP expression (Figure 7B). Notably, this screen also confirmed that: (a) NKX2-1-GFP+ cells are EPcam positive (Supplemental Figure 7A); (b) CPM, recently published as a marker of iPSC-derived NKX2-1+ cells (16) and highly associated with NKX2-1 in our single-cell RNA-Seq (Figure 6D), indeed coexists most NKX2-1-GFP+ cells (Figure 7B), although it is also associated with NKX2-1 hepatic cells (54) that emerge at low levels in this protocol (Figure 6F and Supplemental Figures 5B and 6D); and (c) ALCAM (CD166) and MUC1 (CD227) are 2 additional candidate markers that selectively identify NKX2-1-GFP+ cells at this stage (Figure 7B). Importantly, our antibody screen also suggested CD26 as a negative-selection marker since the brightest CD26+ cells were lower in expression of the NKX2-1-GFP reporter (Figure 7B). When sorting day 13–15 cells based solely on CD47+CD26+ gating, we found significant enrichment for NKX2-1+ cells: 89% ± 4.1% of cells (mean ± SD; n = 11 runs) expressed NKX2-1 nuclear protein as well as the NKX2-1-GFP reporter compared with CD47+ cells, which were depleted of NKX2-1 expression (Figure 7, C–F). These findings were validated for both human ESCs (RUES2) as well as multiple iPSC lines (C17, BU3, 100-3, RC202, and RC204; refs. 18, 19, 55 and data not shown), despite varying efficiencies of NKX2-1+ induction in any given differentiation run (Supplemental Figure 7, C and D). For example, regardless of whether a line differentiated to lung with low or high efficiency (e.g., 13% vs. 56% NKX2-1+ in 3 separate runs for C17; or 50% for RUES2), in each case CD47+CD26+ gating provided significant enrichment in NKX2-1+ cells (7-fold vs. 2-fold enrichment for C17 and 2-fold for RUES2), resulting in populations approximately 90% pure for NKX2-1 expression in each run. Furthermore, human ESCs or human iPSCs sorted solely on CD47+CD26+ gating produced predominantly NKX2-1+ spheroids in 3D culture that expressed lung differentiation markers including pro-SFTPC protein and SFTPC mRNA at levels similar to those of sorted NKX2-1-GFP+ cells (Figure 7, D and G).

Discussion

Our results indicate that iPSC-derived lung epithelial cells originate from identifiable NKX2-1+ progenitors. Through the use of an NKX2-1-targeted GFP reporter these progenitors can be sorted and then further differentiated without mesenchymal coculture support in 3D Matrigel culture. Importantly, human NKX2-1+ progenitors derived with our methods undergo efficient distal SFTPC differentiation and proliferation after recombinant culture with primary distal embryonic mouse LgM. We have labeled these NKX2-1+ cells, which emerge between days 8 and 15 of iPSC differentiation, primordial progenitors because they express a transcriptome that includes the earliest transcripts known to emerge during the endodermal and primary lung bud stages of mammalian development (NKX2-1, SFTA3, SOX9, and SOX2), but they are otherwise lacking in transcripts associated with differentiated/maturing lung epithelia, most of which emerge during the later pseudoglandular stage of lung development.

Our findings support a paradigm in which the human lung epithelium derives directly from NKX2-1+ endodermal progenitors rather than from alternate cells, because sorting human NKX2-1+ cells at the primordial stage highly enriches for cells competent at further differentiating into lung epithelia, while depleting this population significantly depletes cells competent at forming lung. Given the difficulty in accessing and tracking live human fetal cells in vivo during the earliest stages of lung development (approximately 1 month of human gestation), our in vitro model enables the purification, tracking, and visualization of cells undergoing the earliest moments of human lung cell fate decisions, a time period in human lung development that remains elusive to scientific study.
We provide evidence that the genetic control of early human lung development is similar to that in mouse. Indeed, our finding that human iPSC-derived lung epithelial progenitors respond to inductive differentiation cues provided by developing mouse lungs suggests that an evolutionarily conserved biology is common to early mouse and human lung development. Furthermore, our stage-dependent transcription factor signatures for developing lung, thyroid, and forebrain revealed by our in vitro iPSC model provides important validation in a human system of many of the gene ensembles previously identified in mice. For example, we found that many genes and transcription factors of murine lung development are expressed in iPSC-derived human lung progenitors, including NKX2-1, SHH, FOXA1, GATA6, SOX2, SOX9, GRHL2, IRX1, IRX2, NFIA, NFIB, FOXP2, HNF4A, ELF5, and ELF3. Our developmental stage–dependent signatures also suggest novel genes requiring further study, and the ability to employ the human iPSC model system should now provide a tractable developmental human system to examine the roles of these and other genes in human lung lineage specification. Partnered future work focused on this early period of lung lineage specification in vivo in mice is likely to provide a further understanding of the phenotype and biology of primordial lung progenitors across mammalian species, and should enable isogenic head-to-head comparisons of iPSC-derived progenitors with their in vivo counterparts. It is important to point out that the differentiation kinetics of our human iPSC in vitro model is generally faster than that observed in vivo developing human endoderm and lungs. Thus, we cannot exclude the possibility that cells in the in vitro developmental model take a slightly different or alternate path towards lung fate cells, and we propose that further comparisons of our cells to in vivo developing cells, both mouse and human, will be important to address this possibility.

Given the primordial nature of early NKX2-1 lung progenitors, whether derived from iPSCs/ESCs or emerging in vivo embryos (2, 56), specific lung progenitor markers have not been previously identified with certainty to enable their prospective isolation. While FOXP2 in mice has been proposed as a lung primordial marker and should enable isogenic head-to-head comparisons of iPSC-derived progenitors with their in vivo counterparts. It is important to point out that the differentiation kinetics of our human iPSC in vitro model is generally faster than that observed in vivo developing human endoderm and lungs. Thus, we cannot exclude the possibility that cells in the in vitro developmental model take a slightly different or alternate path towards lung fate cells, and we propose that further comparisons of our cells to in vivo developing cells, both mouse and human, will be important to address this possibility.

In summary, we have purified human lung progenitors derived from iPSCs, and these cells are reminiscent of early stages of lung developmental differentiation. Our profiling of these cells as well as their precursors and progeny during the time course of directed differentiation has resulted in an understanding of their global transcriptomic programs at the single-cell level and provides a validated set of cell surface markers and transcription factors selectively enriched in these cells. It should now be possible to test whether cells of similar primordial lung progenitor phenotype remain in the lung postnatally or can be rederived in patients during responses to injury. Given the broad differentiation repertoire of the primordial progenitors, we anticipate that access to pure populations of these cells should facilitate basic developmental studies as well as clinical applications focused on disease modeling, drug development, and potentially future regenerative therapies.

Methods

Human PSC maintenance and gene editing. Previously published PSC lines iPSC17 (18), BU3 (19), and WA09 (H9 ESC) were maintained in feeder-free conditions on Matrigel (Corning) in mTeSR1 (Stem Cell Technologies) and passaged with Gentle Cell Dissociation Reagent (Stem Cell Technologies). In order to generate NKX2-1 knockin reporter PSC lines, TALENs or CRISPR-based technologies were employed as detailed in the Supplement to introduce a DNA double-stranded break into the second intron of NKX2-1 (Supplemental Figure 1A). A donor matrix containing a splice acceptor, NKX2-1 exon 3, 2A-eGFP, andloxP-flanked PGK-puroΔTK selection cassette was integrated by homologous recombination and targeted PSC clones resistant to puromycin underwent Cre-mediated excision of theloxP-flanked puroΔTK selection cassette (Supplemental Figure 1B), followed by confirmation of cassette excision, karyotyping, and characterization as detailed in the supplement.

Human iPSC directed differentiation. Neuroectodermal NKX2-1 cells were generated using STEMdiff Neural Induction Medium (STEMCELL Technologies) according to the manufacturer’s protocol. On day 6 of differentiation, 2 μM purmorphamine (Stemgent) was
added to the base media. NKX2-1- cells were sorted on days 12–14. Thyroid NKX2-1GFP- cells were derived using our recently published protocol (19) and lung NKX2-1GFP- cells were generated by adapting published protocols (8, 9). For both lung and thyroid differentiations we first induced definitive endoderm using a STEMdiff Definitive Endoderm Kit (STEMCELL Technologies) according to the accompanying protocol. After approximately 72 to 84 hours, we harvested and analyzed cells by flow cytometry for efficiency of definitive endoderm induction by the coexpression of the surface markers c-KIT and CXCR4 (see supplemental experimental procedures). After definitive endoderm induction cells were plated in small clumps at approximately 50–150,000 cells/cm² on Matrigel-coated plates in complete serum-free differentiation media (CSFDM) supplemented with 2 μM dorsomorphin (Stemgent) and 10 μM SB431542 (Tocris) for 72 hours. Y-27632 (10 μM, Tocris) was added for the first 24 hours. To specify thyroid epithelium, differentiation medium was changed on day 6 to CSFDM supplemented with 250 ng/ml rhFGF2 (R&D Systems), 100 ng/ml rhBMP4 (R&D Systems), and 100 ng/ml heparin sodium salt (Sigma-Aldrich) according to our published methods (19). To specify lung epithelium, differentiation medium was changed on day 6 to CFKBRa: CSFDM supplemented with 3 μM CHIR99021 (Tocris), 10 ng/ml rhFGF10, 10 ng/ml rhKGF, 10 ng/ml rhBMP4 (all from R&D Systems), and 50–100 nM Ra (Sigma-Aldrich) (9). Day 15 cells were dissociated with 0.05% trypsin (Thermo Fisher Scientific) followed by resuspending as small clumps in CSFDM supplemented with 3 μM CHIR99021, 10 ng/ml rhFGF10, and 10 ng/ml rhKGF (CFK medium) and plated on freshly-coated Matrigel (Corning, 354277) plates. Y-27632 (10 μM) was added to CFK medium for the first 24 hours. On day 22 the medium was changed to CFK+DCL: CFK medium plus 50 nM dexamethasone (Sigma-Aldrich), 0.1 mM 8-bromoadenosine 3’,5’-cyclic monophosphate (8-Br-cAMP) sodium salt (Sigma-Aldrich), and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich).

Sorting iPSC-derived lung progenitors and organoid generation. On day 15, live cells identified by propidium iodide (PI) exclusion were sorted by flow cytometry based on GFP expression for downstream applications including RNA analysis and generating organoids. To generate unsorted organoids, day 15 cells were dissociated with trypsin and pelleted cells were resuspended in Matrigel (Corning, 356230). CFK medium was then added to each well, supplemented with 10 μM Y-27632 medium for the first 24 hours. To generate sorted NKX2-1GFP- or NKX2-1GFP+ organoids, we resuspended the relevant sorted populations in Matrigel at a density of 50,000 cells per 50 to 100 μl Matrigel and allowed to gel as above. Further methods are detailed in the supplement.

Single-cell RNA-Seq analysis of day 15 iPSC-derived lung progenitors. Day 15 NKX2-1GFP- and BU3 unsorted cells were generated using the lung protocol, dissociated, and sorted as described above. Fluidigm C1 integrated fluidics circuits (IFCs) were used to capture individual live cells, lyse, convert polyA+ RNA into full-length cDNA, amplify and generate cDNA according to the manufacturer’s protocol (“Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing,” Fluidigm, PN-100-7168). Paired-end 50-bp reads were aligned with an average of 2.8 million reads per cell per end. Aligned, mapped reads were statistically analyzed using methods detailed in the supplement. The clustering, PCA, and significance testing were performed using SCICAST (details and a walkthrough can be found at https://github.com/iandriver/SCICAST) with additional hierarchical clustering linkages, Pearson’s correlation coefficients, ANOVA, and FDR-adjusted }
ing mouse lung recombinant cultures were approved by the IACUC of Cincinnati Children’s Medical Center.

For details of the methods used for immunostaining, mRNA in situ hybridization, and genomic DNA Southern blotting please see the supplemental procedures.

**Author contributions**

FH, PK, BRD, and DNK conceived the work, designed the experiments, and wrote the manuscript. PK, AMC, and BRD designed the targeting strategy and generated NKX2-1GFP iPSCs/ESCs. FH, AJ, KBM, and DCT performed the lung-directed differentiation experiments. FH, AAK, ANH, and DNK developed the human thyroid differentiation. JMS performed the mouse LgM recombination experiments. SN, BGW, and ASK performed the time-lapse microscopy. SG provided human fetal lung samples. SXH provided critical technical assistance. NS assisted with the microarray analysis. ID and JRR performed the single-cell RNA-seq analysis.

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