Productive Infection of Human Embryonic Stem Cell-Derived NKKX2.1+ Respiratory Progenitors With Human Rhinovirus

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ABSTRACT

Airway epithelial cells generated from pluripotent stem cells (PSCs) represent a resource for research into a variety of human respiratory conditions, including those resulting from infection with common human pathogens. Using an NKKX2.1-GFP reporter human embryonic stem cell line, we developed a serum-free protocol for the generation of NKX2.1+ endoderm that, when transplanted into immunodeficient mice, matured into respiratory cell types identified by expression of CC10, MUC5AC, and surfactant proteins. Gene profiling experiments indicated that day 10 NKX2.1+ endoderm expressed markers indicative of early foregut but lacked genes associated with later stages of respiratory epithelial cell differentiation. Nevertheless, NKX2.1+ endoderm supported the infection and replication of the common respiratory pathogen human rhinovirus HRV1b. Moreover, NKX2.1+ endoderm upregulated expression of IL-6, IL-8, and IL-1β in response to infection, a characteristic of human airway epithelial cells. Our experiments provide proof of principle for the use of PSC-derived respiratory epithelial cells in the study of cell-virus interactions.

SIGNIFICANCE

This report provides proof-of-principle experiments demonstrating, for the first time, that human respiratory progenitor cells derived from stem cells in the laboratory can be productively infected with human rhinovirus, the predominant cause of the common cold.

INTRODUCTION

Human pluripotent stem cells (PSCs) and their differentiated derivatives represent a platform for examining the biology of previously inaccessible or unobtainable human cell types (reviewed in [1]). The potential of this system has provided impetus for the rapid development of methods for differentiating PSCs into a variety of different lineages, including epithelial derivatives of the respiratory system [2–4].

Respiratory epithelial cells (RECs) are key players in a variety of prevalent conditions that affect the lung, including chronic bronchitis, emphysema, and cystic fibrosis. In addition, the airway is particularly susceptible to infection by a variety of human pathogens, including human rhinovirus (HRV), which is responsible for the majority of upper respiratory tract infections (common colds). HRV has also been implicated in illnesses of the lower respiratory tract, such as pneumonia, and can exacerbate respiratory diseases, such as asthma [5] and chronic obstructive pulmonary disease [6]. In many cases, RECs play a major part in disease progression. Despite the growing prevalence of respiratory disease, there is a dearth of effective treatments, a deficiency only paralleled by the scarcity of knowledge surrounding the RECs themselves. This state of affairs is in no small part due to the absence of appropriate models; there are sufficient differences between the organization of mouse and human respiratory systems to make rodent models of limited value. In this landscape, there is a clear need for new and relevant models for understanding human REC biology and physiology. RECs derived from the in vitro differentiation of human pluripotent stem cells represent a potential avenue to generate sufficient numbers of this cell type for medical research.

RECs are derived from foregut endoderm. In the mouse, the region of the ventral foregut destined to become the respiratory system is marked...
by the expression of Nkx2.1 (TTF-1/TITF1) [7]. Although not required for initial lung specification, Nkx2.1 is crucial for the correct branching morphogenesis of the organ [8]. Mouse knockout studies have shown that lung development does not progress past the beginning of the pseudoglandular phase and yields a severely dysmorphic lung, consisting of a main-stem bronchi and no distal structures [8]. In the human, NKX2.1 mutations have been associated with respiratory conditions, hypothyroidism, and neurological deficiencies [9], with the last two phenotypes reflecting other sites in which this gene is expressed.

We used an NKX2.1 knockin human embryonic stem cell (hESC) reporter line [10] to identify, purify, and characterize NKX2.1+ human respiratory progenitors. When cocultured with embryonic mouse lung tissue under the kidney capsule of immunodeficient mice, NKX2.1 progenitors were able to contribute to a differentiated respiratory epithelium marked by expression of CC10, MUC5AC, P63, and surfactant proteins. In vitro, NKX2.1 progenitors supported productive replication of HRV1b, providing proof of principle for the development of this system as a platform for studying cell-virus interactions. Our study demonstrates that hESC-derived NKX2.1+ cells generated by our differentiation protocol represent a progenitor pool that can give rise to mature respiratory epithelia and can propagate functional HRV.

## MATERIALS AND METHODS

### Culture and Differentiation of hESCs

Undifferentiated hESCs (TypLE Select adapted NKX2.1GFP/w HES3) were cultured as previously described [11]. Briefly, cells were maintained on mitotically inactivated mouse embryonic fibroblasts (StemCore, Melbourne, Victoria, Australia, http://www.stemcore.com.au) in a medium consisting of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium, 20% knockout serum replacement, 10 ng/ml basic fibroblast growth factor, 2 mM GlutaMAX, 10 mM nonessential amino acids (NEAA), 1 mM penicillin-streptomycin, and 50 mM β-mercaptoethanol (all reagents from Gibco, Invitrogen, Grand Island, NY, http://www.invitrogen.com). Cells were passaged with TripLE Select (Gibco, Invitrogen) as described previously [11]. NKX2.1GFP/w hESCs were differentiated as spin embryoid bodies (EBs) [12] by seeding 3 \times 10^5 cells per well into each well of a 96-well tray in the fully defined medium, APEL, or in BPEL, a version of APEL in which bovine serum albumin (BSA) was substituted for recombinant human albumin [13]. APEL or BPEL medium was supplemented with activin A (StemRD, Burlingame, CA, http://www.stemrd.com) and BMP4 (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com) at the concentrations indicated. After 4 days of differentiation, medium was changed to APEL/BPEL supplemented with 100 ng/ml fibroblast growth factor 1 (FGF1; PeproTech, Rocky Hill, NJ, http://www.peprotech.com) and 2 μg/ml heparin solution (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) as indicated. After a further 3 days, EBs were transferred well for well to gelatin-coated, tissue culture-treated 96-well trays containing a variation of APEL/BPEL medium in which the polyvinyl alcohol (PVA) had been replaced with an additional 0.25% Albucult (AEL medium) or BSA (BEL medium).

### Reculture and Transplantation Studies

For experiments involving reculture of cells isolated by FACS, cells were reaggregated using a modification of the spin EB protocol (5 \times 10^5 per well) in APEL supplemented with 200 ng/ml FGF10 and 10 μM ROCK inhibitor Y27632 [Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com] [14]. Following 24 hours, the medium was changed to APEL supplemented with 200 ng/ml FGF10 but lacking ROCK inhibitor and embedded in a 1:1 ratio of growth factor reduced-Matrigel (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com) and PVA-free medium. Additional growth factor supplements were included as indicated. For transplantation experiments, EBs or reaggregates were grafted under the kidney capsule of NOD/LtSz-scid IL2Rγ null mice [15] as described previously [16]. In some instances, hESC-derived material was cotransplanted with embryonic day 13.5 mouse distal lung fragments isolated by dissection 1 day prior to grafting. Mice were killed between 59 and 61 days post-transplantation, and the kidney and associated graft was removed, fixed in 4% paraformaldehyde, and processed for histological analysis by Monash histology services (Monash University, Clayton, Victoria, Australia). Hematoxylin- and eosin-stained sections were examined using an Olympus BX51 light microscope (Olympus, Center Valley, PA, http://www.olympusamerica.com). Images were captured using an Olympus DP70 camera and software (Olympus). Experiments involving animals were conducted under the auspices of the Monash University School of Biomedical Sciences-A animal ethics committee.

### Flow Cytometry, Histochemistry, and Immunofluorescence

For flow cytometry, EBs were dissociated using TrypLE Select (Gibco, Invitrogen) as described [13]. Mouse primary antibodies reacting to human cell surface antigens, and the concentrations at which they were used are shown in supplemental online Table 1. Unconjugated antibodies were detected with allophtocyanin-conjugated goat anti-mouse IgG (BD Biosciences (supplemental online Table 1). A subset of paraffin sections was stained with hematoxylin and eosin (H&E) to enable identification of organized structures within the grafts. For immunofluorescence analysis, sections were dewaxed for 3 minutes in each xylene (×2), 100% ethanol (×2), 90% ethanol, 70% ethanol followed by deionized water. Antigen retrieval was performed in 10 mM sodium citrate (pH 6.0; Sigma-Aldrich), followed by 5-minute washes once in deionized water and twice in PBS-T (0.1% Triton X-100 in phosphate-buffered saline). The sections were blocked for 30 minutes in PBS-T supplemented with 0.5% BSA and 5% goat serum at room temperature, before overnight incubation (at 4°C) with the following primary antibodies: mouse anti-TUBBIV (AbCam, Cambridge, U.K., http://www.abcam.com; 1:100), rabbit anti-CDX2 (Cell Signaling Technology, Beverly, MA, http://wwwCELLSIGNAL.com; 1:200), rabbit anti-ECAD (Cell Signaling Technology; 1:100), goat anti-CC10 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com; 1:200), mouse anti-MUC5AC (Millipore, Billerica, MA, http://www.millipore.com; 1:500), chicken anti- GFP (AbCam; 1:400), rabbit anti-MUC1 (AbCam; 1:100), mouse anti-P63 (Millipore; 1:200), rabbit anti-proSP-C (Millipore; 1:1,000), mouse anti-smooth muscle actin (Dako, Glostrup, Denmark, http://www.dako.com; 1:200), rabbit anti-SOX2 (Cell Signaling Technology; 1:100), rabbit anti-SFTPB (Millipore; 1:500), and rabbit anti-SFTPd (Millipore; 1:1,000). After overnight incubation, the sections were washed three times for 5 minutes in PBS-T and incubated with corresponding fluorescent secondary antibodies (Alexa 488 or 568).

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Gene Expression Analysis

Day 10 EBs were dissociated and sorted according to the expression of NKX2.1-GFP using a BD Influx cell sorter (BD Biosciences). Total RNA was extracted using the RNeasy Mini Kit according to the manufacturer’s instructions (Qiagen), then amplified, labeled, and hybridized to human HT12 v4 BeadChips according to Illumina standard protocols at the Australian Genome Research Facility. Initial data analysis was performed using GenomeStudio version 2010.2 (Illumina), using average normalization across all the samples. Further data analysis was performed on non-normalized data in R/BioConductor (R Development Core Team 2010) using algorithms within the lumi package [17]; function: bgAdjust.affy and quantile normalization [18]. Subsequent data analysis was performed using MultiExperiment Viewer [19]. Hierarchical clustering of genes was performed using Pearson correlation with average linkage clustering. Overlap analysis was performed using Venny 2.0 (Computational Genomics, Madrid, Spain, http://bioinfogp.cnb.csic.es).

Rhinovirus Infection Protocol

Day 10 FACS-sorted NKX2.1-GFP+ progenitor cells were seeded at a density of 250,000 onto 96-well plates and infected with HRV1b (provided by Dr. Peter Wark, Hunter Medical Research Institute, New Lambton Heights, New South Wales, Australia) at multiplicity of infection (MOI) of 12 and incubated at 37°C for 24 hours. MRC-5 cells were seeded onto 12-well plates with appropriate growth medium until 90% confluence. Prior to infection, growth medium was replaced with basal medium free of serum and growth factors, and cells were then infected with HRV-1B at MOI 12 for 24 hours. At the specified time point, supernatant and cells were collected for downstream analysis. MRC-5 cell was cultured in DMEM supplemented with 10% (vol/vol) fetal calf serum, 1% (vol/vol) L-glutamine, 1% (vol/vol) NEAA, and 1% (vol/vol) penicillin/streptomycin. All cells were grown at 37°C in an atmosphere of 5% CO2/95% air.

Viral Receptor and Viral Replication

Total RNA was extracted using PurelinkRNA Mini Kit (Life Technologies, Rockville, MD, http://www.lifetech.com) and quantified using Nanodrop (Thermo Fischer Scientific, Waltham, MA, http://www.thermofisher.com). For low density lipoprotein receptor (LDLR) gene expression, cDNA was synthesized using random hexamers and Multiscribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) as previously described [20]. The cDNA was then used in a quantitative real-time polymerase chain reaction (qPCR) using LRDR primers (forward primer: 5′-GAC ATG AGC GAT GAA GTT GG-3′; reverse primer: 5′-ATT GCA GAC GTG GAA ACA G-3′) in the ABI Prism 7300 (Applied Biosystems), and signals were analyzed by the ABI Prism Sequence Detection System Software. Expression of LDLR was measured by enzyme-linked immunosorbent assay. IL-6 was measured using time-resolved fluorometry detection system as previously used and described [20–22] (PerkinElmer Life and Analytical Sciences, Waltham, MA, http://www.perkinelmer.com). Cytokine production was then normalized to the viable cells.

RESULTS

A Three-Step Protocol for Generating Respiratory Endoderm

We investigated the concentrations of activin A and bone morphogenetic protein 4 (BMP4) required for induction of NKX2.1+ endoderm in light of reports that demonstrate that the patterning of mesendodermal germ layers can be influenced by BMP and transforming growth factor β (TGFβ) signaling [23–27]. We did this by performing a heatmap analysis analogous to that described previously for the optimization of cardiac and thymic endoderm differentiation from hESCs [28, 29]. In this procedure, spin embryo bodies (EBs) [12] were set up in round-bottomed low attachment 96-well plates such that each EB formed in the presence of a distinct combination of BMP4 and activin A. Flow cytometry analysis (n = 3) at differentiation day 10 revealed that GFP+ cells were most frequent in EBs induced with a range of growth factor concentrations centered at approximately 10 ng/ml BMP4 and 160 ng/ml activin A (Fig. 1B). These results were consistent with visual observations of individual EBs using fluorescence microscopy (Fig. 1C).

We also tested the ability of a range of growth factors previously implicated in respiratory development to promote the generation of NKX2.1+ endoderm. We found that only FGF1 (100 ng/ml) in the presence of heparin significantly and reproducibly increased the frequency of NKX2.1-GFP+ cells (from 1.94% ± 0.42% to 10.26% ± 2.20%, n = 7) (Fig. 1D). Interestingly, FGF2 supplementation was found to be neither necessary nor beneficial (results not shown). Optimal timing and duration of FGF1 treatment coincided with the 3-day period between the removal of activin A and BMP4 at day 4 and the commencement of adherent culture at day 7. Addition of FGF1 after day 7 was found to be detrimental (data not shown). Thus, through a series of iterative experiments, we established a three-step protocol that routinely generated NKX2.1-GFP+ cells at a frequency of approximately 5%–10%, and occasionally as high as 30% (Fig. 1E).

Characterization of Early NKX2.1-GFP+ Endoderm

Using this protocol, we examined the time course of NKX2.1-GFP induction in relation to cell surface markers associated with epithelial and mesenchymal cell populations [30, 31]. After 4 days of activin A and BMP4 treatment, a proportion of the differentiating cells coexpressed PDGFRα and EpCAM, indicative of mesendodermal precursors [32, 33]. By day 7 almost all cells were EpCAM+PDGFRα+, indicating that these conditions favored the survival of EpCAM+ endodermal cells at the expense of PDGFRα+ mesoderm derivatives (Fig. 1E). NKX2.1-GFP expression was detected as early as day 7 of differentiation.
predominantly within the EpCAM⁺PDGFRα⁻ population. The frequency of NKX2.1⁺GFP⁺ cells decreased significantly from day 10; however, we could not conclude whether this was due to extinction of NKX2.1⁺GFP⁺ cells or the expansion of the GFP⁻ population.

During development, NKX2.1 is expressed in neural lineages of the ventral forebrain [34, 35], as well as within the endoderm by thyroid and respiratory progenitors [7, 36]. In order to more clearly define the identity of NKX2.1⁺GFP⁺ cells, we performed gene expression microarray analysis of cells isolated by flow cytometry on differentiation day 10. We analyzed these data in a number of different ways. First, we compared the average global gene expression of the GFP⁺ and GFP⁻ populations. This analysis identified a subset of genes whose expression, on average (n = 3), was upregulated 10-fold in the GFP⁺ population relative to the GFP⁻ cells (Fig. 2A; supplemental online Fig. 1). In addition to NKX2.1 itself, this subset included a number of other genes (FOXP4, FOXJ1, VIL1, and FGFR4) known to be expressed in the foregut or its derivatives. Similarly, the GFP⁻ population showed upregulation of genes also associated with foregut, but those normally expressed in NKX2.1⁻ regions. For example, SOX2 transcripts were enriched in GFP⁻ cells, consistent with the idea that mutually exclusive expression of NKX2.1 and SOX2 is associated with separation of the foregut into esophagus and trachea [37–39]. In addition, the GFP⁻ fraction also expressed genes associated with nonendodermal cell types, such as VE-cad, PECAM1.
FGFR4 and SOX18 (endothelial) and COL3A1, COL5A2, BMP4, CD93, FOXF2, and CDH11 (mesenchymal). Finally, relative to undifferentiated HEsCs, GFP+ populations expressed high levels of endodermal associated genes such as FOXA1 (296×), FOXA2 (1,330×), SOX17 (150×), CXCR4 (76×), GATA4 (15×), and GATA6 (11×). Interestingly, mRNAs encoding the thyroid-associated transcription factor PAX8 were not detected in the GFP+ population, suggesting that specified thyroid progenitors were not generated by our differentiation protocol.

In order to more clearly define the composition of the GFP+ population, we searched for genes that were consistently upregulated across three independent experiments. This analysis identified a set of 123 genes that were upregulated fivefold in the GFP+ fraction relative to the GFP- population (Fig. 2B; supplemental online Fig. 1). Some genes identified in the scatter plot analysis (average of 10-fold increase) were also represented within this subset. These genes included TPPP3, NKX2.1, SFTA3, FGFR4, and FOXL, which are associated with respiratory endoderm. In addition to this group, the set of consistently upregulated genes included HHEX, ONECUT2, HNF1B, DHRS3, and DDC, which are associated with foregut-derived organs.

In addition to the above analysis, we sought to determine the relationship between the gene expression profile of the GFP+ population and bone fide respiratory derived endodermal cells. To this end, we performed hierarchical cluster analysis based on the list of genes that were on average fivefold upregulated in GFP+ fractions, together with gene expression data derived from the GFP- fractions, undifferentiated HEsCs, primary lung epithelial cells (Lung, Lung CF), and the small airway epithelial cell line, A549 (549) (supplemental online Fig. 1A). This analysis showed that, even with this restricted set, GFP+ cells were more closely related to the GFP- fraction than to mature respiratory epithelium. This distinction between the in vitro and in vivo derived material was reinforced by a similar analysis performed using the set of genes upregulated on average 10-fold in the GFP+ fraction (supplemental online Fig. 1B). Overall, these studies suggest that although the GFP+ population had some characteristics of early respiratory fated foregut endoderm, it lacked many of the key markers associated with definitive respiratory epithelium, such as CC10, MUCSAC, TUBB4, and surfactant proteins.

**NKKX2.1-GFP+ Cells Give Rise to Mature Respiratory Cells**

We reasoned that definitive respiratory markers may become evident if NKKX2.1-GFP+ cells could be recultured under conditions that promoted ongoing development. To this end, aggregates of NKKX2.1-GFP+ cells purified by FACS were cultured in the presence of growth factors previously implicated in the differentiation and branching of the mouse respiratory epithelium [40, 41]. However, irrespective of which growth factor combinations were included, NKKX2.1-GFP expression was rapidly downregulated following isolation and reaggregation (Fig. 3A–3D), and by as early as 7 days postisolation, GFP expression was no longer detectable. Nevertheless, aggregates treated with FGFs (keratinoctye growth factor [KGF] and FGF10) enlarged and appeared branched after 30 days of culture (Fig. 3E, 3F). Examination of histological sections of these aggregates revealed epithelia of both a secretory and squamous nature (Fig. 3G–3I). Immunofluorescence analysis of aggregates demonstrated uniform expression of the epithelial marker E-cadherin (ECAD) (Fig. 3K). Expression of MUCIN (MUC1) was also detected and was localized to lumen-like structures within the expanding reaggregates (Fig. 3J), whereas a substantial proportion of cells reacted with antibodies against CDX2 and SOX2 (Fig. 3L, 3M). In summary, purified NKKX2.1-GFP+ cells did not retain NKKX2.1 expression after extended in vitro culture but instead upregulated expression of transcription factors associated with dorsal foregut (SOX2) and posterior secretory endoderm (CDX2). These results indicated that our in vitro conditions could not maintain the identity of purified NKKX2.1-GFP+ cells and suggested that additional cues would be required to promote either the proliferation or differentiation of cells within the respiratory lineage.

Given that isolated GFP+ cells failed to retain their identity in vitro, we next investigated whether the EBs from which they were derived contained sufficient instructive information to facilitate ongoing respiratory differentiation. To test this, we transplanted day 7 EBs under the kidney capsule of immunodeficient mice, a site previously shown to support the development of lung allografts [42]. Irrespective of the induction regimen (activin A at 40 or 150 ng/ml for the first 4 days), grafts harvested after 59–61 days always included areas containing NKKX2.1-GFP+ cells (n = 9). In
some instances, these cells were associated with large fluid-filled cysts, whereas on other occasions NKX2.1-GFP+ cells appeared to be organized into discrete structures (Fig. 4A–4C). Examination of H&E-stained histological sections of these grafts revealed the presence of lumen lined with simple, columnar, and secretory epithelium (Fig. 4D–4F). Immunofluorescence staining demonstrated the presence of cells that expressed respiratory markers, such as MUC5AC (goblet cells), CC10 (clara cells), p63 (basal cells), and β-tubulin IV (ciliated cells) (Figs. 5G–5J). This, together with the presence of smooth muscle surrounding the NKX2.1-GFP+ structures (Fig. 4K), suggested that day 7 EBs contained sufficient information to support the ongoing development of an organized respiratory epithelium. Nevertheless, these grafts also included separate areas of secretory epithelium that were CDX2+, implying that intestinal epithelial progenitors were present within the day 7 EBs (Fig. 4L).

In pilot experiments designed to test the potential of NKX2.1-GFP+ cells, we purified GFP+ cells by flow cytometry and, following aggregation, transplanted this population under the kidney capsule of immunodeficient mice. However, we were unable to recover grafts from these transplant experiments, suggesting that this environment did not provide factors that could support the survival, expansion, or differentiation of the transplanted population. To address this possibility, we adopted an approach previously used to investigate the differentiation potential of in vitro derived pancreatic progenitors whereby cells were cotransplanted with mouse fetal tissue representing the lineage under investigation [43]. To this end, reaggregates of FACS-purified NKX2.1-GFP+ cells were cotransplanted with embryonic day 13.5 mouse fetal lung tissue. Examination of grafts 2 months post-transplantation revealed the presence of bright NKX2.1-GFP+ structures in four of five mice (Fig. 5A–5C).

Figure 3. Reaggregates of purified NKX2.1-GFP+ rapidly lose NKX2.1-GFP expression and develop into structures that express markers of dorsal and posterior endoderm. (A–D): Bright field (BF) and green fluorescent protein (GFP) fluorescence images of reaggregated fluorescence-activated cell sorting (FACS)-purified NKX2.1-GFP+ cells 1 and 2 days postpurification showing the rapid loss of GFP expression. (E, F): BF images of reaggregates of FACS-purified NKX2.1-GFP+ cells following 28 days of culture in Matrigel supplemented with FGF10 and KGF. No remaining GFP+ cells were observed at this stage. (G–I): H&E-stained sections of late stage (day 28) reaggregates revealed the presence of squamous (asterisk) and secretory epithelia (arrow) structures. (J–M): Immunofluorescence images of day 28 NKX2.1-GFP+ cell reaggregates showing the presence of MUC1+ ductal structures (arrowhead) within convoluted ECAD+ epithelial structures that contained subdomains that expressed CDX2 and SOX2. Scale bars = 50 μm. Nuclei were counterstained with DAPI (blue). Abbreviations: DAPI, 4’,6-diamidino-2-phenylindole; ECAD, E-cadherin; H&E, hematoxylin and eosin.
Histological analysis showed that grafts comprised a mixture of columnar and pseudostratified epithelia (Fig. 5D–5F). Immunofluorescence staining revealed NKX2.1-GFP+ cells were frequently incorporated into epithelial structures and GFP expression overlapped with that of MUC5AC, CC10, and β-tubulin IV, as well as surfactant proteins SFTPB, proSP-C, and SFTPD (Fig. 5G–5L). However, grafts from cotransplants of NKX2.1-GFP+ cells and mouse fetal lung also contained a substantial number of cells that expressed differentiation markers but were GFP− (red arrows in Fig. 5G, 5H). Most likely, these GFP− cells represent derivatives of the cotransplanted mouse fetal lung tissue rather than derivatives of the NKX2.1-GFP+ cells, because such cells were not observed in epithelial structures derived from transplanted day 4 embryoid bodies (Fig. 4). Taken together, these results suggest that, with appropriate inductive cues, NKX2.1-GFP+ cells isolated at differentiation day 10 have the capacity for ongoing development along the respiratory lineage.

NKX2.1-GFP+ Cells Support Respiratory Rhinovirus Replication

Mature cell types generated from the in vitro differentiation of pluripotent stem cells have been used in a variety of contexts, including disease modeling, drug screening, and the analysis of gene function (reviewed in [1]). In addition, a potentially important application of this system is as a platform to examine interactions between human cells and pathogens [44–48]. HRV is a nonenveloped single-stranded RNA virus that is the most common causative agent of respiratory tract infection in young children and infants (reviewed in [49]). It is of particular clinical interest because HRV infection is a significant exacerbating factor...
in existing respiratory conditions such as childhood asthma and cystic fibrosis [20, 50]. In this context, we tested whether hESC-derived NKX2.1+ endodermal progenitors could support infection with HRV1b, a rhinovirus subtype that enters cells following binding to the LDLR [51]. Day 10 differentiated cultures of NKX2.1GFP/w hESCs were subfractionated on the basis of NKX2.1 expression, and the resultant GFP+ and GFP+ populations were aggregated as described above. Aggregates were then infected with HRV1b at a MOI of 12 and, 24 hours postinfection, harvested and assayed for the quantity of HRV1b RNA using qPCR. This analysis indicated that HRV1b could replicate in the NKX2.1+ cells and that, surprisingly, the NKX2.1+ population also supported substantial viral replication as determined by the level of viral RNA (Fig. 6A). Furthermore, this cycle of infection led to upregulation of the viral receptor, LDLR, a commonly observed response to HRV1b infection (Fig. 6B). In addition, HRV1b-infected NKX2.1+ cells increased production of proinflammatory cytokines IL-6, IL-1B, and IL-8 (Fig. 6C), mirroring the response of endogenous airway epithelial cells to challenge with this pathogen.

In addition to supporting viral replication, productive infection requires cells to generate viable particles that can propagate the virus to adjacent host cells. To test this, NKX2.1+ and NKX2.1− cells were infected as described above, harvested 24 hours later, and then subjected to 3 rounds of freeze-thawing to release newly generated viral particles. These lysates were then applied to MRC-5 cells, a human fetal lung fibroblast line that is permissive for HRV infection and has been widely used for screening clinical isolates. MRC5 cultures treated with either lysate exhibited an altered cell morphology consistent with a cytopathic effect (Fig. 6D) and allowed viral replication, as assayed by qPCR for the presence of viral genome within infected cells (Fig. 6E). Collectively, these data provide evidence that

Figure 5. Fluorescence-activated cell sorting-purified NKX2.1-GFP+ cells give rise to respiratory epithelium in vivo. (A–C): Gross morphology of grafts arising from NKX2.1-GFP+ cells cotransplanted with embryonic day 13.5 mouse respiratory tissue under the kidney capsule of immunodeficient mice. (D–F): Examination of histological sections of grafts stained with hematoxylin and eosin revealed the presence of pseudostratified and secretory epithelia. (G–L): Immunofluorescence analysis revealed that GFP was coexpressed (white arrows) with markers of goblet cells (G), Clara cells (H), and ciliated cells (I). (J–L): Staining for surfactant proteins revealed the presence of SP-B (J), proSP-C (K), and SP-D (L). Red arrows point out GFP+ epithelium, which is most likely derived from cotransplanted mouse fetal lung tissue. Scale bars = 50 μm. Nuclei are counterstained with DAPI (blue). Abbreviations: BF, bright field; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; H&E, hematoxylin and eosin.
NKX2.1+ hESC-derived respiratory progenitors support HRV replication and therefore might be further developed to investigate the biology of this important pathological relationship.

**DISCUSSION**

We devised a simple protocol for generating respiratory endoderm from human embryonic stem cells and show that these cells can support productive infection of the human respiratory virus, HRV1b. Consistent with prior studies reporting efficient definitive endoderm induction from ESCs [52–54], we observed that optimal respiratory endoderm differentiation occurred when embryoid bodies were formed in the presence of high concentrations of activin A (Fig. 1B). In the protocol of D’Amour et al. [53], hESCs were differentiated as monolayers in the presence of 100 ng/ml activin A, Wnt3a, and low levels of serum. Because our aim was to achieve differentiation in a fully defined system, we did not formally assess the effects of serum. In addition, pilot experiments indicated that inclusion of Wnt3a was not required (data not shown). Instead, our heatmap analysis suggested that low concentrations of BMP4 were also advantageous, consistent with more recent protocols used for the generation of other endodermal derivatives from hESCs [27, 55–58]. In its first iteration, our method included BSA as the source of albumin. Under these conditions, the frequency of NKX2.1-GFP+ cells obtained was workable but inconsistent (0.5%–30%). When recombinant albumin was substituted for BSA, we found it necessary to include FGF1 to obtain reliable differentiation outcomes. This finding parallels our previous observations surrounding the generation of mouse ESC (mESC)-derived pancreatic endoderm, in which the same substitution (Albucult for BSA) revealed a requirement for FGF2 [59]. In the current study, FGF1 was found to promote respiratory endoderm formation, whereas the effects of FGF2 addition were variable. However, consistent with the recent report of Mou et al. [3], we found that NKX2.1 was robustly induced without the need for inhibiting BMP and TGFβ signaling, a condition suggested to be necessary for anteriorizing embryonic stem cell (ESC)-derived foregut endoderm and included in a number of recently published protocols for generating RECs from PSCs in vitro [2, 4, 25, 60, 61].

Gene expression analysis revealed that the cells at differentiation day 10 expressed markers of anterior ventral foregut endoderm but did not express genes associated with later stages of respiratory epithelium. Nevertheless, low-level expression of some markers of ciliated cells (SOX17 and FOXJ1) was observed, suggesting that tracheal or goblet cell progenitors may have been present within the NKX2.1+ population [62].
Unlike mESC-derived NNX2.1+ cells reported by Longmire et al. [60], human NNX2.1+ cells generated in our study did not attach nor proliferate on gelatin-coated plastic. Instead, we found that embedding aggregates of these cells in Matrigel provided an environment that supported their survival and proliferation. Nevertheless, purified NNX2.1-GFP+ cells cultured in vitro, in the presence or absence of Matrigel, rapidly lost NNX2.1 expression. Furthermore, we were unable to identify a growth factor combination that sustained NNX2.1-GFP expression. For example, GFP+ reaggregates exposed to FGF10 and KGF developed into GFP+ epithelial-like structures that lacked respiratory markers (not shown) but expressed genes associated with esophageal (SOX2+) and intestinal (CDX2+) cell types. This result suggests that the NNX2.1-expressing cells generated in our protocol may require additional cues to maintain their ventral phenotype and to undergo continuing respiratory differentiation. Interestingly, treatment of NNX2.1+ aggregates with FGF2, which supports the growth of purified mESC-derived respiratory endoderm [60], resulted in rapid loss of NNX2.1-GFP expression and the emergence and proliferation of a population with fibroblast-like appearance (data not shown).

Despite the observation that NNX2.1+ cells cultured in vitro rapidly lost their identity, the presence of respiratory epithelia in renal capsule grafts of day 7 EBs demonstrated that respiratory progenitors were being specified under these conditions (Fig. 3). Furthermore, we showed that day 10 purified NNX2.1+ cells were able to give rise to respiratory epithelia (Fig. 4) containing a variety of different lung cells when cotransplanted with mouse fetal lung tissue. Experiments in the mouse [7, 60, 63, 64] demonstrated that NNX2.1 marks one of two cell populations in human ventral foregut endoderm that can give rise to the respiratory epithelia. In particular, Longmire et al. [60] showed that mouse ESC-derived Nkx2.1+ cells could give rise to both respiratory and thyroid epithelia, with the latter marked by expression of PAX8. Although expression of this marker was not observed in our microarray data (nor by qPCR; data not shown), further investigation may be required to establish whether the human NNX2.1+ cells generated from our protocol could be instructed to give rise to cells with a thyroid phenotype given the correct environment, such as coagrafting together with embryonic thyroid mesenchyme.

A major impetus for generating RECs from pluripotent stem cells has been the potential to use these cells for the study of human conditions such as cystic fibrosis, where mutations in the CFTR gene result in defective chloride handling (reviewed in [65]). Recent work by a number of groups raises the possibility of using induced PSC-derived CF RECs as a substrate to identify drugs that could potentially correct misfolding that is molecular consequence of the great majority of CF mutations [3, 4, 61, 66]. In this study, we explored a different use of in vitro derived RECs; namely, their potential to act as a host for human respiratory virus HRV1b. NNX2.1+ REC progenitors proved permissive for viral infection and replication and responded to infection by upregulating expression of inflammatory cytokines, mirroring the response of primary airway epithelial cells [49]. However, the efficiency of viral replication in our REC progenitor population appears to be significantly lower than that observed for primary airway epithelial cells (S.M. Stick, A. Kicic, unpublished observations), perhaps underlining the importance of developing methods that promote the later stages of REC differentiation. Nevertheless, combined with recent advances in deriving more differentiated respiratory lineage cells from hPSCs [2–4], our results raise the prospect of using PSC systems to probe key events during the HRV infection of human RECs.

In vitro derived RECs would have a number of advantages over using primary patient-derived epithelial cells. First and foremost is that they would permit the development of a single standard that could serve as a reference point for those interested in HRV-REC interactions. In addition, PSCs can be readily genetically manipulated, making it possible to generate genetically matched controls that would enable the impact of single variables (such as the status of cystic fibrosis mutations) to be assessed. Furthermore, genetic manipulation also opens up the possibility of probing the function of cellular genes during the viral life cycle. Lastly, recent history shows that human PSC differentiation protocols can evolve quickly, to the point where large numbers of uniform populations can be generated in vitro. In this context, the availability of large numbers of highly similar cells would make it feasible to examine the kinetics of viral infection using similarly large numbers of different viral isolates.

**Conclusion**

We have developed a protocol for the generation of human respiratory progenitors that can serve as a host for the replication of the respiratory pathogen HRV1b. Effective antiviral drugs against HRV have not yet been developed, in part because of the vast number of distinct serotypes of HRV and the lack of appropriate models with which to test compounds and elucidate disease mechanisms. We hypothesize that PSC-derived RECs represent an accessible pathway for generating new knowledge concerning HRV infection and for the development new therapeutics that may curtail the progress of this common respiratory infection.

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**Author Contributions**

R.A.J., C.H., and K.-M.L.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; S.M.L., A.L.G., S.J.M., T.L., and E.S.N.: collection and/or assembly of data, manuscript writing, final approval of manuscript; A.K., A.G., A.G.E., and E.G.S.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; S.M.S. and A.T.: conception and design, manuscript writing, final approval of manuscript.

**Disclosure of Potential Conflicts of Interest**

The authors indicated no potential conflicts of interest.
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