Efficient Generation of Lung Progenitor Cells From Human Pluripotent Stem Cells

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INTRODUCTION

Respiratory diseases are leading causes of death in the world with five of the 30 most common causes of death being involved with the lung[1]. The use of animal and primary airway models has advanced our understanding of lung development and diseases. Although the use of primary human lung cells is ideal because of their physiological relevance, they often fall short on accessibility, cell life span, and their utility in gene editing. The generation of functional proximal and distal airway epithelial cells from human pluripotent stem cells (hPSCs) provides a novel tool to model respiratory diseases and lung development in vitro, and facilitates future drug development as well as autologous cell-based or gene therapies.

In the lung field, NKX2.1 is known to be an important transcription factor, as it is the earliest progenitor marker of lung lineage commitment; however, PSC-derived NKX2.1$^+$ cells may not be lung-lineage specific, as NKX2.1 is not exclusively expressed in the lung but is also expressed at an early stage of thyroid and ventral forebrain development (Figure 1). Nevertheless, purifying lung progenitors by using fluorescence-activated cell sorting (FACS) for intracellular progenitor marker NKX2.1 has limited use, which led to the discovery of another lung-specific marker called carboxypeptidase M (CPM) by Gotoh et al.[2] Importantly, CPM is located at the cell surface, can be utilized for live cell sorting and is highly co-expressed with NKX2.1.

Currently, there are several protocols for generating lung progenitors from hPSCs, but with varying efficiency and lack of robustness across multiple cell lines. To standardize the generation of hPSC-derived airway organoids, we developed STEMdiff™ Lung Progenitor Kit, a serum-free medium kit for the efficient differentiation of human embryonic stem (ES) and induced pluripotent stem (iPS) cell lines into lung progenitor cells. In summary, STEMdiff™ Lung Progenitor Kit supports efficient generation of bipotent lung progenitors and is reproducible across multiple hPSC lines. Cells differentiated using this optimized medium kit and protocol express key markers, including NKX2.1 and its surrogate marker CPM, and will provide researchers with a standardized tool to differentiate these lung progenitors into more mature lung cells, using published protocols for studying lung development and respiratory disorders.

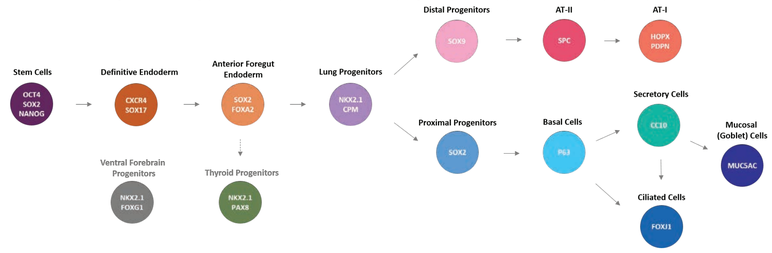

FIGURE 1. Schematic of Different Stages in Lung Development from Human Stem Cells to Specific Airway Epithelial Cells, along with NKX2.1-Expressing Non-Lung Cell Types

Proximal or distal lung cells are generated by sequential lineage commitment of stem cells to endoderm, then into anterior foregut endoderm, which further differentiates into bipotent lung progenitors. Typical markers of each individual stage of development are featured in circles. During branching morphogenesis, the developing proximal-distal axis can be distinguished by SOX2 expression in the proximal endoderm progenitor lineage and SOX9 expression in the distal endoderm progenitor lineage. Upon further maturation, proximal progenitors will become P63$^+$ basal cells, which are the somatic stem cells of the large and small airway, while distal progenitors will become SPC$^+$ alveolar type 2 (AT-II) cells, the somatic stem cells of the alveolar region, that can self-renew upon injury and differentiate into alveolar type 1 (AT-I) cells.

METHODS

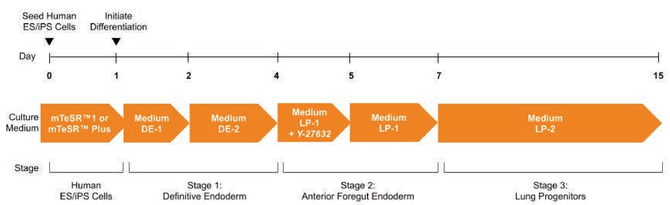

FIGURE 2. Overview of STEMdiff™ Lung Progenitor Kit Workflow

Human ES and iPS cell lines, previously maintained in mTeSR™1 or mTeSR™ Plus, were seeded into Corning® Matrigel®-coated 96- or 24-well plates to be taken through a simple three-stage process to generate lung progenitor cells. On Day 1, differentiation is initiated with Medium DE-1 (STEMdiff™ Endoderm Basal Medium containing Supplement MR and Supplement CJ). Subsequently, on day 2 and 3, the medium is changed to Medium DE-2 (STEMdiff™ Endoderm Basal Medium containing Supplement CJ) for definitive endoderm patterning. On Day 4, to initiate anterior foregut endoderm patterning, the endoderm monolayer is passaged in Medium LP-1 (STEMdiff™ Lung Basal Medium, Lung Supplement (10X), and Supplement 1) and Y-27632. Finally, on Day 7, the cells are differentiated into the lung progenitor stage using Medium LP-2 (STEMdiff™ Lung Basal Medium, Lung Supplement (10X), and Supplement 2).

RESULTS

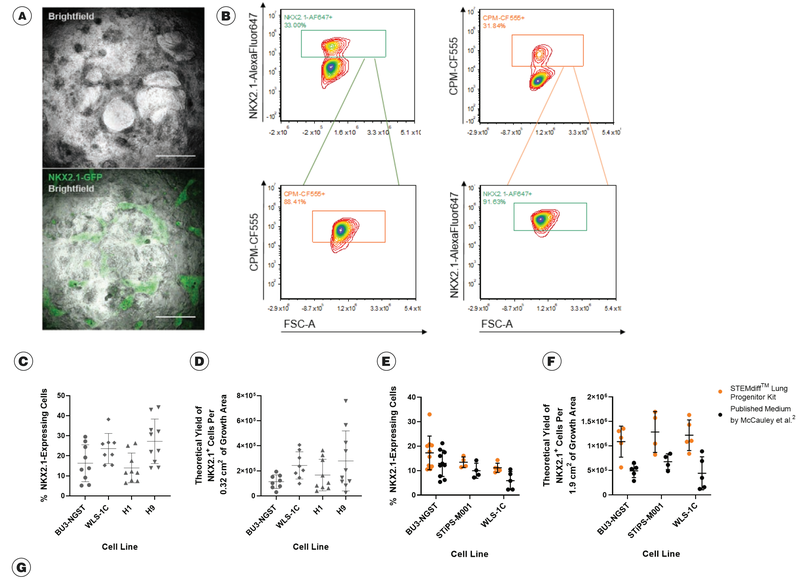

FIGURE 3. Efficient Differentiation of hPSCs into NKX2.1$^+$ /CPM$^+$ Lung Progenitor Cells Using STEMdiff™ Lung Progenitor Kit

(A) Bright-field microscopy of Day 15 lung progenitors derived from H9 ES cells (top) and live cell fluorescence microscopy of Day 15 lung progenitors derived from BU3-NGST (bottom), a fluorescent reporter iPS cell line provided by Darrell Kotton, Boston University. This reporter line has GFP and tdTomato inserted at the endogenous NKX2.1 and SPC loci, respectively. Scale bars = 1000 µm. **(B)** Flow cytometry analysis of Day 15 lung progenitors indicating co-expression of lung progenitor marker NKX2.1 and its surrogate cell surface marker CPM. **(C)** Cumulative quantitative data for NKX2.1 expression of Day 15 lung progenitors generated from multiple iPS (BU3-NGST and WLS-1C) and ES (H1 and H9) cell lines (mean ± SD; n=8 - 10 per cell line in triplicate). **(D)** Cumulative quantitative data for theoretical NKX2.1$^+$ cell yield in multiple differentiated ES and iPS cell lines per well (0.32 cm^2 growth area) of a 96-well plate from Stage 1, if all cells were plated during the midpoint passage on Day 4 of protocol to generate Day 15 lung progenitors (mean ± SD; n=8 - 10 per cell line in triplicate). **(E)** Quantitative data for NKX2.1 expression and **(F)** theoretical NKX2.1 cell yield on Day 15, generated from multiple iPS (BU3-NGST, STiPS-M001, and WLS-1C) cell lines using either STEMdiff™ Lung Progenitor Kit or homemade medium as published by McCauley et al.[3] (mean ± SD; n ≥ 4 per cell line in duplicate). **(G)** Fluorescent immunocytochemistry analysis of BU3-NGST iPS cells differentiated for 15 days co-express lung progenitor marker NKX2.1 and its surrogate cell surface marker CPM along with epithelial marker EPCAM. A CPM cell sorting strategy enables purification of lung progenitors for downstream applications and further differentiation into region-specific lung epithelial cells. Scale bars = 50 µm.

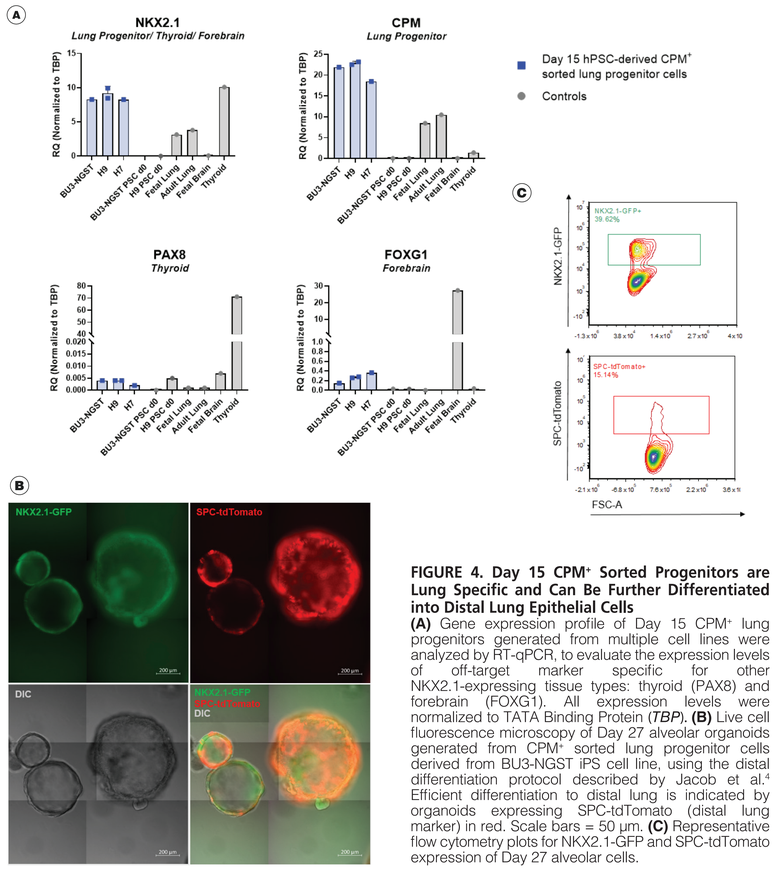

FIGURE 4. Day 15 CPM$^+$ Sorted Progenitors are Lung Specific and Can Be Further Differentiated into Distal Lung Epithelial Cells

(A) Gene expression profile of Day 15 CPM$^+$ lung progenitors generated from multiple cell lines were analyzed by RT-qPCR, to evaluate the expression levels of off-target marker specific for other NKX2.1-expressing tissue types: thyroid (PAX8) and forebrain (FOXG1). All expression levels were normalized to TATA Binding Protein (*TBP*). **(B)** Live cell fluorescence microscopy of Day 27 alveolar organoids generated from CPM$^+$ sorted lung progenitor cells derived from BU3-NGST iPS cell line, using the distal differentiation protocol described by Jacob et al.[4] Efficient differentiation to distal lung is indicated by organoids expressing SPC-tdTomato (distal lung marker) in red. Scale bars = 50 µm. **(C)** Representative flow cytometry plots for NKX2.1-GFP and SPC-tdTomato expression of Day 27 alveolar cells.

Summary

- STEMdiff™ Lung Progenitor Kit promotes efficient and reproducible generation of NKX2.1$^+$/CPM$^+$ lung progenitor cells across multiple ES and iPS cell lines
- NKX2.1$^+$/CPM$^+$ progenitors generated using STEMdiff™ Lung Progenitor Kit are lung-lineage specific and exhibit low expression levels of off-target tissue lineages such as thyroid and forebrain
- Cells generated using STEMdiff™ Lung Progenitor Kit are capable of maturing toward SPC-expressing alveolar cells following a published protocol
- STEMdiff™ Lung Progenitor Kit consists of serum-free media and supplements with a simple three-stage differentiation protocol

References
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2. Gotoh S et al. (2014) Stem Cell Reports 3(3): 394–403.
3. McCauley KB et al. (2017) Cell Stem Cell 20(6): 844–57.e6.
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