A Reproducible Protocol for the Generation of Branching Lung Organoids from Human Pluripotent Stem Cells

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INTRODUCTION

The ability to recapitulate lung development in vitro from human pluripotent stem cells (hPSCs) enables the study of human lung development, as well as the advancement of innovative strategies in respiratory disease modeling, drug discovery, and regenerative cell-based or gene therapies. While current protocols for the derivation of lung organoids from hPSCs are highly variable, their approach is to sequentially follow the lung developmental stages in vivo. During the embryonic stage, the lung bud is formed in the foregut endoderm and the hallmark gene for lung progenitors, NKX2.1, is expressed on the ventral side of these endodermal cells. Branching morphogenesis begins during the pseudoglandular stage and marks the establishment of the proximal-distal axis, with regionalized expression of proximal marker SOX2 and distal marker SOX9. Each region gives rise to other differentiated cell types during the canalicular and saccular stages, with alveoli forming and maturing by the alveolar stage. To standardize the generation of lung organoids, STEMdiff™ Branching Lung Organoid Kit offers an efficient and reproducible protocol for the generation of lung organoids that express key lung markers that align with developmental patterning.

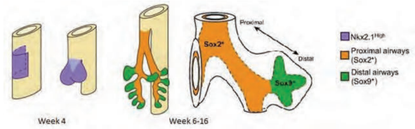


FIGURE 1. Schematic Representation of Key Events During Lung Development
Adapted from Herriges & Morrisey.[1]

METHODS

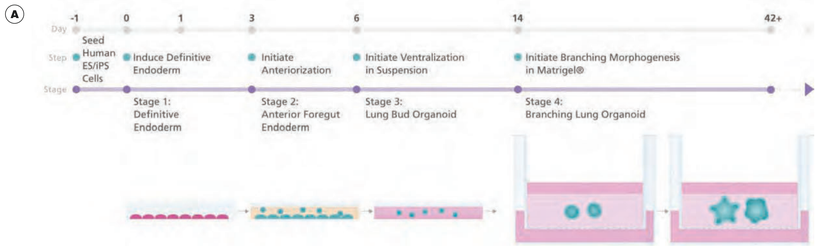


FIGURE 2. Generation of Branching Lung Organoids using STEMdiff™ Branching Lung Organoid Kit
(A) Protocol schematic. **(B)** Representative morphologies observed at each stage.

Human embryonic stem (ES) and induced pluripotent stem (iPS) cell lines were cultured in mTeSR™1 or mTeSR™ Plus for 7 days prior to clump passaging and seeding into a 24-well plate at 3000 clumps/well. Using STEMdiff™ Branching Lung Organoid Kit, the cells underwent four stages of differentiation. In Stage 1, definitive endoderm was induced, followed by anteriorization in Stage 2 for the generation of anterior foregut endoderm buds. Excess buds were cryopreserved in CryoStor® CS10 for later use, while the remaining were collected into suspension culture for Stage 3 to initiate ventralization and generation of lung bud organoids. The lung bud organoids were embedded into a Matrigel® sandwich for Stage 4, where they underwent branching morphogenesis over the course of 28 days. Remaining branching lung organoids were cultured for at least 105 days using STEMdiff™ Branching Lung Organoid Maturation Kit.

RESULTS

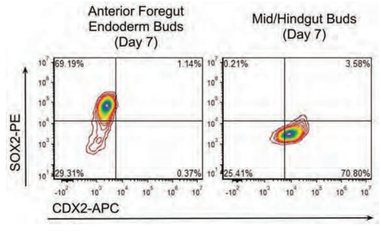
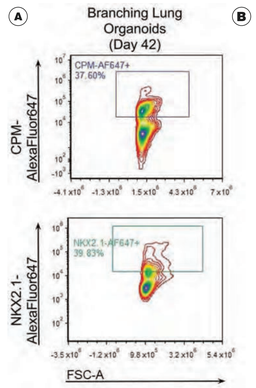


FIGURE 3. STEMdiff™ Branching Lung Organoid Kit Generates Self-Releasing Buds that are Highly Enriched for Anterior Foregut Endoderm Marker SOX2
Anterior foregut endoderm buds generated at the end of Stage 2 (Day 7) demonstrate high expression of anterior foregut marker SOX2, and absence of mid-/hindgut marker CDX2, measured by flow cytometry. Biological negative control of H9 ES cells differentiated to mid-/hindgut were simultaneously stained with antibodies against SOX2 and CDX2.



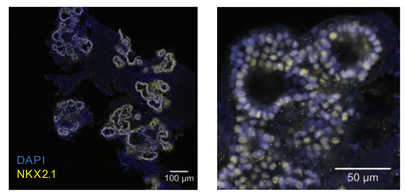


FIGURE 4. Branching Lung Organoids Generated from STEMdiff™ Branching Lung Organoid Kit Express Lung Progenitor Markers
(A) Flow cytometric analysis showing cells from branching lung organoids express NKX2.1 and its surrogate cell surface marker CPM on day 42. **(B)** Branching lung organoids express lung progenitor marker NKX2.1 throughout their branching structures, confirmed through immunohistochemistry on day 42.

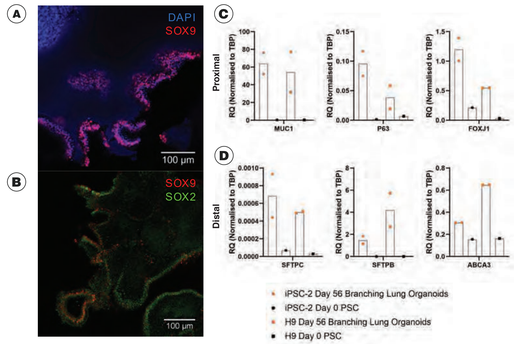


FIGURE 5. STEMdiff™ Branching Lung Organoid Kit Directs Differentiation Along the Proximal-Distal Axis
(A) Organoids derived from iPSC-2 undergo proximodistal differentiation by differential expression of SOX9 and **(B)** SOX2, shown through immunohistochemistry on day 42. **(C)** Branching lung organoids express proximal airway genes for mucous-secreting, basal, and ciliated cells by MUC1, P63, and FOXJ1 expression, respectively, and **(D)** distal airway genes associated with alveolar type II cells. Gene expression was quantified through RT-qPCR in one ES (H9) and one iPS (iPSC-2) cell line after 56 days of differentiation. RQ-values are normalized to housekeeping gene TBP.

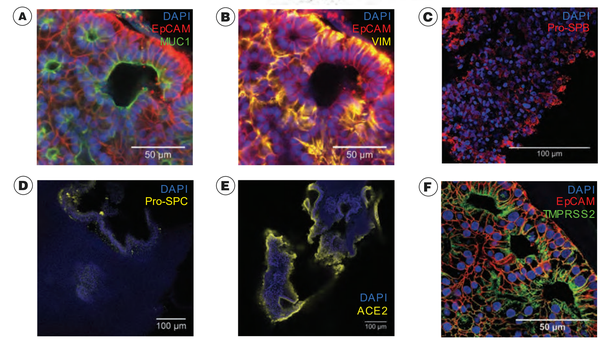


FIGURE 6. STEMdiff™ Branching Lung Organoid Kit Supports Expression of Key Lung and SARS-CoV-2 Markers
(A) The branching structures express epithelial marker EpCAM with luminal expression of MUC1, indicating early proximal airway development through the presence of mucous secretion. **(B)** Organoids are surrounded by VIM-expressing mesenchyme. **(C)** The presence of pro-surfactant protein B (Pro-SPB) and **(D)** pro-surfactant protein C (Pro-SPC) demonstrates the presence of alveolar type II-like cells. **(E)** The organoids express proteins associated with SARS-CoV-2 entry, ACE2, and **(F)** TMPRSS2. Marker expression is visualized on day 42 through immunohistochemistry in four cell lines (iPSC-1, iPSC-2, H1, & H9).

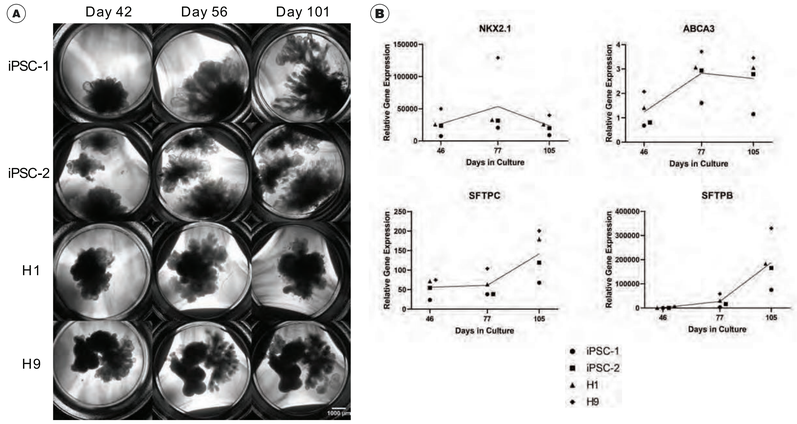


FIGURE 7. Branching Lung Organoids Cultured in STEMdiff™ Branching Lung Organoid Maturation Kit for Extended Periods Express More Mature Lung Markers
(A) Efficient generation of lung organoids with improved branching tip morphology over three different time points (days 42, 56, and 101) across four cell lines (iPSC-1, iPSC-2, H1, & H9). **(B)** The expression levels of more mature distal markers ABCA3, SFTPC, and SFTPB increase over time, while lung progenitor marker NKX2.1 levels remain constant over the course of differentiation. Morphology and gene expression of branching lung organoids cultured up to day 105 were assessed by RT-qPCR. RQ-values were normalized to TBP, then normalized to the gene expression in each undifferentiated hPSC cell line (n ≥ 1). A cycle number of 40 was assigned to genes that had undetectable expression.

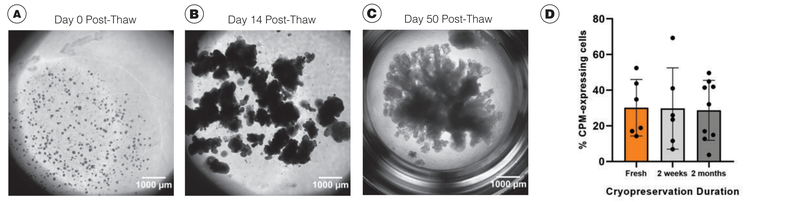


FIGURE 8. STEMdiff™ Branching Lung Organoid Kit Offers Experimental Flexibility Through Cryopreservable Anterior Foregut Endoderm Buds
(A) Previously cryopreserved anterior foregut endoderm buds generated from one ES line (H9) were thawed into a Matrigel® dome. **(B)** After 14 days in the Matrigel® dome, the organoids began to undergo branching morphogenesis and were ready to be transferred to the Matrigel® sandwich. **(C)** Branching lung organoids generated from cryopreserved anterior foregut endoderm buds developed branching airway epithelial structures after culture for 50 days. **(D)** After 2 months of cryopreservation, the resulting branching lung organoids had comparable CPM expression to its fresh control, measured by flow cytometry (mean ± SD, n ≥ 6).

Summary

STEMdiff™ Branching Lung Organoid Kit offers the following:

- A physiologically relevant model system that recapitulates in vivo airway branching morphogenesis and proximodistal specification
- Robust medium that supports efficient differentiation of human ES and iPS cell lines to branching lung organoids
- A tool for modeling infectious diseases such as COVID-19, since branching lung organoids express SARS-CoV-2 entry factors ACE2 and TMPRSS2
- Defined and serum-free medium system optimized to work with mTeSR™1 and mTeSR™ Plus
- Convenient format with a cryopreservable intermediate stage for experimental flexibility

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Reference
1. Herriges M & Morrisey EE. (2014) Development 141(3): 502–13.