Generation of Fully Differentiated and Functional Human Small Airway Cultures

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INTRODUCTION

To date, scientific applications of air-liquid interface (ALI) and organoid cultures have focused primarily on modeling the human bronchial epithelium, but increasing evidence[1,2] implicates the small airway epithelium in the pathogenesis of major lung disorders, such as cystic fibrosis (CF). Pathology of CF starts early in life, leading to progressive obstructions in the small airways caused by mucins,[3] acute and chronic bacterial infections,[4] and neutrophilic and inflammatory responses.[5,6] Due to the regional differences between large and small airways, physiologically relevant small airway research requires specific culture conditions to support in vitro modeling of the distinct biology of the small airway. To address this, we have developed an efficient workflow for the culture of small airway epithelial cells in 2D and 3D formats.

METHODS

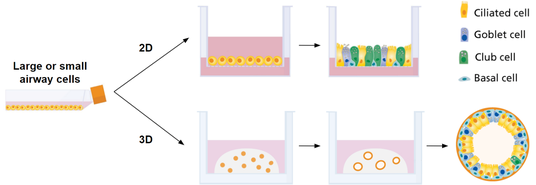

FIGURE 1. Schematic for the 2D and 3D Culture of Large and Small Airway Epithelial Cells.
Commercially available human bronchial and small airway epithelial cells were initiated in a 2D expansion workflow using PneumaCult™-Ex Plus Medium. Passaged single cells were differentiated in ALI cultures using PneumaCult™-ALI Medium and PneumaCult™-ALI-S Medium for large and small airway, respectively. Additionally, cells were used to generate 3D basal cell spheroid cultures with PneumaCult™ Airway Organoid Seeding Medium (AOSM), then differentiated in PneumaCult™ Airway Organoid Differentiation Medium (AODM) and PneumaCult™-ALI-S for large and small airway, respectively.

2D ALI Culture: Large and small airway epithelial cells were expanded in PneumaCult™-Ex Plus until passage 3 (P3), then differentiated at ALI for 28 or 35 days in PneumaCult™-ALI and PneumaCult™-ALI-S, respectively, to achieve mucociliary differentiation. Large and small airway ALI cultures were fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). Barrier function of ALI-differentiated airway cells was assessed by measuring transepithelial electrical resistance (TEER).
3D Organoid Culture: P3 large and small airway epithelial cells were cultured in Corning® Matrigel® domes for 4 - 6 days in PneumaCult™ AOSM. The resulting basal cell spheroids were differentiated as organoids for 21 days in PneumaCult™ AODM and PneumaCult™-ALI-S, respectively.
Differentiation Efficiency: Dissociated ALI and organoid cultures were counted for ciliated cell composition (expressed as a percentage of total cells) using a hemocytometer.
Fluorescence Staining: Briefly, 2D and 3D cultures were processed for whole-mount immunocytochemistry according to STEMCELL Technologies Inc. protocols 'ICC Staining Protocol for Monolayer or Air-Liquid Interface Cultures' and 'Performing Immunocytochemical Staining of Epithelial Organoids', respectively.
Electrophysiology Measurements: Cystic fibrosis transmembrane conductance regulator (CFTR) activity of healthy large and small airway epithelial cells was measured by electrophysiology during the sequential addition of amiloride (epithelial sodium channel [ENaC] inhibition), IBMX + forskolin (CFTR activation), genistein (CFTR potentiation), CFTRinh-172 (CFTR inhibition), and uridine triphosphate (UTP; activation of calcium-activated chloride channels [CaCCs]). Short-circuit current (Isc) was measured by comparing peak CFTR induction and inhibition post-drug addition with resting Isc baseline pre-drug addition.
Forskolin-Induced Swelling Assay: Organoid swelling was induced by treatment with amiloride, forskolin, and genistein and measured at 0 hours and 6 hours after treatment. Quantification of organoid swelling 6 hours post-treatment is based on the percent increase in organoid surface area measurements relative to Time 0.

RESULTS

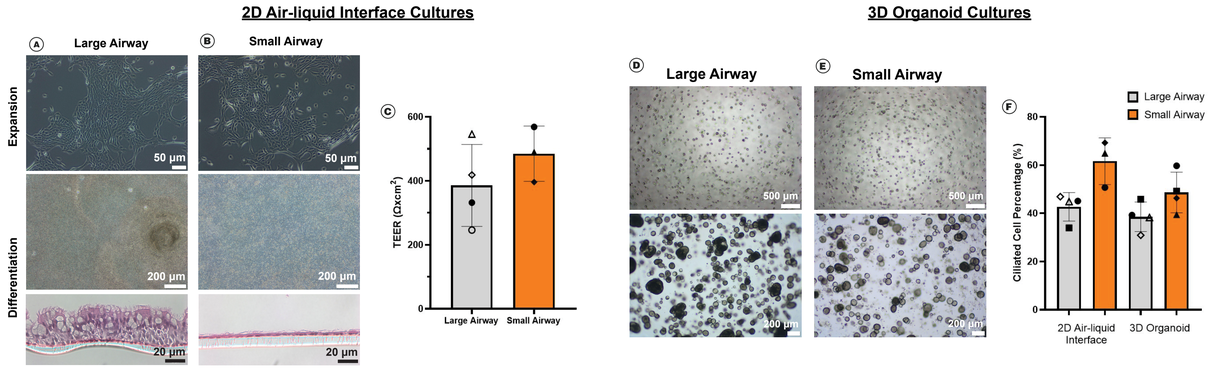

FIGURE 2: 2D and 3D Cultures of Large and Small Airway Cells Displayed Mature Differentiation Characteristics.
Live culture morphology of **(A)** large and **(B)** small airway epithelial cells expanded in PneumaCult™-Ex Plus shown at the end of P3 (top). These cells displayed > 25 cumulative population doublings over the course of 6 and 7 passages, respectively (data not shown). After ALI differentiation, P3 large and small airway cells achieved mucociliary differentiation (middle). Large and small airway ALI cultures also displayed a regional-specific pseudostratified or cuboidal epithelial layer, respectively, confirmed by H&E staining (bottom). **(C)** Large and small airway ALI cultures displayed TEER values of 386 ± 128 Ωxcm^2 (mean ± SD, n = 4) and 485 ± 86 Ωxcm^2 (n = 3), respectively, suggesting appropriate barrier function was achieved in both cell types. For 3D cultures, **(D)** large and **(E)** small airway epithelial cells formed basal cell spheroids (top) after 4 - 6 days of dome culture. After 21 days of differentiation, the resulting organoids displayed visibly beating cilia inside a hollow lumen (bottom). **(F)** In 2D ALI cultures, 43 ± 6% (mean ± SD, n = 4) and 62 ± 10% (n = 3) of large and small airway cells were ciliated, respectively. In 3D organoid cultures, 39 ± 6% (n = 4) and 49 ± 8% (n = 4) of large and small airway cells were ciliated, respectively.

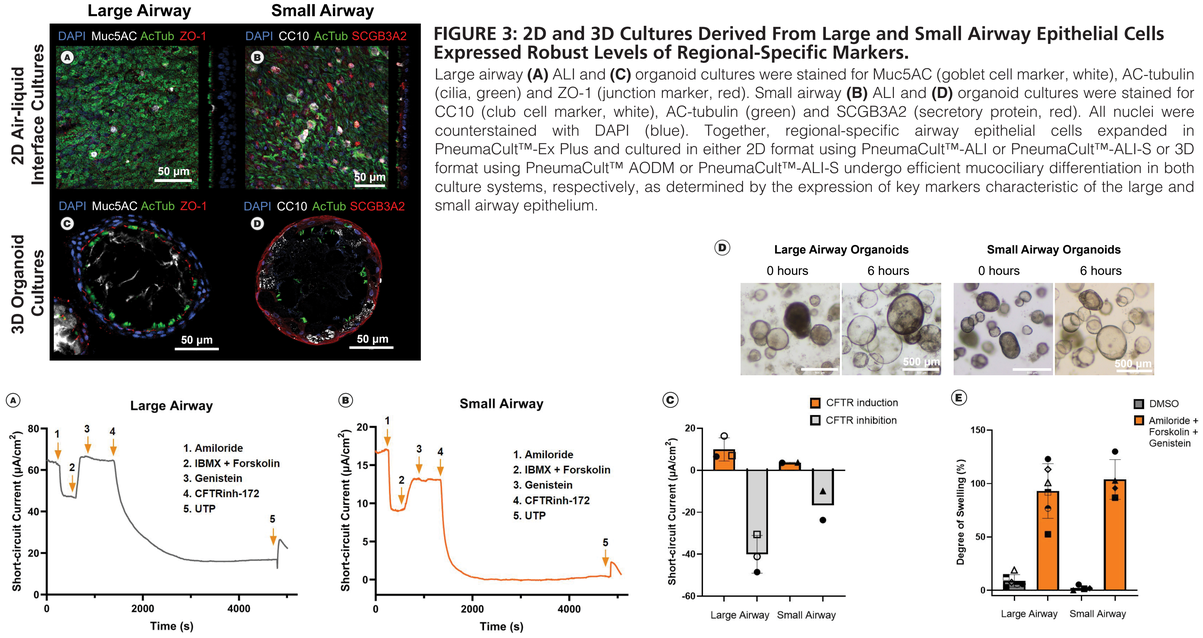

FIGURE 3: 2D and 3D Cultures Derived From Large and Small Airway Epithelial Cells Expressed Robust Levels of Regional-Specific Markers.
Large airway **(A)** ALI and **(C)** organoid cultures were stained for Muc5AC (goblet cell marker, white), AC-tubulin (cilia, green) and ZO-1 (junction marker, red). Small airway **(B)** ALI and **(D)** organoid cultures were stained for CC10 (club cell marker, white), AC-tubulin (green) and SCGB3A2 (secretory protein, red). All nuclei were counterstained with DAPI (blue). Together, regional-specific airway epithelial cells expanded in PneumaCult™-Ex Plus and cultured in either 2D format using PneumaCult™-ALI or PneumaCult™-ALI-S or 3D format using PneumaCult™ AODM or PneumaCult™-ALI-S undergo efficient mucociliary differentiation in both culture systems, respectively, as determined by the expression of key markers characteristic of the large and small airway epithelium.

FIGURE 4: 2D and 3D Cultures Derived From Healthy Large and Small Airway Epithelial Cells Displayed CFTR Activity by Electrophysiology and Forskolin-Induced Swelling.
Representative electrophysiology traces of **(A)** large and **(B)** small airway ALI cultures. Arrows 1 - 5 indicate time points at which compounds were added to induce ENaC inhibition, followed by CFTR activation, potentiation, inhibition, and lastly, activation of CaCCs. **(C)** Characterization of large and small airway ALI culture CFTR activity by short-circuit current (Isc). Large airway cultures exhibited an IBMX + forskolin ΔIsc induction of 10 ± 6 µA/cm^2 (mean ± SD, n = 3) and a CFTRinh-172 ΔIsc inhibition of -40 ± 9 µA/cm^2. Small airway cultures exhibited an induction of 4 µA/cm^2 (n = 2) and an inhibition of -17 µA/cm^2. **(D)** Bright-field images show visible swelling of large and small airway organoids 6 hours after treatment with amiloride, forskolin, and genistein. **(E)** After 6 hours of treatment, large airway organoids increased in surface area by 93 ± 26% (mean ± SD, n = 6) and small airway organoids increased in surface area by 104 ± 19% (n = 4), indicating that healthy 2D and 3D cultures derived from large and small airway epithelial cells expressed functional CFTR proteins.

Summary

- Serum- and BPE-free PneumaCult™-ALI-S Medium is suitable for culturing human small airway epithelial cells as 2D ALI or 3D organoid cultures.
- 2D and 3D cultures of large and small airway epithelial cells generated by the PneumaCult™ media workflow undergo extensive mucociliary differentiation, express regional-specific markers, and exhibit functional CFTR activity, which can be modeled through compound-induced changes in electrophysiology in 2D cultures, as well as forskolin-induced swelling in 3D cultures.
- The complete PneumaCult™ media workflow supports efficient expansion and differentiation of large and small airway cultures in both 2D and 3D formats, resulting in physiologically relevant airway model systems that are suitable to study cystic fibrosis or airway physiology.

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