Sphere Culture of Differentiated HBECs with PneumaCult™-ALI

Introduction

Cultures of primary human airway (nasal, tracheal and bronchial regions) epithelial cells are instrumental in studying basic and applied aspects of airway biology, disease and therapy. While several types of culture systems have been developed, only a subset support the generation of cultures that display the pseudostratified mucociliary phenotype observed in vivo. Among these methods, air-liquid interface (ALI) culture is a commonly used technique that allows for in vitro morphological and functional recapitulation of the in vivo human airway. ALI cultures are formed by plating primary human bronchial epithelial cells (HBECs) onto porous cell culture inserts and allowing the cells to achieve confluence prior to removing the culture medium from the apical surface of the cells (a procedure known as ‘air-lifting’). In conjunction with specialized culture medium, exposing the apical surface of the cells to air triggers their differentiation to a pseudostratified mucociliary epithelium. The use of porous cell culture inserts enables ALI culture to readily support applications such as measuring the transport and metabolism of drugs across the cell layer. However, the requirement of porous culture inserts can limit the application of ALI culture to smaller scale experiments, thus largely precluding high-throughput drug screening of differentiated epithelial cultures. High-throughput culture systems employing other types of epithelial cells, such as prostate1,2 and mammary3 epithelial stem cells, have been achieved using suspension culture of spheres or cellular aggregates. Recently, this method has been adapted to facilitate the culture of differentiated HBECs as spherical aggregates,4-6 thus providing a tool for high-throughput study of the differentiated human airway.

PneumaCult™-ALI, a defined, bovine pituitary extract (BPE)-free medium, was initially developed to support efficient mucociliary differentiation of primary HBECs in ALI culture. PneumaCult™-ALI is now validated to support the generation of differentiated spherical structures of HBECs, known hereafter as bronchospheres, using this sphere culture system.

Results

To enable the formation of bronchospheres, HBECs must be plated on top of a semi-solid medium that prevents their contact with the surface of the cultureware. This can be achieved by coating the tissue culture vessel with a layer of 40% Corning® Matrigel®. Upon solidification of the Matrigel® layer, a single cell suspension of HBECs suspended in 5% Matrigel® in PneumaCult™-ALI Maintenance Medium is plated on top of the semi-solid medium (Figure 1). The HBECs may migrate into the 40% Matrigel® layer or rest on top. HBECs will grow into spheres within approximately one week, continually increasing in size throughout the entire four week culture period. Between week one and two, a lumen will become visible within some of the bronchospheres (Figure 2). By two weeks, the cells within the wall of each bronchosphere will start to differentiate and self-organize to create a pseudostratified epithelium with the apical surface of goblet and ciliated cells pointing into the lumen. By three weeks, visualization under bright field microscopy will reveal beating cilia (Refer to Video 1).

Figure 1. Schematic of Sphere Culture Method Optimized for PneumaCult™-ALI Medium.
Histological analyses of bronchospheres at 28 days post-seeding confirmed that the wall of the sphere is comprised of a pseudostratified epithelium (Figure 3). Hematoxylin and eosin (H&E) staining revealed the presence of goblet cells in the wall of the bronchosphere. The lumen contained a network of mucus, indicating that the goblet cells were capable of secreting mucus into the luminal environment.

A benefit of this protocol over ALI culture is that it allows for the generation of differentiated bronchospheres from later passage HBECs (P4) that may not successfully differentiate in an air-liquid interface culture. Although plating large numbers of HBECs into a single well will create bronchospheres that are not clonal in origin, this sphere culture method may also be amenable to clonal sphere generation by plating many fewer cells per well.

Video 1. Visualization of Bronchospheres.
Light microscope visualization (20X Objective) of two bronchospheres at 21 days of culture. By adjusting the focal plane of the microscope, the video pans through the bronchosphere to reveal beating cilia located in the wall of the bronchosphere and facing towards the lumen. Visit www.stemcell.com/bronchosphere to view video.

Protocol
The instructions below describe an optimized procedure for use with 24-well tissue culture plates. If using alternative cultureware, adjust volumes accordingly. HBECs can be cultured in a serum- and BPE-free expansion medium (e.g. PneumaCult™-Ex, catalog #05008) in T-25 flasks according to the instructions on the Product Information Sheet (PIS). The following procedure should be initiated with HBECs (P1-P4) that are approximately 70-90% confluent in PneumaCult™-Ex.

1. Prepare PneumaCult™-ALI Complete Base Medium by adding 50 mL PneumaCult™-ALI 10X Supplement to 450 mL PneumaCult™-ALI Basal Medium. This Complete Base Medium can be aliquoted and stored at -20°C for up to 6 months. Avoid additional freeze/thaw cycles.
2. Prepare a 200X (96 μg/mL) hydrocortisone (Catalog #07910) and 1 mg/mL Soybean Trypsin Inhibitor (Sigma Catalog #T2884) stock solution (refer to PIS for Catalog #05008 for details).
3. Prepare PneumaCult™-ALI Maintenance Medium by adding the following components per 1 mL PneumaCult™-ALI Complete Base Medium:
   - 10 μL PneumaCult™-ALI Maintenance Supplement (100X)
   - 2 μL hydrocortisone (of a 2 mg/mL stock solution; Catalog #07980)
   - 5 μL hydrocortisone (of a 96 μg/mL stock solution)

   NOTE: Prepare only enough PneumaCult™-ALI Maintenance Medium needed for immediate use.
4. Prepare a sufficient volume of a 40% Matrigel® solution by mixing the following components (require 500 μL/well of a 24-well plate):
   - 25 μL cold Matrigel® (Corning® Catalog #354277)
   - 475 μL cold PneumaCult™-ALI Maintenance Medium
   - 2 μL heparin (of a 2 mg/mL stock solution; Catalog #07980)
5. Aliquot 500 μL 40% Matrigel® solution per well of a 24-well plate.
6. Incubate the plate at 37°C for 30 minutes to allow for the Matrigel® layer to solidify.
7. Prepare a sufficient volume of a 5% Matrigel® solution by mixing the following components (require 500 μL/well of a 24-well plate):
   - 500 μL cold PneumaCult™-ALI Maintenance Medium
   - 200 μL cold Matrigel® (Corning® Catalog #354277)
5. Aliquot 500 μL 40% Matrigel® solution per well of a 24-well plate.
6. Incubate the plate at 37°C in a humidified incubator at 5% CO2 for 30 minutes to allow for the Matrigel® layer to solidify. Steps 7 through 13 can be completed during this 30 minute incubation.
7. Prepare a sufficient volume of a 5% Matrigel® solution by mixing the following components (require 500 μL/well of a 24-well plate):
   - 475 μL cold PneumaCult™-ALI Maintenance Medium
   - 25 μL cold Matrigel® (Corning® Catalog #354277)
8. Warm 5% Matrigel® solution, Ca2+-Mg2+-free PBS, 0.025% Thyrsin-EDTA (1/2 dilution of 0.05% Thyrsin-EDTA [Catalog #07910]) and 1 mg/mL Soybean Trypsin Inhibitor (Sigma Catalog #T6592) at 37°C.
9. Wash each T-25 flask of HBECs twice with 2 mL warm Ca2+/Mg2+-free PBS. Aspirate the Ca2+/Mg2+-free PBS and add 2 mL warm 0.025% Thyrsin-EDTA to each flask.
10. Incubate flask at 37°C for 3 - 5 minutes, until the cells can be dislodged with gentle tapping of the flask. Neutralize the Thyrsin-EDTA by adding an equivalent volume of 1 mg/mL warm Soybean Trypsin Inhibitor to each T-25 flask.
11. Collect the cell suspension into a 15 mL conical tube and centrifuge at 350 x g for 5 minutes.
12. Remove the supernatant and resuspend the cell pellet in 1 - 2 mL warm 5% Matrigel® solution.
13. Perform a viable cell count using Trypan Blue (Catalog #07050) and a hemocytometer.
14. Prepare cells at a concentration of 180,000 cells/mL in 5% Matrigel® solution. Plate 500 μL cell solution per well of a 24-well plate (80,000 cells/well or approximately 50,000 cells/cm2). NOTE: This seeding density has been optimized for use with P4 cells. If using lower passage number cells, the optimal seeding density may be lower.
15. Incubate at 37°C, 5% CO2, in a humidified incubator. Medium changes should be performed three times per week (e.g. Monday/Wednesday/Friday) by gently aspirating the medium above the semi-solid Matrigel® layer and replacing with 500 μL fresh warm 5% Matrigel® solution.
Summary

Here we describe a procedure for recapitulating the human airway in vitro by culturing HBECs as bronchospheres. Primary HBECs seeded on top of a solidified 40% Matrigel® layer are able to form bronchospheres within 2-4 weeks. Each bronchosphere forms a self-organized pseudostratified epithelium that contains goblet cells capable of secreting mucus into the lumen and ciliated cells facing into the lumen. This culture technique provides an alternative method for in vitro human airway modeling that does not require cell culture inserts, thus facilitating high-throughput culture.

Product Information

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<th>PRODUCT</th>
<th>QUANTITY</th>
<th>CATALOG #</th>
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<tbody>
<tr>
<td>PneumaCult™-Ex</td>
<td>500 mL kit</td>
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<td>PneumaCult™-ALI</td>
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<td>0.2% Heparin Sodium Salt in PBS</td>
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<td>Hydrocortisone</td>
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Advantages of Sphere Culture Method over ALI Culture

- Easily generate large numbers of differentiated bronchospheres.
- Successfully differentiate later passage HBECs.
- Method amenable for high-throughput studies.
- May support clonal bronchosphere generation to facilitate enumeration of stem cells.

References