Application of organotypic *in vitro* human cell culture models for research and development of inhalation pharmaceutical formulations targeting the proximal airways

Organotypic human airway tissue models are potentially useful tools for the early phases of inhalation pharmaceutical development programs

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Introduction

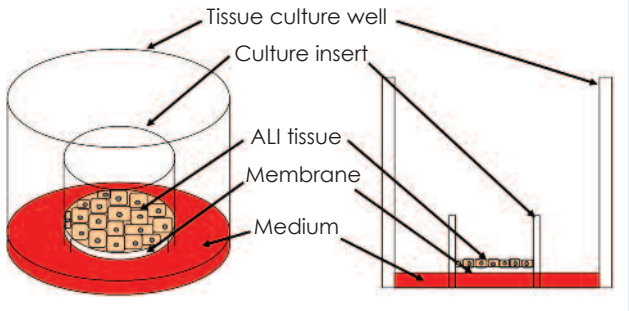
Delivery of pharmaceuticals via inhalation is a potentially useful alternative to intravenous injection for drugs that are unstable or poorly absorbed after oral administration; for chronic disease conditions that require frequent administration; or therapeutics for which the airways are the intended target.[1,2] The proximal airways are common targets for drugs intended to treat inflammatory conditions associated with rhinitis, asthma and chronic bronchitis. In addition, intranasal delivery of vaccines and systemically-targeted drugs has been the focus of significant recent interest.[3] However, inhalation drug delivery via the proximal airways faces challenges in achieving effective doses due to rapidity of mucociliary clearance as well as potential irritation/toxicity issues. These issues are best addressed early in formulation development prior to costly animal studies or clinical trials. This article briefly reviews the application of organotypic *in vitro* tissue culture models for assessing drug disposition, toxicity and efficacy of inhalable formulations intended for administration via the proximal airways. More detailed recent reviews of this topic have been published elsewhere.[4-6]

General features of organotypic *in vitro* human airway models

Culture medium capable of supporting *in vitro* growth of normal (non-cancer-derived) human tracheobronchial epithelial cells (hTBEC) or bronchial epithelial cells (NHBE) was first established by Lechner et. al.[7] These cell cultures have been tremendously useful over the past 30 years for numerous applications in basic human airway biology and toxicology research.[a] However, the traditional methods of submerged cell culture were developed to promote growth and proliferation of these cells and do not reproduce the fully-differentiated phenotype of the *in vivo* airway epithelium. More recently, tissue culture methods have been developed that provide better reproduction of the three-dimensional (3D) organotypic differentiated state of *in vivo* tissues.[8-10] For epithelial tissues of the airway, 3D organotypic differentiation typically involves culturing the epithelial cells on microporous membrane supports at the air-liquid interface (ALI) (Figure 1).[11] Organotypic ALI models provide more realistic, *in vivo*-like structure, barrier properties, metabolic functions and dosing capabilities compared to submerged monolayer cultures.

Figure 1

Air-liquid interface culture on microporous membrane inserts



a A recent Google search on NHBE cells returned 21,800 hits.

Originally developed in academic laboratories,[8-10] organotypic human tissue models are now available as ready-to-use commercial products. The first commercially available ALI airway tissue model, EpiAirway, was introduced by MatTek Corporation (Ashland. MA, USA) in 2000. A second commercial model of differentiated human airway cultures, MucilAir from Epithelix (Geneva, Switzerland), was established in 2006. EpiAirway cultures are produced from normal (i.e. non-cancer-derived, non-transformed) hTBEC cells using serum-free medium, standardized protocols and current Good Manufacturing Practices (cGMP). They may be maintained in culture for at least three months, as necessary to accommodate long-term experiments.[11] The mucociliary, pseudostratified epithelial structure of the EpiAirway tissue model produced from hTBEC is shown in Figure 2. Based on similarities in structure and functional attributes, the cultures are commonly utilized as models of human nasal as well as tracheobronchial epithelium. Models produced from cells isolated exclusively from nasal turbinates or bronchiolar regions are also possible. In addition, the use of normal human cells allows for study of variability in the human population, including individuals with smoking history or diseases such as rhinitis, asthma, COPD or cystic fibrosis.

A key feature of *in vitro* organotypic airway tissue models that is important for inhalation pharmaceutical development applications is the presence of functional tight junction (TJ) complexes between adjacent epithelial cells.[12] These TJs are responsible for producing an *in vivo-like* epithelial barrier that is characteristic of the nasal or tracheobronchial regions. The epithelial cultures should also contain cells with functional beating cilia, as well as non-ciliated Clara and mucus-secreting goblet cells.[b,11] Finally, the cultures should express transporter proteins as well as drug metabolizing enzymes found in human airway epithelium *in vivo*.[11,13]

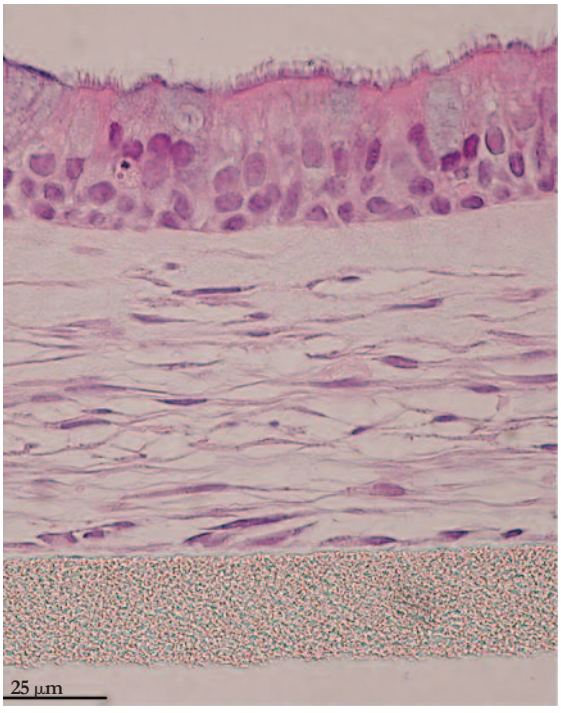
Another key feature of organotypic airway tissue models that is important for inhalation pharmaceutical development studies is the air-liquid interface (ALI) culture format. The ALI format involves culture of the organotypic epithelial tissue on a microporous membrane support, such that the tissue is fed from the basolateral side by culture medium through the pores of the membrane, while the apical epithelial surface possessing beating cilia and active mucus secretion is exposed to the atmosphere (Figure 1). The ALI culture format allows realistic exposure and dosing options compared to cell models that are cultured on solid plastic substrates and submerged in liquid culture medium. Thus, dosing with various types of liquid emulsions, aerosol sprays or dry powder formulations, and determination of drug delivery across the epithelial barrier are readily accomplished.

Air-liquid interface culture models may also be produced from a number of cancer-derived or virally-transformed immortal cell lines such as Calu-3, BEAS-2B, A549, H1299 and H441. These models can also provide useful information in many cases.[6,14] However, models derived from immortal cell lines typically suffer from deficiencies in structure and/or functional activities, including accumulation of genetic changes with extended use, lack of sufficient barrier, cilia or drug metabolizing activity.[14,15] In addition, they will not recapitulate the full spectrum of cell types (i.e. ciliated, basal, goblet and Clara cells) found in the *in vivo* proximal airway epithelium.

Given their ready availability and favorable attributes, normal hTBEC cells represent a preferable choice as a source for production of organotypic human airway models. Nevertheless, immortalized cell lines are often considered as a less expensive option compared to normal human cells. It should be borne in mind, however, that cell costs are a relatively minor fraction of the cost of producing and maintaining organotypic models. Culture media and supplements, microporous membrane culture supports and technical labor may significantly outweigh cell costs when the extended culture time required for producing and maintaining these models is considered. The relative pros and cons of each cell source with respect to the specific goals of the research and development program should be carefully considered when choosing a cell source for organotypic airway model production.

Figure 2

EpiAirway-FT Organotypic *In Vitro* Human Airway Model



b Cultures derived from nasal or tracheal epithelial cells will contain relatively few Clara cells, while the percentage of Clara cells will progressively increase in cultures derived from smaller diameter bronchial regions.

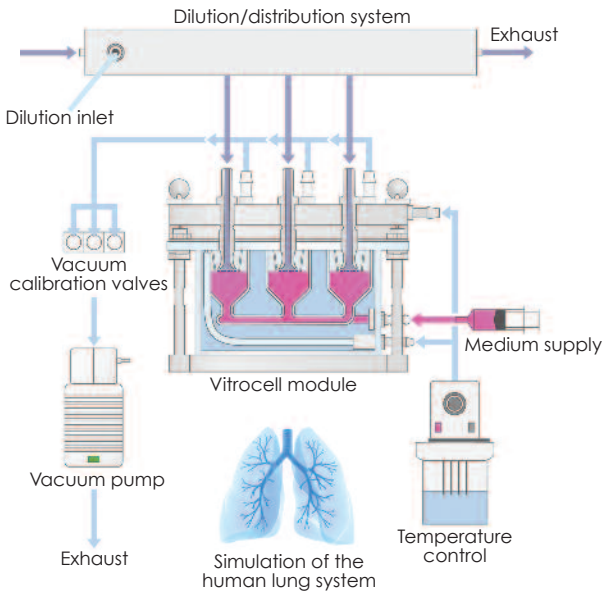
While organotypic *in vitro* tissue models of the proximal airways produced from nasal, tracheal or bronchial epithelial cells are now routinely available, establishment of similar models of the distal airways has proven to be more problematic. Due to difficulties in propagation of normal alveolar type I and type II epithelial cells in culture, organotypic alveolar models must be produced from freshly-isolated human or animal alveolar epithelial cells.[14] The difficulty of preparing freshly-isolated cells from *in vivo* sources has limited the widespread utility of *in vitro* alveolar epithelial tissue models for routine inhalation pharmaceutical development applications. Several immortalized cell lines are commonly utilized as surrogates for alveolar epithelial cells. However, deficiencies in structure and/or function of models produced from cell lines such as the A549 cell line significantly diminish their utility.[6,14,15]

Drug delivery applications

Utilization of *in vitro* organotypic tissue models for drug delivery applications generally involves performing a combination of barrier, drug delivery and toxicity measurements. Drug formulations may be manually applied to the apical surface of the organotypic tissues by means of pipetting or spray devices. Alternatively, more sophisticated exposure systems, such as the VitroCell exposure system (Waldkirch, Germany), may be employed (Figure 3). These systems are designed to accommodate ALI tissue culture formats of various sizes including high-throughput plate formats. Aerosol generators for use with liquids or powder materials, as well as microbalance components for measurement of formulation dosing are available for the Vitrocell exposure system.

Figure 3

Vitrocell Exposure System for Organotypic ALI Tissues



Following application of active pharmaceutical ingredient (API) formulations to the apical (luminal) surface of the *in vitro* organotypic tissue, delivery of the API across the epithelial layer into the basolateral medium compartment (i.e. receiver fluid) is periodically measured. This is accomplished by liquid chromatography/mass spectrometry (LC-MS) or other analytical technique appropriate for the specific analyte being evaluated. The permeation rate is determined by calculating the change in API concentration in the basolateral medium over time. The apparent permeability constant, P_{app} (cm/s), across the epithelial tissue is calculated according to the following equation:

$P_{app} = (V_b/(A \times C_a)) \times (\Delta C_b/\Delta_t)$ where V_b = volume of basolateral medium (cm^3), A = membrane surface area (cm^2), C_a = apical concentration of API at the start of experiment ($mg.cm^{-3}$) and $\Delta C_b/\Delta_t$ = change in concentration of API in basolateral medium over time ($mg.cm^{-3}s^{-1}$).[5,12,16]

The amount of API that partitions into the epithelial cell layer can also be extracted and quantified. Therefore, the ability of prototype formulations to deliver API into or across the proximal airway can be compared and optimized.

It is important to monitor the viability of the organotypic epithelial tissue in conjunction with permeation measurements to make certain that any apparent API permeation is not due to toxic effects and disruption of tissue integrity caused by the API formulation. Tissue viability evaluation may be monitored by a variety of methods. These include the MTT viability assay, a colorimetric assay based on metabolic conversion of the substrate to a purple formazan dye,[17] or assays coupled to cell-death-dependent release of enzymes such as lactate dehydrogenase[18] or adenylate kinase.[19]

The barrier function of organotypic epithelial models is easily monitored by measuring transepithelial electrical resistance (TEER) using an epithelial voltohmmeter equipped with an Endohm electrode chamber or chopstick electrodes (World Precision Instruments, Sarasota, FL). *In vivo* proximal airway epithelium TEER of various species, including human, has been estimated to be in the range of approximately 120-200 Ωcm^2.[20-22] Permeation of hydrophilic molecules rapidly increases as TEER falls below this range. The EpiAirway model typically produces TEER in the range from 300-500 Ωcm^2.[11] An alternative method of monitoring barrier integrity involves addition of fluorescently-labeled (e.g. fluorescein-dextran (FITC)-dextran) or radiolabeled hydrophilic molecules to the apical surface and monitoring the ability of the epithelium to

restrict permeation through the tissue into the basolateral medium. FITC-dextran ≥ 4 kilodaltons should be effectively restricted from permeation across the *in vitro* organotypic airway epithelium.

Experiments utilizing *in vitro* organotypic airway models indicate that the epithelial barrier may be transiently modulated without causing significant epithelial toxicity or cell death. For example, excipients specifically designed to open tight junctions without causing cellular toxicity are being developed to allow transepithelial permeation of large hydrophilic molecules, including peptides and small proteins, via the paracellular pathway.[12,16] Case studies describing the use of the EpiAirway model for development of tight junction modulating formulations have been described.[12,16,23-25] *In vitro* EpiAirway permeation data were found to correlate well with *in vivo* bioavailability data obtained following intranasal dosing in rats.[16] Alternatively, excipients such as cyclodextrins have been developed to facilitate permeation of small hydrophilic models via transcellular pathways.[26,27] *In vitro* human epithelial tissue models such as EpiAirway, together with tissue viability, barrier function and API permeation assays, have demonstrated significant utility for advancing development of novel excipients formulations. Therefore, while the use of organotypic *in vitro* models is at an early stage with respect to inhalable drug delivery applications, these models represent potentially important tools to aid in the development process.

Disease models/Efficacy screening

Common respiratory diseases that represent major targets for inhalation pharmaceutical development include asthma, allergic rhinitis, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). Characteristic features of these diseases include inflammation and excessive mucus production.[28-30] Organotypic *in vitro* human airway tissues can be adapted to model these disease states and may thereby be utilized for early stage development and testing of candidate therapeutics and formulations. For example, an imbalance in T-helper type 2 (Th2) T-cell-derived cytokines is commonly associated with respiratory allergy conditions. Exposure of EpiAirway tissues to Th2 cytokines such as interleukin-13 (IL-13) or interleukin-4 (IL-4) reproduces the epithelial disease state characterized by goblet cell hyperplasia and excessive mucus production (Figure 4).[11] In addition, inflammatory conditions characterized by production of inflammatory signaling molecules can be reproduced by exposure of the *in vitro* tissue model to various stimuli such as tobacco smoke.[31,32] *In vitro* organotypic tissues have also demonstrated utility as a model system for development of antiviral or antibiotic therapeutics.[33-37] Finally, *in vitro* organotypic epithelial tissues models that are produced from asthmatic, COPD and CF-derived cells are also available. While development of *in vitro* asthma and COPD models is still at an early stage, recent reports suggest that the organotypic models can maintain diseased aspects *in vitro*.[32,38] Consequently, these human-disease-derived models may also provide important applications in respiratory disease therapeutics programs.

Summary

In vitro organotypic human airway tissue models are potentially useful tools for the early phases of inhalation pharmaceutical development programs. These *in vitro* tissues reproduce many features of *in vivo* human respiratory epithelium, including 3D epithelial structure, functional cilia, mucus secretion, barrier properties and metabolic activity. They represent a promising approach for *in vitro* evaluation of drug delivery, as well as drug discovery/development studies related to respiratory diseases, such as asthma, rhinitis, COPD, CF and respiratory infectious diseases of viral or bacterial origin.

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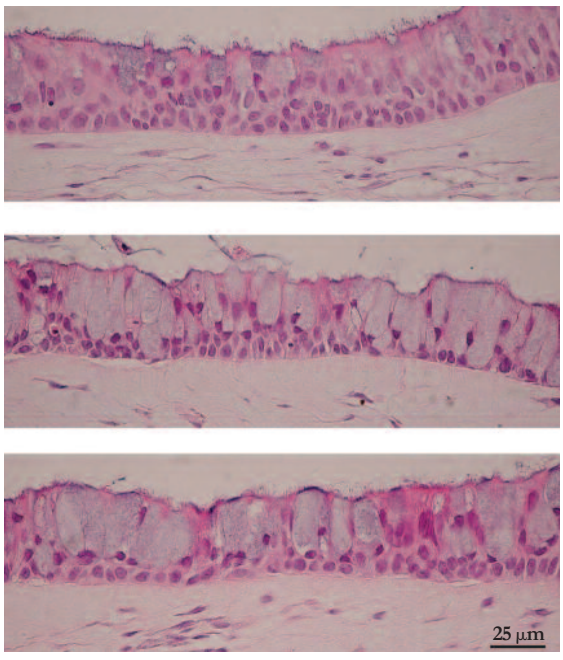
Figure 4

Reproduction of goblet cell hyperplasia in the EpiAirway-FT *In Vitro* Human Respiratory Tissue Model. This condition is characteristic of allergic diseases such as chronic rhinitis or asthma. EpiAirway-FT tissues were exposed to interleukin-13 (IL-13) or interleukin-4 (IL-4) (10 ng/ml) for 6 days. Images show control, IL-13 and IL-4, respectively at a scale of 25 µm.



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