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Airway and alveolar epithelial cells in culture

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Take home message

Airway and alveolar epithelial cells play a key role in health and disease. This review provides an introduction into the principles of epithelial cell culture, emerging developments and foreseeable future applications.

Plain language summary

Epithelial cells line the airways and the alveoli. During inhalation, the airways transport air to the alveoli where the gas exchange occurs, and remove exhaled air. Therefore, these epithelial cells are exposed to the micro-organisms and toxic substances that may be present in inhaled air. The epithelial cells play an important role in preventing infections and injury from exposure to inhaled toxic substances, and are involved in a large variety of lung diseases. Airway and alveolar epithelial cells can be cultured in the laboratory, and this way their function can be studied in detail. In this article, methods for culturing these epithelial cells are explained, including new and future developments.

Abstract (250 words)

Airway and alveolar epithelial cells are daily exposed to large amounts of inhaled air that contains pollutants and pathogens. Local epithelial defence systems are in place to prevent injury, but epithelial cells also play a central role in various lung diseases. The function of these cells in health and disease can be studied in human lung tissue, in animal models and using cell culture. Cell culture offers the important advantage that isolated cells can be exposed under controlled conditions to disease-relevant stimuli, and can be manipulated using a variety of techniques. In this article, we introduce the principles of culturing airway and alveolar epithelial cells, as well as recent new and future developments. Advantages and disadvantages of using cell lines and primary cells isolated from tissue are discussed. In addition, culture of epithelial cells at the physiologically relevant air-liquid interface is described, as well as new culture systems such as lung organoids and the microfluidics lung-on-chip. Finally, genetic editing of cultured cells is discussed. By providing an introduction into epithelial cell culture, we aim to provide a better insight into how these cultures can be used to study the role of epithelial cells in health, disease pathogenesis, drug discovery and evaluation, inhalation toxicology, as well as regenerative medicine.

Why study cultured airway and alveolar epithelial cells?

Epithelial cells that line the airways and the alveoli are the first structural cell targets of inhaled substances. Airway epithelial cells were originally described as providing protection to the underlying tissue, forming an intact barrier protecting the underlying tissue from inhaled substances and by providing mucociliary clearance; alveolar epithelial cells were known for their role in gas exchange and surfactant production. Nowadays, these cells are recognized to have a far wider range of functions (1-3), being key in respiratory host defence through a variety of mechanisms, including metabolism of inhaled toxicants, fluid and ion transport, production of a range of molecules, including antimicrobial peptides, cytokines, chemokines, reactive oxygen and nitrogen intermediates, and lipid mediators. In addition to their role in innate immunity, these cells also link innate and adaptive immunity, for example by transport of polymeric IgM and IgA (airways), and by instructing dendritic cells and innate lymphoid cells

(all zones). The airway epithelium is composed of multiple functionally distinct cell types, including basal, secretory club and goblet, ciliated, neuroendocrine and pulmonary brush cells (also named tuft cells based on their shared features with intestinal tuft cells) and the recently discovered ionocytes (4). The alveolar epithelium comprises alveolar epithelial type I cells (AEC1) and AEC2. AEC1 are attenuated, thin cells that facilitate gas transport, whereas AEC2 act as local progenitor cells, and also produce surfactant and present antigen in their role in host defence and immune regulation (3).

Epithelial cells are both a target for injury as well as a central regulator in disease. Alterations in epithelial cell function are a hallmark of a large variety of acute and chronic lung diseases, including lung cancer. The function of these cells can be studied in human lung tissue, in animal models and in cell culture (5). Cell culture has the advantage that isolated cells can be exposed to disease-relevant stimuli, and can be manipulated by a variety of techniques, including genetic editing. Isolated cell culture was predicated by culture of isolated organs and tissues over a century ago. A modern variant of these techniques is the precision-cut lung slice (PCLS). In the mid 20th century, major developments allowed the culture of isolated cells (mainly for virology studies), whilst techniques to culture epithelial cells began to be introduced in the 1970's. Since then, improved culture techniques have boosted the use of cultured lung epithelial cells for developing disease-relevant models, drug testing, inhalation toxicology, studies on lung development and regenerative medicine.

The aim here is to provide the reader with an introduction and overview on principles of epithelial cell culture, emerging developments and foreseeable future applications. The focus of this review is on human lung epithelium, but many of the fundamentals equally apply to the culture of lung epithelial cells from other species.

How are airway and alveolar epithelial cells cultured?

Sources of epithelial cells include immortalized or tumour cell lines, primary cells isolated from lung tissue, or differentiated pluripotent stem cells; they are cultured adherent to an immobilized surface, often facilitated by coating with extracellular matrix components such as collagens. Since the life span of primary cells is limited, immortalized or tumour-derived cell lines are widely used for convenience and relatively low costs. However, most cell lines share only a limited number of features with epithelial cells *in situ*, and do not show normal differentiation patterns. Primary cells offer the clear advantage that they have not been modified to promote proliferation. In the correct conditions, primary airway epithelial cells show good differentiation into the cell types and profile that constitute the human airway epithelium *in vivo*. In contrast, purified primary AEC2 rapidly differentiate into AEC1 *in vitro*, depending on growth conditions (see below) which complicates primary alveolar epithelial cell studies (6). Primary cells have the additional advantage that cells can be obtained from specific patient populations. Interestingly, some disease-specific features are epithelial cell-intrinsic and persist in culture, providing patient models for genetic airway diseases, such as cystic fibrosis, but also for diseases such as asthma, where cultured airway epithelial cells demonstrate lowered anti-rhinovirus defences (7).

Airway epithelial cell culture. Adult tracheal, bronchial and small airway epithelial cells can be isolated from donor lungs available from transplant programs, from surgically resected tissue, or from bronchial

brushes/biopsies obtained during bronchoscopy. Nasal epithelial cells can be obtained in a minimally invasive way by nasal brushing. Airway epithelial cells are also commercially available as frozen vials or cultures from companies such as Lonza and Epithelix. Epithelial cells are usually dissociated by protease treatment (to detach the cells from each other and the extracellular matrix, and from unwanted cells). Selective media are used to inhibit outgrowth of other cell types, such as fibroblasts. Essential growth factors are included to allow expansion of cells, supporting mesenchymal cells may be used to facilitate growth, and antibiotics are included to prevent microbial overgrowth of the cultures. Most procedures utilise cells submerged in medium and cultured tissue culture plastic that is coated with extracellular matrix, usually collagens (Figure 1). However, culture of primary airway epithelial cells under such submerged conditions results in loss of the differentiated luminal cells (secretory and ciliated cells), and mainly shows a basal cell phenotype. Culturing epithelial cells in Transwells on microporous membranes at an air-liquid interface (Figure 1) promotes airway basal epithelial cell differentiation into a mucociliary epithelial culture that resembles the airway epithelium *in situ* (8), whilst enabling relevant exposure protocols to study airborne substances (9).

Alveolar epithelial cell culture. Normal-appearing tissue following surgery is the main source of adult lung tissue for collection of alveolar epithelial cells. Isolation of AEC2 includes additional steps (e.g. differential adherence, magnetic bead sorting) to separate these AEC2s from other cells such as macrophages and fibroblasts (10, 11). This is critical, as AEC2 can only be maintained in culture for a limited period (3-7 days depending on conditions) before differentiation to AEC1, and, in contrast to airway epithelial cells, cannot be expanded by passaging. Although primary alveolar epithelial cells are also available from commercial suppliers, their value is limited by the discussed constraints in the use of alveolar epithelial cells. Furthermore, for all studies of alveolar epithelial cells *in vitro*, cell characterization and temporal differentiation into AEC1 needs to be monitored to ensure the desired cells (AEC2, AEC1) are being investigated.

Culturing lung epithelial cells in Transwells not only allows culture at the air-liquid interface, but is also used to set-up co-cultures, e.g. with endothelial cells grown on the basal side of the membrane, or on the bottom of the culture plate. In addition, epithelial cells can be co-cultured with immune cells to study their cellular crosstalk. Finally, epithelial cells can be grown on a layer of collagen in which fibroblasts are embedded. The support of airway epithelial cells by fibroblasts has been extensively demonstrated, but a recent study shows that such a design is also beneficial for culturing human AEC2, especially in combination with a Rho-associated protein kinase (ROCK) inhibitor (12, 13).

A variety of techniques are used to characterize epithelial cell cultures based on structure/morphology and expression of unique cell-specific markers, including electron and confocal microscopy, immunostaining and gene expression analysis by RT-PCR. Unique functional characteristics include ciliary beat frequency for airway cells, and surfactant synthesis for AEC2. Novel technology for gene expression analysis, such as single cell RNA sequencing (scRNA-Seq), has identified previously unknown cell types in the airway epithelium, as shown by the recent discovery of a CFTR-expressing, rare cell type, the pulmonary ionocyte (4). In addition, immunochemical methods, RT-PCR, -omics technologies, as well a range of functional assays can be used to study the response of epithelial cell cultures to exposures.

In addition to using epithelial cell cultures of specific patient populations, disease modelling can also be achieved by exposing cultures to mediators. For instance, IL-13 and cigarette smoke exposures have been widely used to mimic features of the airway epithelium in allergic asthma and COPD respectively (14), whereas TGFβ treatment has been used to induce epithelial-mesenchymal transition (EMT) in fibrosis studies in human alveolar and airway epithelial cells (15). An example of the use of human lung epithelial cell culture in studying lung cancer development is provided by a study in which deregulation of SOX2 with simultaneous knockdown of p53 was found to recapitulate features of bronchial dysplasia in early squamous lung cancer (16).

Recent developments in epithelial cell culture

A number of important improvements in epithelial cell culture have recently been introduced. These aim to develop better models to study lung development and repair, disease modelling and inhalation toxicology, and for therapeutic use of cultured epithelial cells in regenerative medicine (9, 17-19). These include for example, the *extended expansion* of cultured primary airway epithelial cells using fibroblast feeder layers combined with ROCK inhibition (20) or strategies using inhibitors of multiple pathways (21, 22), to overcome the senescence associated with passaging epithelial cells. Improved methods for immortalization of epithelial cells represent another advancement in cell culture. Finally, the use of *induced pluripotent stem cells* (iPSC), generated by reprogramming cells from adult tissue, for airway and alveolar epithelial cell culture is an important step (23, 24). In addition, the introduction of organs-on-chips and organoid cultures are recent major advances in the range of culture systems used, and therefore discussed separately.

Organs-on-chips. Organs-on-chips (OOC) technologies use microfluidics, allowing a continuous supply of fresh nutrients and growth factors to cells, and simultaneous removal of waste under flow conditions, as exist *in vivo* (Figure 1). The first lung-on-a-chip model was published in 2010 (25) and demonstrated the feasibility of creating a functional alveolar-capillary interface on a chip. By cyclic stretching the membrane on which the cells are cultured, the impact of the mechanical forces of breathing can be studied in the lung-on-chip (25, 26). The combined use of iPSC and OOC technology (27) and multi-organ chips to build a “body-on-chip”, as illustrated by the three tissue OOC system comprised of liver, heart and lung (28) represents further advances in mimicking lung tissue *in vitro*.

Organoids. Organoids are another new culture technology, popular in studying lung epithelial cell function (recently reviewed (18, 19)) (Figure 1). They are three-dimensional (3D) structures that originate from stem/progenitor cells (from adult or embryonic tissue, or from iPSC) that are embedded in a matrix, such as Matrigel, which self-organise into tissue-like structures containing tissue-specific cells. Lung epithelial organoids have been derived from airway basal cells (29) and from alveolar cells (30), as well as from iPSC-derived airway or alveolar cells (18, 19). Co-cultures with mesenchymal cells are used to study their function as niche cells and possible impairment in disease (31), and co-cultures of T cells with lung cancer organoids have been used to study the induction of tumour-reactive T cells and T cell-mediated tumour killing (32). Conversely, co-culture studies have also revealed that healthy lung epithelium may prevent unwanted mesenchymal activation (33). Recently, long-term culture of airway organoids from biopsies or bronchoalveolar lavage fluid was reported, allowing analysis of CFTR function

by organoid swelling (34). Culturing organoids derived from iPSC or adult stem cells on chips (35) is an example of the possibilities to combine new culture systems.

Genetic and epigenetic editing. Another step forward has been the use of the CRISPR-Cas9 system for genetic editing of cultures (36). This is especially important since siRNA technology is poorly suited to modify gene expression in air-liquid interface cultures because of the duration of the cultures and the limited accessibility of the various cells in differentiated cultures. Another new development is targeted epigenetic editing to control mucin production in cultured lung epithelial cells (37).

Future outlooks

Lung epithelial cell cultures are increasingly used as an alternative to animal experiments. The introduction of novel research tools such as iPSC, organs-on-chips and organoids affords better representation of epithelial cell function *in situ* in cell culture models. Laboratories can now use the multiple available cell sources and culture methods to select the best model for the research question to be addressed. Several more in-depth reviews are available for the interested reader (9, 14, 17-19, 27, 38). Further refinement of controlled methods for exposure to airborne substances are required. For toxicology studies, it is important that the use of culture methods will be accepted by regulatory authorities as a (partial) replacement for animal studies. Finally, applying these promising developments in cell culture methods in personalised regenerative medicine approaches will help to fulfil the promise that regenerative medicine holds for patients with severe and end-stage lung disease.

Figure legend

Figure 1. Various culture systems are used for the culture of airway or alveolar epithelial cells. The cells depicted in this figure are airway epithelial cells, but similar techniques are used for alveolar epithelial cell culture. Cells can be grown as primary cells following their isolation from lung tissue. Classically, cells are cultured on plastic as submerged cultures, but air-liquid interface culture allows differentiation. The lung-on-a-chip combines air-liquid interface exposure with microfluidics technology, whereas 3D organoid culture relies on the self-organisation of cells in a matrix. See text for details.

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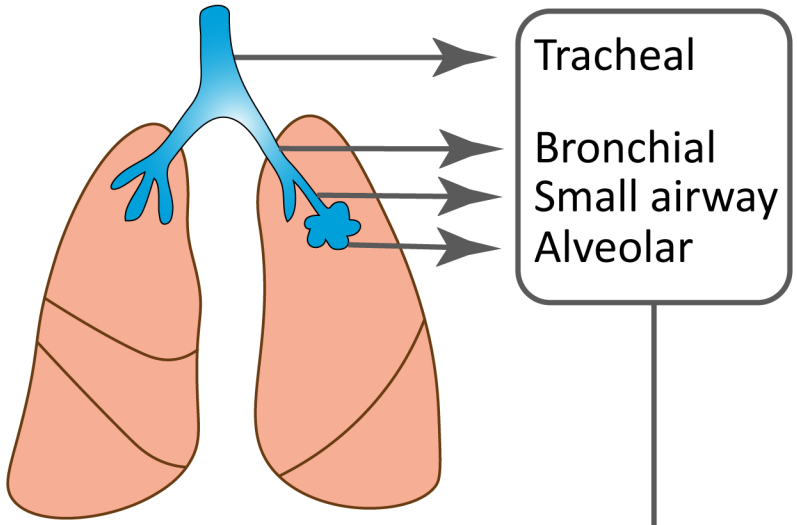
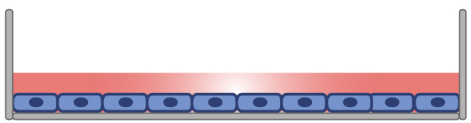
Figure 1



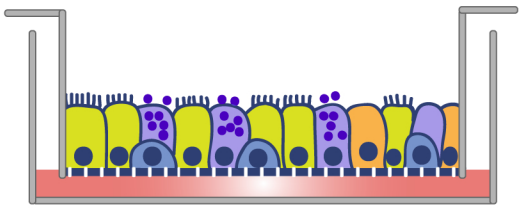
Tracheal

Bronchial
Small airway
Alveolar

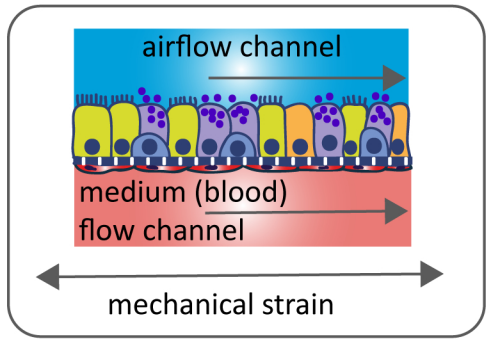
Submerged culture



Air-liquid interface



Lung-on-chip



Organoid