Developing accurate models of the human airways

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Abstract.

Particle delivery to the airways is an attractive prospect for many potential therapeutics, including vaccines. Developing strategies for inhalation of particles provides a targeted, controlled and non-invasive delivery route but, as with all novel therapeutics, *in vitro* and *in vivo* testing are needed prior to clinical use. Whilst advanced vaccine testing demands the use of animal models to address safety issues, the production of robust *in vitro* cellular models would take account of the ethical framework known as the 3Rs (Replacement, Reduction and Refinement of animal use), by permitting initial screening of potential candidates prior to animal use. There is thus a need for relevant, realistic *in vitro* models of the human airways. However, the human respiratory tract is a complex, multi-cellular organ with anatomical regions of differing physiological function and cellular complexity that complicate the development of realistic models. Our laboratory has designed and characterised a multi-cellular model of human airways that takes account of the conditions in the airways and recapitulates many salient features, including the epithelial barrier and mucus secretion. Our pulmonary models recreate many of the obstacles to successful pulmonary delivery of particles and therefore represent a valid test platform for screening compounds and delivery systems.

Developing relevant, accurate models of human airways.

This article aims to consider the features of the human airways that it is possible to develop using *in vitro* cell culture methods and how our laboratory is focussed on increasing the complexity of cellular models. In order to do this, we will firstly consider the anatomy of the normal human airways and which features we are able to effectively mimic in the lab.

Desirable features of a pulmonary model.

The human lung is a complex, multi-cellular organ that is lined by epithelial cells of several different types (Figure 1); these face the lumen where air is entering the body and are supported by the sub-epithelial, parenchymal tissues basally (1).

The epithelial cells of the human respiratory tract fall into three major categories: non-ciliated secretory columnar, ciliated columnar, and basal epithelial cells. Overall, the bronchial epithelium is organised as a pseudostratified cell layer comprised of these three cell types. The secretory cells, such as goblet cells and serous cells, contribute to the secretion of airway mucus and they are present in the apical surface of the pseudostratified epithelial cell layer. In the conducting airways, the goblet cells are most numerous and are the main source of airway mucus. They are characterised by the electron-lucent appearance of secretory granules and morphologically show microvilli expression on the cell surface. Additionally they take part in inflammatory responses by rapidly increasing mucus secretion after exposure to bacterial infection, for example (3, 4). A thin mucus layer lines the epithelium as an innate defence mechanism; this entraps any inhaled particles, other foreign molecules, bacteria and viruses. This mucus layer can also provide a further challenge for particle delivery to the airways, so a realistic model needs to contain the appropriate airways mucins in order to effectively model this component. Serous cells are another type of secretory cell, resembling mucus goblet cells but with a difference in granule content where it is seen to be electron-dense. The proportion of these cells in the airways varies with species, with very low numbers found in the mouse (5), but serous cells have been observed in the small airways of human lung (6).

Ciliated epithelial cells make up 50 % of all epithelial cells in the airways, these reach the airways lumen but still attach to the basement membrane and provide important defence via the

mucociliary escalator. Here, the beating movement of the cilia, the apical hair-like projections, propels the mucus raft towards the pharynx, effectively removing entrapped particles from the airways (7). The ciliated epithelial cells form tight junctions with other columnar epithelial cells in the pseudostratified layer and form desmosomes to allow their attachment to adjacent cells and the basal cell population (1, 8). These cellular junctions form a tight but selective barrier in the paracellular space between epithelial cells, separating the lumen of the airways from the underlying tissue. Tight junctions are located closest to the lumen and form a belt like appearance where adjoining cells are closely connected (8). Tight junctions are therefore responsible for the selectively permeable barrier function of the airways that provides protection from inhaled insults, including pathogens and toxins (9).

Historically, basal cells were thought to be the origin of stem cells in the airway epithelium, giving rise to ciliated and secretory columnar cells in larger airways (1). In addition to their possible progenitor role and attachment of superficial cells to the basement membrane, basal cells also secrete a number of active molecules including cytokines, chemokines, and growth factors. In the pseudostratified epithelium, all cells rest on the basement membrane but basal cells do not reach the lumen and do not contribute to the apical epithelial surface. It is apparent that the basal epithelial cell population forms a necessary, vital component of a pulmonary model given their roles in anchorage of the columnar epithelial cells to the basement membrane, regeneration of the airways epithelium following injury, regulation of inflammation and defence functions (10). The underlying basement membrane or lamina propria is comprised of collagen, elastin and proteoglycans and this forms the basic scaffold to which the basal epithelial cells are attached (11). In the conducting airways, the basement membrane allows direct interaction between the epithelium and the sub-epithelial pulmonary fibroblasts (12).

In addition to the epithelial cells, there are other supporting cell types that need to be taken into account when developing accurate models. The pulmonary fibroblasts were long thought to be simply an inert structural supporting cell, responsible for deposition of the basement membrane components within the lung (13) but growing evidence indicates that pulmonary fibroblasts can directly, actively contribute to pulmonary inflammation (14). Indeed, interstitial fibroblasts in the lungs account for about 40 % of all lung cells (15). Together with the epithelium, the extracellular matrix and neural tissue, the pulmonary fibroblasts make up the mesenchymal trophic unit (16). This unit has been shown to be important during airways growth and branching (17) and is therefore vital for early lung development. However, dysregulation of the cellular components of the mesenchymal trophic unit can have pathological consequences. For example, in asthma, it has been shown that abnormal interactions within the mesenchymal trophic unit leads to increased collagen deposition, cellular proliferation and the damaging fibrosis that is typical of asthmatic airways (18).

In addition, other cells found in the human respiratory system are the immune and inflammatory cells; this includes the alveolar macrophages, neutrophils, eosinophils, mast cells and dendritic cells, all of which can migrate to the airways through the basement membrane to support epithelial cell function and provide protection against inhaled “insults” (7).

This indicates that any useful, realistic model of the human respiratory tract demands inclusion of the structural cells and the supporting cells, and that monitoring changes in the activity and functions of both epithelial and non-epithelial cells can provide powerful information regarding the activation state of the tissue following particle/vaccine delivery to these models.

Cell culture conditions can drive epithelial differentiation

The simplest version of cell culture is under conditions where cells of one defined phenotype are cultured on the base of tissue culture ware; submerged in the appropriate nutrient medium. Submerged cell culture is used in research on airways disease and physiology, but it appears to be losing favour recently as it is obvious that submerged bronchial epithelial cells do not closely mimic the *in vivo* physiology and morphology of normal airways. A more relevant alternative method is to use specialised cell culture inserts, such as Corning's Transwells® that permit culture at the air-liquid interface (ALI), where cells are exposed to the atmosphere apically and remain effectively submerged in medium basally. One human airways cell line that has demonstrated important culture-dependent differences is the Calu-3. These are a well characterised human bronchial cell line derived from an adenocarcinoma, which display characteristics of the serous cells of airway submucosal glands and therefore are extensively used in pulmonary research (19). However, recently, there have been some questions raised over their use as a model of the epithelial cells (20). It is clear that culture of Calu-3 under submerged conditions produces a less suitable model of the tracheobronchial epithelium compared to cultures grown at ALI (21). Several other reports comparing submerged cultures to ALI have reported similar findings; that responses of cells cultured under the two culture methods are often different, and that cytokine expression (22), or the susceptibility of cells to infection (23) appears more physiologically accurate in cells cultured at ALI.

An important feature of culture at ALI is the promotion of a fully differentiated cell population. Airway epithelial cells cultured under submerged conditions are only poorly differentiated and show a squamous phenotype which is not representative of the pseudostratified columnar epithelial cells described above and typical of the airways *in vivo*. Culture of airway epithelial cells at ALI allows mucociliary differentiation, this is a complex process shown to involve cell-matrix and cell-cell interactions, differentiation of serous cells and the establishment of correct ion flow properties but it is yet to be fully elucidated. However, despite the complexity of its origins, the salient features of a differentiated airways epithelium are relatively straightforward to assess and many can be done throughout the culture period without compromising cellular viability or function. Routinely, our laboratory monitors permeability and trans-epithelial electrical resistance (TEER) to assess the barrier properties of the cell layer, immunohistochemical staining for proteins of physiological interest such as zonula occludens 1 (ZO-1; tight junctions) and the cytokeratins (differentiation markers) and finally, we have used scanning electron microscopy on fixed sections to confirm ciliogenesis in epithelial cultures grown at ALI (figure 2).

One further important advantage of culture at ALI is the independent analysis of apical and basal compartments that becomes possible. This reveals polarised secretion of cytokines and gives a vital insight into likely downstream consequences of treatments or interventions. For example, whilst RNA analysis may reveal global increases in cytokine expression, it is only by analysing ALI cultures that it becomes apparent that this is manifest as an enhanced, directed, apical release of the neutrophil chemoattractant, interleukin-8 (24).

These culture-dependent differences produce important distinctions to be taken into account when developing relevant cellular models of the airways, but most of these studies employ mono-cultures of the epithelial component of the airways and we need to increase the cellular complexity of these models if we are to effectively model the cellular complexity of the human airways.

Mono- and co-culture systems for airway related research

ALI cultures of primary cells and transformed cell lines for airway related research mimic, as described above, the *in vivo* morphology and physiology of airway epithelial cells closer than submerged cell cultures. Mono-cultures, employing epithelial cells in isolation, have been extensively used in many areas of research focussing on the airways. For respiratory disease research there are many epithelial cell mono-culture models that all focus on broadening the understanding of lung pathophysiology with different objectives, such as inflammatory responses to the infection with bacteria (25) or bacterial products (26), bacterial adherence to epithelial cells (27) and more general characterisation studies looking at tight junction properties and paracellular integrity (28).

However, *in vivo* the airways are not a simple mono-culture of epithelial cells and instead are a complex and multi-cellular organ, as detailed above, and it has long been known that the different cell types all play their role in tissue homeostasis through direct cell-cell interactions, as well as through autocrine and paracrine communication via secreted growth factors and cytokines (1, 29, 30). Epithelial cell interactions with sub-epithelial fibroblasts have been reported to be important for moderation of cell behaviour, proliferation and differentiation of the epithelium (17). Epithelial wound repair is dependent on epithelial cell activity, but also on epithelial cell interactions with the extracellular matrix and on the cytokine milieu, which is established by the epithelial cells and also other surrounding cells in the airways, such as the fibroblasts, which secrete cytokines and modulate epithelial cell function (31). Much attention has been paid to sub-epithelial fibroblasts in asthma research and certain growth factors and interleukins (IL) have been detected in airways, which derive from epithelial cells as well as fibroblasts, including IL-8, IL-6, hepatocyte growth factor (HGF) and several members of fibroblast growth factors (FGF) (18, 30, 32), further reinforcing the active role of the “supporting” cells in airways functionality and making a case for inclusion of this cell type in a functioning model of the airways.

A relatively simple and straightforward co-culture model that we have employed uses pulmonary fibroblasts underlying the epithelial cells. This is not a particularly new idea, but is novel in that we are using proliferating, normal, human pulmonary fibroblasts in our models to provide a further level of integrity. In contrast, other epithelial-fibroblast co-culture models have placed the fibroblasts in an inert, supporting role. For example, one approach employs mitomycin C-treated fibroblasts in the bottom of a 24-well plate, used as feeder layers (33). This not only prevents normal fibroblast function or response, it also prevents direct cell contact with the epithelial cells and therefore underestimates the contributions of fibroblasts to airways reactivity. There is a vital communication network between epithelial cells and sub-epithelial fibroblasts that is overlooked by models that inactivate the fibroblast component. It has been shown that sub-epithelial fibroblasts establish a suitable environment for human bronchial epithelial cell differentiation (34) and this is supported by the data from our laboratory (figure 3). We have shown that epithelial mono-cultures and epithelial-fibroblast co-cultures secrete MUC5AC (figure 3A), the gel-forming mucin predominantly secreted by goblet cells (35); that this secretion occurs preferentially to the apical compartment of cultures grown at ALI and indeed, no mucin secretion is detectable from fibroblasts in mono-culture or when bronchial epithelial cells are cultured under non-physiological, submerged conditions. We also showed that the barrier function of the co-cultures is maintained in the presence of human pulmonary fibroblasts (figure 3B). Again, fibroblasts in mono-culture do not form a tight barrier, as is expected given their structural role and function in the airways, but we see that epithelial cells alone, or epithelial cells in co-culture with proliferating pulmonary fibroblasts, when cultured at ALI,

will form an electrically resistant barrier, as evidenced by an increased transepithelial resistance, and corresponding decreased permeability, over time.

Increasing the complexity of the models.

It is apparent that inclusion of more cell types in these pulmonary models will allow more precise and representative assessment of the functionality of the airways, in order to permit more accurate screening of likely candidates, be it drugs or vaccines, and also allows us to reduce animal usage. The endothelium plays an important role in inflammation in general and in the airways, circulating inflammatory cells are required to move through the endothelium to access the airways surface in order to facilitate clearance of inhaled pathogens/particulates (36). Activation of endothelial cells can be monitored by assessing the expression of adhesion molecules such as E-selectin, which is rapidly induced by inflammatory stimuli (37). In the airways *in vivo*, the endothelium is orientated such that the basolateral surface of the endothelial cells is in close proximity to the basolateral surface of the epithelial cells; and this creates a challenge *in vitro*. There has been some limited success with the inclusion of endothelial cells into models of the airways, for example, efforts have been made to seed endothelial cells in a Transwell® insert with epithelial cells attached to the underside of the insert (36, 38). Whilst this approach does manage to model the proximity of the cell populations and therefore may be useful for permeability and cell migratory studies, it does not allow culture of the epithelial cells at the ALI and therefore the epithelial cells remain undifferentiated and thus non-representative of the airways *in vivo*.

Whilst the inclusion of a pulmonary endothelial layer in the appropriate orientation to a differentiated airways epithelium still remains elusive, it seems more possible to include inflammatory cells in the models. Of particular relevance in the study of pulmonary delivery are the alveolar macrophages and the dendritic cells (DC). Some co-culture models have been established employing airways epithelial cells with alveolar macrophages or DC and these have been used to investigate the effects of particulate matter exposure (39, 40). However, there are limitations to these models thus far- one co-culture employed submerged culture methods throughout the study and was therefore not recreating a differentiated epithelium (39). The other model used a more sophisticated approach; here monocyte-derived macrophages were added to the apical surface of airways epithelial cell lines, with monocyte-derived DC cultured in the basal compartment (40). There is still an issue over the differentiation of the epithelial cells in this study, however, with monolayers of 10μm observed for the epithelial cell cultures, whereas we measure an average thickness of around 30μm for our pseudostratified, differentiated epithelial mono-cultures. One approach that we are currently developing builds on the existing complexity of our human pulmonary fibroblast and airways epithelial co-culture model, and uses the fully differentiated, mucus secreting, tight epithelial barriers, with the addition of macrophages introduced apically and DC basally. Human pulmonary DC are rare, are extremely difficult to isolate in the absence of stress or activation (41) and even the use of the more accessible monocyte-derived DC can lead to donor variation and limited cell numbers that would impact on the scope of the studies. Thus we use a more convenient and reliable approach in our laboratory. The human THP-1 monocytic cell line is routinely used in our laboratory (42) and can be driven to differentiate to DC or macrophage phenotypes *in vitro*. In our hands, the THP-1 cells are malleable and can become strongly phagocytic for microbial and apoptotic cells; we can exploit this to allow the generation of tolerogenic responses to dying 'self' cells or immunogenic responses that can activate T lymphocytes.

Furthermore, THP-1 derived phagocytes show altered interaction with different liposomal adjuvant formulations, a feature that highlights their use as potential in vitro predictors of in vivo vaccine efficacy (43).

These multi-cellular combinations not only mimic human airways more effectively, but also permit analysis of mixed cellular responses to the delivery of "inhaled" particles (including vaccines) or challenges, such as bacterial infections. We can monitor epithelial function independently of immunogenic potential; we can assess the extent of any airways remodelling; we can also assess the T_{H1}/T_{H2} cytokine balance that potential vaccines have evoked and therefore can use these models, at the very least, as an early screen for cell-mediated versus humoral immunity provoking vaccination strategies.

The need for pulmonary vaccine delivery.

Broadly speaking, there are two streams to pulmonary vaccine research. The first is in developing strategies to deliver a vaccine targeted to a respiratory condition (e.g. tuberculosis; 44). The second is the wider exploitation of the pulmonary system as a convenient portal of entry to the body. Here, for example, intranasal delivery of recombinant HIV-1 vaccines has been shown to enhance mucosal immunity in mice (45). Recently, there have been advances in immunotherapy for lung cancer, the leading cause of cancer deaths. One group has shown that a synthetic peptide vaccine demonstrates significant improvements in overall survival- although this was delivered as sub-cutaneous injection (46). Intranasal delivery of a peptide vaccine for the major cause of respiratory disease in young children, the respiratory syncytial virus (RSV), has shown promise in animal models, but these have yet to be tested in humans (47). Similarly, intranasal immunisation of mice with genetically modified, recombinant influenza virus was shown to drive protective humoral and cellular anti-viral immune responses and was effective even in immunocompromised host animals (48). Pulmonary delivery has proved more successful for human disease control for measles, although here the immune responses are dependent on the formulation, with dry powder eliciting lower immunity than intra-muscular injections (in an animal model) and the age of the population, since clinical trials using nebulised liquid vaccine was shown to be less effective in younger children (49).

However, it is not the purpose of this article to review successes and failures in pulmonary vaccines, as this has been done comprehensively elsewhere (e.g. 50) and instead we shall consider the features that are necessary for effective pulmonary targeting of and where we believe our human pulmonary cell culture models may potentially fit to improve the efficiency of the screening procedure.

Considerations for delivery by the pulmonary route.

Pulmonary drug delivery has been practiced for several centuries, particularly in the widely common (and mostly illegal) practice of inhaling narcotics (51). Desirable formulation characteristics of a pulmonary administered therapeutic agent include stability, ease of handling and dose administration, but there are also various physiological characteristics of the lungs which make pulmonary administration an attractive choice for targeting and delivery. The large total surface area of the airways, which, at approximately 140 m^2 is nearly 40 times more than the external body surface area (52, 53, 54), the relatively low concentration of metabolic enzymes (55) and the highly vascular composition of the alveoli that permits effective gaseous exchange (56, 57) and also allows rapid equilibration of blood and alveolar fluid proteins (58, 59), are all features that allow rapid entry

of inhaled therapeutics to the systemic circulation. Specifically for vaccines for pulmonary diseases, an inhalation route of administration is attractive from the point of view of the patient (no needles required!) and the clinician, since delivery is via the equivalent route of entry as the pathogen. Pulmonary diseases represent a global problem. For example, the World Health Organisation states that pneumonia is responsible for the deaths of 1.1 million children under 5 years old every year. Whilst vaccines for viral and bacterial pulmonary infections exist or are under development (60), these tend to be delivered in the "traditional" method by intra-muscular or sub-cutaneous injection and several have a limited efficacy. Development of an inhaled form of vaccine may improve protective responses and, importantly, may also increase patient compliance (61) and therefore enhance health outcomes overall by promoting "herd immunity".

In addition, for pulmonary vaccine formulations, size does matter! Generally, it is thought that particles with aerodynamic diameter greater than 5 μm mainly deposit by inertial impaction in the upper airways, principally at or near airway bifurcations, where flow velocities are high and change direction sharply. Particles with aerodynamic diameter between 1 and 5 μm are mainly deposited by sedimentation in the lower respiratory tract (i.e., bronchial tree and alveoli), where the air velocity progressively decreases. To reach the alveolar tissue specifically, the aerodynamic diameter of the particles need to be in the range of between 1 and 3 μm. In addition, deposition increases with residence time in the respiratory tract but decreases as the breathing rate increases (62). Below an aerodynamic diameter of 0.5 μm, particles are under Brownian motion, which may result in deposition by diffusion, especially in small airways and alveoli. However, particles of this diameter are mostly exhaled by the expiratory airflow. Overall, for effective particle delivery to the respiratory tract, the recommended aerodynamic size has long been suggested to be between 1 – 5 μm (63, 64). We have shown that developing a bioactive particle with adjuvant properties, within this size range is possible (43). The role of the accessory cells in the airways becomes important in these considerations- whilst development of a vaccine particle of a specific size may permit region-specific delivery to the airways, particle size also impacts on the fate of the particle and subsequent immune response. It has been shown that delivery of 5μm microspheres of encapsulated Hepatitis B surface antigen (HBsAg) elicits significantly higher immune responses than 12 μm microspheres and further *ex vivo* analysis indicated that the smaller microspheres were more effectively taken up by the macrophages (65). We would propose that, whilst our multi-cellular models cannot model the pattern of deposition throughout the entire respiratory tract, inclusion of immune cells in these models will allow investigation of the cellular fate of inhaled vaccine and would tell us which particles/formulations were most effectively taken up by airways macrophages or dendritic cells, with a great potential to reduce animal use.

Despite these recent promising advances, there remain several issues to overcome for successful pulmonary delivery. For example, in order to develop effective immune responses, very small solid or liquid micro-particles (usually between 1 and 5 μm) are required (66), de-aggregation mechanisms are needed to improve the delivery of solid micro-particles to the airway by inhalation devices, a strategy or protection mechanism to avoid degradation by proteases resident in the lungs, and a mechanism to overcome the various protective clearance mechanisms of the respiratory system (67) have to be taken into account. However, it is apparent that most of these obstacles are due to the normal physiology of healthy airways (e.g. the barrier function of the airways, mucus secretion, ciliary activity etc.) and all of which can be present in a differentiated, multi-cellular model. This

means that whilst our accurate, relevant *in vitro* models cannot model deposition *per se*, they will permit screening of delivery systems designed to administer appropriate-sized particles to the normal airways and therefore can enhance and accelerate the likelihood of a successful strategy for therapeutic and prophylactic particle delivery.

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Figure legends.

Figure 1: An illustration of the epithelial structures of the human airways*. a) Airway epithelial cells of the trachea and large bronchi where ciliated and mucus-producing goblet cells predominate with numerous basal cells present to provide anchorage and to act as progenitor cells. b) Airway epithelial cells of the bronchioles where ciliated and non-ciliated secretory epithelial (Clara) cells predominate. Goblet and serous cells decrease distally and are absent in the terminal bronchioles. Adapted from (2).*

Figure 2. Culture of human bronchial epithelial cells at ALI promotes features of a differentiated epithelial cell layer representative of the normal human airways. *A: shows that the permeability of the epithelial layer decreases over time at ALI, demonstrating the production of a functional, semi-permeable barrier under these culture conditions. B: indicates immunohistochemical staining of the apical tight junction protein ZO-1, in cells cultured at ALI. Scale bar is 16 µm. C: Immunohistochemical staining for the cytokeratins, epithelial markers. CK5 defines the basal, regenerative and reparative cell population whilst CK8 is a marker of differentiated epithelium. Importantly both epithelial populations are present after culture of bronchial epithelial cells at ALI. D: Scanning electron micrographs of a confluent Calu-3 mono-culture showing that these cells are covered in cilia-like projections on the apical surface after culture at ALI. This image also shows the cobblestone morphology that typifies airways epithelium. Scale bar is 5 µm.*

Figure 3. The inclusion of normal, human pulmonary fibroblasts in the model of the airways promotes and supports normal airways function. A: *is an image of a dot blot that shows that the apical release of airways mucin (MUC5AC) by epithelial cells is enhanced in the presence of fibroblasts (lanes 1 and 2) compared to mucin release by epithelial cultures alone (lanes 3 and 4) and additionally, that mucin production is specific to culture models employing epithelial cells (lanes 5 and 6 are secretions from mono-cultures of pulmonary fibroblasts).* ***B:*** *demonstrates that the inclusion of fibroblasts in the co-culture model (HPF and Calu-3 Co) does not compromise the development of a tight barrier, indicated by increasing transepithelial electrical resistance (TER) with time.*

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496 The authors declare that they have no conflicts of interest to disclose.

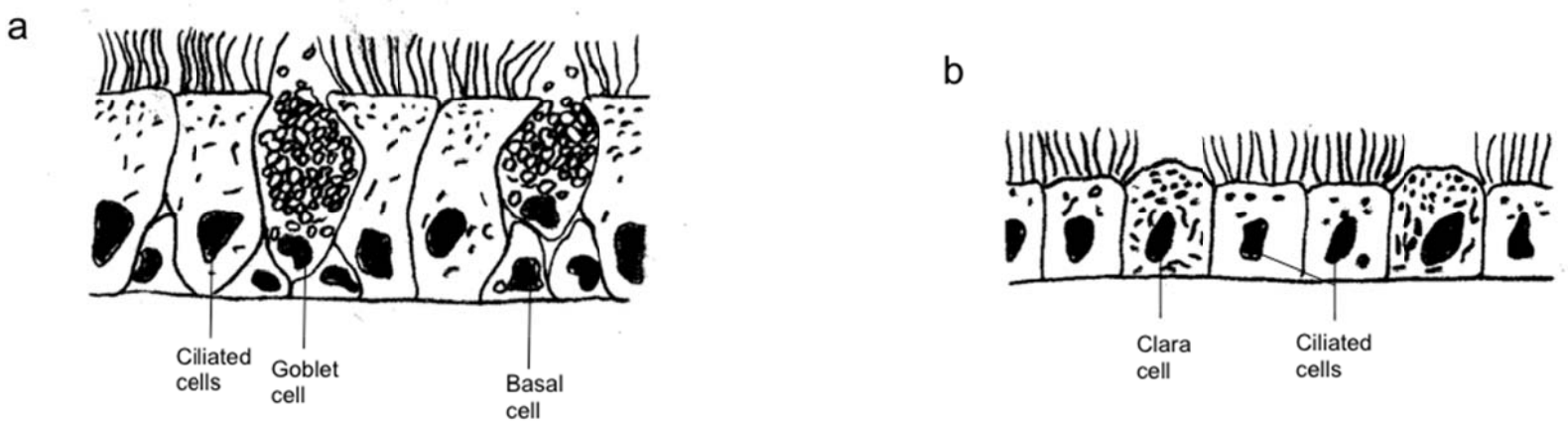
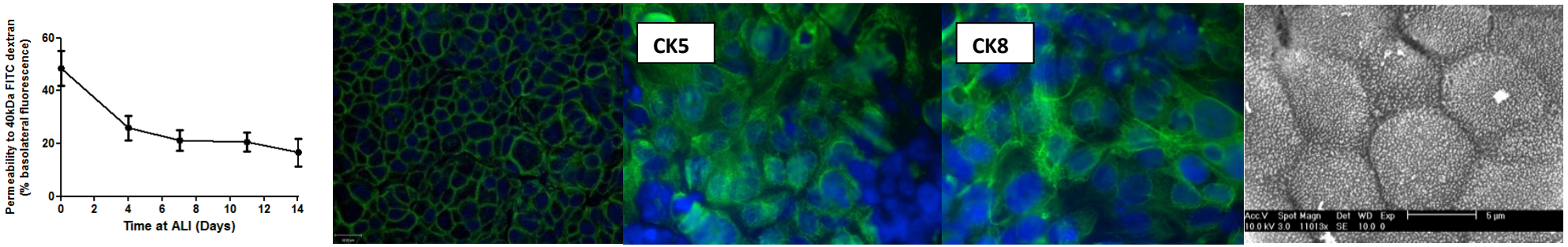
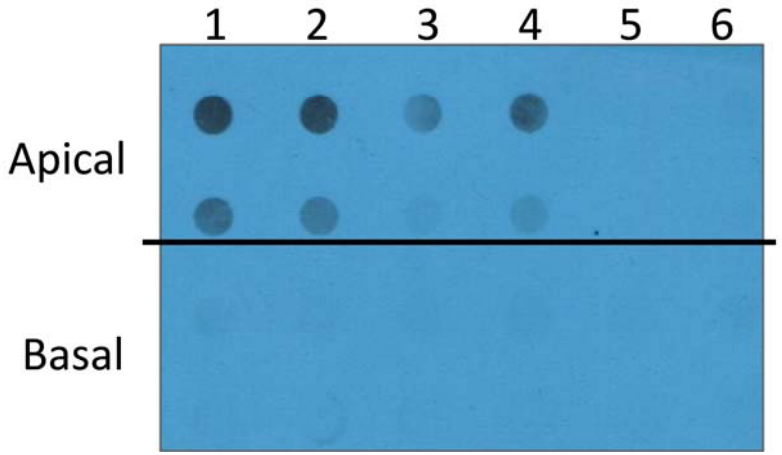

a
Ciliated cells
Goblet cell
Basal cell
b
Clara cell
Ciliated cells

Figure 1

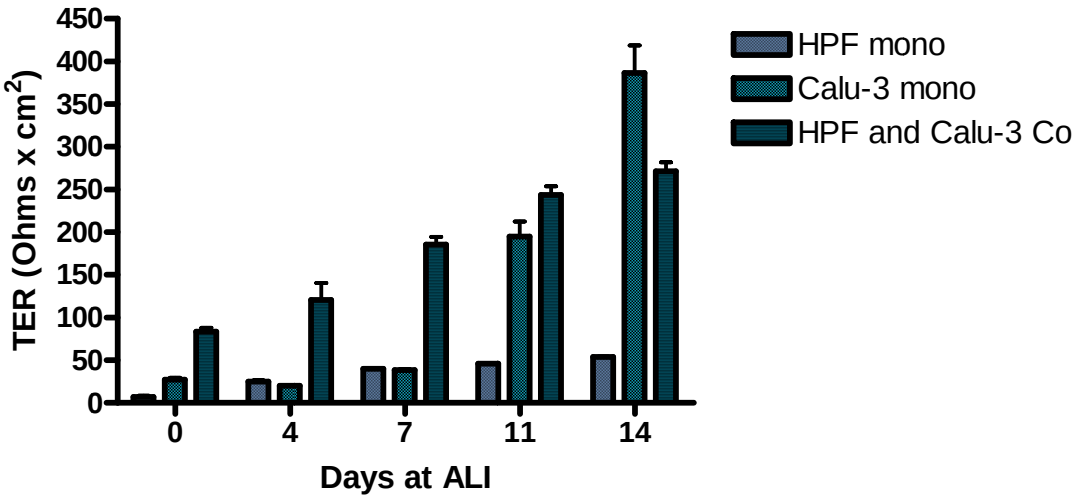


A **B** **C** **D**

Figure 2.


1
2
3
4
5
6
Apical
Basal

A


TER (Ohms x cm2)
450
400
350
300
250
200
150
100
50
0
0
4
7
11
14
Days at ALI
HPF mono
Calu-3 mono
HPF and Calu-3 Co

B