

1 **Developing accurate models of the human airways**

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## 8 **Abstract.**

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

## 23 **Developing relevant, accurate models of human airways.**

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

## 28 **Desirable features of a pulmonary model.**

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

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

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

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

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

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

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

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

94

## 95 **Cell culture conditions can drive epithelial differentiation**

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

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

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

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

137 **Mono- and co-culture systems for airway related research**

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

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

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

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

#### 184 **Increasing the complexity of the models.**

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

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

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

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

### 235 **The need for pulmonary vaccine delivery.**

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

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

### 257 **Considerations for delivery by the pulmonary route.**

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

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

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

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



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

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- 458

459 Figure legends.

460

461 **Figure 1: An illustration of the epithelial structures of the human airways.** a) Airway epithelial cells  
462 of the trachea and large bronchi where ciliated and mucus-producing goblet cells predominate with  
463 numerous basal cells present to provide anchorage and to act as progenitor cells. b) Airway epithelial  
464 cells of the bronchioles where ciliated and non-ciliated secretory epithelial (Clara) cells predominate.  
465 Goblet and serous cells decrease distally and are absent in the terminal bronchioles. Adapted from  
466 (2).

467

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

479

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

489

490

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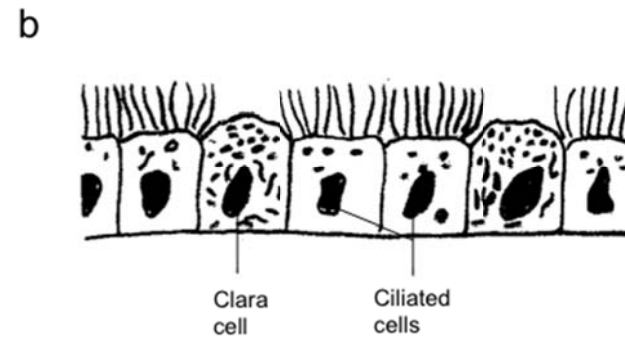
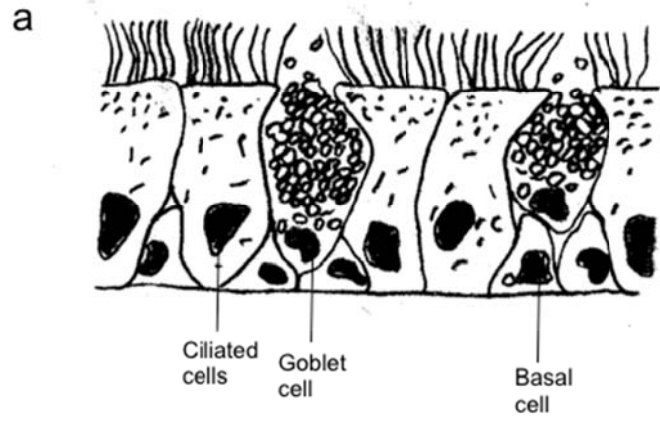
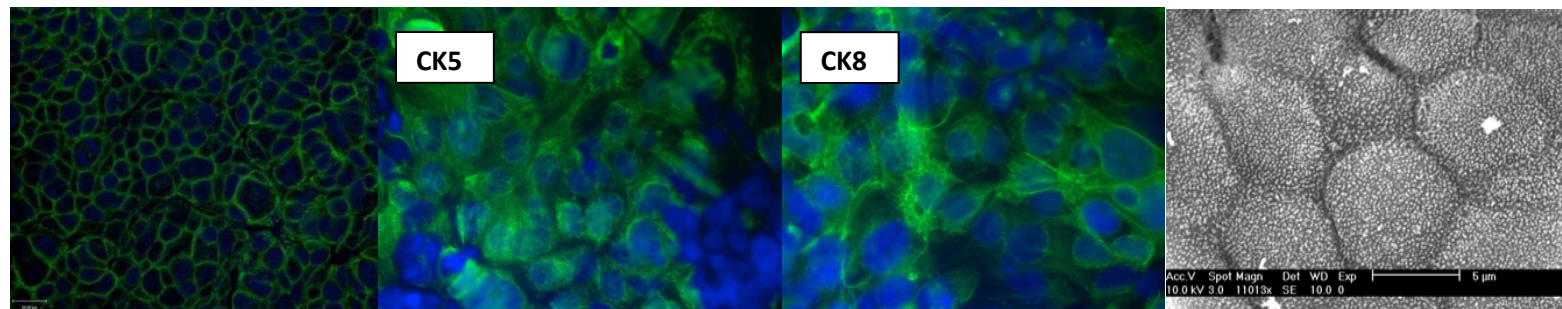
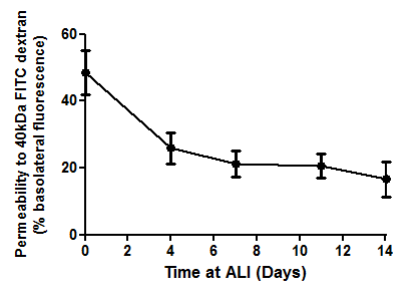


Figure 1





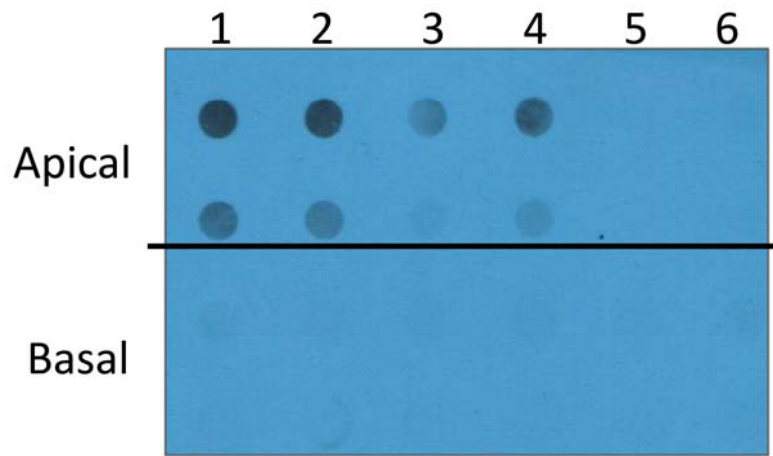
**A**

**B**

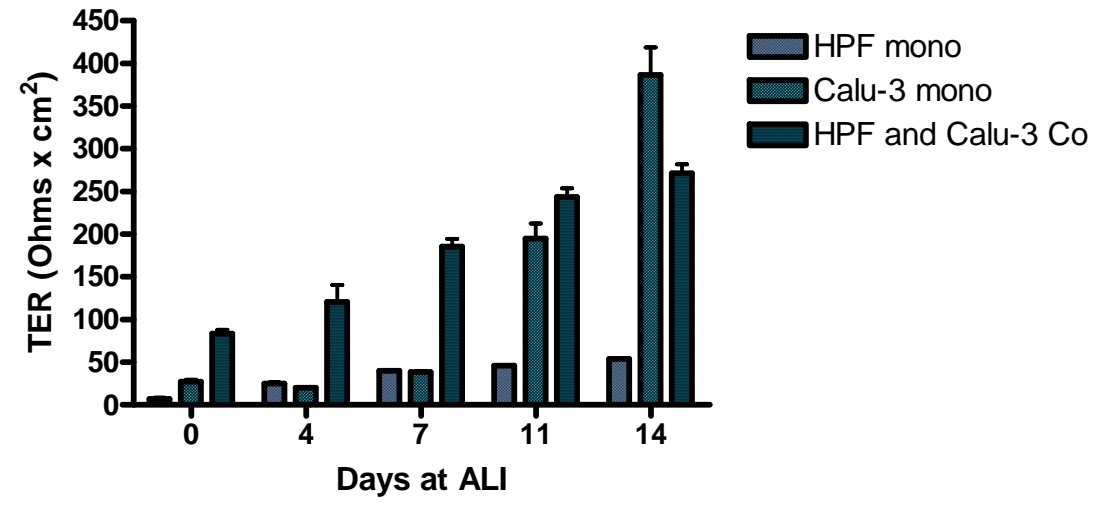
**C**

**D**

Figure 2.



**A**



**B**