Stem cell-derived respiratory epithelial cell cultures as human disease models

Jessica C. Orr, BSc¹ and Robert E. Hynds, PhD²

¹) Lungs for Living Research Centre, UCL Respiratory, Division of Medicine, University College London, London, U.K.

²) UCL Cancer Institute, University College London, London, U.K.

*Corresponding Author:

Dr. Robert. E. Hynds (rob.hynds@ucl.ac.uk)

UCL Cancer Institute, 72 Huntley Street, London, WC1E 6BT, United Kingdom

Keywords: respiratory epithelium, epithelial cells, cell culture, stem cells, cloning, single cells

Running title: Human epithelial models of lung health and disease

Author contributions: J.C.O. wrote and edited a draft manuscript and prepared figures. R.E.H. edited a draft manuscript and approved the final manuscript.
Abstract

Advances in stem cell biology and the understanding of factors that determine lung stem cell self-renewal have enabled long-term in vitro culture of human lung cells derived from airway basal and alveolar type II cells. Improved capability to expand and study primary cells long-term, including in clonal cultures that are recently derived from a single cell, will allow experiments that address fundamental questions about lung homeostasis and repair, as well as translational questions in asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, and lung cancer research. Here, we provide a brief history of post-natal lung epithelial cell culture and describe recent methodological advances, including some culture systems that now permit clonal cell culture. We further discuss the applications of primary cultures in defining ‘normal’ epithelium, modelling lung disease and in future cell therapies.
**Introduction**

Over the past decade, rapid progress has been made in our understanding of both the composition of human lung epithelia and the stem cell hierarchies that contribute to their maintenance and renewal following injury. In alveoli, alveolar type II (AT2) cells sustain differentiated AT1 cells (1). In the airways, basal cells replenish the pseudostratified epithelium in the trachea and throughout most generations of the intrapulmonary conducting airways (2). Basal cells have the capacity to self-renew as well as differentiate into the major secretory and multiciliated luminal cell types (3) and likely also minor populations such as tuft cells, ionocytes and pulmonary neuroendocrine cells (PNECs) (4, 5).

The differentiation trajectories of human airway epithelial cells have been refined by single cell RNA sequencing (scRNAseq) approaches. Both *in vivo* (6, 7) and *in vitro* (4, 8) studies converge on a model in which basal cells differentiate through a suprabasal progenitor population to give rise to club-like cells that can contribute to both goblet and multiciliated lineages. The lineage history of minor cell types is more difficult to ascertain in current datasets, but basal cell-descended tuft-like cells might give rise to ionocytes and PNECs, based on transcriptional similarities (5). Subsets of basal cells, distinguishable by proliferation or differentiation status, have been identified in these studies (9), as have a population of basal cells – ‘hillock’ basal cells – that might uniquely contribute to squamous stratified regions of epithelium (7). Cells residing in airway submucosal glands can be distinguished from surface epithelium based on their distinct gene expression profiles (5, 10). Smoking skews airway epithelial differentiation towards mucosecretory cell production, inhibits ciliogenesis and globally alters the airway epithelial transcriptome.
Differentiation is also altered during disease pathogenesis; in asthma, ciliated cells begin to express mucosecretory genes characteristic of goblet cells (12) and COPD pathogenesis involves epithelial senescence (13) and altered AT2 differentiation (14). Abnormal airway epithelial cell states have also been described (in addition to impaired AT2 cell differentiation and AT2 senescence phenotypes) in pulmonary fibrosis (15-17).

Our understanding of airway cell biology informs in vitro models of the epithelium and vice versa. Cell culture methods have been developed to recapitulate lung epithelial proliferation, differentiation and responses to environmental stressors, such as cigarette smoke and air pollution. These approaches also allow researchers to study airway damage and disease in controlled experimental conditions, including the epithelial response to putative therapies. Here, we review advances in cell culture methodology that allow sustained expansion of lung epithelial cells in culture, with an emphasis on recent advances that allow the study of ‘clones’, or cell cultures derived from individual cells.

A brief history of lung epithelial cell culture

Primary human bronchial epithelial cells (HBECs) have been cultured from various clinical samples, including rejected or excess transplant tissue, lobectomy tissue, induced sputum samples, bronchiolar lavage and endobronchial forcep or brush biopsies. In order to optimize HBEC culture conditions, extensive studies were performed to define the calcium and growth factor concentrations in growth media (18). Key factors include epidermal growth factor (EGF), insulin, transferrin, hydrocortisone, phosphoethanolamine and ethanolamine. Growth media compositions were further modified to increase the
initial success and duration of culture; retinoic acid was included in epithelial culture media as it was found to reduce or reverse the generation of squamous metaplasia in culture (19). Serum-free LHC-9 medium was a further refinement that saw the addition of bovine pituitary extract, epinephrine and 3,3’,5-triiodo-L-thyronine (T3) to increase proliferation (20). The generation of bronchial epithelial growth medium (BEGM) and several commercially available alternatives built upon these previous media, allowing serial culture of primary HBECs and the initiation of cultures from cryopreserved cells (21, 22). Air-liquid interface (ALI) culture of HBECs in differentiation media allows basal cells to form a confluent, polarized monolayer before generating luminal cell types. In similar medium, three-dimensional (3D) differentiation of basal cells can be achieved in either suspension culture (23) or by embedding cells in extracellular matrices to form cyst-like organoids commonly called ‘tracheo-’ or ‘bronchospheres’ (24).

Even in optimized cell culture media, few HBECs could be expanded in 2D cultures from small clinical biopsies for functional studies in either ALI or 3D cultures. Serial culture was only possible for a limited number of population doublings over which time cells progressively lost their differentiation potential. Three different cell culture strategies have extended primary lung cell culture lifetime and decreased reliance on cancer cell lines to model normal epithelium (Table 1): immortalization, improved 2D cell culture conditions and improved 3D culture conditions (Figure 1).
Figure 1: A timeline of lung epithelial cell culture methodology.

Blue boxes indicate advances in 2D primary cell culture, orange boxes indicate strategies involving immortalized lung epithelial cells and green boxes indicate advances in lung epithelial cell differentiation in cell culture. Created with BioRender.com.
First, immortalized cell lines have been developed by introducing immortalization factors into primary cells (Table 1) (25, 26). As an example, the partial transformation of primary HBECs with a hybrid adenovirus-simian virus 40 (SV40) virus created the BEAS-2B (27) cell line which no longer underwent replicative senescence in long-term culture, retained epithelial morphology but was unable to form tight junctions. On the other hand, 16HBE14o cells – which were immortalized with the SV40 large T antigen – could form tight junctions and also retained a cobblestone appearance in culture, expressed the cystic fibrosis transmembrane conductance regulator (CFTR) and could be differentiated to ciliated epithelium in ALI cultures (28, 29). Unfortunately, since the SV40 large T antigen inhibits the function of multiple tumor suppressor genes including TP53 and Rb, these cells can become tumorigenic with serial passaging (30). However, immortalization can also be achieved without the need for viral oncoproteins. Induction of cyclin-dependent kinase 4 (CDK4) and human telomerase reverse transcriptase (hTERT) expression enabled generation of the HBEC-KT cell lines (31). These cells retain a stable phenotype over serial passaging and are able to differentiate into both ciliated and goblet cells in ALI or in 3D culture (32, 33). Longer-term culture of basal cells with multipotent differentiation potential can also be achieved by overexpression of BMI-1 – a polycomb complex protein that acts as an oncogene through regulation of cell cycle inhibitors – either alone, achieving around 20-25 passage before senescence (34, 35), or in combination with hTERT, achieving at least 30 passages (36). Robust protocols exist for obtaining primary AT2 cells from human lung tissue but alveolar epithelial cell culture results in the transdifferentiation of cells to an AT1-like phenotype within two weeks (37). Immortalization of alveolar epithelial cells led to an AT1-like cell line (38, 39) while
immortalization of iPSC-derived AT2 cells maintained an AT2 phenotype for up to 30 passages (40).
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Derivation</th>
<th>Characteristics</th>
<th>References</th>
</tr>
</thead>
</table>
| 16HBE14o- | Primary HBECs immortalized with SV40 large T antigen | • Retains epithelial "cobblestone" morphology  
• Forms tight junctions  
• Upon differentiation multiciliated cells and microvilli are observed  
• No mucin secretion upon differentiation  
• Expresses CFTR  
• cAMP dependent Cl⁻ transport | (28, 41, 42)                                                                 |
| A549       | Lung adenocarcinoma                              | • Expresses surfactant proteins  
• Lamellar bodies present  
• Ineffective barrier formation due to defective tight junctions  
• Cancer driver mutations in KRAS and STK11  
• Abnormal hypotriploid karyotype, structural abnormalities | (43)                                                            |
| BEAS-2B    | Primary HBECs transduced with SV40 large T antigen | • Forms confluent monolayer with epithelial "cobblestone" morphology  
• No tight junction formation  
• No mucin secretion upon differentiation  
• Spontaneous transformation at late passages | (27, 29, 44)                                                                |
| Calu-3     | Bronchial adenocarcinoma                         | • Forms confluent monolayer  
• Forms tight junctions  
• Expresses CFTR  
• cAMP dependent Cl⁻ transport  
• Produces cilia-like projections and secretes mucins upon differentiation  
• Cancer driver mutation in TP53 | (41, 45-47)                                                               |
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal hypotriploid karyotype, structural abnormalities</td>
<td>• Now superseded as a model of normal bronchial epithelium by the ability to use non-cancerous cells</td>
<td></td>
</tr>
<tr>
<td>H441</td>
<td>Lung papillary adenocarcinoma</td>
<td>• Morphological features of club cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Does not express mature surfactant protein B protein or contain lamellar bodies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Forms monolayers with barrier function, effective tight junctions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Derived from a metastatic site</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cancer driver mutations in KRAS and TP53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Abnormal hyperdiploid karyotype, structural abnormalities</td>
</tr>
<tr>
<td>HBEC-KTs</td>
<td>Primary HBECs transduced with CDK4 and hTERT</td>
<td>• Expresses basal cell marker p63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Expresses p16 and has an intact p53 pathway</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Goblet and ciliated cells observed upon differentiation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Adherent junctions observed</td>
</tr>
<tr>
<td>HPLE</td>
<td>Primary distal lung epithelial transfected with SV40 large T antigen</td>
<td>• Epithelial morphology observed; club and AT2 cell phenotype</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Multivesicular bodies and incomplete multilamellar body-like structures observed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Expresses keratins and club cell specific protein</td>
</tr>
<tr>
<td>hSABCi-NS1.1</td>
<td>Primary small airway basal cells</td>
<td>• Expresses basal cell markers p63 and keratin 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Forms tight junctions</td>
</tr>
</tbody>
</table>

References: (48, 49, 31-33, 50, 51)
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSAEC1-KT</td>
<td>Primary human small airway epithelial cells transduced with CDK4 and hTERT. Expresses basal cell marker p63, expresses p16 and has an intact p53 pathway, contact inhibition of growth, fails to form soft agar colonies or tumors in immune-compromised mice.</td>
</tr>
<tr>
<td>LIMM-NBE1</td>
<td>Primary lung cells transduced with hTERT. Lung epithelial origin, transcriptional profile of bronchial epithelial cells, some karyotypic changes detected.</td>
</tr>
<tr>
<td>LL-iPSC-AEC II</td>
<td>iPSCs induced to AT2 cells transduced with hTERT and BMI-1. Forms monolayer with epithelial “cobblestone” morphology, lamellar bodies and microvilli observed, expresses keratins and surfactant protein C, uptake and release of surfactant protein B, secretion of phospholipids, able to differentiate into AT1 cells.</td>
</tr>
<tr>
<td>NuLi</td>
<td>Primary human airway epithelial cells transduced with hTERT and HPV-16 E6/E7. Retains Na⁺ transport upon differentiation, forms polarized epithelium; cAMP dependent Cl⁻ transport observed but declines over passages, goblet and ciliated cells observed upon differentiation, proportions vary over passages.</td>
</tr>
<tr>
<td>TT1</td>
<td>Primary AT2 cells transduced with hTERT and SV40 large T antigen. AT1-like cell phenotype with no lamellar bodies and flattened morphology.</td>
</tr>
</tbody>
</table>
Table 1: A list of pulmonary epithelial cell lines.

Second, co-culture of primary HBECs with mitotically-inactivated 3T3 fibroblasts allowed the expansion of basal cell cultures derived from single epithelial cells (56, 57), a method first demonstrated and optimized for epidermal keratinocytes (58, 59). The ability to form colonies of different morphologies in these conditions can be used as a surrogate for stem cell potential (57, 60). The introduction of a Rho-associated protein kinase (ROCK) inhibitor, Y-27362, improved the plating efficiency and culture duration of HBECs, among other epithelial cell types (61, 62). Nasal, tracheal, bronchial and small airway epithelial cells have been cultured using this methodology (63, 64). Basal cell differentiation capacity is preserved over a greater number of in vitro population doublings in bulk cultures (63, 64), while single cell-derived clonal lines can proliferate for variable timeframes (3-20 passages) while retaining multipotent differentiation potential (65). The co-culture of alveolar epithelial cells in these conditions enabled in vitro proliferation of AT2 cells but passaging of cells resulted in AT1-like differentiation (66). Further modifications to the 3T3-J2 co-culture approach in HBECs have combined BEGM, Y-27632 and lower oxygen conditions (67). While lung epithelia are exposed to a partial pressure of oxygen (PO₂) of approximately 13-14%, stem cell niches typically experience

<table>
<thead>
<tr>
<th>Cell Line Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>• No expression of AT2 cell markers alkaline phosphatase, pro–surfactant protein C or thyroid transcription factor-1</td>
</tr>
<tr>
<td>• Expresses the AT1 cell markers caveolin-1 and receptor for advanced glycation end products (RAGE)</td>
</tr>
<tr>
<td>• Poor tight junction formation</td>
</tr>
</tbody>
</table>
less than 6% PO$_2$ (68). Reducing cell culture oxygen levels to 2% led to improved basal cell culture longevity with multipotent differentiation competency preserved for up to 10 passages in bulk cultures and short-term in cultures derived from single cells (67).

Other improvements to 2D culture methodologies have included manipulation of the media composition. Analysis of signaling pathways present in the airway epithelium revealed that SMAD pathway activation was high in luminal epithelial cells but low in basal cells (69). Applying this knowledge to cell culture resulted in a dual SMAD inhibition protocol whereby basal cell culture lifetime was extended with cells retaining the ability to differentiate. Consistent with this, TGFβ pathway inhibition using A83-01 in combination with ROCK inhibition and isoproterenol supplementation supported long-term growth and multiple rounds of single cell cloning in the absence of feeder cells (70). Addition of the mammalian target of rapamycin (mTOR) inhibitor rapamycin to TGFβ and ROCK inhibition extended growth of basal cells from neonatal tracheal aspirates (71).

Third, important instructive signals – including the extracellular matrix (ECM) and the presence of differentiated cells – are missing from 2D cell cultures. 3D culture of HBECs by encapsulation within ECM substrates results in ‘tracheo-’ or ‘bronchospheres’, organoids containing both basal and luminal cell types (3, 24). Here, investigation of clonality by mixing labelled and unlabeled HBECs identified a clonal seeding threshold of $\leq$ 75 cells per well of a 384-well plate, beyond which spheres also formed by aggregation (24). Conditions for the long-term culture of airway cells in 3D organoids have been developed whereby a polarized pseudostratified epithelium containing basal, secretory and ciliated cells can be maintained for over a year of serial culture (72). 3D co-culture of human AT2 cells with the human embryonic lung fibroblast cell line MRC5 (1, 73) or adult
human lung fibroblasts (74) generates ‘alveolospheres’ containing both AT1 and AT2 cells. Recently, long-term, feeder-free conditions have been described that enable propagation of alveolar epithelial organoids in the absence of fibroblasts (75, 76). Beyond Matrigel-based organoid cultures, lung-on-a-chip devices have also been developed that allow the integration of primary epithelial cells with other lung cell types, mechanical force and flow (77).

**Isolating single epithelial cells for clonal expansion**

The conditions and specific factors that allow extended bulk cell cultures likely target the ability of basal or AT2 cells to survive, proliferate, self-renew, and/or differentiate. In improved culture conditions, new insights can be gained by isolating single stem cells in culture to ensure that all cells in a culture are derived from a recent common ancestor cell (Figure 2). Single cell culture is more challenging as stem cells rely on paracrine signals from other stem cells or differentiated progeny in bulk cultures which are no longer present in single cell or sparse culture conditions. To achieve clonal expansion, cells need to be isolated and cultured in a way that prevents anoikis and cross-contamination with cells from other lineages, which might include the use of inactivated feeder cells or conditioned medium from bulk epithelial or stromal cultures.
Figure 2: Methods to expand clonal epithelial cell cultures from lung tissue.

Most simply, cells can be serially diluted to low density with either one (78) or several (65) cells per well and taking wells in which a single colony forms forwards for culture. Alternatively, a mixed population can be cultured at low density and individual colonies picked for further expansion in the expectation that a single cell initiates colony growth (60, 79, 80). Magnetic bead purification can also be used to enrich for the cell type of interest from primary tissue, for example positive selection using EPCAM or negative selection for immune (CD45+) and endothelial (CD31+) cells. Similarly, flow cytometry allows marker-specific cells to be deposited into individual wells. The ubiquitous basal cell markers keratin 5 (KRT5) and transformation protein 63 (p63) are intracellular and can therefore not be used for live cell sorting but surface proteins such as integrin alpha 6 (ITGA6), nerve growth factor receptor (NGFR) and podoplanin (PDPN) are expressed by a majority of basal cells (3, 81), while CD66 was recently proposed as a marker for basal cells primed for secretory differentiation (9). Anti-HTII-280 antibodies target a surface protein on human alveolar type II cells with high specificity (82) and an antibody targeting Transmembrane 4 L Six Family Member 1 (TM4SF1) has recently been reported to identify a progenitor subset of human AT2 cells that respond to Wnt signals (73).

Identification of novel surface marker proteins for lung epithelial stem/progenitor cells would assist in bead or flow cytometric sorting strategies, although this is complicated by donor- and disease-variable expression patterns. Microfluidic technologies can also be used for single cell trapping (83), these may be gentler than exposure to flow cytometry protocols but cell types cannot be isolated based on their surface markers.

**Translational applications of improved primary cell culture methods**

*Defining ‘normal’ lung epithelium*
In lung cancers, smoking results in distinct mutational signatures in tumors. Molecular abnormalities accumulate during cancer progression from histologically normal epithelium through pre-invasive disease towards invasive cancers (84-86) and are detectable in histologically normal epithelium surrounding tumors in lung cancer patients (87). Since expanded clonal cultures can reveal information about the genome of the founder cell, a recent study applied whole-genome sequencing to cultured epithelial cells. Colonies were initiated by flow sorting single epithelial cells from the histologically normal airways of smokers, ex-smokers and never smokers, and cultured for two passages ex vivo. This experiment revealed the predicted higher mutational burden in smokers and ex-smokers but also found that the genomic impact of smoking on the epithelium is heterogeneous (78). In smokers or ex-smokers, some basal cells have a mutational burden comparable to those in never smokers. Interestingly, the proportion of basal cells with low mutational burden increased in ex- compared to current smokers (78) suggesting that their expansion is actively repressed during smoking or promoted following cessation.

Since this clonal culture strategy expanded colonies of basal cells from single cells, it is possible that other genomic differences, such as copy number alterations, might be present in differentiated cells that could not form colonies in vitro. The accumulation of somatic mutations in physiologically normal tissues during aging has been demonstrated in multiple other organs (83, 88-92), including using cell culture approaches to give single cell resolution within hematopoietic, gastrointestinal and liver tissues (89, 93, 94).

Cumulatively, these data suggest that the lung epithelium is highly dynamic over the life course and in response to injury. Genomic differences might provide a partial explanation for the inter-individual variability observed within primary lung cell cultures and suggests
that the clonal composition of widely used normal lung cell lines should be more deeply characterized. It will be interesting to explore how somatic evolution varies in smoking-associated chronic lung diseases such as COPD and pulmonary fibrosis, where the epithelium is known to play a key role in pathogenesis. In lung cancer, questions remain about how oncogenic mutations influence airway epithelial cell behavior, how mutant cells interact with surrounding non-mutant cells, the potential for immune pruning of mutant cells and the impact of cancer therapies upon normal lung epithelial dynamics. Clonal cell culture of normal lung epithelial cells now provides a tractable system in which to test hypotheses associated with these open questions, for example by looking at direct cell competition between isogenic wild-type and mutant clones.

*Modelling disease initiation and pathogenesis*

Genetic lung diseases can be caused by single variants: diverse mutations in *CFTR* disrupt ion transport and cause cystic fibrosis (CF), those in *SERPINA1* cause alpha-1 antitrypsin deficiency and mutations within a range of genes that affect the structure or function of motile cilia cause primary ciliary dyskinesia (PCD). Steps towards personalized medicine have been achieved in cystic fibrosis by screening primary biopsy-derived intestinal organoids. The fact that forskolin treatment increases intracellular cyclic AMP, activates CFTR, and results in luminal fluid secretion has been exploited in an organoid swelling assay (95, 96). This assay allows the assessment of an individual's response to currently available treatments and will thus be particularly relevant for patients with rare and poorly characterized mutations. The adaptation of the forskolin-induced swelling assay to airway organoids will likely reduce the requirement for gastrointestinal tract biopsies in these patients (72). Primary cell culture from nasal brush
biopsies has allowed individualized cell cultures from PCD patients as differentiated ALI cultures retain the ciliary defects seen in patients (97, 98). Improved culture methods have allowed miniaturization of ALI cultures to at least 96-well plate format (99, 100) and even higher throughput might be achievable through optimization of plate design. Mechanistic studies of alpha-1 antitrypsin deficiency pathogenesis in the lung might also benefit from new alveolar epithelial cell culture methods since SERPINA1 is prominently expressed by AT2 cells (101).

Beyond monogenic diseases, large genome-wide association studies have revealed germline variants at many loci involved in the susceptibility to and pathogenesis of chronic lung diseases, while acquired somatic mutations have been associated with chronic diseases in other organs (102, 103). In both cases, primary cell culture methods offer a valuable opportunity to test the functional impact of variants in epithelial cells, for example by using clustered regularly interspaced short palindromic repeats-associated nuclease Cas9 (CRISPR-Cas9)-based genome editing approaches. Lentivirus-mediated knockout of *MUC18*, which is upregulated during lung inflammation, was achieved in primary nasal epithelial cells (104) demonstrating proof-of-principle for this approach. While the puromycin selection strategy that was used here resulted in a heterogeneous bulk population of edited cells, recent advances in clonal expansion will allow the selection of a single genotype and facilitate characterization of possible off-target effects. Alternatively, gene editing can be performed once airway cells have differentiated in ALI cultures (105).

Epithelial cell culture can also be used to study disease initiation in several contexts, providing a window into a disease stage that is impossible to study in patients. For
example, air-liquid interface and organoid cultures have provided a system to study epithelial cell infection with respiratory viruses such as influenza (106, 107), respiratory syncytial virus (72, 108) and SARS-CoV-2 (75, 109-111). Clonal primary human lung epithelial cell expansion also offers a unique opportunity to study early carcinogenesis as multiple cancer-associated genetic events can be experimentally introduced into lung epithelium in a step-wise fashion. Clonal derivation of lung cancer organoids is also feasible using similar methods to primary airway organoids (72, 112, 113). Such cultures offer opportunities to study intra-tumor heterogeneity, monitor on-going mutational processes *in vitro*, and study tumor-immune cell interactions in co-cultures.

The abnormal differentiation phenotypes, such as goblet cell hyperplasia, that are associated with asthma and COPD can be studied either by exposing cells from healthy donors to relevant growth factors, cytokines or small molecules or by studying primary cells derived from patients with those diseases. A human recombinant protein screen using ‘bronchospheres’ identified that EGF promoted basal cell proliferation while interleukin 13 (IL-13) and IL-17a caused goblet cell metaplasia in a Notch2 dependent manner (24). While individual organoids in these assays are likely to have been clonally derived, analyses were performed at the well-level and thereby represent mixed lineage cultures. Future experiments might expand this approach using clonally derived cell cultures of known genotypes. Alternatively, cell culture methods can also be applied to epithelium from patients with respiratory diseases. Evidence from bulk cultures suggests that phenotypes of asthma, COPD and pulmonary fibrosis are maintained in culture. For example, differences in epithelial barrier formation (114), delayed wound repair (115) and reduced innate defense against bacterial infection (116) persist in COPD cultures.
Recently, RNA sequencing of clonal airway epithelial cell cultures expanded from normal and COPD lung tissue revealed four clusters of basal cell variants. These four variant clones had distinct differentiation phenotypes; resulting in normal epithelium, goblet cell metaplasia, squamous cell metaplasia and inflammatory squamous cell metaplasia, respectively (80). The metaplastic basal cell phenotypes were present at low levels in normal adult and fetal lungs but abnormally expanded in COPD airways and persisted after as many as 25 passages (80), suggesting that the phenotypes were at least partially epigenetically stable in culture. It will be interesting to unravel the extent to which the predominance of these four clonal subtypes determines the interindividual variability in bulk patient cultures, how variable transcriptomic subtypes are in other respiratory diseases and whether specific transcriptomically-defined subtypes respond differently to cigarette smoke or infection.

Cell therapy

Cell therapies aiming to directly replace epithelial cells have been proposed for lung diseases including bronchopulmonary dysplasia (BPD), bronchiolitis obliterans, COPD, pulmonary fibrosis and cystic fibrosis (117). There is precedent for cell therapy using cultured epithelial cells in the context of epidermal and limbal burns injuries (59, 118). In the airways, epithelium from split-skin grafts has been used to provide ‘biological inhibition’ in the context of laryngotracheal stenosis (119, 120), where chronically damaged epithelium might contribute to fibrotic reactions and recurrence of stenoses. Clinical applications of cultured autologous cells have been limited. In tissue-engineered grafts used in airway transplantation cases (121, 122), the epithelial components are not thought to have engrafted, with any epithelialization instead attributed to in-growth from
surrounding host cells (123); this view is supported by difficulties in engrafting airway epithelium in pre-clinical models (124). Two bronchiectasis patients also received cultured basal cells via bronchoscopy in a pilot clinical study (125); the fate of these cells is unknown and additional pre-clinical work is required before further human trials.

Nevertheless, existing pre-clinical data are promising. Human bronchial basal cells (126) and developmental precursors derived from induced pluripotent stem cells (iPSCs) (127) transplanted into the injured airways of immunocompromised mice can engraft and differentiate into both secretory and ciliated cell types, restoring the airway epithelium at least in the short-term.

In the future, epithelial cell therapies might be used to treat genetic lung diseases through the replacement of impaired cells with functional gene-corrected cells. Primary epithelial cells from CF (65) and PCD (99) expand and differentiate similarly in culture to those from healthy control donors making this a plausible approach and induced pluripotent cell-based alternatives are also under development (128). Advances have been made towards functional gene correction in both CF and PCD. Using CRISPR-Cas9 approaches, CFTR function has been restored in intestinal organoids (129, 130) and CF iPSCs guided towards a lung epithelial lineage (131). Promisingly, the most common CF CFTR mutation – ΔF508 – has been corrected using CRISPR-Cas9 delivery in airway basal cells that retained the ability to differentiate into pseudostratified epithelium in ALI culture (132). The protocol did not use cell selection or cloning to preferentially expand the gene corrected cells, resulting in a heterogeneous cell population in terms of CFTR function. In PCD, transcription activator-like effector nuclease (TALEN)-mediated gene editing allowed correction of DNAH11 mutations with the wild-type sequence in bulk cell
cultures, restoring ciliary function (133). The use of clonal epithelial cultures might reduce the heterogeneity of on-target phenotype modification and simultaneously limit the extent of off-target safety concerns of these approaches (134).

However, a number of hurdles remain. Firstly, estimates for the number of cells required for therapeutic airway epithelial cell engraftment converge in the range of 50-100 million cells (64, 65). Given the small number of basal cells that are recovered from bronchoalveolar lavage (<2000; (69)), induced sputum (<2000; (69)), bronchial brush biopsy (10,000-200,000; (135)) or bronchial forcep biopsy (5,000-200,000; (135)) samples, and the difficulty to manipulate isolated cells without culture, it is likely that such a therapy will require cell expansion in culture. It remains to be seen whether clonal, gene-corrected cells from CF patients can be expanded to this extent while retaining the capacity to regenerate the airway epithelium long-term. Secondly, available gene-editing technologies face the issue of off-target effects. Here, clonal cell culture methodologies will facilitate quality control steps to determine whether obvious somatic or integration-related aberrations preclude transplantation. A further consideration is that preconditioning of the patient is likely to be required (136) but the methods used to generate injury experimentally in mice are unlikely to be acceptable clinically.

**Future directions**

Historically, airway epithelial cells have been more readily cultured than their alveolar counterparts. Nevertheless, recent advances make clonal cell culture of alveolar epithelium possible (111), albeit in organoids that lack many of the morphological aspects of alveoli. This breakthrough makes a number of experiments – such as determining the
genomic landscape of individual AT2 cells in normal distal lung epithelium during aging and following tobacco exposure, recapitulating human AT2 to AT1 differentiation *in vitro* and determining the engraftment potential of cultured human alveolar cells in mouse injury models – achievable in the near-term. A future application of alveolar organoids might be in cellular therapy but substantial challenges exist even in comparison to those for basal cells; the alveolus has a complex 3D architecture including precise cellular architecture that will prove difficult to recapitulate by repopulating with cells. However, cell-matrix scaffolds in combination with more extensive partial tissue replacement should be prioritized for investigation.

Several aspects of lung epithelial biology discovered in the mouse remain uncertain in human lungs. Basal cells are increasingly sparse in the distal-most human airways as the epithelium becomes simple columnar rather than pseudostratified. Mouse studies suggest the involvement of club cells as stem cells in simple columnar airway epithelium but human cell culture studies suggest that human club cells arise from basal cells (137). Epithelial cell culture can shed light on the cell biology of this region in humans since multiple lines of evidence suggest that region-specific phenotypic differences persist *in vitro*: transcriptomic differences are found between cultured basal cells from nasal, tracheal or small airway epithelium (56), immortalized cell lines can retain small airway-like differentiation capacity (51) and air-liquid interface cultures from nasal, large and small airways retain differential sensitivity to viral infection (110). Cell culture might also help to ascertain whether airway epithelial cells can acquire greater plasticity following severe injury than is seen during normal cell turnover, as is seen when club cells contribute to the basal stem cell pool (138, 139). Similarly, future studies might address
whether human AT2 and club cells pass through a transition state characterized by KRT8 expression and TP53 pathway activation before reaching full AT1 differentiation, as has been observed in mice and suggested by analyses of human disease states (140-142).

Gene editing technology advances applied to model organisms allow in vivo clonal analyses under experimental control. In one example that might be applicable to cultured lung epithelial cells – Lineage and RNA recovery (LARRY) – cells are transduced with a lentivirus containing an eGFP construct with a DNA barcode in the 3’ UTR region. After brief culture to allow cell division, a proportion of these cells are analyzed by scRNAseq with the remaining cells either re-plated in vitro or transplanted to assess in vivo differentiation. Analysis of scRNAseq data from before or after plating/transplantation allows mapping of progenitor transcriptomes to their cell fates through barcode matching, potentially determining cell states associated with self-renewal, differentiation and/or engraftment. Entirely in vivo systems might also be deployed. For example, the CARLIN (CRISPR array repair lineage tracing) mouse model barcodes cells in order to track their progeny (143). A doxycycline-inducible Cas9, guide RNAs and an array of their target sites sit within the Col1a1 locus and allow temporally controlled and/or sequential editing of the array to generate diverse barcodes trackable using scRNAseq. Although initially used to reconstruct lineages and investigate functional heterogeneity in the hematopoietic system (143), CARLIN-edited gene transcripts are observed in lung tissue. Improvements to this mouse line that allow greater barcode diversity would provide an opportunity to trace cell lineages through recovery after lung injury or infection in adult mice.
**Conclusion**

Primary human lung epithelial cells cultures represent powerful tools for understanding lung health and disease. Improvements to cell culture methodologies allow prolonged culture of both airway and alveolar epithelial cells, including as clonal sub-cultures. In combination with other technological advances – particularly in gene editing and next-generation sequencing – these will help to further dissect the molecular heterogeneity of the lung epithelium in patients and pre-clinical models (Figure 3). Future cell therapies might also benefit through the capacity to expand cells of a single known genotype to therapeutic quantities.
Figure 3: Translational applications of clonal lung epithelial cell culture.

Created with BioRender.com.
Abbreviations

3D – three-dimensional
ALI – air-liquid interface
AT1 – alveolar type I
AT2 – alveolar type II
BEGM – bronchial epithelial growth medium
CARLIN – CRISPR array repair lineage tracing
CDK4 – cyclin-dependent kinase 4
CF – cystic fibrosis
CFTR – cystic fibrosis transmembrane conductance regulator
COPD – chronic obstructive pulmonary disease
CRISPR – clustered regularly interspaced short palindromic repeats
ECM – extracellular matrix
EGF – epidermal growth factor
HBEC – human bronchial epithelial cell
hTERT – human telomerase reverse transcriptase
IL – interleukin
ITGA6 – integrin alpha 6
iPSC – induced pluripotent stem cell
KRT5 – keratin 5
LARRY – lineage and RNA recovery
mTOR – mammalian target of rapamycin
NGFR – nerve growth factor receptor
Acknowledgements

The authors apologize to authors whose work it was not possible to cite here due to space constraints. We also thank Dr. Eva Grönroos (The Francis Crick Institute, London, U.K.) for proof-reading a draft manuscript and colleagues at the Great Ormond Street Institute of Child Health (University College London, U.K.) and UCL Respiratory (University College London, U.K.) for feedback on the revised manuscript.

Funding information

J.C.O. is funded by a PhD studentship from the Longfonds BREATH lung regeneration consortium. R.E.H. is a Wellcome Trust Sir Henry Wellcome Fellow (WT209199/Z/17/Z) and is supported by the Roy Castle Lung Cancer Foundation, the James Tudor Foundation and the Cancer Research UK Lung Cancer Centre of Excellence. R.E.H. is a member of the UK Regenerative Medicine Platform (UKRMP2) Engineered Cell Environment Hub (MRC; MR/R015635/1).
Conflict of interest

The authors declare no conflict of interest.
References:


47. Grainger CI, Greenwell LL, Lockey DJ, Martin GP, Forbes B. Culture of calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. *Pharm Res* 2006;23(7):1482-1490.


