Stem Cell–derived Respiratory Epithelial Cell Cultures as Human Disease Models

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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, pulmonary fibrosis, and lung cancer research. Here, we provide a brief history of postnatal lung epithelial cell culture and describe recent methodological advances. We further discuss the applications of primary cultures in defining “normal” epithelium, in modeling lung disease, and in future cell therapies.

Keywords: respiratory epithelium; epithelial cells; cell culture; stem cells; cloning

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 (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 data sets, but basal cell–descended tuft-like cells might give rise to ionocytes and pulmonary neuroendocrine cells, 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 toward mucosecretory cell production, inhibits ciliogenesis, and globally alters the airway epithelial transcriptome (5, 11). Differentiation is also altered during disease pathogenesis; in asthma, ciliated cells begin to express mucosecretory genes characteristic of goblet cells (12) and chronic obstructive pulmonary disease (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).

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Our understanding of lung 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 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. To optimize HBEC culture conditions, 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 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 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 either in suspension culture (23) or by embedding cells in

extracellular matrices to form cyst-like organoids commonly called “tracheospheres” 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 possible for only a limited number of population doublings during which cells progressively lost their differentiation potential. Three main 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).

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, because 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 CDK4 (cyclin-dependent kinase 4) and hTERT (human telomerase reverse transcriptase) 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 organoid 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 passages 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 these cells to an AT1-like phenotype within 2 weeks (37). Immortalization of alveolar epithelial cells led to an AT1-like cell line (38, 39), whereas immortalization of induced pluripotent stem cell (iPSC)-derived AT2 cells maintained an AT2 phenotype for up to 30 passages (40).

Second, coculture of primary HBECs with mitotically inactivated 3T3 fibroblasts allowed the expansion of basal cell cultures derived from single epithelial cells (41, 42), a method first demonstrated and optimized for epidermal keratinocytes (43, 44). The ability to form colonies of different morphologies in these conditions can be used as a surrogate for stem cell potential (42, 45). The introduction of a ROCK (Rho-associated protein kinase) inhibitor, Y-27632, to culture medium improved the plating efficiency and culture duration of HBECs, among other epithelial cell types (46, 47). Nasal, tracheal, bronchial, and small airway epithelial cells have been cultured using this methodology (48, 49). Basal cell differentiation capacity is preserved over a greater number of *in vitro* population doublings in bulk cultures (48, 49), whereas single cell–derived clonal lines can proliferate for variable timeframes (3–20 passages) while retaining multipotent differentiation potential (50). Culturing alveolar epithelial cells in these conditions enabled brief *in vitro* proliferation of AT2 cells, but passaging of cells resulted in AT1-like differentiation (51). Further modifications to the 3T3-J2 coculture approach in HBECs have combined bronchial epithelial growth medium, Y-27632, and lower oxygen conditions (52). While lung epithelia are exposed to a partial pressure of oxygen of ~13–14%, stem cell niches can experience less than 6% *in vivo* (53). 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 (52).

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

Table 1. A List of Human Pulmonary Epithelial Cell Lines

Cell Line	Derivation	Characteristics	References
16HBE14o-	Primary HBECs immortalized with SV40 large T antigen	<ul style="list-style-type: none"> 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, 131, 132)
A549	Lung adenocarcinoma	<ul style="list-style-type: none"> Expresses surfactant proteins Lamellar bodies present Ineffective barrier formation due to defective tight junctions Cancer driver mutations in <i>KRAS</i> and <i>STK11</i> Abnormal hypotriploid karyotype, structural abnormalities 	(133)
BEAS-2B	Primary HBECs transduced with SV40 large T antigen	<ul style="list-style-type: none"> Forms confluent monolayer with epithelial “cobblestone” morphology No tight junction formation No mucin secretion upon differentiation Spontaneous transformation at late passages 	(27, 29, 134)
Calu-3	Bronchial adenocarcinoma	<ul style="list-style-type: none"> 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 <i>TP53</i> Abnormal hypotriploid karyotype, structural abnormalities Now superseded as a model of normal bronchial epithelium by the ability to use noncancerous cells 	(131, 135–137)
H441	Lung papillary adenocarcinoma	<ul style="list-style-type: none"> Morphological features of club cells Does not express mature SPB protein or contain lamellar bodies Forms monolayers with barrier function, effective tight junctions Derived from a metastatic site Cancer driver mutations in <i>KRAS</i> and <i>TP53</i> Abnormal hyperdiploid karyotype, structural abnormalities 	(138, 139)
HBEC-KTs	Primary HBECs transduced with CDK4 and hTERT	<ul style="list-style-type: none"> Expresses basal cell marker p63 Expresses p16 and has an intact p53 pathway Goblet and ciliated cells observed upon differentiation Adherent junctions observed 	(31–33)
HPLE	Primary distal lung epithelial transfected with SV40 large T antigen	<ul style="list-style-type: none"> Epithelial morphology observed; club and AT2 cell phenotype Multivesicular bodies and incomplete multilamellar body-like structures observed Expresses keratins and club cell-specific protein 	(140)
hSABCI-NS1.1	Primary small airway basal cells transduced with hTERT	<ul style="list-style-type: none"> Expresses basal cell markers p63 and keratin 5 Forms tight junctions Ciliated, club, mucus, neuroendocrine, ionocyte, and surfactant-positive cells observed upon differentiation 	(124)
HSAEC1-KT	Primary small airway epithelial cells transduced with CDK4 and hTERT	<ul style="list-style-type: none"> 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 	(31, 141)

(Continued)

Table 1. (Continued)

Cell Line	Derivation	Characteristics	References
LIMM-NBE1	Primary lung cells transduced with hTERT	<ul style="list-style-type: none"> • Lung epithelial origin • Transcriptional profile of bronchial epithelial cells • Some karyotypic changes detected 	(142, 143)
LL-iPSC-AEC II	iPSCs differentiated to AT2-like cells and transduced with hTERT and BMI-1	<ul style="list-style-type: none"> • Forms monolayer with epithelial “cobblestone” morphology • Lamellar bodies and microvilli observed • Expresses keratins and SPC • SPB uptake and release, secretion of phospholipids • Able to differentiate into AT1 cells 	(40)
NuLi	Primary airway epithelial cells transduced with hTERT and HPV-16 E6/E7	<ul style="list-style-type: none"> • 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 	(144)
TT1	Primary AT2 cells transduced with hTERT and SV40 large T antigen	<ul style="list-style-type: none"> • AT1-like cell phenotype with no lamellar bodies and flattened morphology • No expression of AT2 cell markers alkaline phosphatase, pro-SPC, or thyroid transcription factor-1 • Expresses the AT1 cell markers caveolin-1 and receptor for advanced glycation end products • Poor tight junction formation 	(38, 39)

Definition of abbreviations: AT1 = alveolar type I; AT2 = alveolar type II; CDK4 = cyclin-dependent kinase 4; CFTR = cystic fibrosis transmembrane conductance regulator; HBEC = human bronchial epithelial cell; HPV = human papillomavirus; hTERT = human telomerase reverse transcriptase; iPSC = induced pluripotent stem cell; p63 = tumour protein p63; SPB = surfactant protein B; SPC = surfactant protein C; SV40 = simian virus 40.

activation was high in luminal epithelial cells but low in basal cells (54). 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, TGF- β 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 (55). Addition of the mTOR inhibitor rapamycin to TGF- β and ROCK inhibition also extended growth of basal cells from neonatal tracheal aspirates (56).

Third, important instructive signals—including the extracellular matrix and the presence of differentiated cells—are missing from 2D cell cultures. 3D culture of HBECs by encapsulation within extracellular matrix substrates results in “tracheospheres” or “bronchospheres,” organoids containing both basal and luminal differentiated cell types (3, 24). Investigation of clonality in bronchospheres by mixing labeled and unlabeled HBECs identified a clonal seeding threshold of ≤ 75 cells per well of a 384-well plate, above which density

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 (57). 3D coculture of human AT2 cells with the human embryonic lung fibroblast cell line MRC5 (1, 58) or adult human lung fibroblasts (59) 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 (60, 61). 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 (62).

Isolating Single Epithelial Cells for Clonal Expansion

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 cells in bulk cultures that are no longer available 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 promotes survival, proliferation and stem cell maintenance, which might include the use of inactivated feeder cells or conditioned medium from bulk epithelial or stromal cultures.

Most simply, cells can be serially diluted to low density with either one (63) or several (50) cells per well and taking wells in which a single colony forms forward for culture. Alternatively, a mixed population can be cultured at low density and individual colonies picked for further expansion assuming that a single cell initiates colony growth (45, 64, 65). 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, fluorescence-activated cell sorting allows marker-specific cells to be deposited

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. 2D = two-dimensional; 3D = three-dimensional.

into individual wells. The commonly used basal cell markers KRT5 (keratin 5) and p63 (tumor protein p63) are intracellular and can therefore not be used for live cell sorting, though surface proteins such as ITGA6 (integrin α 6), NGFR (nerve growth factor receptor), and PDPN (podoplanin) are expressed by a majority of basal cells (3, 66). CD66 was recently revealed as a surface 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 (67) and an antibody targeting TM4SF1 (Transmembrane 4 L Six Family Member 1) has recently been reported to identify a progenitor subset of human AT2 cells that respond to Wnt signals (58). 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 (68); these may be gentler than 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

Molecular abnormalities accumulate during lung cancer progression from histologically normal epithelium through preinvasive disease toward invasive cancers (69–71). Tobacco smoking results in distinct mutational signatures in the genomes of both tumours and the histologically normal epithelium surrounding them (72). To reveal information about the genome of the founder cell, a recent study applied whole-genome sequencing to clonal epithelial cell cultures. 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 (63). 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-smokers compared with current smokers (63) suggesting that their expansion is actively repressed during smoking or promoted following cessation. Because this clonal culture strategy expanded colonies of basal cells from single cells, it is theoretically 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 (73–78), including using cell culture approaches to give single cell resolution within hematopoietic, gastrointestinal, and liver tissues (74, 79, 80).

Figure 2. Methods to expand clonal epithelial cell cultures from lung tissue. Created with BioRender.com. FACS = fluorescence-activated cell sorting.

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 interindividual 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. With regard to early lung cancer, questions remain about how oncogenic mutations influence airway epithelial cell behavior, how mutant cells interact with surrounding nonmutant cells, the potential for immune pruning of

mutant cells, and the impact of cancer therapies on normal lung epithelial dynamics. Clonal cell culture of normal lung epithelial cells now provides a tractable system in which to investigate these open questions, for example, by looking at direct cell competition between isogenic wild-type and mutant clones.

Modeling Disease Initiation and Pathogenesis

Genetic lung diseases can be caused by mutations in single genes: 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 toward personalized medicine have been achieved in CF 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 (81, 82). 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 (57). Primary cell culture from nasal brush biopsies has allowed individualized cell cultures from patients with PCD as differentiated ALI cultures retain the ciliary defects seen in patients (83, 84). Improved culture methods have allowed miniaturization of ALI cultures to at least 96-well plate format (85, 86), 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 because *SERPINA1* is prominently expressed by AT2 cells (87).

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, whereas acquired somatic mutations have been associated with chronic diseases in other organs (88, 89). 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 CRISPR-based genome editing approaches. Knockout of *MUC18* (90), which is upregulated during lung inflammation, or of transcription factors involved in epithelial fate decisions (5) has been achieved in primary cells, demonstrating proof of principle for this approach. Although the strategies used so far have resulted in heterogeneous bulk populations 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 (91).

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, ALI and organoid cultures have provided a system to study epithelial cell infection with respiratory viruses such as influenza (92, 93), respiratory syncytial virus (57, 94) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (60, 95–97). 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 stepwise fashion. Clonal derivation of lung cancer organoids is also feasible using similar methods to primary airway organoids (57, 98, 99). Such cultures offer opportunities to study intratumor heterogeneity, monitor ongoing mutational processes *in vitro*, and study tumor-immune cell interactions in cocultures.

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 IL-13 and IL-17a caused goblet cell metaplasia in a Notch2-dependent manner (24). Although 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 some phenotypes associated with asthma, COPD, and pulmonary fibrosis are maintained in epithelial cell cultures. For example, differences in epithelial barrier formation (100), delayed wound repair (101), and reduced innate defense against bacterial infection (102) 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 clone types had

distinct differentiation phenotypes, resulting in normal epithelium, goblet cell metaplasia, squamous cell metaplasia, and inflammatory squamous cell metaplasia, respectively (65). The metaplastic basal cell phenotypes were present at low levels in cultures from normal adult and fetal lungs but abnormally expanded in those from COPD airways and the phenotypes persisted after as many as 25 passages (65), suggesting at least partial epigenetic stability in culture. It will be interesting to unravel the extent to which the predominance of these four clone types determines the interindividual variability in bulk patient cultures, how variable transcriptomically defined clone types are in other respiratory diseases, and whether specific clone types 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, bronchiolitis obliterans, COPD, pulmonary fibrosis, and CF (103). There is precedent for cell therapy using cultured epithelial cells in the context of epidermal and limbal burns injuries (44, 104). In the airways, epithelium from split-skin grafts has been used to provide “biological inhibition” in the context of laryngotracheal stenosis (105, 106), 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 (107, 108), the epithelial components are not thought to have engrafted, with any epithelialization instead attributed to in-growth from surrounding host cells (109); this view is supported by difficulties in engrafting airway epithelium in preclinical models (110). While two patients with bronchiectasis also received cultured basal cells via bronchoscopy in a pilot clinical study (111), the fate of these cells is unknown and additional preclinical work is required before further human trials.

Nevertheless, existing preclinical data are promising. Human bronchial basal cells (112) and developmental precursors derived from iPSCs (113) 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 (50) and PCD (85) expand and differentiate similarly in culture to those from healthy control donors, making this a plausible approach, and iPSC-based alternatives are also under development (114). Advances have been made toward functional gene correction in both CF and PCD. Using CRISPR-Cas9 approaches, CFTR function has been restored in intestinal organoids (115, 116) and in CF iPSCs that could be guided toward a lung epithelial lineage (117). Promisingly, the most common CF *CFTR* mutation— $\Delta F508$ —has been corrected using CRISPR-Cas9 delivery in airway basal cells that retained the ability to differentiate into pseudostratified epithelium in ALI culture (118). 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, TALEN (transcription activator–like effector nuclease)-mediated gene editing allowed correction of *DNAH11* mutations with the wild-type sequence in bulk cell cultures, restoring ciliary function (119).

However, a number of hurdles remain. First, estimates for the number of cells required for therapeutic airway epithelial cell engraftment converge in the range of 50–100 million cells (49, 50). Given the small number of basal cells that are recovered from BAL (<2,000 [54]), induced sputum (<2,000 [54]), bronchial brush biopsy (10,000–200,000 [121]), or bronchial forcep biopsy (5,000–200,000 [121]) samples, and the difficulty to manipulate freshly isolated cells, it is likely that such a therapy will require cell expansion in culture. It remains to be seen whether clonal, gene-corrected cells from patients with CF can be expanded to this extent while retaining the capacity to regenerate the airway epithelium long term. Second, available gene-editing technologies can cause unintended off-target effects. The use of clonal cell cultures might reduce the heterogeneity of on-target phenotype modification and simultaneously limit the extent of off-target safety concerns associated with these approaches (120).

A further consideration is that preconditioning of the patient is likely to be required (122) 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 (97), 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 in addition to those outlined above for basal cells; the alveolus has a complex 3D structure including precise cellular architecture that will prove difficult

to recapitulate using exogenous 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 (123). Epithelial cell culture can shed light on the cell biology of this region in humans because 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 (41); immortalized cell lines can retain small airway-like differentiation capacity (124); and ALI cultures from nasal, large, and small airways retain differential sensitivity to viral infection (96). 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 in mice (125, 126). 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 has been suggested by analyses of human disease states (127–129).

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' untranslated region. After brief culture to allow cell division, a proportion of these cells are analyzed by scRNAseq with the remaining cells either replated *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 to track their progeny (130). 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 (130), 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.

Conclusions

Primary human lung epithelial cell cultures represent powerful tools for understanding lung health and

Figure 3. Translational applications of clonal lung epithelial cell culture. Created with BioRender.com.

disease. Improvements to cell culture methodologies allow prolonged culture of both airway and alveolar epithelial cells, including as clonal subcultures. 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 preclinical models (Figure 3). Future cell therapies might also benefit through the capacity to expand cells of a single known genotype to therapeutic quantities. ■

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