Induced pluripotent stem cells for treating cystic fibrosis:
State of the science

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Abstract
Induced pluripotent stem cells (iPSCs) are a recently developed technology in which fully differentiated cells such as fibroblasts from individual CF patients can be repaired with [wildtype] CFTR, and reprogrammed to differentiate into fully differentiated cells characteristic of the proximal and distal airways. Here, we review properties of different epithelial cells in the airway, and the in vitro genetic roadmap which iPSCs follow as they are step-wise differentiated into either basal stem cells, for the proximal airway, or into Type II Alveolar cells for the distal airways. The central theme is that iPSC-derived basal stem cells, are penultimately dependent on NOTCH signaling for differentiation into club cells, goblet cells, ciliated cells, and neuroendocrine cells. Furthermore, given the proper matrix, these cellular progenies are also able to self-assemble into a fully functional pseudostratified squamous proximal airway epithelium. By contrast, club cells are reserve stem cells which are able to either differentiate into goblet or ciliated cells, but also de-differentiate into basal stem cells. Variant club cells, located at the transition between airway and alveoli, may also be responsible for differentiation into Type II Alveolar cells, which then differentiate into Type I Alveolar cells for gas exchange in the distal airway. Using gene editing, the mutant CFTR gene in iPSCs from CF patients can be repaired, and fully functional epithelial cells can thus be generated through directed differentiation. However, there is a limitation in that the lung has other CFTR-dependent cells besides epithelial cells. Another limitation is that there are CFTR-dependent cells in other organs which would continue to contribute to CF disease. Furthermore, there are also bystander or modifier genes which affect disease outcome, not only in the lung, but specifically in other CF-affected organs. Finally, we discuss future personalized applications of the iPSC technology, many of which have already survived the “proof-of-principle” test. These include (i) patient-derived iPSCs used as a “lung-on-a-chip” tool for personalized drug discovery; (ii) replacement of mutant lung cells by wildtype lung cells in the living lung; and (iii) development of bio-artificial lungs. It is hoped that this review will give the reader a roadmap through the most complicated of the obstacles, and foster a guardedly optimistic view of how some of the remaining obstacles might one day be overcome.

KEYWORDS
basal stem cell, bystander genes, ciliary cell, club cell, goblet cell, lung-on-a-chip, modifier genes, neuroendocrine cell, NOTCH, type I alveolar cell, type II alveolar cell
Induced pluripotent stem cells (iPSCs) are a recently developed technology in which fully differentiated cells such as fibroblasts can be reprogrammed to resemble the least differentiated embryonic stem cells (ESCs). Subsequently iPSCs can be coaxed to differentiate into pulmonary, cardiac, neural, and other lineages, and finally into fully differentiated cells. Recent studies show that ESCs and iPSCs are genetically identical when developed from the same genetically matched donor cells. Genomic instability in human iPSCs can occur due to replication stress, which can be controlled, for example, by nucleoside supplementation and increases in checkpoint kinase 1 (CHK1). Nonetheless, among the most attractive properties of iPSCs is that they can proliferate rapidly, and can apparently propagate indefinitely. Thus iPSCs can provide large amounts of cells for personalized regeneration of a diseased tissue. This technology thus seems tailor-made for repairing genetic diseases such as cystic fibrosis (CF) in which mutations in the CFTR gene are responsible for a life-limiting clinical phenotype in the CF lung. Indeed, the timely matching of iPSC and genome editing technologies has recently been described as “forecasting experiments for the next decade.” For example, if the [508del]CFTR mutation from a CF patient’s iPSCs could be replaced with a [wildtype]CFTR sequence, perhaps using CRISPR/Cas9 or other editing procedures, then the “corrected” iPSCs might be reprogrammed to replace epithelial cells in the diseased lung. This is the ultimate therapeutic promise for iPSC technology.

In vitro, the positive news is that gene editing has been successfully deployed to correct the [508del]CFTR to [wildtype]CFTR in iPSCs derived from a CF patient’s fibroblasts. Furthermore, it has been determined that [wildtype]CFTR function and protein can be detected in these now normal cells when they have been differentiated at the air-liquid interface to form proximal airway epithelial cells. As proof of normal function, Firth et al were able to detect cAMP-activated chloride conductance by patch clamp, and mature CFTR protein “C” band by Western blot. CFTR is a cAMP-activated chloride channel, which, when properly glycosylated, trafficks to the apical plasma membrane of epithelial cells as a higher molecular weight “C” band. Importantly, equivalent experiments have been done by other investigators as well, not only for lung, but also for CF pancreatic duct and CF cholangiocytes. Thus for treating CF lung disease with iPSCs, the vision for this workflow would be as follows: (i) to obtain fibroblasts from a CF patient; (ii) to induce iPSCs from the fibroblasts; (iii) to correct the CFTR mutation, either with CRISPR/Cas9 or zinc-finger nucleases (ZFNs); (iv) to grow up unlimited numbers of corrected iPSCs; and (v) to induce iPSCs to differentiate; and (vi) to deploy them back into the CF patient who donated the original fibroblast as personalized medicine. However, as we shall see below, the deployment part of this algorithm remains a significant challenge.

By focusing attention on the CF airway, a second profound challenge is understanding exactly what histological structures are being reconstituted, by which cells, and in which order. Which are the cells to which the iPSCs should be induced to develop, and how pure must the iPSCs and their induced progeny be? To develop iPSCs into therapies, should we be trying to use fetal development of the lung as a roadmap for repair and reconstitution, or is there another way? A final question is how to “administer” the iPSC-based therapy. Suggestions have ranged from whether to introduce cells as individuals or as pre-grown sheets of mature endothelium through the airway; or to re-seed bronchi and bronchioles that have been stripped of mutant epithelium; or to inject the spheroids of mature lung cells of cadaveric origin intravenously, and let them spontaneously find their way to the lung.

However, there are clinical, structural, and physiologic barriers which must be overcome to move from these in vitro proof-of-principle experiments to deploying the technology therapeutically in CF patients. Many outstanding articles and comprehensive reviews have been published in the last few years that specifically address the lung. Here, we will assess important challenges that the lung, and the CF disease itself, erects against different therapeutic strategies. In addition, there is much more to cystic fibrosis than the effects on the lung. We will discuss this aspect of CF below. Yet it is in the lung where the threat-to-life is most manifest, and where clinical work-arounds have been most challenging. We, therefore, focus primary attention in this review on replacing CF epithelial cells lining the CF airway, and on the challenges of the present state of the science. On a positive note, it has been calculated that by delivering just 25% of wildtype CFTR to the CF epithelial surface one might observe normal rates of mucus transport on the CF epithelium. This calculation would thus seem to set a lower limit to the kind of success that added iPSCs might bring to a chimeric [wildtype] + [mutant] CFTR airway.

### 2 WHAT SYMPTOMS MUST BE SUPPRESSED BY A DURABLE THERAPY IN THE CYSTIC FIBROSIS LUNG?

#### 2.1 Thick, sticky mucus

The physiology of the CF lung seems to be focused on failure of the cAMP-activated CFTR chloride channel to secrete chloride into the airway, with consequent dehydration of the airway. If the channel had been active, first sodium and then water would have followed the chloride, and hydration of the airway would have been assured. The consequences of failure are catastrophic, especially when concatenated with the concomitant failure of the airway epithelial cells to discriminate between septic bacterial and viral infection, and aseptic damage such as dehydration, free neutrophil elastase, or an acidic environment. Thus in response to a damage stimulus, the CF goblet cells secrete mucus into a dehydrated airway. However, secreting mucus into a dehydrated environment limits mucin distribution, and results in thick, sticky mucus. Physiologically, this makes sense to the body because the function of the mucus is to protect airway cells by binding to bacteria and viruses. Therefore, on the explicit assumption that microbial invaders have arrived, and that there is a need to deflect them from their intended epithelial cell targets, mucus will be available to bind them. The ciliary cells will then ferry the mucin-bound microbes up and out of the lung. But because the CF
airways are poorly hydrated, the mucus, along with the invaders stuck to it, becomes immobilized within the airway. This leads to failure of mucociliary clearance, especially in the small airways that are denied air due to being blocked off by mucin plugs. These are now sites of potential infection. Impaired PGE2-stimulated chloride and bicarbonate secretion in submucosal glands also contribute to cystic fibrosis airway disease. The importance of bicarbonate is further manifest by a recent report of data from the CF rat, in which correction of low pH and bicarbonate transport is a specific target for restoring mucus clearance. Further regarding oxygen detection in mucin-blocked airways, neurosecretory cells at bronchial bifurcations signal to the brain through their vagal nerve innervation that CO2 levels are high and O2 levels are low. Vagal innervation which contributes to the cough reflex in humans, may be very important to the CF patient for helping clear blocked airways. In the hypoxic mutant CFTR condition, it has been shown that hypoxia-induced 5HT (serotonin) release from neuroendocrine cells is likely suppressed, as well as the secretory response to high potassium. In conclusion, even if immune cells do respond by entering into mucin-blocked, low oxygen regions, they do not function well in an environment that is additionally low in chloride because of mutant CFTR, and low in pH because of low bicarbonate. Bacteria and viruses can then grow in these mucin plug blocked places where infection is further enhanced by denial of access to blood-born antibiotics. An important target for iPSC-based CF therapies would therefore be either suppression of mucin production or increased hydration, or both, in the CF submucosal glands and airways.

2.2 Inflammation

CF airway epithelial cells chronically secrete high levels of chemokines such as IL-8 and cytokines such as IL-6, IL-1β, and TNFα. The tendency to secrete high levels of IL-8 occurs under both baseline and activated conditions. The causative mechanism in CF is based on an intrinsically constitutive activation of NFκB signaling. Subsequent experiments with the potent anti-inflammatory drug digitoxin have shown that digitoxin blocks NFκB activation in CF lung epithelial cells by inhibiting the interaction between TNFR1 and its first intracellular adaptor TRADD (TNFα-associated Death Domain). TRADD is an obligatory mediator of TNFa/NFκB signaling. However, only recently has the biological mechanism of NFκB-driven chronic activation in CF lung been identified. Briefly, under normal conditions, CFTR on the apical plasma membrane binds to the digitoxin target TRADD, and directs it to the proteasome for destruction. However, a functionally inactive mutant CFTR, such as [G551D]CFTR, or an absent mutant CFTR, such as [AF508]CFTR, is unable to execute this process. The result is that TRADD is now free to drive constitutive activation of NFκB, and also drive expression of NFκB-driven downstream proinflammatory cytokines and chemokines such as IL-8, IL-6, IL-1β, and many others. Consistently, a recent placebo-controlled, dose escalation trial of digitoxin in CF patients found that proinflammatory signaling was suppressed in biopsied nasal epithelia. In addition, neutrophil elastase in the sputum was significantly suppressed. This study had been initially planned by one of this review’s authors, and executed at Johns Hopkins University (NCT00782288, clinicaltrials.gov). Concurrently, new potentiator drugs such as VX-770 and correctors such as VX-809 and VX-661 were being developed for administration individually or in combinations. It has been reported that they can suppress proinflammatory signaling in some CF patients. Not unexpectedly, rescue of CF cells with [wildtype] CFTR can suppress these constitutive chemokine and cytokine elevations. Thus, suppression of the hyper-proinflammatory CF phenotype in the lung will be a necessary target for lung-centric iPSC-based therapies.

2.3 Immune cell dysfunction

A third lung-centric CF problem is that CFTR mutations affect immune cell functioning. Examples include neutrophils, macrophages, and lymphocytes in the immune system. Importantly, failure of neutrophil function is intrinsic to CF damage in the lung. For example, there is an abundance of IL-8 in the CF airway, which successfully attracts circulating CF neutrophils into the lung. The neutrophil’s purpose there would be to consume bacteria and other microorganisms. However, the CF neutrophils are physiologically defective. Neutrophils normally use the chloride conducting properties of CFTR to bring ambient chloride into the phagolysosome where myeloperoxidase converts chloride to bacteriocidal HOCl. However, ambient chloride concentration is low in the CF airway, and the chloride conducting properties of mutant CFTR are reduced. Therefore HOCl production is limited and bacterial killing becomes inefficient. Another problem is that these crippled CF neutrophils chronically release ("dribble") neutrophil elastase (NE) into the airway, thus contributing to bronchial damage and bronchiectasis, reactive and sustained mucin secretion, and finally lung failure. However, it is possible that an iPSC-based therapy might reduce hypersecretion of IL-8 from the CF lung, thereby reducing the attractive signals for circulating CF neutrophils. It is already known that by reducing the absolute concentrations of circulating neutrophils by approximately 50% with high dose-ibuprofen, the chronic influx of CF neutrophils can be controlled, with positive therapeutic consequences for lung function in those CF patients able to sustain this therapy.

2.4 Chronic infection

In the CF lung, there is an age-dependent progression of different types of chronic bacterial infections, starting with Staphylococcus aureus, and Haemophilus influenza, and progressing to Pseudomonas aeruginosa (controlled by intermittent tobramycin) and finally Burkholderia cenocepacia complex. The Cystic Fibrosis Foundation has also compiled detailed records on infection incidence in the entire US population. This problem has not necessarily gone away with the advent of correctors and potentiating drugs: while there is on average a prompt reduction in infection, in some patients there is a later rebound. Thus an important target for iPSC therapy would be a profound change of the lung micro-environment such that it no longer preferentially accommodates the survival of these bacteria in the CF
lung. Prospectively, perhaps iPSCs could also be used to make mesenchymal CD34+ stem cells, which would be used to permanently repair the mutant CFTR gene in the CF immune cell population. However, one can envision the possibility that if the chemotaxtractive production of IL-8 could be suppressed by iPSC-based repair of mutant CFTR, massive invasion and damage to the CF lung by crippled neutrophils and macrophages might be minimized.

2.5 | The bystander/modifier gene problem

It has been increasingly realized that there is a great variation in disease severity for CF patients even with the same mutation. Environmental effects from smoking or second hand smoke became immediately apparent.52 For the CF patient, the combustion products of smoke in the lung actually cause a significant reduction in CFTR gene expression.53 Thus whatever residual function might still be available to the CF patient is diabolically suppressed by smoke exposure. More recently it has become apparent that a higher average temperature, found closer to the equator, enhances CF disease severity.54 The mechanism is not known. However, this observation is reminiscent of the observation by Paul Di Sant’Agnese that in New York City during the hot and humid summers of 1948-1949, CF babies suffered from heat prostration, and sweated more chloride than normal babies.55 The result was the classic sweat test for CF. On the other hand, increased chloride in inspired ocean air, as originally noted by a surfer with CF in Australia, reduces symptoms, leading to now-routine administration of inhaled sodium chloride.56

However, there are also problems in CF which are not defined by the CFTR gene itself. Instead they are defined by non-CFTR bystander or modifier genes, that directly affect not only disease severity,57 but in select cases also the capacity to respond to CF drugs.41 We have estimated from a comprehensive survey of the literature that there may be as many as 56 such candidate genes. Variants associated with most of these genes significantly affect disease severity with respect to lung disease. A few examples of such lung-centric genes, many associated with inflammation,58 include TGFβ1 (Transforming Growth Factor beta 159), TNFα (Tumor Necrosis Factor alpha60,61), MBL2 (Mannose Binding Lectin 262,63), and MUC5AC (Mucin 5AC64). Additionally, variants in other bystander/modifier genes significantly affect disease severity in other CF organs. A few examples of such genes include CLCA4 (Chloride channel accessory 4/Ca++ activated chloride channel 4, promoting intestinal disease65); A1AT (Alpha 1 Anti-Trypsin, promoting CF Liver Disease [CFLD]66); GSTP1 (glutathione S-transferase pi 1; promoting increased osteoporosis67), TCF7L2 (transcription factor 7-like 2; promoting increased incidence of CF related diabetes [CFRD]68), and SLC26A9 (solute carrier family 26 member 9; promoting pancreatic damage, CF related diabetes, [CFRD], and meconium ileus69). Thus if iPSC-dependent therapies were directed exclusively to the lung, they might render effects of lung-centric bystander/modifier genes moot. But other non-lung CF organ pathologies would remain intact. Thus these non-lung CF organs would still be susceptible to enhanced CF disease severity by the non-lung-specific bystander/modifier genes.

2.6 | Other CF affected organs in the body

However, as noted above, CF is a type of chronic injury that affects functions not only in the lung, but also elsewhere in the body. Although the principal focus of this review is directed to iPSCs for repair of proximal airway epithelium in the CF lung, it should be appreciated that the disease associated with CFTR mutations affects more than epithelia in the lung. For example, mutations in CFTR also affect smooth muscles in the lung which control the diameters of small airways. In the CF pig model, CFTR is associated with the sarcoplasmic reticulum (SR) and functions to increase the efficiency of calcium uptake. Consistently, in the cystic fibrosis SR, calcium uptake is reduced.70 Consequently, elevated calcium in the cytosol leads to enhanced contractility of the smooth muscle. By contrast, Ivacaftor®/VX-770 administration to CF patients with the [G551D] mutation enhances airway distensibility and reduced vascular tone both in small airways and in tissues outside the lung.71

In the gastrointestinal tract, cells affected directly by CFTR mutations include cholangiocytes (the cells lining the biliary ducts), pancreatic ductal cells, and epithelia in the small and large intestine. This recalls the historical situation that before pancreatic enzymes and bile salts became available for CF patients, CF was first and foremost a failure-to-thrive disease based on malnutrition. In fact, as iterated in the introduction, the use of iPSCs to reconstitute [wildtype]CFTR in CF cholangiocytes has been reported.15,16 Similarly, iPSCs have also been developed to reconstitute [wildtype]CFTR expression in pancreatic ductal cells.14 In addition, inflammation in the pancreas might still have consequences for lung disease, independent of mutant CFTR. Although the mechanism is unknown, there is a well known clinical phenomenon connecting inflammation in the pancreas with pulmonary inflammation.72 Thus pathology in these separate organs are linked in some manner, possibly by the shared vagus nerve.

Other systems sensitive to CFTR function include the male urogenital system, where congenital hypoplasia or aplasia of the vas deferens and seminal vesicles may occur either bilaterally or unilaterally.73 Consequently, most CF males are phenotypically sterile. Finally, in skin, the dysfunction of the sweat glands is life-threatening in hot, humid weather, and is patho-mnemonic for CF.74,75 Mechanistically, mutant CFTR prevents the sweat glands from reabsorbing chloride. However, while these non-lung centric CF cell disorders remain unresolved, most would agree that the central threat to life for the CF patient lies squarely in the cells lining the proximal airway.

3 | EPITHELIAL CELLS IN THE LUNG

There are at least five different types of epithelial cells in the pseudostratified squamous cell epithelium of the proximal airway: club cells, ciliated cells, goblet cells, neuroendocrine cells, and basal stem cells. These cells line the trachea, bronchi, bronchioles, submucosal glands, and smaller airways. Figure 1 shows a representation of cell types in this epithelium, and some limited information about genetic markers and patterns of differentiation. Figure 2H-K shows that basal cells can become club cells, ciliated cells, neuroendocrine cells, and goblet cells. Although most of the detailed information we know about...
these cells comes from the mouse, where possible most investigators have been careful to test for parallels between mouse and humans. Remarkably and fortunately, the parallels have proved to be quite substantial.

3.1 | Proximal airway development, in vivo and in vitro

During fetal development in mouse and human, the foregut endoderm begins to form lung buds, which further undergo stereotypical branching morphogenesis. The initial epithelium is composed of proximal progenitors, including basal cells, which closely adhere to the basal lamina. Basal cells have been presumed to be the stem cells for the rest of the pulmonary epithelium, based on studies dating back more than 25 years, and more recently shown definitively. The definitive evidence included the ability of purified basal cells from mouse trachea to form tracheospheres, populated by columnar cells identifiable as club cells (SCGB1A1+), neuroendocrine cells (CGRP+), and goblet cells (MUC5AC+). Basal stem cells were marked by TRP63+, KRT5+, and KRT14+. Basal stem cells were found to divide to either form additional basal stem cells, or to differentiate into club cells when induced to do so. The signal for basal stem cells to begin differentiation may include high levels of cytokines and chemokines. As indicated in Figure 2, NOTCH signaling has been shown to be critical for decisions by basal stem cells as to which differentiation process to follow. Loss of function experiments in both mouse and human basal stem cells show that NOTCH signaling is required for differentiation, but not for self-renewal.

3.2 | Differentiation of neuroendocrine cells

During fetal development, the first epithelial cells to emerge from the progenitor basal stem cell compartment are the neuroendocrine cells. These cells occur singly or in clusters, called neuroepithelial bodies, which appear to localize near bifurcations in the developing airway tubes. The function of neuroepithelial bodies is to detect local hypoxia/high CO₂ levels, and to signal this information through the vagus nerve to the brain. Development of neuroendocrine cells from basal stem cells is stimulated by NOTCH-hes1 signaling. The mature neuroendocrine cells store serotonin, calcitonin, calcitonin-gene related peptide (CGRP), and other bioactive amines in dense core vesicles. These cells also provide serotonin to both circulating platelets and to clusters of platelet-generating megakaryocytes where they occur in infected lung. Neuroendocrine cells, originating either from basal stem cells or club cells, can also self-replicate. Neuroendocrine cells are innervated from the basal side by the vagus nerve and can be uniquely destroyed by diphtheria toxin. However, whether iPSCs can also reestablish connections between neuroepithelial cells and the vagus nerve is not yet known.

3.3 | Basal stem cell differentiation

Consistent with the data from Rock et al and as summarized in Figure 1A, the basal stem cells are able to differentiate into club cells,
ciliated cells, and goblet cells. Club cells themselves are able to replicate, as well as differentiate into ciliated cells with inhibition of NOTCH signaling. Club cells can also differentiate into goblet cells with increased NOTCH signaling. In both mouse and human, club cell differentiation into goblet cells is also accompanied by SPDEF and FOXA3 expression following allergen exposure. Finally, intermittent de-programming has been applied to club cells to generate a less differentiated state with the classical four component cocktail consisting of: Oct3/4, Sox2, Klf4, C-Myc. The result is rapid expansion of a cell population that can be redifferentiated with 50% efficiency in vitro to a large club cell population. This redifferentiated club cell population can be used to repopulate denuded tracheal tissue in vivo, or in vitro at the air liquid interface, to yield a totally reconstituted epithelial surface, complete with apical expression of CFTR.

3.4 | Submucosal glands

Submucosal glands are found in the trachea in mouse, and in both the trachea and many generations of bronchiolar bifurcations in the human lung. These glands are the result of substantial in-pocketing of the airway surface epithelium, which has the effect of vastly increasing surface area of the lung. It has been appreciated for many years that progenitor cells of the adult human airway contribute to submucosal gland development. In the case of CF, where mucus is thickened, and clearance is profoundly compromised, the ducts connecting the submucosal glands to the airway surface epithelium are blocked by thickened mucus. Thus, it has been readily appreciated that cells in the submucosal glands may not have efficient access to medicinal agents administered to the airway, if the agent cannot easily navigate through mucin-blocked ducts. In fact, the basal stem cells in the submucosal glands are in a specialized niche, where they are protected from toxic inhaled gases, particulates and microorganisms, and thus serve as a reserve to repopulate the airway epithelium. Wine and Joo hypothesized that the thickened mucus was due to loss of CFTR's cAMP-dependent chloride conductance, and consequent reduction of water flow into the gland. Physiologically, CF submucosal glands have been found to have lost control of their cAMP-dependent mucin secretion response and, instead, are rendered solely and completely responsive to Ca++. This dependency of mucin secretion on cAMP may be independent of the cAMP-activated CFTR chloride channel. Consistent with physiologic differences between airway surface epithelium and submucosal gland epithelium, Lynch and Engelhardt recently reported that basal stem cells in the two compartments have different but overlapping properties involving Wnt signaling in physiological conditions.
response to regeneration following denuded tracheal xenografts. Epithelial proliferation rates in the submucosal glands are apparently greater than in airway surface epithelium, when presented with equivalent regeneration challenges.

### 3.5 Variant club cells as origin of type II and type I alveolar cells

Toward the end of gestation, the tips of distal branches further differentiate to form Type II Alveolar cells, which in turn give rise to Type I Alveolar cells, and thus the complex responsible for O₂ and CO₂ exchange. The transition between the proximal and distal airways may differ in some detail between mouse and human, but this is an area of lung biology that is still not fully understood. Importantly there is little evidence that gas exchange, per se, is a critical problem for the CF patient. However, as further summarized in Figure 1B, club cells, or variant club cells (marked by SCGB1A1) at the bronchio-alveolar duct junction (BADJ), may differentiate into Type II Alveolar epithelial cells in the distal airway. It has been suggested that variant club cells might dedifferentiate back into basal stem cells. From this perspective it is these differentiated basal cells, or dedifferentiated club cells, which may be the true origins of the Type II Alveolar epithelial cells. Dedifferentiation of club cells into functional basal stem cells has been reported following ablation of airway stem cells by doxycycline-inhalation-enabled diphtheria toxin expression. The question mark in Figure 1B indicates the need for further work. Type II Alveolar epithelial cells can further differentiate into Type I Alveolar epithelial cells, thus providing the complete functional structure of the distal alveolar lung. These remarkable examples of pluripotency for club cells has led some investigators to consider the club cell as yet another type of pluripotent stem cell, just like the basal stem cell. The context seems important since under homeostatic conditions, turnover in the lung is slow. However, in the event of major catastrophe, such as airway denudation or partial pneumonectomy, the remaining lung can rapidly enter a high production state to uniformly regenerate the lost functional capacity. Except for ciliary cells, all the epithelial cells are capable of cell-division, and club cells can either dedifferentiate into basal stem cells, or differentiate into ciliated cells, goblet cells, or Type II Alveolar cells. These concerns become important, as will be seen below, when addressing the question of how to engineer replacement of CF lung cells with their otherwise identical [wildtype]CFTR replacements.

### 4 IPSCs TO RESCUE CF LUNG DISEASE

As shown in Figure 2A-E, it has become possible to isolate iPSCs from a CF patient, convert the CFTR mutation to the wildtype...
condition, and then differentiate the corrected iPSCs to basal stem cells. The basal stem cell is a reasonable target, based on the information just summarized about this cell’s apparent conditional capacity to form all known epithelial cell types in the lung. By way of a caveat for the foregoing, it is clear that the different cell populations are at best ca. 50% “pure,” and sometimes substantially less. The actual pathway for differentiating iPSCs to definitive adult lung epithelial cells is full of many zigs and zags, well summarized in the review by Wong and Rossant. Thus the in vitro proof-of-principal example given in the introduction represents more of a beginning than an end. The message seems to be that exactly what cells are being determined at any one time is stochastic or probabilistic, and is very dependent upon context. We have tended to agree that “...The lung can be considered a highly plastic and "democratic" tissue, in which a broad diversity of quiescent cell lineages can be induced to proliferate, dedifferentiate, or re-differentiate, and even change phenotype to repair an injured region.”

### 4.1 Definitive endoderm and anterior foregut endoderm

As shown in Figures 2A and 2B, it had been known from earlier embryological studies that high levels of Activin-A biomarked cells that had become definitive endoderm, and that NKX.2.1 biomarked cells that had become anterior foregut endoderm. Green et al. were the first to use this information for inducing iPSCs first to form definitive endoderm by incubating with high levels of Activin-A, and then to convert definitive endoderm to anterior foregut endoderm. Figures 2B and 2C show that the latter goal was achieved by incubating these cells simultaneously in low levels of TGFβ (suppressed by NOGGIN), and by low levels of BMP. More recently, increases in sonic hedgehog (SHH) signaling have been shown to enhance the efficiency of the reaction. These changes were accompanied by elevation of SOX2, a marker for the foregut; by reduction in CDX2, a marker for the hindgut; and by expression of the now classical NKX.2.1 marker for anterior foregut endoderm. Several important controls, mentioned in explicit detail in a later publication, include the demonstration that even though NKX2.1 marks other anterior structures such as thyroid and forebrain, thyroid lineage markers Tg and Pax8, and the neuroectoderm marker Olig2 could not be detected in the NKX2.1+ definitive endoderm (Figure 2C). Importantly, posterior endoderm markers were also absent: Alb (liver), Pdx1 (pancreas), and Foxa3 (posterior endoderm). Consistently, pure Nkx2.1 cells, marked by Nkx2.1 mCherry from fetal mice, form clonal spheroids in semi-solid culture that can differentiate into polarized epithelium with multiple types of lung epithelial cells.

### 4.2 Basal stem cells

After reaching the state of anterior foregut endoderm, this tissue can then be induced to differentiate into either Type II Alveolar Cells by a medium including retinoic acid (RA) (Figures 2C and 2G), or lung endoderm (Figures 2C and 2D) by alternatively adding WNT3a, BMP4, and FGF2. However, adding KGF, NOGGIN (to lower TGFβ) and retinoic acid to lung endoderm does finally yield a preparation enriched in basal stem cells (Figures 2D and 2E). Thus the Anterior Foregut Endoderm may stand at the bifurcation of development, leading either to proximal airway progenitor cells such as the Basal Stem Cell and its progeny, or to the distal airway, comprising the Type II Alveolar Cell and its Type I Alveolar Cell progeny.

Additional progress was also made in terms of increasing the efficiency of these development pathways by Mou et al., who used a similar differentiation protocol, but differently timed, on both mouse and human iPSCs. Remarkably, these investigators showed that mouse and human progenitors were able to form respiratory epithelium in situ when injected subcutaneously in nude mouse models. Because NKX2.1 is also known as thyroid transcription factor-1, Longmire et al. were motivated to find a way to able to separately purify lung and thyroid NKX2.1 progenitors. They did so by inclusion of FGF2 in the differentiation medium (see Figures 2B and 2C). These pathways details are for the most part still definitive, although significant progress in increasing efficiency has been accomplished. Thus with preparations of basal stem cells in hand, it has now become possible to ask personalized questions regarding the metaplastic properties of the iPSC-derived cells, and to compare the results with data inferred from experiments with naturally abundant basal stem cells.

### 4.3 Human lung organoids (HLOs) in 3-D culture

A perceived limitation of the iPSC approach to therapy is that directed differentiation of iPSCs do not lead to a complete lung. For example, current studies describe production of basal stem cells and their progeny, but not fibroblasts, smooth muscle and other mesenchymal elements. However, Dye et al. have found that while 3-D cultures of anterior foregut endoderm contain 85-95% endodermal cells, the remaining cells were actually mesodermal. Furthermore, these investigators found conditions in the 3-D cultures in which the anterior foregut endoderm could be induced to resemble structures reminiscent of the early embryonic lung. These structures, termed human lung organoids (HLOs), included proximal lung cells such as basal cells, ciliated cells, and club cells. Neuroendocrine cells were not mentioned. However, HLOs also included smooth muscle cells, myofibroblasts and fibroblasts. In addition, HLOs also included myeloid immature alveolar airway-like structures. Thus the frequently observed heterogeneity of the iPSC-derived cultures may not be as fundamentally problematic as initially anticipated. Instead the heterogeneity may indicate a network of interacting iPSCs and their differentiated products, with an intrinsic ability to form differentiated cells and structures typical of the entire lung.
5.1 Patient iPSCs as a tool for personalized drug discovery

As predicted by the inventors and developers of this technology, iPSCs could be deployed as a tool, either for analysis of a personalized CF disease phenotype, or as a personalized CF drug discovery platform. Wong et al were the first to use iPSC-derived epithelium from a homozygous [delF508]CFTR CF patient to show that expression of mature [ΔF508]CFTR C-Band, defined by Western blot, could be induced by incubation with 10 μM C-18, an older VX-809-like compound. Cyclic AMP-activated iodide-efflux, a marker for CFTR chloride channel function, could also be detected in the presence of C-18. It is therefore possible that bystander/modifier genes than affect CFTR function in pulmonary epithelia may also be functionally detectable and, therefore, modifiable by gene editing or other drug regimens. In prospect, it would appear that the study of specific drug actions on these personalized iPSC-derived CF epithelial cells will be critical in both understanding patient-specific disease progression for different CFTR mutations, and in developing the best drug treatments for individual patients.

The concept of developing "lab-on-a-chip" (LOC) technology for CF has only recently begun to mature. A LOC is a microfluidics device that integrates several laboratory functions on a single integrated circuit to achieve automation and high throughput. For our present purposes, however, "lab-on-a-chip" has become "lung-on-a-chip," in which cyclic mechanical stress can be applied to either proximal or distal lung epithelial cells. For example, it has been possible to mimic what might be happening in emphysema, where wall stresses might be great, or ventilator-induced lung injury, where flow and stretch conditions can vary profoundly from normal states. In pre-"lung on a chip" era studies on cyclic stretch effects on lung cells, it was found that stretch (15% strain for 4 h at 20 cycles/min), as induced by a ventilator-induced lung injury, could affect both glutathione biochemistry and secretion of IL-6 and IL-8 in the adenocarcinoma A 549 cell model of Type II Alveolar Cells. Another early study in the same cell system showed that cyclic stretch could not only enhance proliferation, but also mitigate the detrimental effect of hypoxia on cell proliferation and viability.

However, Figure 3A-E demonstrates properties of a more recently developed sophisticated lung-on-a-chip, constructed by Guenat and coworkers, that is possibly more CF-relevant. Figure 3A shows how a bioartificial alveolar membrane can be induced to stretch by a microdiaphragm, which is itself actuated by an electro-pneumatic driver. Figure 3B is a schematic for a triple "lung-on-a-chip," which consists of three microfluidic cell culture wells, each identical to the units described in Figure 3A. Figure 3C shows the actual physical assembly in which the lower chambers are filled with food dye for greater visibility. Figure 3D shows the xy-projection from a confocal microscope image of a confluent monolayer of normal human bronchial epithelial cells (16HBE14o-), growing as a monolayer on a bioartificial membrane ("a") driven by an electro-pneumatic driver (not shown). Pink spaces represent culture medium. An epithelial cell layer is depicted growing on the upper surface of the bioartificial membrane. B, Schematic for a lung-on-a-chip. The lung-on-a-chip is composed of two parts: the upper microfluidics and the lower pneumatic assembly. C, Photograph of the lung-on-a-chip. Three systems are on one pneumatic assembly. Lower assembly is filled with dye to distinguish parts. A 96 feature lung-on-a-chip is described. D, Monolayer of epithelial cells growing in the lung-on-a-chip. A confocal image of confluent 16HBE14o- cells. These cells are a derived line of [wildtype]CFTR human lung epithelial cells, and are imaged in the xy plane. The red fluorescence is E-Cadherin. The blue fluorescence is nuclei. E, IL-8 secretion from 16HBE14o- cells growing in the lung-on-a-chip. Cyclic stretching (11 excursions/min) and lengthy time (2 days) in culture stratifies the difference between static and dynamic effect on IL-8 secretion (P < 0.001). Images of the lung-on-a-chip were originally published by Stucki et al, also available under the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/). Original, high quality images from this work were generously shared for this review by Dr Olivier Guenat.
top of the bioartificial membrane in Figure 3A. Here, the red fluorescence marks E-Cadherin (adherens junctions) and the blue fluorescence marks cell nuclei. Figure 3E shows the effect of static versus dynamic culture conditions, and of exposure time, on the secretion of IL-8 from 16HBE14o- cells. These experiments show that long periods of stretching, at a frequency approximating the normal breathing rate (10-12 breaths/min), and normal mechanical strain (5-12% linear elongation), significantly stratifies the level of IL-8 secreted in a dynamic culture over a 48 h period from a static culture over the same time period. In terms of deploying CF patient-derived iPSCs and their differentiated cellular products, this “lung-on-a-chip” platform could therefore provide a complete way to study drug effects and lung biology for individual CF patients, especially patients with the less common CFTR2 mutations.112–115 Relevantly, tidal breathing has been shown to affect phasic motion-induced shear, with consequences for release of nucleotides to the extracellular space and regulation of pericellular liquid homeostasis in CF patients.116 The lung-on-a-chip concept also permits a more global physiological view of CF, in which the disease is more than just a CFTR mutation. Rather it provides a more physiologically relevant context for analysis.117

5.2 Replacement of mutant lung cells by wildtype lung cells in the living lung

The so-far unresolved challenge has been how to use the iPSC technology for therapy: the replacement of mutant lung epithelial cells with their wildtype equivalents. We give several examples here to illustrate that this approach has been tested in different ways, and for different indications. One approach has been to attempt to perform the replacement in the otherwise diseased but still functional lung. One strategy has been to administer iPSCs directly by the intravenous route.118–120 For example, acute lung injury (ALI) was induced in mice by administering endotoxin by the endotracheal route.118 Mouse iPSC cells were then delivered into the mouse by tail vein injection. The consequences were that iPSC incorporation was enhanced; histopathologic changes, NFκB and neutrophil accumulations were reduced; and hypoxemia and pulmonary function were rescued. In these cases, iPSCs were apparently being used as a drug. In another strategy, immuno-competent mice, that had been treated with bleomycin to induce a model of interstitial fibrosis, were injected with cultured allogeneic and syngeneic adult lung spheroids.17 Progression of fibrosis and inflammation were suppressed, without eliciting significant immune rejection. A human trial is said to be planned. In an alternative to the intravenous injection route, lungs of mice and pigs, whose lungs had been pre-injured/pre-conditioned with 2% polidocanol (PDOC), mature human airway epithelial cells were administered intra-tracheally.121 Based on a fluorescent label, cell retention/ “engraftment” 2 days later was ca. 10% in mouse and ca. 22% in the pig. The purpose of the PDOC, an FDA-approved local anesthetic and anti-pruritic, was to temporarily remove the surface airway epithelial cells, thereby opening up a place for epithelial cell engraftment, and activating proliferation of normally quiescent epithelial cells by enhancing cytokine and chemokine expression in the airway.

The “pre-injured/pre-conditioned strategy” has also been attempted in studies with direct CF-centric relevance. In an early study with bone marrow (stem) cells (BMC), Wong et al122 administered mild injury to mouse airways with intraperitoneal (IP) naphthalene to destroy club cells and ciliary cells, and then intratracheally delivered cultured BMCs. Expression of epithelial markers were detected for club cell secretory protein and surfactant protein C, and retention of BMCs were observed for at least 120 days. While the presence of the BMCs was thought to be responsible for awakening proliferation of the remaining original residents of the host lung, some of the BMC label was also found in epithelial cells, suggesting transdifferentiation. More recently, but still using the older BMC technology, Duchesneau et al123 were able to implant wildtype BMCs in the trachea of CFTR-null (CFTR⁻) mice which had had their club cells and ciliated cells preliminarily destroyed by intraperitoneal naphthalene treatment. Recalling that transdifferentiation of BMCs was infrequent, nonetheless, apical [wildtype]CFTR protein was detected in the reconstituted airway; P aeruginosa infection was delayed; and survival was increased. The benefit lasted for more than 6 months. Thus for those patients who are null for CFTR, and are therefore unlikely candidates for the newer corrector and potentiator drugs, this kind of approach, possibly with wildtype iPSCs instead of BMCs, may eventually be to their benefit.

5.3 Decellularized lungs and artificial scaffolds

In response to the fact that there are so few lungs available for transplant, and so many patients in need, the idea of artificial lungs has developed significant appeal.124,125 One more recent CF-relevant idea has been to use a patient’s corrected iPSCs to repopulate a decellularized lung, and then to replace the diseased lung with the re-cellularized lung. The object of studies on lungs, ranging from mouse126 to a non-human primate (Rhesus macaque),127 has been to isolate an intact scaffold made up of a passive extracellular matrix, which can be repopulated with autologous stem or progenitor cells. The first problem has been to remove as much cellular material as possible, without the entire scaffold/lung falling apart. The solution has been threefold: first, to wash the lung as completely as possible with divalent cation-free aqueous solutions; second, to extract as much as possibly with a succession of detergents such as sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC), and CHAPS126; and third, to then fix the entire organ in formaldehyde. Following many additional steps, a somewhat flexible scaffold is generated, upon which cellular re-population can hopefully be achieved. Presently, the experiments have been limited to 350 micron thick sections of the scaffold, which have been used to test the ability of stem cells to grow and assemble into a continuous proximal and distal lung. However, there is still much to learn even from this simple kind of experiment.

For example, in a recent CF-relevant experiment, Shojaie et al97 used stem cell-derived definitive endoderm, in this case differentiated from ESCs (embryonic stem cells), to form NKX2.1+, SOX2+ progenitors. They then incubated the cells with thick sections of mouse or rat decellularized lung scaffolds. The result was that the
progenitor cells bound to the scaffold surface and differentiated into mature airway epithelia, including ciliated cells (FOXJ1+, TUBB4A+), club cells (SCGB1A1+), and basal cells (TRP63+, KRT5+). CFTR protein was present, and was also functional, as defined by cAMP-dependent iodide efflux. Pit-like structures upon the surface could be discerned by optical microscopy. These were interpreted as nascent submucosal glands, and confirmed by scanning EM. Importantly, the epithelial differentiation process proved to be dependent upon the presence of heparinase I-sensitive heparin sulfate proteoglycans in the decellularized matrix. This paper also emphasized an important control: showing that decellularized kidney sections, a mesoderm-derived tissue, could not replace decellularized lung sections as a matrix for constitution of the airway epithelium. Thus this heavily washed and fixed scaffold is still biochemically “alive.” Nonetheless, the lung physiologist reading this will think of many more organismic properties that this bioengineered lung must display in order to be durable and operational.

6 | CONCLUSION

Based on the state of the science, it appears that the best use of iPSCs for CF lies in using them from individual CF patients in their differentiated states for personalized medicine. For example, the impact of different bystander/modifier genes on drug responses can be studied today using a lung-on-a-chip approach. Thus the ability of specific variants in a patient’s bystander/modifier genes can be assessed in terms of how a specific drug might affect CFTR trafficking and CFTR function, including cAMP-activated chloride transport or inflammation. However, in thinking of using iPSCs to create a durable physical therapy for CF lung disease, we quote the opinion of Shinya Yamanaka, the 2012 Nobel Laureate for the development of the iPSC technology, who concluded that “the potential is enormous, but many obstacles remain before this technology can become converted to therapy.”

7 | GLOSSARY

This glossary is provided as an aid to following the different workflows. Some of these entries are described in depth in the text. Unreferenced information has been abstracted from either Wikipedia or GeneCards.

16HBE14a: an immortalized human bronchial epithelial cell line that is widely used to model barrier function of the airway epithelium, and to study respiratory ion transport as well as the function of CFTR. A1AT: Alpha 1 Anti-Trypsin. A1AT is encoded by the SERPINA1 gene, and functions as a protease inhibitor. In the lung, it is best known as an inhibitor of neutrophil elastase. Variants are associated with increased risk of CF Liver Disease (CFLD).

Activin-A: Activin-A belongs to the TGFBeta-protein superfamily, and regulates branching morphogenesis in prostate, lung and kidney. Upregulation drives iPSCs into a mesoendodermal fate. It had been noticed that high levels of Activin-A marked embryonic cells that had become definitive endoderm. Therefore Activin-A was used to drive the first step of converting iPSCs into proximal lung epithelium. Like TGFβ and BMPs, Activin-A receptors activate transcription factor SMADs, to induce gene expression.

ALI: Air Liquid Interface refers to an in vitro cell culture conditions that models the interface between epithelial cells lining the airway and air in the airway.

Alveolar cells: Type I Alveolar cells are cells in distal airway for gas exchange. Type II Alveolar cells are cells in distal airway containing surfactant. They are also progenitor r cels for Type I Alveolar cells.

Anterior Foregut Endoderm (AFE): AFE gives rise to cell types such as esophagus, salivary glands, lung, thymus, parathyroid and thyroid glands. This tissue became the target for differentiation from iPSCs, and its heterogeneity could be an obstacle for trying to create lung-specific cells.

AQP: aquaporin. Aquaporins are members of a 13 member gene family which regulates water flow across membranes. AQP 1, 3, and 4 are expressed in the lung. AQP3 and AQP4 are specifically expressed in airway epithelia. AQP5 are expressed in Type I alveolar epithelial cells, submucosal gland acini, and a subset of airway epithelial cells.

BADJ: broncholaveolar duct junction. The BADJ is a branch point between a terminal bronchiolce and the distal airways alveolar sac. Cells responsible for this transition may be variant club cells, marked by SCGB1A1.

Basal Luminal Precursor: a transitional form of the basal stem cell as it differentiates into a more lumenally located epithelial cell. It is marked by changes from KRT14 or – gene expression, to express KRT5 and KRT8.

Basal stem cells: are named for their proximity to the underlying basal lamina. They are multipotent stem cell population of pseudo-stratified airway epithelium, which are marked by the TRP63 gene. These cells can give rise to all of the cell types in the epithelium, and can be back dedifferentiated from club cells. Basal stem cell markers shared by mouse and human include TRP63, SNA12, NGFR, EGFR, PDPN, KRT5, KRT14, AQP3, CDH3, ITGB6.

BMP: Bone morphogenetic proteins comprise a family of 7 genes, which are a group of growth factors that orchestrate tissue architecture including foregut and hindgut. BMPs are members of the TGFβ superfamily and activation mobilizes SMAD5. BMP4 plays a role in development of the lungs, liver, teeth and facial mesenchyme cells. BMP7 is an inhibitor of TGFβ1 signaling.

CDH3: cadherin 3, also called P-cadherin. CDH3 is one of 6 cadherin genes located on the long arm of chromosome 16. CDH3 is a calcium-dependent cell-cell adhesion glycoprotein composed of five extracellular cadherin repeats, a transmembrane region and a highly conserved cytoplasmic tail. CDH3 is a biomarker for differentiated epithelial cells.
CDX2: Caudal type homeobox 2. CDX2 is a biomarker for hindgut, and plays a role in early embryonic development of the intestinal tract.

CGRP: Calcitonin gene-related peptide. CGRP is a potent vasodilator and is found throughout the body, including the lung, where it is a biomarker for neuroendocrine cells. High circulating levels are associated with migraine headache and sepsis.

CFRD: Cystic Fibrosis Related Diabetes. CFRD is a variant of Type I diabetes and is the most significant extra-pulmonary comorbidity in CF. It is increasingly common in older patients and is associated with lung decline.132 CFRD is caused by β cell loss, and immune cell infiltration of the islet.133 CFTR may play a role in normal first phase insulin secretion from the β-cell.

CFTR: cystic fibrosis transmembrane conductance regulator. [ΔF508]CFTR (also termed [F508del]CFTR) is found in 80% of CF patients, and therefore the most common CFTR mutation. This mutation is missing phenylalanine (F) at position 508 in the CFTR protein. This mutation is also referred to as F508del-CFTR. [G551D]CFTR (p.GLY551ASP)CFTR is the next most common CF mutation, in which glycine (G) at position 531 in the CFTR protein is replaced by aspartic acid (D).

Chemokine: Chemokines are a family of small proteins in the size range of 8-10 kDa which are secreted by cells in order to attract target cells. Their full name is chemotactic cytokines, and are found in 4 families based on patterns of cysteine location in the sequence (viz, CXC, CC, CX3C, and XC; C is one letter code for cysteine; X is any amino acid). An example of a CXC chemokine is IL-8, which can be secreted from lung epithelial cells and attracts neutrophils.

Ciliary cell: Ciliary cells are columnar epithelial cells that are principally characterized by densely distributed apical cilia, and function to propel the mucin lining the airway surface up and out of the lung or down into the stomach for destruction. Ciliary cells can be destroyed by intraperitoneal naphthalene. Naphthalene administration has been used as a strategy to provide a cell-free region for iPSC-derived epithelial cells to grow.

CLCA4: Chloride channel accessory 4/Ca++ activated chloride channel 4. CLCA4 is a bystander/modifiers gene for CF that promotes intestinal disease.

Club cell: Club cells are also known as broncholar exocrine cells, and were originally known as Clara cells.134 They are dome shaped cells with microfilli that are found in the pseudostratified airway epithelium of the lung. Their function is to detoxify chemicals with cytochrome p450 enzymes, and to act as auxiliary stem cells to the basal stem cells by differentiating into ciliated cells and goblet cells. They can also dedifferentiate into basal stem cells. They synthesize protective glycosaminol glycans and antibiotic lysozyme. Variant club cells may also generate Type II Alveolar cells. Club cells can be destroyed by intraperitoneal naphthalene.

Corrector drugs: These drugs help some mutant CFTR proteins to traffick to the membrane. Examples are VX-809 and VX-661.

Cytokines: Cytokines are small proteins produced by a broad range of cells, including epithelial cells and immune cells, and are partially responsible for the host cell's response to infection. In general they activate cell division in target cells. They may include chemokines such as IL-8, interferons, interleukins such as IL-6, lymphokines and tumor necrosis factors such as TNFα.

Definitive endoderm: The definitive endoderm forms during gastrulation from extra-embryonic visceral endoderm, located along the ventral surface of the embryo.

Distal airway: Refers to that portion of the lung involved in gas exchange via Type II and Type I Alveolar Cells.

EGFR: Epidermal growth factor receptor. EGFR is a cell surface protein that binds to epidermal growth factor. Binding of the protein to a ligand induces receptor dimerization and tyrosine autophosphorylation, and leads to cell proliferation.

ESC: Embryonic stem cell. ESCs are pluripotent stem cell which are derived from the inner cell mass of a blastocyst, an early stage pre-implantation embryo.

Endoderm: forms during gastrulation and replaces the extra-embryonic visceral endoderm. It also contributes to morphogenesis of the gut tube and visceral organs.135

EMT: Epidermal-mesenchymal-transition. EMT is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties. Classically, the process is marked by loss of E-cadherin and gain of vimentin. Signaling pathways such as TGFβ, FGF, EGF, HGF, Wnt/beta-catenin and NOTCH, and hypoxia induce EMT.

FGF: Fibroblast growth factor. FGF is a family of 22 members which binds to FGF receptors with mostly mitogenic consequences, but different functions. Curiously, the crystal structure resembles IL-1β. FGF2 is secreted from lung fibroblasts in response to TGFβ1.136

FOXJ1: Forkhead box protein J1. FOXJ1 is a member of the Forkhead winged helix (Fox) family of transcription factors. It is involved in ciliogenesis and is expressed in ciliated cells in the lung and elsewhere. FoxJ1 regulates NFKB activity by regulation of IKKB.

GATA6: Transcription factor GATA-6. GATA6 is a zinc finger transcription that controls the late differentiation stages of alveolar epithelium and aquaporin-5 promoter activation. The name of this transcription factor is based on its bolded bases in the consensus binding sequence (T/C)GATA(A/T)(A)/cSOX2).

Gene: Gene can refer to the DNA, or it can refer to the cognate messengerRNA (mRNA). In both cases the abbreviation is in italics. When written in plain text the word gene refers to the protein that is made from the corresponding mRNA.

Goblet cell: Goblet cells are columnar epithelial cells, shaped like a wine goblet, that contains membrane-bounded mucus granules. The goblet cell secretes mucus by exocytosis, and epithelial lining fluid which is used as the medium for mucociliary clearance.

GSTP1: glutathione-S-transferase pi 1 gene. Glutathione S-transferases (GSTs) are a family of enzymes that play an
important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. The addition of glutathione is the detoxifying step. **Homeobox**: Homeobox genes are members of a gene family with an estimated 235 functional genes. Members of this gene family directs formation of many body structures during embryonic development.

**IL-6**: Interleukin 6. IL-6 is a pro-inflammatory interleukin and cytokine. In general, cytokines activate cell division.

**IL-8**: Interleukin 8. IL-8 is a pro-inflammatory chemokine, the principle attractor of neutrophils to the lung. In general, chemokines attract immune cells to the chemokine source.

**IL1β**: Interleukin 1β. IL-1β is cleaved from its precursor by caspase 1 (interleukin 1 beta convertase). It is a lymphocyte mitogen and pyretic, and is induced by COX-2.

**iPSCs**: induced pluripotent stem cells. iPSCs are products of a recently developed technology in which fully differentiated cells such as fibroblasts can be reprogrammed to resemble the least differentiated embryonic stem cells (ESCs).

**ITGB6**: Integrin subunit beta 6. Integrins are members of a superfamily comprising 24 genes that function as transmembrane cell adhesion receptors. Cytoplasmic tails interact with cytoskeletal proteins. TGFβ1 activates ITGB6 expression in the lung.

**KGF**: Keratinocyte growth factor. KGF is also known as FGF7. KGF binds to FGF Receptor 2b, and functions as an epithelial cell-specific growth factor associated with morphogenesis of epithelium.

**KRT**: Keratins. Keratins, also termed cytokeratins, comprise a family of 30 genes. KRTs are a structural part of intermediate filaments which dynamically connect the nucleus to the plasma membrane, and are responsible for the structure of epithelial cells. Key KRT genes for basal stem cell development are KRT 5, KRT8, and KRT14.

**Lamellar bodies**: Lamellar bodies are surfactants synthesized in Type II Alveolar cells. Lamellar bodies are mostly composed of phosphatidylcholine. Surfactants are secreted into the alveolar space and function to prevent collapse of alveoli during expiration. See surfactant, SFTPC.

**Lung-on-a-chip**: a microfluidics device that applies cyclic mechanical stress to cultures of either proximal or distal lung epithelial cells automatically and with high throughput.

**Lung endoderm**: The respiratory endoderm develops from a small cluster of cells located on the ventral anterior foregut. This population of progenitors generates the myriad epithelial lineages required for proper lung function in adults.

**MUC5AC**: Mucin5AC. Mucin5AC is a glycoprotein in tracheobronchial and gastric tissue that protects the mucosa from infection and chemical damage by binding to inhaled bacteria and viruses, or particles, that are subsequently removed by the mucociliary escalator, either up and out by expectoration or swallowed into the stomach for destruction.

**MBL2**: Mannose binding lectin 2. MBL2 belongs to the class of calcium-dependent lectins (collectins), in the C-type lectin superfamily, whose function appears to be recognition of oligosaccharide structure or lipids that are on the surfaces of bacteria, viruses, protozoa, and fungi. The result is activation of the lectin pathway of the complement system, and death of the microbe.

**Neuroendocrine cell**: Neuroendocrine cells are lung cells that receive neuronal input from the vagus nerve, and secrete bioactive amines such as serotonin (5HT), calcitonin, CGRP, gastrin-releasing peptide (GRP), and cholecystokinin. Their function is to act as a chemoreceptor for hypoxia and as a source of 5HT for nascent circulating platelets. Neuroendocrine is also spelled neurendocrine in the United Kingdom.

**NFκB**: Nuclear Factor Kappa B. NFκB is a 5 member family of genes that that is held inactive in the cytoplasm by binding to IkBa. For example, in the case of NFκB_p65, phosphorylation of IkBa frees the NFκB_p65 protein to enter the nucleus to drive expression of IL-8 and other proinflammatory genes.

**NGFR**: Nerve Growth Factor Receptor; also Tumor necrosis factor receptor superfamily member 16. NGFR is part of a 29 member TNF Receptor superfamily.

**NKX2.1**: NK2 Homeobox1. NKX2.1 is also known as TTF-1 (thyroid transcription factor1. NKX2.1 is a biomarker shared by lung, thyroid, and diencephalon during embryonic development. NKX.2.1 is a biomarker for anterior foregut endoderm.

**NOGGIN**: encoded by the NOG gene. NOGGIN was originally named due to its ability to produce embryos with large heads, and regulates bone morphogenetic protein (BMP) expression during embryonic development. The function of NOG is to bind and inactivate TGFβ superfamily signaling proteins such as TGFβ1 and Activin-A. It also acts downstream of WNT and Sonic Hedgehog (SHH) to antagonize BMP4.

**NOTCH**: a gene named after a Drosophila mutation causing a notch in the wing. NOTCH binds to 4 possible notch receptors. Intracellular interactions occur with CFB1 and mastermind to activate transcription. NOTCH is crucial for embryonic epithelial mesenchymal transition (EMT) induction, and formation of pancreas gut and lung from endoderm. NOTCH and most of its ligands are transmembrane proteins, so the cells expressing the ligands typically must be adjacent to the Notch expressing cell for signaling to occur.

**PDNP**: Pedoplanin, also known as T1a. PDNP is a mucin-like protein that may function as an influenza virus receptor. However, its function remains ill defined. It is a convenient biomarker for Type I Alveolar cells.

**Potentiator drugs**: Potentiator drugs are cystic fibrosis drugs that function by opening the hitherto closed, mutant CFTR chloride channel in the membrane. An example potentiator drug is VX-770 (Ivacaftor/Kalydeco). An example target CFTR mutation is G551D.CFTR.

**Retinoic acid (RA)**: is an active metabolite of vitamin A. It binds to the retinoic acid receptor (RAR), which then binds to...
retinoic response elements (RAREs) on target DNA promoters. It is a potent morphogen in hind brain development and in the heart.

SCG1A1: Secretoglobin family 1A member 1. This gene is also known as Uteroglobin. It is a biomarker for club cells. Variant club cells are also marked by SCGB1A1, and are located at branch points between a terminal bronchiole and the distal airways. It is hypothesized that they are the progenitors of Type II Alveolar Cells. Alternatively, they may dedifferentiate into basal stem cells, which are then the actual progenitors for Type II Alveolar Cells.

SFTPC: Surfactant protein C. SFTPC is a membrane protein in Type II Alveolar cells involved in synthesis of pulmonary surfactant. Example of a product includes SFTP C1.

SHH: Sonic Hedgehog. SHH is a critical morphogen during embryonic lung development, and regulates the interaction between epithelial and mesenchymal cell populations in proximal and distal lung.\(^ {138}\)

SLC26A9: solute carrier family 26 member 9. A variant in SLC26A9 promotes pancreatic damage, CF related diabetes, [CFRD], and meconium ileus in CF patients, and can attenuates CF drug efficacy (see section 2.5).

SMAD: Small Mother’s Against Dodecaplegic. SMADs are members of a family of 9 genes which are downstream effectors of TGF\(\beta\) and BMP signaling.

SOX2: (SRY) SOX determining region Y)-box 2. SOX2 is one of 4 transcription factors used to generate iPSCs, and to drive embryonic development. SOX2 regulates emergence of lung progenitor cells by regulating TRP63, and bronchoalveolar stem cells, by regulating GATA6.

SPEDF: Sam-pointed domain containing Ets Transcription factor. SPEDF is needed for the differentiation of both pulmonary and intestinal goblet cells.

Surfactant: a lipoprotein complex formed by Type II Alveolar cells that prevent collapse of the alveoli at end of expiration.

T1a: See PDPN.

TCF7L2: transcription factor 7-like 2. A variant in the TCF7L2 gene promotes increased incidence of CF related diabetes (CFRD) in CF patients.

TGF\(\beta\): Transforming growth factor beta. TGF\(\beta\) is a member of a superfamily that itself includes 4 TGF\(\beta\) isoforms. Signaling depends on activation of SMADs which mediate downstream signaling by translocating to the nucleus. Under normal conditions TGF\(\beta\) blocks the cell cycle at G1. To activate proliferation during development, inhibition is mediated by NOGGIN (See Figure 2).

TNF\(\alpha\): Tumor Necrosis Factor alpha. TNF\(\alpha\) is part of a 16 member superfamily of type II transmembrane proteins. TNF\(\alpha\) and certain other members of this superfamily can be released from the cell membrane by extracellular proteolytic cleavage, where the freed protein fragment can function as a cytokine.

TRADD: TNF\(\alpha\) Receptor Associated Death Domain. TRADD is the first intracellular adaptor to the

**TNF Receptor 1/TNF\(\alpha\) complex.** The function of this binding activity is to activate IKK\(\alpha\)\(\beta\), downstream NF\(\kappa\)B signaling, and inflammation. In the presence of wildtype CFTR, TRADD is directed to the proteasome, NF\(\kappa\)B is not activated, and no inflammation occurs.\(^ {37}\)

**Transcription Factor:** Transcription factors bind to DNA sequences in a gene promoter, or to other transcription factors bound to promoter DNA, and either drive or inhibit gene expression. An example of a driver is NF\(\kappa\)B. An example of an inhibitor is the glucocorticoid receptor.

TRP63: Transformation-related protein p63, also known as TP63. TRP63 is an evolutionary founding member of the p53 superfamily, and is regulated by SOX2 to induce the formation of basal stem cells.

TUBB4A: Tubulin beta-4A. TUBB4A is a form of tubulin (TUB), a major cytoskeletal protein, which together with TUBB4B is said to be preferentially and highly expressed in the central nervous system. Curiously, it is also a biomarker for ciliated cells.

UCHL1: ubiquitin carboxy-terminal hydrolase L1. UCHL1 is mostly found in the brain and testes/ovary. However, it is also found in lung and is a biomarker for neuroendocrine cells.

**Vagus nerve:** The vagus nerve is the tenth cranial nerve and originates in the area postrema. It provides both sympathetic and parasympathetic control of the heart, lungs and digestive tract. Conduction pathways in the vagus nerve are bothafferent (going to the brain) and efferent (going away from the brain). In the lung, the neuroendocrine cells provide information on local hypoxia at airway bifurcations.

**WNT:** a combination name of the wingless gene and the Interr 1 gene, first discovered in Drosophila, but highly conserved. It forms three (3) signaling pathways, all activated by binding to one of the family of Frizzled receptors, which passes the information to an internal Dishevelled protein. Pathways in embryology include cell fates, proliferation, and migration. An example is WNT3a.

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