When Is an Alveolar Type 2 Cell an Alveolar Type 2 Cell? A Conundrum for Lung Stem Cell Biology and Regenerative Medicine

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Abstract

Generating mature, differentiated, adult lung cells from pluripotent cells, such as induced pluripotent stem cells and embryonic stem cells, offers the hope of both generating disease-specific *in vitro* models and creating definitive and personalized therapies for a host of debilitating lung parenchymal and airway diseases. With the goal of advancing lung-regenerative medicine, several groups have developed and reported on protocols using defined media, coculture with mesenchymal components, or sequential treatments mimicking lung development, to obtain distal lung epithelial cells from stem cell precursors. However, there remains significant controversy about the degree of differentiation of these cells compared with their primary counterparts, coupled with a lack of consistency or uniformity in assessing the resultant phenotypes. Given the inevitable, exponential expansion of these approaches and the

probable, but yet-to-emerge second and higher generation techniques to create such assets, we were prompted to pose the question, what makes a lung epithelial cell a lung epithelial cell? More specifically for this Perspective, we also posed the question, what are the minimum features that constitute an alveolar type (AT) 2 epithelial cell? In addressing this, we summarize a body of work spanning nearly five decades, amassed by a series of “lung epithelial cell biology pioneers,” which carefully describes well characterized molecular, functional, and morphological features critical for discriminately assessing an AT2 phenotype. Armed with this, we propose a series of core criteria to assist the field in confirming that cells obtained following a differentiation protocol are indeed mature and functional AT2 epithelial cells.

Keywords: type II pneumocyte; induced pluripotent stem cell; embryonic stem cell; surfactant; lamellar body

The Promise of Regenerative Medicine for Understanding and Treating Lung Disease

Chronic lung disease is now recognized as a major cause of global morbidity and mortality (<http://www.who.int/respiratory/en/>). Impaired restitution of dying alveolar epithelial and endothelial cells through either self-renewal or via contributions from endogenous lung progenitor cells results in a loss of gas exchange surface area and/or barrier function, as well as the potential promotion of expansion of

mesenchymal compartments (1). This acquired “stem/progenitor cell failure” from a loss of key cell populations and the resulting aberrant injury/repair represent a central tenet in pathogenesis of degenerative parenchymal lung diseases, such as chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis, as well as in the development and propagation of acute lung injury from a variety of etiologies (2–8). As there is a limitation of present therapies to reverse or correct such losses, regenerative medicine, with the hope of replacing damaged lung

with healthy cells or tissue, represents a new paradigm for the definitive management of parenchymal pulmonary diseases.

The alveolar type (AT) 2 pneumocyte (hereafter AT2 cell) is an endodermally derived, multifunctional, polarized epithelial cell spatially restricted to the distal mammalian adult lung (9). Since the first report of the successful isolation of a surfactant-producing epithelial cell from the lungs of rodents by Kikkawa and Yoneda in 1974 (10), a wealth of data using primary cultured AT2 cells isolated from a

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variety of species has assigned important biosynthetic, secretory, metabolic, host defense, and repair/regenerative functions to this population, rendering this cell critical to the maintenance of alveolar homeostasis (11, 12). In addition, AT2 dysfunction or dropout has been implicated in the pathogenesis of a variety of parenchymal lung diseases, including idiopathic pulmonary fibrosis, Hermansky-Pudlak syndrome-related pulmonary fibrosis, and chronic obstructive pulmonary disease, among others (1, 8, 13–17). However, despite improvements in cell yields and purities over the past 40 years, major barriers to better understanding the role of the AT2 cell in health and disease have persisted from both the inability to culture and maintain *primary* AT2 cells in a well differentiated state, as well as the discouraging reality that stable cell lines (including A549, L2, H441, RLE-6N, and MLE12/15) fail to faithfully recapitulate the morphologic, functional, molecular, proteomic, and/or lipidomic markers of the AT2 phenotype (12, 18). Now, with the rapid emergence of stem cell biology and the use of reprogrammed pluripotent progenitor populations, eternal hope has sprung once again that, a suitable alternative to the use of primary cultured AT2 cells can be found.

Lung Stem Cell Biology and the AT2 Cell: a Clear and Present Conundrum

The promise and power of multipotent adult stem cells, embryonic stem cells (ESCs), and, more recently, induced pluripotent stem cell (iPSC) –based technologies for regenerative and personalized medicine cannot be understated. The first studies that suggested that multipotent adult stem cells could differentiate into lung epithelium involved attempts to use *in vivo* differentiation of injected bone marrow cells to generate populations of either distal lung epithelial precursors or potentially fully differentiated distal lung epithelial cell lineages, both AT2 (19) and AT1 (20). However, these early studies and subsequent reports that leveraged more robust imaging (e.g., confocal microscopy) and labeling protocols determined that both engraftment and *in vivo* differentiation of the injected cells was

extremely low and required the concomitant induction of lung injury using exogenous agents, such as bleomycin or *Pseudomonas* infection (21–24). Recently, endogenous, multipotent lung stem cells have been identified in the distal airways (25, 26). Dubbed either lineage-negative epithelial stem progenitor cells (25) or distal airway stem cells (p63/Krt5) (26), these basally located cells can be isolated as a single rare population distinct from the more prevalent AT2 cells or bronchiolar cells. When transferred to influenza-challenged recipient lungs, lineage-negative epithelial stem progenitor cells or distal airway stem cells (p63/Krt5) can repopulate the areas of severest injury. Although capable of differentiating to several cell types *in vivo* (including AT1, AT2, and bronchiolar cells), the utility of these cells for *in vitro* disease modeling or regenerative therapy remains to be defined.

A second wave of stem cell-based techniques to generate lung epithelia has involved *in vitro* differentiation protocols applied to animal-derived ESCs and a limited number of human ESC (hESC) lines (27–29). The initial proof-of-concept studies performed in the early part of this century demonstrated that, under specific conditions, these primordial precursor populations could be driven to adopt some phenotypic features associated with more terminally differentiated cells normally derived during the course of the fetal development program from all three major definitive embryonic germ layers (ectoderm, mesoderm, and endoderm), including distal airway epithelia. Wang and colleagues (27) used transfection of an undifferentiated hESC line (ES 9.2) with a human surfactant protein (SP-C) promoter–neomycin transgene (3′-hprt-SPCP.NEO) to permit isolation of clonal populations of lung epithelial cells expressing mRNA transcripts of SP-C, cystic fibrosis transmembrane conductance regulator, and α_1 -antitrypsin, as well as proSP-C and surfactant protein A (SP-A) proteins by immunofluorescence. These cells also contained some ultrastructural evidence of lamellar body (LB) formation. Subsequently, a second group has shown that undifferentiated hESCs grown on porous membranes and sequentially cultured in a defined differentiation medium at air–liquid interface were capable of generating a cell population

demonstrating quantitative increases in mRNA (by real-time quantitative PCR) and protein expression (immunohistochemistry [IHC]) of distal lung epithelial markers, Nkx2-1, CC16, SP-C, and aquaporin (AQP) 5 (28). More recently, purification and directed differentiation of primordial lung and thyroid progenitors derived using Nkx2-1^{GFP} knockin reporter mouse embryonic stem cells (ESCs) has been described (29).

Limited in scale-up by a number of barriers (including political, ethical, and pragmatic), the early successes with hESCs have been largely supplanted by a wave of newer iPSC-based methodologies. First described by Takahashi and colleagues (30), pluripotent iPSCs induced from a variety of somatic cells from human and animal donor sources (including skin fibroblasts and peripheral blood mononuclear cells) via overexpression of Oct4, Klf4, Sox2, and cMyc (colloquially referred to as “Yamanaka factors”), have further revolutionized the field of stem cell biology. These reprogrammed cells have opened up the promise of developing disease/patient-specific *in vitro* models to enhance understanding of disease pathogenesis, to promote discovery of personalized therapeutic portfolios, and to provide a source of organ-specific cellular components for future tissue engineering and organ refurbishing strategies.

Although these iPSC approaches have afforded remarkable progress in a number of nonpulmonary fields with proof-of-concept reports of generation of patient-specific and/or disease-specific cardiomyocytes, hepatocytes, and other lineages (31–33), the promise of iPSCs for lung biology has remained somewhat unfulfilled. In the lung, most approaches have entailed taking reprogrammed primordial iPSCs from early embryonic stages through to definitive endoderm, anterior endoderm, and subsequent differentiation toward a distal respiratory epithelial phenotype through *in vitro* sequential exposure to/incubation with combinations of exogenous mediators (growth factors, hormones, and inhibitors) that recapitulate developmental pathways. Using this approach, Ghaedi and colleagues (34) obtained a CD54-positive epithelial cell population from human iPSCs, which expressed molecular markers associated with an AT2 phenotype (proSP-C, proSP-B, SP-A), but bore a limited

ultrastructural resemblance to a fully differentiated AT2. Using a similar *in vitro* strategy, Huang and colleagues (35) generated an iPSC-derived lung epithelium expressing both AT2 (proSP-C, SP-A) and AT1 (AQP5) markers that ultrastructurally appeared similar to human fetal lung epithelium. This preparation was also capable of demonstrating endocytosis of a boron-dipyrromethene (BODIPY) dye-labeled SP-B preparation, a known, but not specific, AT2 functional attribute (36, 37). In a third report, a method to generate random integration-free human iPSCs in which exogenous reprogramming factor transgenes can be subsequently excised, has been described (38). In contrast to the use of prolonged culture strategies, the generated iPSCs have been reported to be capable of rapid differentiation to AT2 cells when cultured on Matrigel for 14 days; however, the molecular and functional endpoints used to assess the AT2 phenotype were incompletely described.

Despite the enthusiasm associated with these approaches touting the successful engineering of differentiated distal lung cell populations from definitive endoderm progenitors (34, 35, 38), as indicated previously here, the resultant iPSC-derived AT2-like epithelia *in vitro* represent a cautionary tale. When placed into the context of the substantial body of data generated from many investigators using primary AT2 cells isolated from a variety of species, *in vivo* proof-of-concept studies using transgenic mouse models, and translational correlations with human specimens, the phenotypic characterization of many of these promising reagents indicates there is still work to be done. Given this current disconnect between the two fields (established AT2 cell biology versus emerging iPSC-derived lung epithelia) and the directions that the rapidly advancing field of iPSC-based *in vitro* modeling and therapeutics is heading, it seems prudent to attempt to establish a clearer consensus of the Holy Grail of what makes an AT2 cell an AT2 cell.

Quacking Like a Duck: What Is an AT2 Cell?

The alveolar sacs of the distal lung are lined by a coupled mosaic of two major epithelial subtypes: the AT1 and AT2 cell. AT1 cells, which represent 8% of total lung cells, are complex branched cells with multiple

cytoplasmic plates that are relatively devoid of organelles (39). This structure permits this numerically modest cell population to provide a major contribution to the gas exchange surface in the alveolus. On the other hand, while contributing only 7% of the alveolar surface area, but comprising 16% of the total parenchymal cell number (39), AT2 cells represent metabolically and functionally complex differentiated epithelia that can be readily isolated from the lung and studied *in vitro* (12). The majority of early published studies of AT2 cell functions were performed with AT2 cells obtained from adult rat sources using improved methods of purification and culture pioneered by Dobbs, Williams, Mason, and others (40–44). Although less frequently used, these methods have been adopted and modified for mouse, guinea pig, rabbit, and bovine sources. In recent years, the ability to sort cells using monoclonal antibodies against AT2 surface markers (45–47) has increased the use of adult human AT2 cells, which has complemented prior work by Ballard's group (48, 49), which successfully reported the isolation of AT2 cells from cultured human fetal lung explants.

When studied *in vitro*, AT2 cells from all these sources represent an experimental challenge, as primary isolates maintained on tissue culture plastic in the presence of serum, typically with 2–7 days of plating, undergo morphological and biochemical changes associated with a loss of AT2 phenotype, including SP mRNAs and LBs (i.e., “dedifferentiation”) (50–52). In some cases a coincidental switch to expression of more AT1-like markers (e.g., T1 α [aka, podoplanin], AQP5, homeodomain-only protein [also known as Hopx]) has been found (i.e., “transdifferentiation”) (41, 47, 51, 53–56). Various efforts, including the use of hormones (dexamethasone), cAMP, growth factors (e.g., keratinocyte growth factor), and three-dimensional culture (Matrigel substrata or organoids) have been shown to delay, but not prevent, these phenotypic changes (48, 49, 57, 58).

Despite these caveats associated with culture-induced phenotypic changes, this model system of primary AT2 cells has been successfully employed for many years to characterize various AT2 functions and, in particular, surfactant metabolism. AT2 cells have been shown to synthesize, secrete, and recycle all components of pulmonary surfactant, the biochemically heterogeneous

complex composed of primarily lipids and protein, which function collectively to augment surface tension at the air–liquid interface along the epithelial lining layer (52, 59). AT2 cells synthesize all subclasses of surfactant lipids (principally phosphatidylcholine [PC] with one [lyso-PC] or two [diplmitoyl (DP)PC] palmitic acid side chains) either *de novo* from precursors, such as palmitate and choline, or via an acylation/reacylation pathway that reuses lipid recycled from the distal airspace (60). In addition to these sources of lipid, within the alveolar niche, lipofibroblasts, which are lipid droplet-containing interstitial fibroblasts located within close proximity to AT2 cells, contribute to the production of pulmonary surfactant by assimilating neutral lipids and transferring them to AT2 cells for final processing of the surfactant (47, 61).

Beyond lipid, biochemical analysis of surfactant has identified four unique protein components, designated SPs: SP-A, SP-B, SP-C, and SP-D (reviewed in Refs. 62–65). AT2 cells again are the principal source of all four proteins expressing mRNA signatures for each. However, in the *adult*, only the expression of SP-C is restricted exclusively to AT2 cells (66, 67), with mRNA for SP-A, SP-B, and SP-D detected in nonciliated airway epithelium (68–71) and rarely in extrapulmonary sites (68). Furthermore, only AT2 cells are capable of the complete biosynthesis of SP-B and -C from proprotein precursors (62, 63, 72–78).

When compared with neuroendocrine cells and many secretory epithelia (exocrine and endocrine) in other tissues, both functionally and morphologically, the AT2 cell is somewhat unique, given the diversity of cellular cargo that must be managed (phospholipids, hydrophobic proteins, multimeric hydrophilic proteins, cytokines). In addition, at the ultrastructural level, AT2 cells lack a classic dense secretory granule, but instead, transmission electron microscopy (TEM) reveals the presence of cytoplasmic, osmiophilic, lamellated organelles (LBs), long recognized as the storage organelle from which surfactant is released into the alveolar lumen (79) (Figure 1B). The presence of lipid within LBs can be easily and rapidly detected using Nile red staining and fluorescence microscopy (Figure 1A). Biochemically, based on a substantial body of work, the basic biology of the lamellar

Figure 1. Examples of methodologies used to define alveolar type (AT) 2 cell phenotypic features. (A) Human AT2 cells prepared from differentiated human fetal explants after 4 days in culture stained for lipid using Nile red to identify lamellar bodies (LBs). N, nuclei. (B) Transmission electron micrograph of human AT2 cell from normal adult lung showing well formed LBs (LB→) containing a dense core granule. In addition, multivesicular bodies (MVBs) and microvilli (mv) are also identified. (C) Immunohistochemistry of human AT2 cell monolayer prepared as in A and stained for pro-surfactant protein (SP) C using anti-NPROSP-C (red) and 4',6-diamidino-2-phenylindole (DAPI; nuclei) showing punctate cytosolic staining indicative of post-Golgi trafficking of proSP-C to LBs. (D) Western blotting with proSP-C antisera of freshly isolated mouse AT2 showing the 21-kD proSP-C primary translation product (arrow) and low-molecular weight processed intermediates (bracket). (E) Western blotting of two separate preparations of human AT2 cells prepared as in A and C using SP-B antisera. The proSP-B primary translation product (thick arrow), 25-kD intermediates (thin arrow), and mature SP-B (bracket) are identified. In addition, dimeric mature SP-B (arrowhead) also appears.

body appears to significantly overlap with that of a number of other lysosome-related organelles in that they are acidic, express lysosomal markers (CD63, lysosomal associated membrane protein [LAMP]-1), but diverges, as they contain surfactant lipids, SP-B, and SP-C, as well as a portion of the intracellular pool of SP-A (80). LBs release surfactant into the alveolus via regulated exocytosis regulated by a variety of signaling pathways (reviewed in Ref. 81).

In addition to biosynthesis, AT2 cells participate in the uptake, catabolism, and reuse of surfactant (82–84). Protein and lipid components are endocytosed via clathrin-dependent and -independent

mechanisms, routed through the central vacuolar system (early endosomes, late endosomes/multivesicular bodies) and sorted to lysosomes for degradation or to LB for resecretion (85, 86). Beyond playing a major role in surfactant homeostasis in the distal lung, there have been a number of other functional, molecular, and metabolic activities associated with an AT2 phenotype. These include, but are not limited to, a role in alveolar ion transport and fluid balance, innate host defense, and, very importantly, as a progenitor population for lung repair, being capable of both proliferative expansion (self-renewal) and of transdifferentiating to AT1 cells (1, 12, 41, 47, 87).

Metrics to Determine “AT2ness”

To avoid “irrational exuberance” regarding the interpretation of current and future results in engineering stem cells for lung biology, it is essential that each new protocol be held accountable for its ability to generate cells that faithfully recapitulate the intended differentiated cell phenotype found *in vivo*. In the case of the distal lung epithelium, “AT2ness” can best be determined by applying a set of standard criteria based on the already well characterized molecular, morphologic, biochemical, and functional markers that have emerged over the past 50 years. These “metrics” could and should include either quantitative comparisons of each criterion present in engineered stem cell populations to those of AT2 cells using freshly isolated, species- and age-matched (fetal, adult) AT2 cells, or that significantly overlap with comparable signals/readouts/endpoints with AT2 cells evaluated *in situ* using well characterized lung specimens.

In addition to incorporating the wealth of data present in the lung biology literature, in assembling this Perspective, we also reached out informally to many experts in the field and have incorporated their viewpoints into a “consensus” list of potential phenotypic endpoints aimed at capturing the quintessential features of what makes an AT2 cell an AT2 cell. These features, summarized in Table 1, can be more broadly grouped thematically as follows:

1. Evidence of a Significantly Mature Surfactant System

- Expression and maintenance of mRNA for key AT2 genes, *SFTPB* (SP-B) and *SFTPC* (SP-C), plus ATP-binding cassette class A3 (*ABCA3*; and potentially *SFTPA* [SP-A]), comparable to AT2 cells from same species.
- Demonstration of biosynthesis and *complete* post-translational processing of proSP-B and proSP-C proteins using Western blotting or metabolic labeling. The presence of detectable levels of low-molecular weight proSP-C intermediates, as well as mature SP-B, is supportive (Figures 1D and 1E).
- Documentation that the resultant cell population can biosynthesize and store surfactant phospholipids (notably, DPPC).

Table 1. Proposed Criteria for Assessment of an Alveolar Type 2 Cell Phenotype

Category	Phenotypic Metric	Methodologies	Reference(s)
Molecular	Expression and maintenance of key surfactant-related AT2 mRNAs, including <i>SFTPC</i> , <i>SFTPB</i> , <i>SFTPA</i> , and <i>ABCA3</i>	qRT-PCR	43, 44, 48, 49, 57, 99
	Expression of other abundant AT2 marker genes (HTII-280; progastrin)	IHC; WB	46, 73
Morphometric	Presence of morphologically well formed lamellar bodies in numbers comparable to those found in native lung	TEM Quantitative morphometry	9, 112
	Spatial fidelity of expression of proSP-B, proSP-C, <i>ABCA3</i> (e.g., a cytosolic vesicular pattern) consistent with correct biosynthetic routing to lamellar bodies	IHC Immunogold EM	74, 75, 78, 89, 91, 93, 98
Biochemical	Evidence of biosynthesis and complete post-translational processing of proSP-B and pro SP-C with resultant production of mature protein isoforms	WB for proSP-C/SP-B Metabolic labeling/immunoprecipitation	72, 74–76, 78, 89, 90, 92
	Evidence that reprogrammed cells biosynthesize and store surfactant phospholipids (notably DPPC) in a lamellar body compartment	Metabolic precursor labeling Lipidomic profiling of cells	49, 60, 106–108
Functional	Demonstration of regulated exocytosis of surfactant lipid and hydrophobic surfactant proteins in response to known secretagogues comparable to AT2	Western blotting Lipidomic profiling of recovered surfactant	49, 107, 109
	Ability to transdifferentiate to an AT1 cell phenotype	Expression of AT1 markers: AQP5, T1- α <i>In vivo</i> lineage trace	41, 55, 56
	Ability to form tight junctions and a resistive monolayer in culture	IHC for junctional proteins TER	41, 55, 56
“Progenitoriness”	Exhibition of capacity for self-renewal and pluripotency*	Organoid culture; <i>in vivo</i> lineage trace	47, 122
	Demonstration of ability to repopulate lung scaffolds or engraft injured lungs	Seeding on decellularized scaffolds <i>in vivo</i> transfer	34, 35
“Omic”	Transcriptomic profile and PCA metric similar to isolated AT2 cell or derived from known single-cell RNA samples	Microarray/qRT-PCR RNAseq Bioinformatics/PCA	e.g., LungMAP [†]
	Protein expression profiles with high degrees of similarity to isolated AT2	LC/MS Bioinformatics/PCA	e.g., LungMAP [†]
	Intracellular and/or secreted lipid profile similar to freshly isolated cells from the same species	Lipidomics	49, 60, 107

Definition of abbreviations: *ABCA3*, ATP-binding cassette class A3; AQP5, aquaporin-5; AT, alveolar type; DPPC, dipalmitoylphosphatidylcholine; IHC, immunohistochemistry; LC/MS, liquid chromatography/mass spectrometry; PCA, principal component analysis; qRT-PCR, quantitative RT-PCR; *SFTP*, surfactant protein gene; SP, surfactant protein; TEM, transmission electron microscopy; TER, transepithelial resistance; WB, Western blot.

*Capacity for self-renewal (may be limited to a subset of AT2 cells; see main text).

[†]LungMAP, available from <http://www.lungmap.net/about/lungmap-team/nhlbi>.

- Demonstration that surfactant phospholipids and hydrophobic proteins SP-B and SP-C are secreted via regulated exocytosis.
- Expression of additional important AT2-related mRNAs/proteins (e.g., progastrin C, napsin A) and exclusion of known AT1 (AQP5, T1 α) and some proximal airway (p63) markers.

Rationale and justification. The expression of three major protein components of the surfactant system and the ability to synthesize, store, and release surfactant phospholipid species and proteins have been strongly associated with an AT2 cell phenotype.

SP-C is the only AT2 epithelial cell-specific protein, and is the most commonly used marker for identification of AT2 cells (Figure 1C). In addition, at least for the mouse, it has been reported that a subset of SP-C⁺ AT2 cells was also found to express low levels of the Clara/Club cell gene, *SCGB1A/CC10*, with these double-positive cells capable of giving rise to AT1 cells (88). Thus, the presence of coexistent CC10 expression does not absolutely preclude identification as an AT2 cell.

There are several important points to consider when evaluating for the expression of SP-C protein. First, the alveolar form of SP-C protein, which appears in surfactant, is actually synthesized as a 191–197 amino acid precursor (proSP-C) that undergoes post-translational proteolytic processing that ultimately results in generation of the 3.7-kD mature product. Importantly the proSP-C primary translation product (21 kD) and its major processing intermediates (6–10 kD) can be readily detected by Western blotting of cell lysates using most available “(pro)SP-C” antibodies (Figure 1D). Second, the 6-kD proSP-C isoform is observed to be enriched in LB subcellular fractions by Western blotting and detectable by IHC (76, 78, 89–93). The observed IHC pattern should reveal a cytosolic, vesicular pattern (see Figure 1C) that colocalizes with other LB markers, such as CD63, ABCA3, or lysosomal associated membrane protein (LAMP)3. This level of analysis has been lacking in many reports, where only a single 21-kD proSP-C band and/or low-power IHC are presented as evidence of complete SP-C biosynthesis and trafficking. Finally, it should also be recognized that the use of commercially available proSP-C antibodies

cannot detect the fully processed mature (~4 kD) isoform; there is, in fact, only one polyclonal “mature” SP-C reagent (WRAB-MSPC; Seven Hills Bioreagents, Cincinnati, OH) produced over 20 years ago, which has limited availability and sensitivity (M.F.B., unpublished data).

SP-B is an 8-kD hydrophobic protein also generated from a larger 42-kD proprotein precursor (73, 74, 77, 94). Given the essential requirement for the presence of intracellular mature SP-B for normal LB genesis (as evidenced by the lack of LB in the lungs of SP-B-deficient mice and humans) (95, 96), identification of this mature isoform via Western blotting of cell lysates is highly supportive of an AT2 phenotype (see Figure 1E). It should be noted that the well known immortalized human Club/Clara cell line, H441, expresses SP-B mRNA and the proSP-B primary translation product, but does not generate mature SP-B or contain LBs (97).

The lipid transporter, ABCA3, plays a critical role in the biogenesis of AT2 cell LBs (98, 99). Studies suggest that ABCA3 functions as an intracellular transporter of cholesterol and phospholipids, including PC, phosphatidylglycerol, phosphatidylserine, and sphingomyelin (100). Although mRNA expression has been found in other tissues, such as pancreas, ABCA3 protein can be clearly identified on the limiting membrane of LB by IHC (101). In addition, as for SP-B, both the *Abca3* knockout mouse models and human ABCA3-null patients die of surfactant-deficient respiratory failure and fail to demonstrate normal LB on TEM, supporting a role for this transporter as one of the critical regulators of LB biogenesis (102).

SP-A, the first SP isolated (103, 104), is the major protein component of pulmonary surfactant. Functions of SP-A in the alveolus include the facilitation of surface tension-lowering properties of surfactant phospholipids, regulation of surfactant phospholipid synthesis, secretion, and recycling, and participation in the innate host response to microbes and particulates in the distal lung (103, 105).

Surfactant phospholipids are synthesized and assembled for secretion into the alveolus exclusively by AT2 cells. A wealth of data in the literature has been generated using metabolic labeling strategies (typically choline or palmitic acid incorporation) (106), and, more recently,

lipidomic profiling of primary AT2 isolates in culture (107) to characterize the biosynthetic pathways, enzymes, and adjunctive factors required for the generation of large intracellular pools of surfactant for release into the alveolar space (reviewed in Refs. 60, 108).

Functionally, AT2 cells have been shown that release both surfactant lipids and the hydrophobic SPs (SP-B, SP-C) in a quantile fashion in response to a variety of secretagogues (calcium, ATP, protein kinase C agonists, protein kinase A agonists) via regulated exocytosis (81, 109–111)

2. Evidence of a Comparable AT2 Cell Organellar Ultrastructure

- Ultrastructural evidence by TEM of LBs and quantitative morphometrics showing that the engineered cell line does achieve this metric at a magnitude equal to AT2 cells from the native lung.
- Evidence for a lysosome-like organelle compartment, which is acidic, rich in phospholipid, and contains mature SP-B/proSP-C proteins.

Rationale and justification. The presence of well formed LBs with dense core bodies (Figure 1B) implies sufficient expression of SP-B/-C, ABCA3, and DPPC, as well as key endoplasmic reticulum chaperones, adapter proteins, and processing enzymes required for their normal biogenesis. Given the biological heterogeneity of isolated and enriched stem cell-derived, differentiated epithelial populations, rigorous and statistically powerful techniques (in lieu of presenting single micrographs) should be employed. These metrics are easily derived from standard ultrastructural methodologies developed by Weibel, Hyde, Ochs, and others in concert with consensus guidelines for lung morphometry as published by the ATS/ERS (60, 112).

As LBs represent a highly specialized subpopulation of lysosome-related organelles, strong supporting evidence of a successfully reprogrammed AT2 cell population would include the presence of acidic, lipid-rich organelles (lysotracker⁺; Nile red⁺, or phosphine 3R⁺ cytosolic vesicles), which also concentrate hydrophobic SPs (Figures 1A and 1C). This is critical, as “SP-C-positive” or “SP-B-positive” IHC should demonstrate the presence of labeled cytosolic vesicles.

3. Evidence of Participation in Maintenance or Repair of Barrier Function

Rationale and justification. Because of their role in barrier function, both as primary constituents of the heterotypic alveolar epithelial monolayer (juxtaposed with AT1) and as potential precursors to AT1, the ability of putative re-engineered AT2 populations to transport sodium, develop tight junctions, and generate high transepithelial resistance *in vitro* and/or express markers of AT1 cells *in vivo* provides additional strong supporting evidence (41, 55, 113–115).

4. Evidence of Progenitor Cell Function

- Evidence that the re-engineered population can exhibit self-renewal and pluripotency.
- Evidence that the re-engineered cells can repopulate lung scaffolds, or engraft injured lungs with reasonable efficiency *in vivo*.

Rationale and justification. Historical data from primate and rat models suggested that SP-C⁺ AT2 cells function as progenitor cells in the alveoli and proliferate and possibly transdifferentiate into AT1 cells (116, 117). Recent genetic lineage-tracing studies in the mouse have provided more definitive evidence by demonstrating that SP-C⁺ AT2 cells, as an unfractionated population, proliferate *in vivo* and give rise to AT1 cells (47). These AT2 cells placed into three-dimensional culture were shown to be able to generate self-renewing lung organoids or “alveolospheres,” which contained both AT2 cells and cells expressing multiple AT1 markers, including *Hopx*, a new marker for AT1 cells (56). The potential for this AT2 population to undergo clonal expansion (i.e., multiple rounds of symmetric cell division) as well as asymmetric expansion to form AT1 cells has also been shown (118).

Additional studies suggest that this property of self-renewal may be limited to only a portion of the AT2 pool. A recent preliminary report by Zacharias and colleagues (119) has identified a subpopulation of lung epithelial cells in mice (Axin2⁺/SP-C⁺) that are capable of self-renewal and of generating

alveospheres composed of both AT2 and AT1 cells in organoid culture. By lineage tracing, this same population can give rise to AT1 in injured lungs *in vivo*. These results extend older published data that suggested that at least two populations of AT2 cells could be isolated from injured lungs based on E-cadherin expression (120). E-cadherin-negative AT2 cells were damage resistant, proliferative, and exhibited high levels of telomerase activity, suggesting that they might represent a transiently amplifying progenitor subpopulation of AT2 cells that could contribute to repopulation and repair of damaged alveolar epithelium.

Although not unique to fully differentiated AT2, the ability to home to and/or engraft in areas of local injury may be advantageous. Prior studies have described the engraftment of lineage-negative or distal lung progenitor populations in injured rodent lungs (25, 26). Similarly, the instillation of whole populations of isolated primary AT2 cells has been shown to limit bleomycin-induced fibrosis in rats, although the exact degree of engraftment was difficult to interpret (121). iPSC-derived distal lung epithelial clones with some phenotypic features of AT2 cells have also been shown to be capable of engraftment on to decellularized lung scaffolds (34).

Taken together, it seems prudent to recommend that the iPSC-generated “AT2” population not only demonstrates traditional molecular, biochemical, morphological, and functional markers of the AT2 phenotype (Table1), but the derived cell population should also show a capacity to contribute to lung repair/regeneration through self-renewal and/or differentiation (which has been shown for AT2 cells). However, the application of strict criteria linking the minimal number of cell divisions and degree of pluripotency awaits additional studies.

5. Broader Quantitative Phenotypic Overlap: Harnessing the Power of “omics”

- Transcriptomic profile and principal component analysis (PCA) metrics similar to that recovered from isolated AT2 cells or determined from known single-cell RNA analyses.

- Protein expression profiles with comparable degrees of similarity.
- Intracellular and/or secreted lipid profiles similar to cells from isolated the same species.

Rationale and justification. To address the contribution of “nonsurfactant functions” in the overall phenotype of stem cell-generated AT2 cells, but for which candidate gene or pathway criteria are currently less developed, the emerging powerful and compelling profiling techniques coupled with bioinformatics analyses should be considered. Using this “big data” approach with bioinformatics tools, comparisons of data from freshly isolated AT2 cells and the derived candidate AT2 cells can be made. PCA of well controlled and adequately powered surveys of AT2 markers at the level of mRNA expression (microarray, single-cell RNAseq), protein expression (e.g., tandem mass spectrometry), or metabolomics/lipidomics between putative engineered AT2 cells and reference populations, is perhaps the most powerful metric. Reference resources that may provide opportunity for such comparisons could include the NHLBI-funded LUNGMap project (<http://www.lungmap.net/about/lungmap-team/nhlbi>). This consortium now has sorted and single-cell RNA profiles and will ultimately have detailed lipidomics and proteomics data from which to make such comparisons (J. Whitsett, personal communication).

Final Thoughts

Despite the power of emerging stem cell technologies, a major limitation for the pulmonary biology community currently is the ability to derive mature, functional epithelial populations of the distal lung. Although the platform offers unparalleled opportunities to model *in vitro* human lung development and/or genetic pulmonary disease, as well as to create potential cell therapeutics for a whole host of acute and chronic lung diseases, the mainstream use of iPSCs for lung biology is squarely at a critical crossroads. To maintain consistency and reproducibility, and to interpret findings in the proper context, it is incumbent upon investigators

in the field to confront the alveolar epithelial cell biology of the past by thoughtfully validating the newly engineered cell lineages to ensure that the pluripotent stem cell they begin with ends up as the *bona fide* AT2 cell they are seeking. ■

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