Lung stem cells: do they exist?

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Running Head: Epithelial stem cells in the adult lung

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ABSTRACT

Recognition of the potential of stem cell based therapies for alleviating intractable lung diseases has provided the impetus for research aimed at identifying regenerative cells in the adult lung, understanding how they are organised and regulated, and how they could be harnessed in lung regenerative medicine. In this review we describe the attributes of adult stem and progenitor cells in adult organs, and how they are regulated by the permissive or restrictive microenvironment in which they reside. We describe the power and limitations of experimental models, cell separative strategies and functional assays used to model the organisation and regulation of adult airway and alveolar stem cells in the adult lung. The review summarises recent progress and obstacles in defining endogenous lung epithelial stem and progenitor cells in mouse models and in translational studies.

Key Words:  airway epithelium; animal models; cell biology; lung regeneration; adult stem cells
"Omnis cellula e cellula"
Rudolf Virchow (1858)

The identity of stem cells, their location and organisation within tissues, and the regulation of their regenerative potential has been the subject of ongoing debate since articulation of modern cell theory in the mid 19th century which recognised that cells are the fundamental building blocks of all living organisms, and that every cell is derived from a pre-existing cell (Omnis cellula e cellula). The term “stem cell” was originally coined in the late 19th century\(^1\) by embryologists to describe embryonic cells thought to give rise to the specialised tissues of multicellular organisms during ontogeny; and by haematologists to describe the hypothesised common precursor of the diverse cell types identified in the bone marrow, peripheral blood and lymphatic system.

Historically, adult organs have been classified as continuously renewing (bone marrow, gut, skin), conditionally renewing (lung, kidney, liver), or essentially non-renewing (nervous tissue, muscle) on the basis of their proliferative capacity and cell turnover in the steady state or following injury\(^2,3\). A continuously renewing tissue like the bone marrow, has the prodigious capacity to generate approximately \(200 \times 10^9\) erythrocytes, \(50 \times 10^9\) white blood cells, and \(125 \times 10^9\) platelets daily in the steady state, and can rapidly augment cell production up to 10-fold in times of stress\(^4\). The gut also generates approximately \(100 \times 10^9\) intestinal epithelial cells daily, replacing the entire intestinal epithelium every 5 days\(^5,6\).

The lung on the other hand, is a conditionally renewing organ. Airway epithelial cell turnover is low (~1% per day) with replacement of the tracheobronchial epithelium...
estimated at approximately 4 months\textsuperscript{7}. However like kidney and liver, the lung is able to undergo compensatory growth, rapidly increasing its rate of regeneration to replace ablated tissue following severe injury\textsuperscript{8}.

**The intrinsic properties of adult stem cells:**

The adult stem cell paradigm is largely underpinned by the analysis of cell regeneration in continuously renewing tissues. Accordingly, adult stem cells are defined as rare, morphologically unrecognisable cells endowed with a high proliferative potential and the life-long ability to (a) maintain and replenish their own kind, (b) generate large numbers of functionally differentiated progeny, and (c) replace senescent and damaged cells in the steady state and following perturbation or injury\textsuperscript{9}.

The classical adult stem cell hierarchy comprises an age-structured continuum of stem and transit amplifying progenitor cells of progressively restricted proliferative and differentiative potential which in turn give rise to mature non-dividing functionally differentiated cells (Figure 1). As a general rule multipotent stem cells at the head of this hierarchy are highly quiescent. Typically, the vast majority are in a G\textsubscript{0} state\textsuperscript{10} and descendent cell lineages are derived from a small number of active clones\textsuperscript{11}, thus ensuring the preservation of a stem cell reserve while mitigating the risk of error-prone stem cell replication and transformation\textsuperscript{12}. Day-to-day demands for replacement of senescent and damaged cells are met by more abundant rapidly cycling lineage-restricted transit amplifying progenitors of limited proliferative potential\textsuperscript{10}. According to this paradigm, stem cells divide asymmetrically to homeostatically regulate stem cell pool size while simultaneously replenishing the more committed transit amplifying progenitor cell compartments, but also retaining the ability to divide symmetrically to expand stem cell numbers in order to meet the increased demand.
for differentiated cells following tissue injury\textsuperscript{13, 14}. Analysis of stem cell compartments in inbred mouse strains\textsuperscript{15}, and the BXD set of recombinant mouse strains derived by crossing C57BL/6J and DBA/2J mice\textsuperscript{16, 17}, has also shown that stem cell variables including pool size, cell kinetic status, sensitivity to cytokines, irradiation and cytotoxic perturbation are genetically determined.

While the organisation of regenerative cells in continuously renewing tissues conforms to this classical model, it is not precisely recapitulated in all organs and tissues. Regional differences in the developmental potential and organisation of regenerative cells have been documented in different compartments of organs including the prostate, skin and the gastrointestinal tract\textsuperscript{18-20} and along the proximal-distal axis of the lung\textsuperscript{7, 21}. A recent study argues against the obligate requirement for a long-lived oesophageal epithelial stem cell reserve\textsuperscript{20, 22}. There is evidence for and against the existence of regional diversity in neural stem cells in the brain\textsuperscript{23}. Sub-populations of differentiated multifunctional cells able to revert to a (facultative) stem cell state have been identified in lung\textsuperscript{24}, liver and pancreas\textsuperscript{25}, while terminally differentiated cardiomyocytes appear capable of regenerating damaged heart tissue by a process of dedifferentiation and redifferentiation\textsuperscript{26, 27}.

However, it is interesting to note that at a time when the delineation and functional analysis of adult stem cells was in its infancy, Till\textsuperscript{28} speculated that they would likely be characterised by the expression of multiple markers including differentiation markers characteristic of later stages of development. Likewise, Lajtha\textsuperscript{10} questioned the imprecision of the concept that stem cells were necessarily the sole precursors of other cell types, and the assumption that they were necessarily undifferentiated\textsuperscript{10}. He speculated: (a) that although stem cells may be less differentiated than their descendents they were nonetheless one of many
differentiated cell types; and (b) that renewal is relative rather than absolute with stem and
transit progenitor cells part of a recognisable age-structure continuum in which both stem
and transit populations could be precursors of other transit populations. In hindsight, these
reflections on the nature and properties of stem cells have proven remarkably insightful,
suggesting that classical and non-classical models of stem cell organisation may have more
in common than might appear at first glance\textsuperscript{25, 29, 30}, and emphasizing the fact that stem and
progenitor cells are defined operationally: by what they do rather than how they are
decorated.

**Stem cells are defined in context – the role of the niche in stem cell specification:**
Importantly, adult stem cells are not solely defined by their intrinsic properties, but also by
the permissive or restrictive microenvironments in which they reside. The importance of the
microenvironment in stem cell specification had long been appreciated in both embryonic
development\textsuperscript{31} and adult haematopoiesis\textsuperscript{32}, but the concept of the stem cell niche was only
articulated by Schofield in 1978\textsuperscript{33} in modelling the regulation of haematopoietic stem cells.
He proposed that adult stem cells occupy specific anatomical sites which preserve their
developmental potential, regulate their replication, inhibit their differentiation and provide
a milieu conducive to the preservation of engrafted cells in a stem cell state. The crosstalk
between the stem cell and its niche profoundly affects stem cell behaviour and
functionality\textsuperscript{3, 34-37}. In the lung, the interdependence of regenerative cells and their niche
microenvironment is evident in both the process of lung epithelial regeneration and repair\textsuperscript{38}
and in the aberrant proliferation and differentiation of airway and alveolar epithelial cells as
a consequence of the pathophysiological remodelling and scarring of the subepithelial
microenvironment in lung fibrosis and asthma\textsuperscript{39-42}.
Problems and pitfalls in assaying stem cells and modelling stem cell hierarchies:

Because stem cells are fundamentally defined by what they do rather than by their morphology or biomarker profile, the assignment of “stemness” relies on the precise analysis of their developmental potential and regenerative capacity. For this reason, the modelling of stem and progenitor cell hierarchies has progressed in lockstep with the development and refinement of:

1. specific antibodies, reagents and multiparameter cell separative protocols for the prospective isolation of highly purified and defined target cells;
2. robust in vitro clonogenic assays for enumerating stem and progenitor cells and measuring their proliferative and differentiative potential;
3. transplant models and assays to measure their regenerative potential in vivo;
4. lineage tracing techniques to mark their spatial location in situ and measure their contribution to tissue maintenance, regeneration and repair; and,
5. genetically-engineered and mutant mouse models to identify specific stem cell regulatory networks.

The delineation of haematopoietic stem and progenitor cells is an outstanding example of the power of these multiparameter strategies to: (a) analyse stem and progenitor cell organisation and regulation, (b) validate assays predictive of their regenerative capacity, and (c) identify stem cell targets around which therapies can be constructed (Figure 2). The characterisation of multipotent mouse mammary stem cells capable of regenerating a functional mammary gland following transplantation of a single cell is another pertinent example. However, because the analysis of the functional attributes of stem cells is context...
dependent, there are many experimental variables which can confound the assignment of stemness and compromise the ability of assays to discriminate closely related stem and progenitor cell subsets and measure their ability to give rise to descendent lineages.

Tissue disaggregation disrupts the relationship between stem cells and their regulatory microenvironment potentially affecting their viability, developmental potential, and signature profile\textsuperscript{46, 47}. Stem cell biomarkers are commonly shared by multiple cell lineages as well as closely related cells of differing developmental potential and regenerative capacity\textsuperscript{48, 49}. The proliferation and differentiation of stem and progenitor cells in vitro is determined by colligative properties of the culture system including cell seeding density, choice of medium\textsuperscript{50}, oxygen tension\textsuperscript{51}, pH, and the conditioning of the medium by factors elaborated by the progeny of the cultured stem cells\textsuperscript{52}. Discordance between proteome and transcriptome as a result of post-translational regulation of the proteome must also be considered in assessing the fidelity of biomarker signature profiles used to characterise and order stem and progenitor cells and their descendent progeny\textsuperscript{53, 54}.

The interpretation of in vivo assays can be similarly confounded. Transplant outcomes are contingent on the number of transplanted cells, their route of delivery, and the modality employed to precondition transplant recipients. Evidence from haematopoietic transplant models also shows that stem cell homing and engraftment is influenced by the cell cycling status of transplanted stem cells\textsuperscript{55}. The regenerative capacity of resident stem cells in acute lung injury models can be confounded by disruption and remodelling of the stem cell niche microenvironment\textsuperscript{7, 56}. And, cell lineage tracing in conditional transgenic reporter mouse models can be confounded by the lack of specificity of promoters and the pharmacokinetics of inducers used to elicit gene expression\textsuperscript{57, 58}.
Characterisation and organisation of lung epithelial stem and progenitor cells

The lung is a complex organ comprising at least 40 cell lineages of endodermal, mesoderm and ectodermal origin which arises from an out-pouching of the foregut. While progress has been made in delineating stem and progenitor cells in all lung compartments, this review focuses on controversies surrounding the nature, properties and organisation of epithelial stem cells in the adult lung.

During development the primordial lung undergoes a precisely orchestrated program of branching morphogenesis culminating in arborisation of the conducting airways and their supporting parenchyma and vasculature, terminating in the gas-exchanging alveolar bed. The trachea and proximal airways are lined by a pseudostratified epithelium comprising basal, secretory, ciliated and neuroendocrine cells. Distal conducting airways are lined by a simple columnar epithelium comprising club (Clara), ciliated, and pulmonary neuroendocrine cells, while alveoli are populated by cuboidal surfactant secreting alveolar epithelial Type II cells and squamous gas-exchanging alveolar epithelial Type I cells. The cell lineages comprising each of these compartments are maintained by regenerative cells characterised by high proliferative activity during foetal and perinatal life, and slow turnover and the capacity to replace senescent and damaged cells in the adult.

The organisation of endogenous epithelial stem and progenitor cells in the adult lung has been extensively reviewed in recent years. Briefly, these studies suggest that regional stem and progenitor cells are responsible for the maintenance of specific epithelial cell lineages in the proximal and distal conducting airways and the alveolar bed (Figure 3). These include p63^pos^Krt5^pos^ and/or Krt14^pos^ basal cells, Scgb1a1^pos^ club (Clara) cells and Krt14^pos^ submucosal gland (SMG) duct cells of the trachea and upper airways.
naphthalene resistant Scgb1a1\textsuperscript{pos}CyP450\textsuperscript{neg} and/or scgb3a2\textsuperscript{pos} variant club (Clara) cells and neuroendocrine cells in the bronchiolar airways\textsuperscript{73-75}, CCSp\textsuperscript{pos}SP-C\textsuperscript{pos} bronchioalveolar stem cells (BASC) at the bronchioalveolar duct junction of terminal bronchioles\textsuperscript{76-78}; and the Type II progenitor of Type I cells in the alveolar bed\textsuperscript{79, 80}. However, the analysis of lung epithelial cell regeneration in genetically engineered mice, and the development of multiparameter cell separative strategies and three dimensional organotypic clonogenic assays for the identification and characterisation of adult lung stem cells in vitro has added another layer of complexity and ambiguity to the understanding of their organisation and spatial localisation.

Lung injury models confirm that the regenerative potential of candidate stem cells is context dependent, and that different lung epithelial stem and progenitor cell cohorts are recruited to regenerate specific airway epithelial cell lineages in the steady state and following severe perturbation\textsuperscript{81, 82}. On the other hand, in vitro clonogenic assay of putative epithelial stem cells, and transcription profiling of their progeny, provides evidence of the existence of multipotent epithelial stem cells in the adult lung\textsuperscript{21, 83-85}. While, cell lineage tracing in genetically engineered conditional mutant mice also reveals plasticity in the differentiative potential of specific lung epithelial stem cell cohorts.

Discordant studies notwithstanding\textsuperscript{86}, this context-dependent diversity in the proliferative and differentiative potential of epithelial stem and progenitor cells has been observed in all anatomical compartments of the airway tree. In the trachea, Krt14\textsuperscript{pos} basal cells adopt a different fate in the normal lung and following naphthalene injury\textsuperscript{71, 87}. Likewise, Hegab et al\textsuperscript{72} have identified multipotent nerve growth factor receptor positive (NGFR\textsuperscript{pos}) α6 integrin positive (α6\textsuperscript{pos}) tracheal SMG duct cells able to regenerate both SMG tubules and secretory...
and ciliated airway epithelial cells following severe ischaemic injury. In the distal lung, pulmonary neuroendocrine and alveolar cells (but not club (Clara) and ciliated cells) have been shown to share a common CGRP\textsuperscript{pos} neuroendocrine cell lineage origin during development, whereas they function as unipotent neuroendocrine progenitor cells in the normal adult lung but are able to renew and generate both club (Clara) and ciliated cells following naphthalene injury\textsuperscript{88}. Other lineage tracing studies also provide evidence of the derivation of alveolar epithelial cells from Scgb1a1\textsuperscript{pos} airway cells following bleomycin injury\textsuperscript{89}, and from normally absent p63\textsuperscript{pos}Krt5\textsuperscript{pos}Krt14\textsuperscript{pos} airway cells following H1N1 virus infection\textsuperscript{90}. In the alveolar bed, it appears that alveolar epithelium is not simply repaired by expansion and differentiation of SP-C\textsuperscript{pos} type II cells following bleomycin injury, but also by SP-C\textsuperscript{neg}α\textsubscript{6}\textsuperscript{pos}β\textsubscript{4}\textsuperscript{pos} cells sporadically distributed in the alveolar wall\textsuperscript{91}.

Modelling lung epithelial stem cell hierarchies: Are we there yet?

The development of powerful animal models\textsuperscript{57} and the refinement of cell separative strategies and in vitro clonogenic assays based on approaches proven effective in characterising haematopoietic stem cells (Figure 2) have underpinned the significant progress made in identifying putative stem cells in the adult lung and the critical factors and pathways regulating their regenerative potential. However, the lack of definitive lung epithelial stem cell biomarkers; the co-expression of cellular and molecular biomarkers by cells of differing regenerative potential, cell lineage origin or maturational age; the limitations of flow cytometric sorting\textsuperscript{47} and in vitro organotypic assays\textsuperscript{92}; and the lack of gold-standard in vivo assays akin to those that have enabled the validation and modelling of the haematopoietic stem cell hierarchy\textsuperscript{93, 94}, have conspired to blur the identity, spatial location and organisation of lung epithelial stem cells in health and disease. In particular, it
is still unclear whether the heterogeneous behaviour of seemingly homogeneous target cells is indicative of co-fractionation of heterogeneous cells expressing common biomarkers, or the stochastic commitment of homogeneous cells to different fates in response to microenvironmental cues. This is especially the case in injury models where modulation of biomarker signature profiles is not necessarily indicative of the ability of regenerative cells to give rise to descendent lineages.

**Translational studies and the identification of therapeutic targets in the human lung**

As yet relatively few studies have attempted to isolate and characterise adult epithelial stem cells in the human lung. These have mostly analysed tracheal and proximal airway epithelia utilising various in vitro assays including air-liquid interface culture and serial propagation of spheroid-forming cells, and the generation of mucociliary epithelium in devitalised tracheal implants xenografted in immune-compromised mice (reviewed in [66]). More recent studies also suggest that human homologues of murine lung epithelial stem cells are also amenable to analysis and characterisation employing similar experimental strategies to those utilised in mice.

Both immortalised human bronchial epithelial cells [95] and freshly isolated human proximal airway epithelial cells [70, 96-98] generate complex 3-dimensional organoids of differentiated airway epithelial cells when cloned in matrigel. Multiparameter flow cytometric analysis shows that these putative human airway epithelial stem cells share a biomarker profile similar to that of their murine counterparts. These include NGFR$^{\text{pos}}$α6$^{\text{pos}}$p63$^{\text{pos}}$ basal cells which generate cystic organoids of Krt14$^{\text{pos}}$p63$^{\text{pos}}$ basal cells, Krt8$^{\text{pos}}$ luminal cells and ciliated cells [70]; and, NGFR$^{\text{pos}}$α6$^{\text{pos}}$ basal and SMG duct cells which can be further resolved on the basis of their aldehyde dehydrogenase (ALDH$^{\text{hi}}$), CD166, CD44, and α6 integrin signature.

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profile to identify cells differing in their ability to generate organoids comprising Krt5\textsuperscript{pos}, Krt14\textsuperscript{pos} and mucous and serous secretory epithelial cells\textsuperscript{97}.

Others have propagated dispersed, heterogeneous human lung cell suspensions in liquid culture to pre-enrich lung epithelial stem cells prior to their prospective isolation and characterisation by flow cytometric cell sorting and functional analysis\textsuperscript{99, 100}. Using this approach, Oeztuerk-Winder et al\textsuperscript{99} have cloned putative E-Cad\textsuperscript{pos}Lgr6\textsuperscript{pos}\alpha6\textsuperscript{pos} lung epithelial stem cells that can be serially passaged in liquid culture, and they have shown that both freshly isolated and propagated E-Cad\textsuperscript{pos}Lgr6\textsuperscript{pos} cells are able to generate differentiated human bronchoalveolar epithelial tissue following transplantation of a single cell under the kidney capsule of recipient immune-deprived (CD-1 nude) mice. While lung cells expressing the stem cell associated antigen c-Kit did not exhibit stem cell potential in this study, another study using this approach\textsuperscript{100} has described a putative multipotent c-kit\textsuperscript{pos} human lung stem cell with broader developmental potential, seemingly capable of generating lung epithelial, endothelial and mesenchymal cell lineages in different contexts\textsuperscript{100} but its status is as yet uncertain\textsuperscript{101}.

This notwithstanding, the delineation of human lung epithelial stem and progenitor cell hierarchies is beset with practical difficulties likely to slow progress. Translational studies aimed at identifying stem cell homologues of those in mice will need to consider differences in the distribution of epithelial cell lineages along the proximal-distal axis of the murine and human airway tree\textsuperscript{62}. Murine cells also have extremely long telomeres\textsuperscript{102}, endowing them with a far more prodigious replicative capacity than human cells. Consequently, human homologues of putative mouse lung epithelial stem cells may have a more restricted proliferative potential and may not be appropriate therapeutic targets in clinical contexts.
Practical issues related to the acquisition of human lung tissue biopsies suitable for stem cell experimentation will impact the development and refinement of optimal strategies for stem cell isolation and characterisation. The modelling of lung epithelial stem cell organisation and regulation will require access to normal lung tissue from different anatomical regions along the proximal-distal axis of the airway tree, and its processing in a timely manner. Regular access to normal lung is rare, and resected “normal” lung tissue and bronchoscopy samples are invariably acquired from patients undergoing procedures for diverse clinical indications. These clinical indications are likely to affect the biomarker profile and attributes of putative stem and progenitor cells and also amplify the intrinsic biological variability of measured human parameters necessitating larger sample sizes for statistical validation of stem cell attributes.

That said, recent clinical studies demonstrating the therapeutic efficacy of tracheobronchial reconstruction using bioengineered recellularised human tracheal matrix scaffolds\textsuperscript{103} is cause for optimism for the future of stem cell based therapies in lung regenerative medicine.

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FIGURE LEGENDS

Figure 1: In the steady state, the classical stem cell hierarchy comprises an age-structured continuum of stem cells, transit amplifying progenitor cells and their progeny of progressively restricted proliferative and differentiative capacity. The majority of stem cells in the hierarchy are highly quiescent, have a low probability of entering cell cycle, and are randomly recruited to replenish transit amplifying progenitor cell pools. Day-to-day requirements for functional differentiated cells are met by the more abundant pool of rapidly cycling committed transit amplifying cells, while the highly quiescent stem cell pool is activated and recruited into cycle to regenerate descendent lineages following severe perturbation or injury.

Figure 2: A haematopoietic “roadmap” illustrating the iterative experimental process for determining and validating the immunophenotypic signature profile of putative stem and progenitor cell sub-populations, and the robustness of surrogate in vitro assays to predict the incidence and regenerative potential of candidate stem cells in the steady state and in perturbation and disease models.

Figure 3: A schematic representation of the spatial location, regional distribution and differentiation potential of lung epithelial stem and progenitor cells along the proximal-distal axis of the airway tree. Regenerative pulmonary neuroendocrine and neuroepithelial cells are scattered or clustered throughout the proximal and distal airways, and there is evidence for the existence of multipotent stem/progenitor cells in all compartments.
Glossary

**Adult stem cell:** Rare, undifferentiated cells within a tissue which are able to renew and to maintain the life-long production of progenitor cells and the diverse mature functionally differentiated non-dividing cells which comprise that tissue, in both the steady-state and following perturbation or injury.

**Progenitor cell:** Relatively undifferentiated transit amplifying cells which arise from stem cells and have a finite lifespan with a more limited proliferative capacity and a more restricted ability to give rise to descendent cell lineages within a tissue.

**Facultative stem cell:** This term describes a class of normally functional differentiated cells in some adult organs which are able to revert to an undifferentiated stem cell state and regenerate diverse damaged cell lineages in response to severe perturbation or injury.

**Regenerative cells:** In this review, this is a collective term used to describe any cells in a tissue or organ which are able to proliferate and replenish descendent cell lineages in the steady state or following injury.

**Pluripotent/multipotent/unipotent stem cells:** These terms describe the relative ability of a stem cell to give rise to diverse differentiated cell lineages. The term pluripotent stem cell is reserved for embryonic stem cells which give to differentiated cells of endodermal, mesodermal and ectodermal origin which comprise the organism. Multipotent stem cells give rise to a multiple, but limited number of cell lineages, usually of a single germ layer origin. Unipotent stem or progenitor cells are only able to differentiate into a single cell type.
Non-dividing Functionally Differentiated Cells

G₀ Probability of cycling

Potential for renewal

Transit Amplifying Cells

Proliferative potential

Morphologically Recognisable Cells

Lineage commitment/differentiation

Non-dividing Functionally Differentiated Cells

Fig 1
Phenotype

“Gold Standard”

Transplant

Assay

in vitro

Clonogenic

Assay

Correlation

Co-expression

Co-fractionation

Does assay readout predict stem cell dysregulation?

Cytotoxic Perturbation and Mutant Mouse Models

Sca-1

C-kit

Fig 2