Editorial

Demise of lung transplants: exposing critical gaps in understanding lung stem cells

Wa Xian, Frank McKeon

Institute of Molecular Medicine, McGovern Medical School, University of Texas Health, Sciences Center, Department of Biology and Biochemistry, Stem Cell Center, University of Houston, Houston, TX, USA

Correspondence to: Wa Xian. Institute of Molecular Medicine, McGovern Medical School, University of Texas Health Sciences Center, Department of Biology and Biochemistry, Stem Cell Center, University of Houston, Houston, TX, USA. Email: wa.Xian@uth.tmc.edu.

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Lung transplantation is the only potentially curative therapy for end-stage chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and cystic fibrosis (CF), which together claim the lives of 150,000 patients each year in the USA (1). Yet less than 2,000 of these procedures are performed each year in the US due to donor availability, the need of specialized surgical and medical teams, and the overall unit costs that are approaching one million dollars. A further drag on this option is the high rates of lethal obliterative bronchiolitis (OB) appearing in these patients across centers (2). This vexing phenomenon largely accounts for low 5-year survival rate of lung transplants relative to other organ transplants, challenges our understanding of the pathophysiology OB, reveals enormous gaps in our knowledge of regenerative processes in the lung, and may ultimately force a reevaluation of strategies for addressing chronic lung disease. OB itself has features typical of organ rejection including inflammation and peribronchiolar and perivascular fibrosis, and ultimately results in small airway occlusion (2-4). However, the lung parenchyma is surprisingly spared in transplant-associated OB, arguing against a generalized allotypic response and perhaps a more specific response to the very cells responsible for the regenerative maintenance of the small airways. Swatek *et al.* (5) couple lung transplants between outbred ferrets known to yield an OB indistinguishable from that seen in human recipients with stem cell clonogenic analyses to reveal an altogether new facet of OB pathobiology. While their key findings that p63+/Krt5+ stem cells are progressively depleted from both small and large airways as well as destruction of submucosal glands (SMG) during lung rejection are detailed in the excellent editorial by Smirnova and Eickelberg (6), here we will focus some of the broader implications of Swatek *et al.* as they pertain to our limited understanding of airway stem cells, the potential of stem cell cloning to fill these gaps, and the very real possibility that such epithelial airway stem cells will form the basis of autologous solutions to the problem of chronic lung disease.

Loss of stem cells during rejection: generalized loss of p63+/Krt5+ airway stem cells

One of the remarkable observations of Swatek *et al.* was that OB impacts the presence and clonogenic potential of all p63+/Krt5+ stem cells of the large and small airways. Furthermore, SMG, which have been suggested to be the reserve source of surface airway basal cells, are destroyed early in the disease process. Their analysis of p63+/Krt5+ stem cell populations is especially relevant as they represent only a fraction of the airway stem cell candidates in play listed by Smirnova and Eickelberg and yet the only ones that fulfill general criteria for stem cells on other somatic

tissues including clonogenicity, long-term proliferative potential, and multipotency (7,8). The observed attrition and loss of proliferative potential of these p63+/Krt5+ stem cells across the airways suggests a more widespread pathology accompanying the rejection process that must be accounted for in this progressive condition. At the same time, the lung parenchyma harboring alveolar structures is largely spared both in their ferret model as well as in human transplant-associated OB (5), and neither shows the inflammatory infiltrates and fibrosis typically linked to allogenic rejection processes. This raises the possibility that these stem cells are either triggering an especially specific allogenic response or somehow breaking tolerance to attract the fury of autoimmune mechanisms (2-4). Either way, the loss of p63+/Krt5 stem cells from their diverse sites is likely to impact distinct regenerative process across the airways in ways we cannot predict at present. What we do know from single cell cloning of p63+/Krt5+ stem cells from nasal, tracheal, and distal airways is that they are distinct at the level of gene expression. Thus, these three "types" of p63+/Krt5+ stem cells differ from one another in the expression of approximately 300 genes of about 20,000 probed. But upon differentiation *in vitro* and following transplantation, the nasal and tracheal stem cells yield an "upper airway" epithelium dominated by ciliated cells with a minority of goblet cells, whereas the p63+/Krt5+ cells of the distal airways (termed "distal airway stem cells" or DASCs) differentiate to Clara cells and Type I and Type II pneumocytes of alveoli *in vitro* and *in vivo* (9,10). Importantly, these three types of p63+/Krt5+ airway stem cells are epigenetically fixated on those fates for upper or lower airway epithelia regardless of how long they have been propagated *in vitro* as single cell-derived clones, and do not get confused or show obvious plasticity in these respective fates. As revealed by Swatek *et al.* (5), the above scheme does not factor in the consequences of losing the p63+/Krt5+ basal stem cells precursors of the SMGs in OB, as their relative fate properties have not been worked out at the clonal level. This gap in accommodating SMG p63+/Krt5+ stem cells in airway regenerative properties underscores what is likely an enormous gap in our understanding of stem cells of the airways in general. Particularly, the concepts of "adult stem cells" of regenerative epithelia are in flux and the airways are no exception. However, if we can extrapolate from emerging data from the human gastrointestinal tract (11), where a continuous spectrum of epigenetically fixed stem cells account for the enormous regiospecificity of the overlying epithelia across the stomach, intestines, and colon, it is almost certain that we are greatly underestimating the stem cells types responsible for regiospecificity across the airway generations. We should add that prior to a detailed clonogenic analysis of stem cells along the human gastrointestinal tract, the general view was behind a pliable "intestinal stem cell" that gave rise to local histological and functional diversity of the gastrointestinal tract by local "niche" influences. This latter concept has now been challenged by a detailed clonal analysis of stem cells from multiple discrete regions of single individuals. Rather than monotonous intestinal stem cell, stem cells from each segment of the intestine and colon had distinct and epigenetically stable gene expression profiles, and upon differentiation *in vitro* formed epithelia identical to the local epithelia from which they were derived (11). What is clear from the example of the gastrointestinal tract is that stem cells are patterned early in development and maintained by epigenetic mechanisms throughout life. Moreover, this commitment pattern does not have distinct boundaries between say ileum and jejunum but rather presents as a spectrum of discrete stem cells that gradually define regional characteristics across the gastrointestinal tract. By analogy, the airway stem cells will likely represent a spectrum of differentiation potentials defined crudely to date as the p63+/Krt5+ stem cells that give rise to proximal and distal airway epithelia, respectively. Defining the properties of this spectrum of stem cells at each generation of the airway is likely to be more than an academic exercise as it could prospectively reveal the requirements and tolerance for such cells in regenerative medicine for airway conditions.

When to move on autologous p63+/Krt5+ epithelial stem cell transplants?

Independent of the rates of associated OB, lung transplants have struggled worldwide to reach 4,000 cases per year, nowhere near the three million COPD deaths in the same interval they might prevent (12). And while much progress into the basis and ultimately prevention of transplant-associated OB is being achieved, it may be important to consider and devise alternatives that circumvent the enormous challenges presented by allogeneic lung transplants to ultimately address a larger fraction of the patient population with end-stage disease.

One alternative to allogeneic lung transplants with arguably the highest probability of success would involve the transplantation of autologous lung epithelial

stem cells. For one, we now know that the lung has an immense capacity for wholesale regeneration and that this regeneration is mediated by p63+/Krt5+ DASCs (9,10). Key support for these notions came from H1N1 influenza infections in mice, which showed that mice sustaining a 60% loss of lung parenchyma undergo a complete recovery in a matter of two months as judged by lung histology and physiology (9). These findings parallel the remarkably rapid and complete recovery of lung structure and function in pediatric patients who have acquired severe necrotizing pneumonia (13,14). Similarly, many cases of moderate to severe acute respiratory distress syndrome (ARDS) in adults are resolved at the level of pulmonary structure and function within six months to a year post treatment (15,16). As important, the underlying stem cell responsible for this highly efficient process in mice has been identified as a p63+/Krt5+ distal airway stem cell (e.g., DASCs) that are absolutely required for lung regeneration. The dynamics of the DASC response to acute lung injury is also telling. Rare in the terminal bronchioles of murine distal airways of normal mice, DASCs undergo a 400-fold proliferative amplification within a week of injury, migrate to damaged regions of lung at day 10–11, and form nascent alveoli by day 14 in a highly distinctive, peribronchiolar pattern (9). Preliminary data indict a very similar kinetics of DASC dynamics in patients infected by H1N1 influenza virus (Xian *et al.*, unpublished observations), as well as patients with more general forms of ARDS. These DASCs are formally required for lung regeneration as genetic models of their diphtheria toxin-mediated ablation during H1N1 influenza infection results in the deposition of fibrosis in lieu of lung regeneration.

A second feature of lung regeneration that bodes well for regenerative medicine is that the underlying stem cell is highly clonogenic, shows unlimited expansion capacity *in vitro*, and readily transplants to form functional alveoli in acutely damaged lung (10). A single p63+/Krt5+ DASC can be cloned, expanded, and transplanted via intratracheal delivery to acutely damaged lungs, where they selectively inhabit damaged regions and differentiate to form Clara cells and alveoli composed of type I and type II pneumocytes. Importantly, these same p63+/Krt5+ DASCs showed no incorporation in mice absent acute lung injury, suggesting that the efficient regenerative properties of these cells are not marred by "off-target" incorporation (10). Lastly, DASC's are readily cloneable from simple bronchoscopic biopsies, from bronchopulmonary lavage, or from transmural biopsies, providing good sources of autonomous stem cells that can be expanded to hundreds of billions of cells in four weeks (10-17). Taken together, the established properties of DASCs, including clonogenicity, expandability, and facility for accurate transplantation obviate many theoretical objections that could have limited their use in regenerative medicine. Still, major hurdles remain before the use of autologous epithelial stem cells becomes practical for either acute and chronic lung diseases.

Critical barriers to autologous p63+/Krt5+ stem cell transplantations

The barriers to autologous epithelial stem cell transplants are distinct for acute and chronic lung diseases. A total of 150,000 cases of ARDS are seen in the US each year represent significant medical challenges with a 20–40% death rate and the potential for long-term consequences such as lung fibrosis in survivors (15,16). Based on experience from DASC transplants in mice, it is possible and likely that the delivery of autologous DASCs could improve survival and limit long-term sequelae including fibrosis. Given the ongoing necrotizing damage in ARDS patients, it would seem an ideal setting to benefit from the addition of exogenous p63+/Krt5+ DASCs to augment and speed the regenerative process. The key technical challenge will be cloning and expanding sufficient numbers of DASCs in the timeframe (e.g., 7–14 days) to benefit patients in the throes of ARDS. The technology for such epithelial stem cell expansion continues to improve (Xian *et al.*, unpublished) and it would seem the feasibility of such trials should be realized in the near term.

For chronic lung conditions, the problem of acquiring sufficient autologous stem cells is obviated by the course of the disease that offers many opportunities to clone and expand populations of DASCs. In the case of cystic fibrosis, this long lead time would enable the correction of mutant CFTR loci by homology-directed recombination (HDR) using genome editing technologies, and similar strategies could conceivably be used to modify loci risk loci implicated in COPD, pulmonary fibrosis, or other lung conditions (18). However, the most significant problem presented by chronic lung conditions is the vast remodeling of these lungs that, despite the pathology, is devoid of active necrotizing domains known to be critical for the regenerative process in lung and almost all other organs known to undergo a regenerative response. Simply delivering populations of autologous stem cells to such patients is unlikely to favorably impact outcome and could

negatively impact enthusiasm for cell-based therapeutics in general (17). Thus, the key challenge for resolving chronic lung conditions will be to devise highly controlled and sterile means of inducing a necrotizing damage of discrete regions of the lung. This will require a means of mimicking the signals that normally recruit innate immune cells to the sites of infection such that discrete regions of remodeled lung will be cleared of fibrotic lesions and other abnormal cells and tissues. Such signals and the pathways of inducing them are well known to immunology community but must be harnessed in a practical and predictable way to leverage the emerging epithelial stem cell technology to resolve chronic lung conditions.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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