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Regenerative Potential of Human Airway Stem Cells in Lung Epithelial Engineering

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Abstract

Bio-engineered organs for transplantation may ultimately provide a personalized solution for end-stage organ failure, without the risk of rejection. Building upon the process of whole organ perfusion decellularization, we aimed to develop novel, translational methods for the recellularization and regeneration of transplantable lung constructs.

We first isolated a proliferative KRT5⁺TP63⁺ basal epithelial stem cell population from human lung tissue and demonstrated expansion capacity in conventional 2D culture. We then repopulated acellular rat scaffolds in *ex vivo* whole organ culture and observed continued cell proliferation, in combination with primary pulmonary endothelial cells. To show clinical scalability, and to test the regenerative capacity of the basal cell population in a human context, we then recellularized and cultured isolated human lung scaffolds under biomimetic conditions. Analysis of the regenerated tissue constructs confirmed cell viability and sustained metabolic activity over 7 days of culture. Tissue analysis revealed extensive recellularization with organized tissue architecture and morphology, and preserved basal epithelial cell phenotype. The recellularized lung constructs displayed dynamic compliance and rudimentary gas exchange capacity. Our results underline the regenerative potential of patient-derived human airway stem cells in lung tissue engineering. We anticipate these advances to have clinically relevant implications for whole lung bioengineering and *ex vivo* organ repair.

Graphical abstract

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AUTHOR CONTRIBUTIONS

SEG designed, conducted, and analyzed all experiments, and prepared the manuscript; JMC constructed the lung bioreactor; XR prepared the endothelial cell population for recellularization; LFT prepared lungs for decellularization and recellularization, and assisted with manuscript preparation; TW assisted with primary cell isolation; DJM assisted with experimental design and data interpretation; HCO oversaw all experimental design, data analysis, and manuscript preparation.

Keywords

Lung Tissue Engineering; Regeneration; Epithelial Repair; Extracellular Matrix; Decellularization; Recellularization

INTRODUCTION

Solid organ bioengineering based on native extracellular matrix scaffolds has fuelled recent enthusiasm for regenerative medicine approaches to treat end organ failure (1). The main approach involves combining regenerative cell populations with corresponding biological matrices to form living, functional grafts. To this end, native solid organ extracellular matrix (ECM) scaffolds can be readily generated by perfusion decellularization with specific detergents, rendering a biocompatible framework as a foundation for regeneration (2–7).

Clinically relevant organ recellularization presents significant challenges, both in terms of identifying an ideal cell source and in the establishment of functional biomimetic organ culture systems to support organ maturation prior to transplantation (8). An optimal cell source would be easily obtained and expanded *in vitro*, while maintaining contact inhibition and cell cycle control. Directed differentiation of induced pluripotent stem cells through key developmental stages presents a promising option for obtaining lung-specified cell populations (9–11), but the length of *in vitro* culture and limited cell number and purity restricts their current utility for large-scale organ engineering. While largely quiescent, adult lung tissue has a remarkable capacity for regeneration, owing to a number of facultative stem/progenitor cell populations that become activated in response to tissue damage (12). Airway basal cells, identified by the transcription factor TP63 and expression of cytokeratin 5 (KRT5), function as multipotent stem cells of the proximal airway epithelium, and are critical for maintaining airway homeostasis during physiological cell turnover and regeneration (13, 14). This essential cell population comprises 30% of the cells in human airway epithelium (15), and early studies of airway regeneration have demonstrated the ability for isolated basal cells to recapitulate a fully differentiated airway epithelium when seeded onto denuded mouse tracheas (16). In response to injury, basal epithelial stem cells can rapidly proliferate and give rise to both ciliated and club cell progeny, confirming their important function in tissue homeostasis and injury repair (17). Basal cells can be isolated (18, 19) and propagated in culture (20), which makes them a useful candidate population for tissue and organ engineering applications. We demonstrate that this population can be readily derived from human cadaveric lung tissue following clinical organ donation and cold ischemia, and expanded *in vitro*. This isolated primary stem cell population also provides an

important tool for studying basic biology and tissue regeneration (13), particularly given their role in lung repair and capacity for multi-lineage differentiation (21, 22). Following injury, basal airways stem cells have been reported to undergo rapid proliferation, migration, and a surprising differentiation toward distal pneumocyte lineages, in order to reconstitute the damaged alveolar-capillary network (23). When delivered to rodent lungs following injury, TP63⁺KRT5⁺ cells have been shown to differentiate to type I and type II pneumocytes, in addition to bronchiolar secretory cells (24). Lung repair and remodelling after influenza or bleomycin injury may also involve a specialized subset of KRT5⁺ cells (25, 26). Although this phenomenon has yet to be investigated in the context of epithelial tissue engineering, these studies highlight the evolving understanding of traditional cell identity, hierarchy, and regenerative ability.

In the present study, we aimed to exploit the capacity for lung basal stem cells to respond to injury and to re-establish epithelial integrity and functional organization (13, 27), by investigating the utility of human donor tissue-derived cells in the context of whole organ engineering. We propose that the architectural and biological niches retained within the native extracellular matrix may provide a valid template to guide cell engraftment and investigate mechanisms of lung tissue repair (28, 29), and in combination with extended biomimetic culture, provide an important platform for the regeneration of human lung constructs.

METHODS

Study Approval

Human donor lungs otherwise unsuitable for transplantation were obtained from the New England Organ Bank (see Supplementary Table 1), following informed consent. All experiments were approved by the Massachusetts General Hospital Internal Review Board (#2011P002433) and Animal Utilization Protocol (#2014N000261).

Cell isolation and Expansion

Donor lung peripheral tissue was gently homogenized and digested in 0.1mg/ml DNase (Sigma) and 1.4mg/ml Pronase (Roche, 11459643001) for 24 hours/4°C (30). Digested tissue was plated onto uncoated culture flasks for 30 minutes/37°C, then non-adherent cells transferred to culture on human Collagen-IV (Sigma-Aldrich C7521)-coated flasks and maintained in Small Airway Growth Media (SAGM, Lonza, CC-3118). Primary endothelial cells were isolated from the large vessels of donor lungs using the same digestion protocol. The endothelial population was sorted for CD31⁺ purity by flow cytometry, and maintained and expanded on Gelatin-coated flasks in EGM2 (Lonza, CC-3162) until utilized for lung scaffold recellularization (See Supplemental Figure 1). For *in vitro* co-culture, endothelial cells were plated onto a 0.4µm transwell insert (Corning, 07200161) placed above the epithelial cell culture, and maintained in SAGM, with or without the addition of 40ng/ml VEGF (Peprotech), for 7 days.

Air-Liquid Interface Culture

Primary epithelial cells at passage 3 were plated onto 0.4µm Transwell inserts coated with collagen IV and maintained in submerged culture with SAGM for 5 days. Media was replaced with PneumaCult™-ALI medium (Stemcell Technologies, 05001) in the basal chamber only, and maintained for 21 days at Air-Liquid interface, with alternate day media changes.

3-Dimensional Sphere Assay

Primary basal epithelial cells at passage 3 were filtered through a 40µm mesh to remove any cell clumps then transferred onto a 0.4µm Transwell insert (5000 cells/90µL of 50:50 matrigel-to-SAGM substrate), following a previously published protocol (31). Single cell suspension was confirmed by light microscopy (40×). Cultures were maintained with SAGM in the basal chamber only for 7 days.

Lung Decellularization

Rat and human donor lungs were decellularized as previously described (2, 6). Briefly, cadaveric rat lungs were explanted from male Sprague-Dawley rats (250–300 g, Charles River Laboratories) and decellularized by perfusion of 0.1% SDS solution through the pulmonary artery at 40mmHg, followed by washing. Human lung decellularization was performed by perfusion of 0.5% SDS solution through the pulmonary artery at a constant pressure between 30 mmHg and 60 mmHg.

Rat Lung Recellularization and Culture

Where indicated, primary pulmonary endothelial cells (CD31⁺, 10×10⁶ total cells) were first delivered to the pulmonary artery in 100ml of media at a constant pressure of 30mmHg. After 90 minutes of static incubation (37°C), 20×10⁶ cells primary lung epithelial cells (passage 4) were delivered to the scaffold airways in 20ml of media by gravity. Constant media perfusion of SAGM with 40ng/ml Vascular Endothelial Growth Factor (VEGF) through the pulmonary artery was maintained at 4ml/min and changed daily. Continuous positive airway pressure (CPAP) of 20cmH₂O with room air (21% FiO₂) was initiated on day 2 of culture and maintained for 2-hours/day. Recellularized lungs (n=3 individual lungs per group) were maintained in culture for 7 days. From each lung n=3 representative tissue samples from the upper, middle, and lower lung were saved for both histologic and mRNA analysis.

Human Lung Recellularization and Biomimetic Culture

A total of 160–240×10⁶ primary pulmonary endothelial cells were first delivered to the vasculature via the pulmonary artery and vein by pump, at a constant pressure of 50mmHg (32). After 90 minutes, a total of 220–280×10⁶ epithelial cells (passage 4) were delivered to the main airway in solution (500ml media) by pump at 50ml/min. For each recellularization experiment, the cells were isolated from a unique donor. A total of n=3 independent lobes were recellularized, in separate experiments. For recellularization, the cells and the scaffold were not donor matched.

Constant perfusion of SAGM with 40ng/ml Vascular Endothelial Growth Factor (VEGF) was maintained for 7 (n=2) or 10 (n=1) days at 20–40ml/min. Perfusion pressure was continuously monitored and maintained within physiologic range (mean = 21.39 ± 4.53mmHg, see Figure 5C). Negative pressure ventilation was generated via chamber pressure oscillations to achieve a breath rate of 6 breath-cycles/minute with a positive end expiratory pressure (PEEP) of 8mmHg. Ventilation was initiated on day 3 of culture and maintained for 2-hours/day. Media samples from the pulmonary artery, pulmonary vein, and chamber were tested by iSTAT cartridge (CG4+/CG8+, Abbott) daily.

Positive pressure ventilation was performed on the final day of culture. Volume-controlled ventilation was applied using a Drager Evita-4 ventilator, with a tidal volume of 150–200mL, a PEEP of 5mmHg, and a respiratory rate of 12 breaths/min. Samples were analyzed after 10 minutes of ventilation with a FiO₂ of 21% and again after 10 minutes with and FiO₂ of 100%, using a GC3+ iSTAT cartridge. On the final day of culture, a 0.05mM Resazurin solution (500ml) was circulated for 90 minutes at a constant flow of 30ml/min. Resazurin reduction was measured by fluorescence in culture media (excitation 544nm, emission 590nm), and compared to a standard curve of known cell numbers *in vitro* (50:50 mixture of epithelial-to-endothelial cells, cultured for 90 minutes in Resazurin-containing media, n=3 replicates) to calculate an estimate of total cell number in recellularized lungs. Tissue samples were fixed in 5% formalin, or saved in RNAlater (Qiagen) for subsequent analysis. From each recellularized lobe (n=3), a total of n=4 representative tissue samples from the upper, middle, and lower lung were saved for both histologic and mRNA analysis.

Quantitative PCR

mRNA was isolated (Qiagen RNeasy Plus Kit) and transcribed to cDNA (Invitrogen SuperScript III). Gene expression was analyzed using Taqman probes and the OneStep Plus system (Applied Biosystems). Each biological sample was analyzed in experimental replicate (n=2 repeated wells of the qPCR reaction) with the Ct value of each replicate averaged and handed as n=1 unique biologic sample. Expression for each sample was normalized to β -Actin (ACTA1) gene expression (Ct) and relative to cadaveric peripheral lung tissue control samples (Ct), with fold change calculated by 2^{-Ct} (33).

Immunostaining

Following de-paraffinization and rehydration, 5 μ m tissue section were permeablized with 0.1% triton-x for intracellular antigens, when appropriate. Cells in culture were fixed with ice-cold methanol prior to staining. All samples were blocked with 1% donkey serum for 1hr. Primary Antibodies (all 1:100): TP63 (Santa Cruz, sc-25268), KRT5 (Abcam, ab24647), E-cadherin (CDH1, BD, 610181), Surfactant Protein-B (Millipore, AB3430), pro-Surfactant Protein-C (Abcam, ab3786), Aquaporin-5 (Abcam, ab92320), Acetylated α -Tubulin (TUBA1B, Abcam, ab24610), α 2 β 1 integrin (Abcam, ab24697), α 3 β 1 integrin (Abcam, ab24696), KI67 (Millipore, AB9260) and CD31 (Dako, M082301-2). Secondary antibodies all (1:400): Alexafluor Donkey anti-Mouse, Rabbit, or Goat, conjugated to 488 or 594 (Life Technologies). Samples were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nucleus and imaged using a Nikon Ti-Eclipse microscope.

Trypan blue (0.4%) staining following ROCK inhibitor Y27632 (10uM) treatment (Supplemental figure 2B) was visualized by bright-field image on the Nikon Ti-Eclipse microscope. β -galactosidase staining following ROCK inhibitor Y27632 (10uM) treatment (Supplemental figure 2C) was performed following the manufacturer's instructions (Cell Signaling Technology #9860), and visualized by bright-field image on the Nikon Ti-Eclipse microscope.

Statistical Analysis

For all experiments, the n value stated represent an independent biological sample. Data was analyzed by Mann Whitney U-test or Kruskal-Wallis with a Dunn's multiple comparison posttest, as appropriate, using GraphPad Software. All statistical significance is reported accordingly. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

RESULTS

We first isolated and characterized a highly proliferative cell population from human cadaveric lung tissue. Robust expansion of a KRT5⁺TP63⁺ basal epithelial stem cell population was reproducible over serial passages in culture, with increased expression of E-Cadherin and loss of pro-SP-B and α Tubulin positive cells. (Figure 1A–B). The proliferative capacity of the isolated cell population was maintained through 3 passages (KI67⁺ cells by staining, $63.4 \pm 8.08\%$, n=3 images quantified per passage), and began to decline by passage 4 (Figure 1C). Phenotypic stability was examined by flow cytometric analysis of passage 1 and passage 4 cells, confirming the expansion of the KRT5⁺TP63⁺ basal stem cell lineage (n=3 unique donor-derived cell lines analyzed. Figure 1D). Gene expression was longitudinally profiled (n=3 donor-derived cell lines analyzed in biologic triplicate at each time point. Figure 1E), additionally confirming the enrichment of the basal airway stem cell population, with a parallel loss of type 1 (*HOPX1*, *AQP5*) and type 2 (*SFTPB/C*) pneumocytes, and Club (*SCGB1A1*⁺) secretory cells. Throughout the culture period, no increase in expression of mesenchymal genes such as vimentin (*VIM*) or smooth muscle actin (*ACTA2*) was found, nor was an increase in the expression of epithelial-to-mesenchymal transition associated transcription factors *ZEB1* and *SNAIL* detected. Maximal cell proliferation at passage 3 was confirmed by quantification of *KI67* gene expression. In our hands, use of ROCK inhibitor Y-27632 (34) during cell isolation and passage did not enhance the basal cell population, or alter proliferation (*KI67*, *PCNA*) or senescence (senescence-associated cyclin-dependent kinase inhibitor 2A, *CDKN2A*) (35) (Supplemental Figure 2) and was therefore not used for *in vitro* expansion. Culture of the isolated basal epithelial stem cells at air-liquid interface demonstrated their capacity for ciliogenesis at day 21 (Supplemental Figure 3A–B). Additionally, culture of single-cell suspensions in matrigel revealed clonal generation of epithelial spheres with preservation of the KRT5⁺TP63⁺ phenotype on day 7 (Supplemental Figure 3C–D).

To investigate the regenerative capacity of this epithelial stem cell population in whole lung engineering, they were first used to recellularize rat lung scaffolds (Figure 2A). The airways were repopulated with human donor-derived cells and maintained in *ex vivo* biomimetic culture for 7 days (Figure 2B, Scaffold⁺Epithelium⁺, n=3 lungs). In parallel experiments,

scaffolds airways were recellularized in combination with primary pulmonary endothelial cells to the vasculature (Scaffold⁺Epithelium⁺Endothelium⁺, n=3 lungs). Constant media perfusion through the vascular network was maintained throughout culture. Airway recruitment was performed daily and continuous positive airway pressure (CPAP) was delivered for 2 hours per day to reduce atelectasis, improve oxygenation, and enhance biomimetic stimulation during regeneration (36) (Figure 2A). Analysis of the regenerated lung tissue confirmed a maintenance of the *TP63⁺KRT5⁺* basal epithelial stem cell lineage on day 4 and day 7 of scaffold culture. A significant increase in adherens junctions E-Cadherin (*CDH1*) was found in lungs recellularized with both epithelium and endothelium. Expression of the ciliated cell marker FOXJ1 was significantly increased in all recellularized lung after biomimetic culture. Continued proliferation, as measured by *KI67* expression, was noted in all recellularized lungs, with a significance increase found in lung tissue recellularized with both pulmonary endothelial cells and basal epithelial stem cells. No increase in the activation of cellular senescence (*CDKN2A*) was found following lung recellularization and culture. All recellularized lung tissue was compared to cells maintained in 2D culture at the same passage for reference (n=3 independent cell lines at passage 4). We also found that co-culture of the basal epithelial stem cell population with pulmonary endothelial cells significantly increased epithelial proliferation *in vitro* (Supplemental Figure 4) which supports the effect observed in whole lung culture. Analysis by immunofluorescent staining further revealed extensive recellularization of both distal alveolar lung tissue (Figure 2Ci–iii) and larger airways (Figure 2C iv–v). Reformation of native tissue morphology and architecture was observed, including both airway epithelium (*TP63⁺KRT5⁺*, *CDH1⁺*) and vessels (*CD31⁺*). Representative whole section scans identifying *KRT5⁺* cells and *CD31⁺* cell distribution in recellularized rat lung scaffolds on Day 7 of culture are presented in Supplementary Figure 5.

To move towards a more clinically relevant matrix and scale, we adapted our human lung bioreactor system to enable large-scale whole organ culture and the recellularization of isolated human lung scaffolds (37) (Figure 3A, used for n=3 independent lung recellularization experiments). We delivered the expanded basal stem cell population via the airways ($220\text{--}280 \times 10^6$ cells), and primary human pulmonary endothelial cells ($160\text{--}240 \times 10^6$ cells, *CD31⁺*) via the native artery and vein vasculature (Figure 3B). The bioreactor maintained a physiologic perfusion range (mean = 21.39 ± 4.53 mmHg) in the *ex vivo* regenerating organ (n=3 experiments, Figure 3C), while cell survival and metabolic activity were monitored in a non-invasive manner throughout the culture. Glucose consumption and lactate production in the perfusate was measured every 48 hours as a non-invasive indicator of cell growth (38), which gradually increased over the culture period (repeated measures, n=2 independent recellularization experiments, Figure 3D–E). Negative pressure ventilation of the lung construct was achieved at 6 breaths/minute by oscillating between set chamber pressure targets, in order to facilitate airway recruitment and oxygenation during culture (Figure 3F). This resulted in a median peak trans-mural pressure of 15.88 mmHg (14.42–21.73 mmHg, n=2447 breaths), and a median tidal volume of 138.08 ml/breath (78.08–183.32 ml, n=2447 breaths) (Figure 3G–I). Positive pressure ventilation was performed as an end-point test of potential organ function (Figure 3J) and oxygenation transfer to the perfusate media was measured following ventilation at *FiO₂* of 21% and 100% for 10

minutes. A resulting pO_2 of 72mmHg ($PaO_2/FiO_2=343$ mmHg) was measured, which increased to 412mmHg ($PaO_2/FiO_2=412$ mmHg), with a corresponding pCO_2 of 17.5mmHg and 24.9mmHg, respectively (Figure 3K). This suggests that the regenerated human lung construct can support rudimentary organ function and gas transfer following recellularization and culture.

Tissue recellularization and cell viability was visually assessed following metabolism of Resazurin-containing perfusate, noting the metabolism to a pink colour by viable cells (Figure 4A). Extent of coverage and cell morphology was investigated by histologic staining across multiple areas of each lobe (Figure 4B). Broad cell distribution throughout the repopulated scaffold, from the upper airways to the distal lung region with cell alignment in accordance with the preserved matrix architecture was found. The ability of reintroduced cells to continue expansion within the matrix was confirmed. Based on Resazurin reduction assay (39), the total cell number contained within each scaffold at the end of culture was calculated (Figure 4C). An average of 9.0×10^8 cells was calculated ($HL1 = 7.20 \times 10^8$, $HL2 = 1.07 \times 10^9$, $HL3 = 9.07 \times 10^8$), representing an continued increased compared to total cells seeded on Day 0. By staining, a robust fraction of epithelial cells remained proliferative ($61.7\% \pm 10.4$ KI67⁺) at the end of organ culture ($n=3$ representative areas were analyzed per lung, with 4 images quantified per area. Figure 4D–E). A preserved KRT5⁺TP63⁺ basal stem cell phenotype was observed throughout the regenerated lung tissue (Figure 4Fi), with a very minor contribution of non-adherent proSPB⁺ cells identified (Figure 4Fii). Heterogeneous endothelial cell (CD31⁺) coverage was observed throughout the vascular compartment, which corresponded with the expected distribution based on the initial cell number seeded. Rudimentary gas exchange units could be identified, represented by single layer endothelial and epithelial cells lining the alveolar-capillary interface (Figure 4Gi) and repopulated vascular conduits were found (indicated by white arrow, Figure 4Gii). The regenerated tissue was further analyzed for gene expression, confirming the maintenance of the basal stem cell population with *TP63* expression greater than 25-fold higher than normal cadaveric lung tissue, ($n=4$ independent tissue pieces analyzed per lung, with expression level of cells *in vitro* presented for reference. Figure 4H). A significant increase in *FOXJ1* and *KI67* expression was also found following human lung recellularization and biomimetic culture, along with a significant decrease in the cellular senescence marker CDKN2A.

Together, this data demonstrates the capacity for the clinically relevant human donor tissue-derived cell populations to recellularize native matrix scaffolds and serve as an important tool for tissue engineering of lung constructs for transplantation.

DISCUSSION

We have isolated a highly proliferative basal stem cell population from an easily accessible tissue source and demonstrated rapid expansion *in vitro*. This cell population, identified by KRT5⁺TP63⁺ expression, has been studied in many animal models of lung repair (40–42) and in human disease (43).

Within the KRT5⁺TP63⁺ population, additional distinct subpopulations of basal stem cells may exist, each with a unique role in tissue homeostasis and repair. This includes the

recently reported lineage-negative epithelial progenitor (LNEP) cells within normal distal lung, which can specifically proliferate following injury (25). It is unclear whether these rare cellular subsets can act in isolation, or if they require combined signalling and action of other cells in the injured tissue milieu. Mathematical models support a heterogeneous basal stem cell population, proposing approximately equal numbers of multipotent stem cells and committed precursors (44). The role of injury, including source, intensity, and duration, is also an important determinant of cell activation and fate, both *in vivo* and in tissue engineering aims.

Following lung injury, the re-establishment of an intact epithelium is critical to quickly restore barrier function and tissue homeostasis (45). In our model of lung repair, we propose the decellularized lung scaffold serves as the provisional matrix for epithelial cell migration, recapitulating the processes activated *in vivo* to cover and repair denuded airway and gas exchange surfaces (46). The physiologic role of basal cells, to help anchor epithelial cells to the matrix and protect the underlying stroma, is aided by their expression of abundant cytoskeletal, junctional and adhesive proteins, which supports their demonstrated utility in re-epithelialization of native lung ECM (47). For clinical utility of this methodology in autologous tissue transplantation, the source of isolated cell population must be considered. For indications with an underlying genetic cause, such as Cystic Fibrosis, it may be possible to correct the mutation in the isolated cell population prior to recellularization of the scaffold. Smoking-associated lung diseases, such as COPD, pose a unique challenge. The basal cell population isolated from COPD patients has been reported to display an altered gene expression profile and diminished capacity for regeneration (48). While the regenerative capacity is reduced in basal cells from smokers (43), it is not a completely lost, suggesting that a regenerative subpopulation remains. It is not yet known how these COPD-associated changes would affect re-epithelialization of healthy lung scaffolds, and if specific subpopulations could be identified that specifically retain a regenerative potential. An important area of future research would include investigation of this question and development of techniques to overcome any regenerative failure. In order to reduce the amount of starting donor tissue required, additional experimental factors including longer *ex vivo* culture time and optimized microenvironment (biomechanics, growth factors) may be developed to enhance the recellularization process. In addition to cell origin, the source of biologic scaffold may also play an important role in regeneration capacity, both in terms of cell distribution and survival in the construct (49).

Rodent models of airway injury have established a timeline of epithelial repair. After epithelial injury, cell spreading and migration occurs as the primary repair mechanisms in the first 12–24 hours, with proliferation beginning after 24 hours and continuing for several weeks (50). This timeline aligns with our model of lung repair in *ex vivo* culture and regeneration. We have also found that proliferation of the epithelial population is enhanced by co-culture with pulmonary endothelial cells. We believe that the capacity for cellular crosstalk is an important element of whole organ regeneration, both in our model and *in vivo*. The influence of other cell types may have a unique effect on epithelial repair and differentiation in the context of the proximal or distal lung niche.

Following proliferation and coverage, the next step in the repair mechanism would be reestablishment of a pseudostratified epithelium, which can take several weeks to generate (51). Our model of culture on human and rat lung scaffolds demonstrated the initial upregulation of FOXJ1 gene expression. Extended bioreactor culture may be required to fully mature the reconstituted airway epithelium, as *in vitro* air-liquid interface (ALI) models require 3–4 weeks to recapitulate the mature airway biology (52). Following recellularization and *ex vivo* culture, we did not identify significant pneumocyte lineages incorporated within our reconstituted epithelium. Longer regeneration time, combined with modulation of key signaling pathways will likely be required to induce any potential pneumocyte differentiation from the delivered airway stem cell population, with animal models demonstrating distal lung regeneration required 50–90 days *in vivo* (24, 25). Further optimization of cell delivery and biomimetic culture procedures are also required areas of future investigation to enhance regeneration.

Organ regeneration based on decellularized scaffolds is a unique model to study injury and test cell potential, as it provides a tool to investigate the essential elements of epithelial cell differentiation and repair. Given the isolated environment, coupled with the biomimetic stimulus provided by the *ex vivo* culture of the regenerating organ, it is possible to directly assess the specific response and capacity of individual cell populations during tissue repair. In the present study, by employing a systematic building-blocks approach, we make a critical step forward by demonstrating that a primary isolated airway stem cell population can accomplish extensive tissue regeneration on an acellular lung scaffold, and can be directed toward both proximal and distal epithelial lineages. Future advances will benefit from combined developments in bioengineering and cell biology to further mature the regenerating lung and translate this technology toward a fully reconstituted, therapeutically relevant, transplantable lung.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Badylak SF, Taylor D, Uygun K. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. *Annual review of biomedical engineering*. 2011; 13:27–53.
2. Guyette JP, Gilpin SE, Charest JM, Tapias LF, Ren X, Ott HC. Perfusion decellularization of whole organs. *Nature protocols*. 2014; 9(6):1451–68. [PubMed: 24874812]
3. Ott HC, Matthiesen TS, Goh SK, Black LD, Kren SM, Netoff TI, Taylor DA. Perfusion decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nature medicine*. 2008; 14(2):213–21.
4. Ott HC, Clippinger B, Conrad C, Schuetz C, Pomerantseva I, Ikonomou L, Kotton D, Vacanti JP. Regeneration and orthotopic transplantation of a bioartificial lung. *Nature medicine*. 2010; 16(8):927–33.

5. Petersen TH, Calle EA, Zhao L, Lee EJ, Gui L, Raredon MB, Gavrilov K, Yi T, Zhuang ZW, Breuer C, et al. Tissue-engineered lungs for in vivo implantation. *Science*. 2010; 329(5991):538–41. [PubMed: 20576850]
6. Gilpin SE, Guyette JP, Gonzalez G, Ren X, Asara JM, Mathisen DJ, Vacanti JP, Ott HC. Perfusion decellularization of human and porcine lungs: bringing the matrix to clinical scale. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation*. 2014; 33(3):298–308.
7. Wagner DE, Bonenfant NR, Parsons CS, Sokocevic D, Brooks EM, Borg ZD, Lathrop MJ, Wallis JD, Daly AB, Lam YW, et al. Comparative decellularization and recellularization of normal versus emphysematous human lungs. *Biomaterials*. 2014; 35(10):3281–97. [PubMed: 24461327]
8. Gilpin SE, Ott HC. Using Nature's Platform to Engineer Bio-Artificial Lungs. *Annals of the American Thoracic Society*. 2015; 12(Suppl 1):S45–9. [PubMed: 25830835]
9. Longmire TA, Ikonomou L, Hawkins F, Christodoulou C, Cao Y, Jean JC, Kwok LW, Mou H, Rajagopal J, Shen SS, et al. Efficient derivation of purified lung and thyroid progenitors from embryonic stem cells. *Cell stem cell*. 2012; 10(4):398–411. [PubMed: 22482505]
10. Mou H, Zhao R, Sherwood R, Ahfeldt T, Lapey A, Wain J, Sicilian L, Izvolsky K, Musunuru K, Cowan C, et al. Generation of multipotent lung and airway progenitors from mouse ESCs and patient-specific cystic fibrosis iPSCs. *Cell stem cell*. 2012; 10(4):385–97. [PubMed: 22482504]
11. Huang SX, Green MD, de Carvalho AT, Mumau M, Chen YW, D'Souza SL, Snoeck HW. The in vitro generation of lung and airway progenitor cells from human pluripotent stem cells. *Nature protocols*. 2015; 10(3):413–25. [PubMed: 25654758]
12. Kotton DN, Morrissey EE. Lung regeneration: mechanisms, applications and emerging stem cell populations. *Nature medicine*. 2014; 20(8):822–32.
13. Rock JR, Randell SH, Hogan BL. Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. *Disease models & mechanisms*. 2010; 3(9–10):545–56. [PubMed: 20699479]
14. Rawlins EL, Hogan BL. Epithelial stem cells of the lung: privileged few or opportunities for many? *Development*. 2006; 133(13):2455–65. [PubMed: 16735479]
15. Mercer RR, Russell ML, Roggli VL, Crapo JD. Cell number and distribution in human and rat airways. *American journal of respiratory cell and molecular biology*. 1994; 10(6):613–24. [PubMed: 8003339]
16. Inayama Y, Hook GE, Brody AR, Cameron GS, Jetten AM, Gilmore LB, Gray T, Nettesheim P. The differentiation potential of tracheal basal cells. *Laboratory investigation; a journal of technical methods and pathology*. 1988; 58(6):706–17.
17. Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, Randell SH, Hogan BL. Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106(31):12771–5. [PubMed: 19625615]
18. Hackett NR, Shaykhiev R, Walters MS, Wang R, Zwick RK, Ferris B, Witover B, Salit J, Crystal RG. The human airway epithelial basal cell transcriptome. *PloS one*. 2011; 6(5):e18378. [PubMed: 21572528]
19. Davidson DJ, Gray MA, Kilanowski FM, Tarran R, Randell SH, Sheppard DN, Argent BE, Dorin JR. Murine epithelial cells: isolation and culture. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*. 2004; 3(Suppl 2):59–62. [PubMed: 15463928]
20. Fulcher ML, Randell SH. Human nasal and tracheo-bronchial respiratory epithelial cell culture. *Methods in molecular biology*. 2013; (945):109–21. [PubMed: 23097104]
21. Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations. *American journal of physiology Lung cellular and molecular physiology*. 2004; 286(4):L643–9. [PubMed: 12871857]
22. Evans MJ, Shami SG, Cabral-Anderson LJ, Dekker NP. Role of nonciliated cells in renewal of the bronchial epithelium of rats exposed to NO₂. *The American journal of pathology*. 1986; 123(1):126–33. [PubMed: 3963147]

23. Kumar PA, Hu Y, Yamamoto Y, Hoe NB, Wei TS, Mu D, Sun Y, Joo LS, Dagher R, Zielonka EM, et al. Distal airway stem cells yield alveoli in vitro and during lung regeneration following H1N1 influenza infection. *Cell*. 2011; 147(3):525–38. [PubMed: 22036562]
24. Zuo W, Zhang T, Wu DZ, Guan SP, Liew AA, Yamamoto Y, Wang X, Lim SJ, Vincent M, Lessard M, et al. p63(+)Krt5(+) distal airway stem cells are essential for lung regeneration. *Nature*. 2015; 517(7536):616–20. [PubMed: 25383540]
25. Vaughan AE, Brumwell AN, Xi Y, Gotts JE, Brownfield DG, Treutlein B, Tan K, Tan V, Liu FC, Looney MR, et al. Lineage-negative progenitors mobilize to regenerate lung epithelium after major injury. *Nature*. 2015; 517(7536):621–5. [PubMed: 25533958]
26. Quantius J, Schmoltdt C, Vazquez-Armendariz AI, Becker C, El Agha E, Wilhelm J, Morty RE, Vadasz I, Mayer K, Gattenloehner S, et al. Influenza Virus Infects Epithelial Stem/Progenitor Cells of the Distal Lung: Impact on Fgfr2b-Driven Epithelial Repair. *PLoS pathogens*. 2016; 12(6):e1005544. [PubMed: 27322618]
27. Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *The American journal of pathology*. 2004; 164(2): 577–88. [PubMed: 14742263]
28. Balestrini JL, Niklason LE. Extracellular matrix as a driver for lung regeneration. *Annals of biomedical engineering*. 2015; 43(3):568–76. [PubMed: 25344351]
29. Daley WP, Peters SB, Larsen M. Extracellular matrix dynamics in development and regenerative medicine. *Journal of cell science*. 2008; 121(Pt 3):255–64. [PubMed: 18216330]
30. Karp PH, Moninger TO, Weber SP, Nesselhauf TS, Launsbach JL, Zabner J, Welsh MJ. An in vitro model of differentiated human airway epithelia. *Methods for establishing primary cultures*. *Methods in molecular biology*. 2002; (188):115–37. [PubMed: 11987537]
31. Barkauskas CE, Crouce MJ, Rackley CR, Bowie EJ, Keene DR, Stripp BR, Randell SH, Noble PW, Hogan BL. Type 2 alveolar cells are stem cells in adult lung. *The Journal of clinical investigation*. 2013; 123(7):3025–36. [PubMed: 23921127]
32. Ren X, Moser PT, Gilpin SE, Okamoto T, Wu T, Tapias LF, Mercier FE, Xiong L, Ghawi R, Scadden DT, et al. Engineering pulmonary vasculature in decellularized rat and human lungs. *Nature biotechnology*. 2015; 33(10):1097–102.
33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25(4):402–8. [PubMed: 11846609]
34. Liu X, Ory V, Chapman S, Yuan H, Albanese C, Kallakury B, Timofeeva OA, Nealon C, Dakic A, Simic V, et al. ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. *The American journal of pathology*. 2012; 180(2):599–607. [PubMed: 22189618]
35. Sharpless NE, Sherr CJ. Forging a signature of in vivo senescence. *Nature reviews Cancer*. 2015; 15(7):397–408. [PubMed: 26105537]
36. Guerin C, Debord S, Leray V, Delannoy B, Bayle F, Bourdin G, Richard JC. Efficacy and safety of recruitment maneuvers in acute respiratory distress syndrome. *Annals of intensive care*. 2011; 1(1):9. [PubMed: 21906333]
37. Charest JM, Okamoto T, Kitano K, Yasuda A, Gilpin SE, Mathisen DJ, Ott HC. Design and validation of a clinical-scale bioreactor for long-term isolated lung culture. *Biomaterials*. 2015; 52:79–87. [PubMed: 25818415]
38. Ozturk, SS.; Jorjani, P.; Taticek, R.; Lowe, B.; Shackleford, S.; Ladehoff-Guiles, D.; Thrift, J.; Blackie, J.; Naveh, D. *Animal Cell Technology: From Vaccines to Genetic Medicine*. Carrondo, MJT.; Griffiths, B.; Moreira, JLP., editors. Dordrecht: Springer Netherlands; 1997. p. 355-60.
39. Ren X, Tapias LF, Jank BJ, Mathisen DJ, Lanuti M, Ott HC. Ex vivo non-invasive assessment of cell viability and proliferation in bio-engineered whole organ constructs. *Biomaterials*. 2015; 52:103–12. [PubMed: 25818417]
40. Teixeira VH, Nadarajan P, Graham TA, Pipinikas CP, Brown JM, Falzon M, Nye E, Poulsom R, Lawrence D, Wright NA, et al. Stochastic homeostasis in human airway epithelium is achieved by neutral competition of basal cell progenitors. *eLife*. 2013; 2:e00966. [PubMed: 24151545]
41. Wansleben C, Barkauskas CE, Rock JR, Hogan BL. Stem cells of the adult lung: their development and role in homeostasis, regeneration, and disease. *Wiley interdisciplinary reviews Developmental biology*. 2013; 2(1):131–48. [PubMed: 23799633]

42. O’Koren EG, Hogan BL, Gunn MD. Loss of basal cells precedes bronchiolitis obliterans-like pathological changes in a murine model of chlorine gas inhalation. *American journal of respiratory cell and molecular biology*. 2013; 49(5):788–97. [PubMed: 23742075]
43. Staudt MR, Buro-Auriemma LJ, Walters MS, Salit J, Vincent T, Shaykhiev R, Mezey JG, Tilley AE, Kaner RJ, Ho MW, et al. Airway Basal stem/progenitor cells have diminished capacity to regenerate airway epithelium in chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine*. 2014; 190(8):955–8. [PubMed: 25317467]
44. Watson JK, Rulands S, Wilkinson AC, Wuidart A, Ousset M, Van Keymeulen A, Gottgens B, Blanpain C, Simons BD, Rawlins EL. Clonal Dynamics Reveal Two Distinct Populations of Basal Cells in Slow-Turnover Airway Epithelium. *Cell reports*. 2015; 12(1):90–101. [PubMed: 26119728]
45. Stripp BR, Reynolds SD. Maintenance and repair of the bronchiolar epithelium. *Proceedings of the American Thoracic Society*. 2008; 5(3):328–33. [PubMed: 18403328]
46. Zahm JM, Kaplan H, Herard AL, Doriot F, Pierrot D, Somelette P, Puchelle E. Cell migration and proliferation during the in vitro wound repair of the respiratory epithelium. *Cell motility and the cytoskeleton*. 1997; 37(1):33–43. [PubMed: 9142437]
47. Evans MJ, Van Winkle LS, Fanucchi MV, Plopper CG. Cellular and molecular characteristics of basal cells in airway epithelium. *Experimental lung research*. 2001; 27(5):401–15. [PubMed: 11480582]
48. Crystal RG. Airway basal cells. The “smoking gun” of chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine*. 2014; 190(12):1355–62. [PubMed: 25354273]
49. Balestrini JL, Gard AL, Gerhold KA, Wilcox EC, Liu A, Schwan J, Le AV, Baevova P, Dimitrievska S, Zhao L, et al. Comparative biology of decellularized lung matrix: Implications of species mismatch in regenerative medicine. *Biomaterials*. 2016; 102:220–30. [PubMed: 27344365]
50. Crosby LM, Waters CM. Epithelial repair mechanisms in the lung. *American journal of physiology Lung cellular and molecular physiology*. 2010; 298(6):L715–31. [PubMed: 20363851]
51. Dupuit F, Gaillard D, Hinnrasky J, Mongodin E, de Bentzmann S, Copreni E, Puchelle E. Differentiated and functional human airway epithelium regeneration in tracheal xenografts. *American journal of physiology Lung cellular and molecular physiology*. 2000; 278(1):L165–76. [PubMed: 10645904]
52. Pezzulo AA, Starner TD, Scheetz TE, Traver GL, Tilley AE, Harvey BG, Crystal RG, McCray PB Jr, Zabner J. The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia. *American journal of physiology Lung cellular and molecular physiology*. 2011; 300(1):L25–31. [PubMed: 20971803]

Figure 1.

Characterization of primary human lung epithelial stem cell expansion *in vitro*. Bright-field and immunofluorescent images of (A) Passage 1 (P1) and (B) Passage 4 (P4) primary human epithelial cells *in vitro*, illustrating colony outgrowth with an enrichment of KRT5⁺TP63⁺ basal stem cells. Scale bar = 100μm (C) Quantification of KI67 positive cells *in vitro* by passage. n=3 images quantified per passage (P1–P4). Kruskal-Wallis analysis compared to Passage 1 (P1), with Dunn's post-test. All error bars represent standard deviation. (D) Flow cytometric quantification of epithelial cell markers at P1 and P4. n=3 cell lines analyzed per passage. (E) Quantitative PCR analysis of gene expression by cell passage. n=3 individual donor-derived cultures presented, analyzed in biological triplicate at each passage with standard deviation. Gene expression normalized to β-actin (*ACTA1*) expression and relative to normal control lung tissue.

Figure 2. Rat lung scaffold regeneration. **(A)** *Ex vivo* biomimetic culture of recellularized lungs with constant media perfusion and constant positive airway pressure (CPAP). **(B)** Gene expression of lungs recellularized with epithelial cells alone versus epithelial plus endothelial cells, analyzed after 4 days (light grey bars) and 7 days (dark grey bars) in biomimetic culture, compared to basal epithelial cells *in vitro* (dashed bar). Each bar represents 3 independent tissue pieces analyzed, with n=3 independent recellularized lungs presented. Expression normalized to β -actin (*ACTA1*) and relative to normal cadaveric lung

tissue control. All error bars represent standard deviation of the n=3 tissue pieces analyzed. Statistical differences determined by Kruskal-Wallis test with a Dunn's multiple comparison post-test compared to basal epithelial cells *in vitro* (n=3 independent cell lines at passage 4). (C) Immunofluorescent images demonstrate tissue morphology and regeneration in (i–iii) distal alveolar structures and (iv–v) larger airways, after 7 days of culture. Scale bars = 50µm.

Figure 3.

Regeneration and culture of human lung scaffolds. **(A)** Schematic of lung bioreactor capable of constant organ perfusion and negative pressure ventilation. **(B)** Single human decellularized lung lobe in bioreactor with access ports for pulmonary vein, airway, and pulmonary artery highlighted. Lobes were recellularized and maintained under constant media perfusion plus periodic negative pressure ventilation. **(C)** Pulmonary artery pressure over 8 days of recellularized lung perfusion culture, $n = 3$ recellularized lobes. Data represents mean pressure $\pm$ SD. Arrows indicate daily fluid sampling. **(D–E)** Change in **(D)** Glucose measurements in media sampled from the lung culture media. Media was changed every 48-hours, and values represent the change in glucose concentration (mg/dL) and **(E)** lactate concentration (mmol/L) after 48-hours of organ perfusion, compared to fresh media. Data from $n=2$ independent lung cultures per time point. Error bars represent standard deviation. **(F)** Representative pressure traces during negative pressure ventilation (breath rate of 6/min). Pressures are simultaneously recorded in the organ chamber, pulmonary artery, PEEP chamber, airway, and pulmonary vein. **(G)** Peak transmurial pressure (mmHg) during ventilation. **(H)** Calculated tidal volume (mL) during ventilation. Box plots represent median, plus the first and third quartile. Whiskers represent the range of data. Outliers (points greater than $1.5 \times IQR$ of the box plot) are represented by a plus sign (+). **(I)** Representative pressure-volume loop generated during negative pressure ventilation in the bioreactor. Traced loop $t=0$ is represented as Blue and $t=final$ is represented as Red **(J)** Endpoint positive pressure ventilation of a single lobe. **(K)** Representative measurement of pH, pO_2 , pCO_2 , and HCO_3^- of perfusate during positive pressure ventilation. Lobe was recellularized and cultured for 7 days prior to testing with 21% and 100% FiO_2 .

Figure 4.

Analysis of recellularized human lung tissue following biomimetic culture. (A) Perfusion of Resazurin containing media to assay cell viability on day 7 of biomimetic culture. Viable cell metabolism of the blue dye is visualized by transition to a pink color, demonstrating cell retention, distribution, and viability after culture. (B) Representative scan of H&E section of recellularized lung tissue (i) scale bar = 5mm and (ii) scale bar = 100μm. (C) Calculated total cell number at the end of lung culture, based on Resazurin reduction in culture media, compared to standard curve of known cell numbers. (D) Immunofluorescent image of continued E-Cadherin⁺ (CDH1⁺) epithelial cell proliferation (KI67⁺) on Day 7 of culture. Scale bar = 50μm. (E) Quantification of cell proliferation in recellularized lung tissue by KI67⁺ staining. Three representative areas analyzed per lung, with 4 images quantified per area. Error bars represent the standard deviation. (F) Immunofluorescent images of recellularized lungs at Day 7 of culture. Scale bars=50μm (G) Localization and organization

of CD31⁺ cells in the vascular capillary compartment of the lung scaffold. Scale bars=50µm. Arrow indicates the vascular network. **(H)** Quantitative PCR analysis of gene expression of lung tissue on final day of culture. Data from 3 independent recellularized lungs (HL1, HL2, HL3) is shown, with n=4 unique tissue samples analyzed per lung. Expression normalized to β-actin (*ACTA1*) and relative to normal cadaveric lung tissue control. Gene expression level for cells maintained *in vitro* is shown for reference. All error bars represent standard deviation. Statistical analysis by Kruskal-Wallis test with a Dunn's multiple comparison post-test compared to *in vitro*.