Enhanced Lung Epithelial Specification of Human Induced Pluripotent Stem Cells on Decellularized Lung Matrix

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Background. Whole-lung scaffolds can be created by perfusion decellularization of cadaveric donor lungs. The resulting matrices can then be recellularized to regenerate functional organs. This study evaluated the capacity of acellular lung scaffolds to support recellularization with lung progenitors derived from human induced pluripotent stem cells (iPSCs).

Methods. Whole rat and human lungs were decellularized by constant-pressure perfusion with 0.1% sodium dodecyl sulfate solution. Resulting lung scaffolds were cryosectioned into slices or left intact. Human iPSCs were differentiated to definitive endoderm, anteriorized to a foregut fate, and then ventralized to a population expressing NK2 homeobox 1 (Nkx2.1). Cells were seeded onto slices and whole lungs, which were maintained under constant perfusion biomimetic culture. Lineage specification was assessed by quantitative polymerase chain reaction and immunofluorescent staining. Regenerated left lungs were transplanted in an orthotopic position.

Results. Activin-A treatment, followed by transforming growth factor-β inhibition, induced differentiation of human iPSCs to anterior foregut endoderm as confirmed by forkhead box protein A2 (FOXA2), SRY (Sex Determining Region Y)-Box 17 (SOX17), and SOX2 expression. Cells cultured on decellularized lung slices demonstrated proliferation and lineage commitment after 5 days. Cells expressing Nkx2.1 were identified at 40% to 60% efficiency. Within whole-lung scaffolds and under perfusion culture, cells further upregulated Nkx2.1 expression. After orthotopic transplantation, grafts were perfused and ventilated by host vasculature and airways.

Conclusions. Decellularized lung matrix supports the culture and lineage commitment of human iPSC-derived lung progenitor cells. Whole-organ scaffolds and biomimetic culture enable coseeding of iPSC-derived endothelial and epithelial progenitors and enhance early lung fate. Orthotopic transplantation may enable further in vivo graft maturation.


At present, transplantation is the only therapeutic option for many patients with end-stage lung disease; yet, limitations in organ availability and complications of immunosuppression and chronic graft dysfunction remain [1]. The successful translation of organ-engineering strategies to regenerate lungs for transplantation would provide a novel organ source and increase the number of lungs available for patients. The ability to create patient-specific organs would be of further benefit, reducing the need for immunosuppression and potentially improving graft survival.

Acellular lung scaffolds can be created from cadaveric organs by multiple approaches, all aiming to remove cellular material while retaining the extracellular matrix scaffold upon which cells can later be reintroduced [2]. Our decellularization methodology uses constant-pressure detergent perfusion through the native lung vasculature and results in a biocompatible whole-organ scaffold [3]. This scaffold can support primary epithelial and endothelial recellularization to regenerate transplantable constructs that could be perfused and ventilated in vivo for up to 1 week [4, 5].

There is great interest in the use of cell populations derived from induced pluripotent stem cells (iPSCs) as tools for patient-specific lung regeneration [6]. Derivation of these cells requires recapitulation of the sequential steps in early lung specification. During development, germ layer restriction first occurs during gastrulation, with cells exposed to strong nodal signalling becoming

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specified to definitive endoderm [7]. In culture, nodal signalling is mimicked with high dose activin-A to generate an enriched definitive endoderm population marked by the transcription factors SRY (Sex Determining Region Y)-Box 17 (Sox17) and forkhead box protein A2 (Foxa2) [8]. The endoderm layer then forms the primitive gut tube from which the respiratory and digestive systems develop through an anterior-posterior division. Lungs and trachea both arise from the anterior foregut endoderm, marked by the transcription factor Sox2 and preservation of Foxa2 [9, 10]. Timed inhibition of the transforming growth factor-β signalling pathway is sufficient to anteriorize iPSC-derived endoderm in vitro [11].

The transcription factor NK2 homeobox 1 (Nkx2.1) is the earliest marker of lung-specified endoderm [12], as well as early thyroid and brain. Mouse embryos carrying a homozygous disruption in the Nkx2.1 locus form a primitive lung structure but fail to undergo branching morphogenesis or epithelial development and cannot support ventilation [13]. Specific growth factors, including Wnt, bone morphogenetic protein (BMP), and fibroblast growth factor (FGF), are critical to ventralization and the generation of Nkx2.1-expressing cells [9]. From this ventral wall, the primary lung buds emerge and separate from the dorsal esophagus, driven by signalling from the surrounding mesenchyme [14]. Directed, step-wise in vitro differentiation protocols have successfully generated airway epithelium and lung pneumocytes [11, 15–17] from human iPSCs by recapitulation of these developmental stages.

The role of matrix–cell interactions in mediating cellular fate decisions is an important but incompletely understood element of lung development. Basement membrane attachment can direct type II pneumocytes to adopt a differentiated type I phenotype [18], and matrix interactions can enhance surfactant protein-C expression by differentiating embryonic stem cells [19]. The 3-dimensional structure of lung matrix [20], along with mechanical stimuli, such as stretch, also provide developmental cues [21, 22]. Moreover, the cross-talk between endogenous cells and the extracellular matrix during tissue repair, involving growth factor production and matrix modification, further highlights this complex relationship [23].

By combining principles of lung development and bioengineering, the aim of patient-specific therapies for end-stage lung disease may be realized through the regeneration of whole-organ scaffolds [24]. The use of patient-derived stem cells and the elucidation of the key developmental stages for recellularization of native scaffolds are important milestones toward functional organ regeneration and transplantation.

Material and Methods

Human iPSC Culture

All experiments used iPSCs generated by messenger RNA transfection (Kruppel-like factor 4 [Klf4], c-Myc, octamer-binding transcription factor 4 [Oct4], Sox2, LIN28) of postnatal fibroblasts [25]. Undifferentiated cells were maintained in feeder-free culture in mTeSR media (STEMCELL Technologies Inc, Vancouver, BC, Canada) on Gelatrex-coated plates (Life Technologies Corp, Carlsbad, CA), and passaged with Accutase (STEMCELL Technologies Inc) at 70% confluency. Differentiation was initiated at 80% confluency.

Human iPSC-Derived Endothelium

Endothelial differentiation used a basal media of Iscove’s modified Dulbecco’s medium, BIT 9500 Serum Substitute (STEMCELL Technologies) nonessential amino acids (Gibco, Carlsbad, CA), L-glutamine (Gibco), monothioglycerol (10%, Sigma-Aldrich, St. Louis, MO), and penicillin/streptomycin. Complete differentiation media was supplemented with BMP-4 (50 ng/mL, PeproTech, Rocky Hill, NJ), FGF-basic (50 ng/mL, PeproTech), and vascular endothelial growth factor 165 (50 ng/mL, PeproTech) for 6 days. Differentiated endothelial cells were expanded in endothelial cell growth medium-2 (Lonza, Basel, Switzerland) supplemented with 20% fetal bovine serum (HyClone/GE Healthcare Life Sciences Laboratories, South Logan, UT) on 0.1% gelatin-coated plates (Appendix Fig 1).

Human iPSC-Derived Epithelial Progenitors

A basal media of Roswell Park Memorial Institute 1640 with Glutamax (Life Technologies) plus 2% B27 serum supplement minus insulin (Invitrogen, Carlsbad, CA) was supplemented to mimic three developmental stages: (1) induction of definitive endoderm with 50 ng/mL activin-A (PeproTech) for 4 days, (2) anteriorization of endoderm with 1μM A8301 (Tocris Bioscience, Bristol, United Kingdom) for 4 days, and (3) ventralization with 10 ng/mL BMP-4 (PeproTech), 100 ng/mL FGF-2 (PeproTech), and 100nM CHIR 99021 (Stemgent, Cambridge, MA) for 4 to 7 days.

Histology and Immunofluorescence

Paraformaldehyde-fixed, paraffin-embedded tissue sections were stained by hematoxylin and eosin or underwent antigen retrieval and permeabilization with 0.1% Triton-X100. Cultured cells were fixed with 100% methanol.

Immunofluorescent staining included (1) blocking by 5% donkey serum, (2) primary antibodies (1:200) overnight at 4°C, (3) washing with 0.1% Tween-20, (4) secondary antibodies (1:400; AlexFluor 488 or 594, Life Technologies) for 1 hour at room temperature, and (5) counterstaining with 4′,6-diamidino-2-phenylindole for 5 minutes. Images were captured with a Ti-PFS inverted microscope (Nikon, Melville, NY).

Quantitative Real-Time Polymerase Chain Reaction

Samples were stored in RNALater (Qiagen, Valencia, CA) until RNA isolation by RNeasy-Plus Mini Kit (Qiagen). RNA was transcribed to complementary DNA by SuperScript III Reverse Transcriptase (Life Technologies). Gene expression was quantified by Taqman assays using the
OneStepPlus system (Applied Biosystems, Foster City, CA). Gene expression was analyzed by the ΔΔCt method with normalization to 18S gene expression.

Lung Tissue Slice Experiments
Decellularized human lung tissue was fixed in 30% sucrose, embedded in optimum cutting temperature compound, cut at 100 μm, and immobilized on tissue culture plates. Slices were washed with 100% and 95% ethanol and then air-dried for 30 minutes. Sections were incubated overnight in phosphate-buffered saline to remove residual detergents and then preconditioned with media for 3 hours before seeding.

On day 2 of ventralization, cells were reseeded directly to lung slices and cultured for 5 days. For cell localization, images at original magnification ×10 were captured on 6 separate cell-matrix cultures. ImageJ software (National Institutes of Health, Bethesda, MD) was used to quantify cells positive for 4',6-diamidino-2-phenylindole localized on or off the matrix. Counts were normalized to total matrix area.

Decellularization, Recellularization, and Whole-Organ Culture
Animal experiments were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and performed in compliance with the Animal Welfare Act. Cadaveric rat lungs were perfusion decellularized as previously described [4]. In brief, cadaveric lungs were explanted from Sprague-Dawley rats (260 to 280 g, Charles River Laboratories International Inc, Wilmington, MA). The pulmonary artery (PA) was cannulated with an 18-gauge dispensing needle (McMaster-Carr), and perfused under constant pressure (50 cm H2O) sequentially with heparinized (10 U/mL) saline, 0.1% sodium dodecyl sulfate (2 hours), deionized water (15 minutes), and 1% Triton-X-100 (100 minutes). Scaffolds were washed with phosphate-buffered saline containing antibiotics for 72 hours (Sigma-Aldrich) to eliminate residual detergent. Removal of cells and residual DNA was routinely confirmed by hematoxylin and eosin staining and by quantitative assay (Quant-iT kit, Invitrogen) [3].

Decellularized lungs were intubated with 16-gauge intratracheal cannulae and placed in a custom bioreactor facilitating continuous perfusion through the PA (2 mL/min). Human iPSC-derived endothelial cells were delivered to the PA (15 to 20 × 10⁶) by syringe in a 10-mL volume at 3 to 5 mL/min. At day 10, a confluent 10-cm plate of ventralized iPSC-derived epithelial progenitor cells was collected and delivered to the Airways by gravity in a 30-mL volume.

Orthotopic Lung Transplantation
Recipient Sprague-Dawley rats (260 to 300 g, Charles River) were anesthetized with 5% isoflurane (Abbott, Abbott Park, IL), intubated with a 16-gauge endotracheal tube (Becton-Dickinson, San Jose, CA), and ventilated with a rodent ventilator (Harvard Apparatus, Holliston MA) supplying 100% supplemental oxygen. Regenerated lungs (n = 5) were removed from the bioreactor, and the left lung was prepared for transplantation using a modified nonsuture external cuff technique [26]. In brief, the donor hilar vessels were isolated and transected to mount cuffs prepared from Venisystems Abbocaths (Hospira, Lake Forrest, IL). Arterial and venous vessels were secured with 8-0 silk to the 18-gauge cuff, and the bronchus was secured to a 16-gauge cuff.

A left anterior thoracotomy was performed on the nonheparinized recipient rats. The hilar vessels were dissected circumferentially and individually clamped with micro-Serreclens clamps. Donor vessels and cuffs were inserted into the recipient vessels and secured with 6-0 silk.

Blood gases were analyzed at 20 and 60 minutes after left lung reperfusion. Samples were drawn from the left pulmonary vein at the anastomosis and analyzed using an iStat Portable Analyzer with CG4+ Cartridges (Abbott). Lungs were then explanted and fixed in 10% formalin or stored in RNAlater.

Results
Human fibroblast–derived iPSCs were differentiated in vitro toward a lung epithelial progenitor phenotype through defined developmental stages (Fig 1A). First, activin A treatment generated definitive endoderm at day 4, as confirmed by loss of OCT4 and induction of SOX17 and FOXA2 (Fig 1B, C). Next, inhibition of TGF-β signalling for 4 days facilitated anteriorization to a foregut fate, indicated by SOX2 upregulation (Fig 1D, E). Stimulation with FGF-2 and BMP-4, in combination with glycogen synthase kinase-3 pathway inhibition, successfully ventralized the anteriorized population toward a lung epithelial progenitor phenotype. Approximately 40% to 60% of cells expressed Nkx2.1, the marker of lung specification, after 12 days of differentiation (Fig 1F); corresponding to an eightfold to 21-fold increase in messenger RNA expression over that of undifferentiated iPSCs (Fig 1G).

Next, ventralized iPSCs (day 10) were seeded onto human decellularized lung slices and maintained in ventralizing media for 5 days. More seeded cells were found localized to the matrix slice than on tissue culture plastic (Fig 2A). Cell viability (>90%) was observed (Fig 2B), and maintenance of a proliferative capacity confirmed by Ki67 expression (Fig 2C). Expression of E-cadherin demonstrated cell–cell interaction and predominance of an epithelial phenotype (Fig 2D). Lung progenitor phenotype was confirmed by Nkx2.1 expression in a subset of cells (Fig 2E). Loss of OCT4 and maintenance of FOXA2 expression was confirmed for the ventralized cell population on day 5 of cell-matrix culture. In addition, expression of mature alveolar epithelial cell markers TFF3 and mucin-1 was detected along with the ciliated cell marker FOXJ1 (Fig 2F). Compared with controls, an increase in Nkx2.1 expression was measured in cell-matrix cocultures (Fig 2G).

Whole-lung constructs were prepared for transplantation by first recellularizing the vasculature with
Fig 1. Differentiation of human induced pluripotent stem cells (iPSCs) to lung epithelial progenitors. (A) In vitro differentiation protocol (BMP = bone morphogenic protein; Nkx2.1 = NK2 homeobox 1; RiPSC = RNA induced iPSCs; RPMI = Roswell Park Memorial Institute; TGF-β = transforming growth factor β.) (B) Generation of definitive endoderm indicated by loss of octamer-binding transcription factor 4 (OCT4; upper right), SRY (Sex Determining Region Y)-Box 17 (SOX17; lower right), and forkhead box protein A2 (FOXA2; upper left), and dual-immunofluorescence indicating single-cell coexpression (yellow, upper left). (DAPI = 4′,6-diamidino-2-phenylindole.) (C) Quantitative polymerase chain reaction (qPCR) analysis on day 4 of differentiation relative to undifferentiated day 0 cells. (D) Anteriorized endoderm population indicated by SOX2 expression on day 8 of differentiation by immunofluorescent staining (red) and (E) qPCR analysis relative to day 0 (light gray) and day 4 (dark gray) (DE = definitive endoderm.) (F) Differentiation to a lung epithelial progenitor population on day 12 indicated by nuclear NK2 homeobox 1 (Nkx2.1) expression (red), and (G) qPCR expressed as fold-increase from day 0 undifferentiated population (n = 3 separate experiments). Gene expression all normalized to 18S expression by ∆∆Ct (n = 3 samples/differentiation in duplicate). The error bars represent the standard deviation. Scale bars = 100 μm.
human iPSC-derived endothelium (Appendix Fig 1). Cells were delivered by the PA and cultured for 3 days under constant perfusion with endothelial media. Next, ventralized (day 10) iPSC were delivered to the airways. Biomimetic culture with ventralizing media containing vascular endothelial growth factor was maintained for 2 or 5 days (Fig 3A, B). After culture, heterogeneous cell distribution was observed within the scaffold, with some areas remaining relatively acellular and others displaying notable cell attachment and retention (Fig 3C). Seeding efficiency was approximately 40% to 50% scaffold coverage, based on histologic assessment. Cell proliferation within whole-lung cultures was confirmed. Conservation of the epithelial progenitor phenotype was demonstrated by nuclear Nkx2.1 expression and the endothelial phenotype confirmed by cluster of differentiation (CD) 31 expression (Fig 3D).

Further specification toward mature lung phenotypes was noted by expression of the alveolar type I pneumocyte marker T1α/podoplanin and Clara cell secretory protein/CC10, thereby indicating an upper airway phenotype (Fig 3E). Quantification of Nkx2.1 revealed increased expression in cells cultured on whole-lung scaffolds (Fig 3F). For recellularized lungs cultured for 2 days in the bioreactor, an average increase in Nkx2.1...
expression of $5.8 \pm 2.8$-fold was measured compared with cells maintained in vitro. For lungs cultured for 5 days, the average expression increased $14.2 \pm 9.2$-fold ($n = 3$ lungs/time point).

After bioreactor culture, single orthotopic left lung transplant was performed ($n = 5$, Fig 4A, B). After reperfusion, blood gases were sampled from the left pulmonary vein just proximal to the anastomosis, representing a mixed venous sample. Samples demonstrated adequate ventilation in the presence of the transplanted graft as indicated by a partial pressure of carbon dioxide maintained within normal limits over the study period; observed partial pressure of oxygen values were compatible with animal survival (Fig 4B). Histologic analysis of the explanted lungs showed red cell perfusion throughout the alveolar capillary network, with occasional alveolar hemorrhage (Fig 4C).

**Comment**

There is continued need for alternate and novel therapies for patients with end-stage lung disease. Although advances in organ preservation, recipient management, and immunosuppression represent significant therapeutic advancements, lung transplantation remains the only effective treatment option [1, 27]. Progress in donor management and organ reconditioning have increased the usable fraction of the donor pool to nearly 50% [28], yet half of all donor lungs are not transplanted, often for logistical reasons.
Decellularization and regeneration would impart new value to these organs from otherwise healthy donors with a structurally undamaged matrix.

Stem cell–based therapies for lung repair is an area of much research involving endogenous and exogenous mechanisms of cellular action. However, effective engraftment or direct enhancement of stem cell populations or their derivatives has not yet been successful in patients. As directed stem cell differentiation toward mature lung phenotype advances, the ability to therapeutically engraft stem cell–derived epithelial cell populations into diseased lungs remains a challenge. We propose that acellular scaffolds can optimally accommodate delivered cells and support the requisition of function. This goal presents many challenges, including the generation of sufficient cell numbers for regenerating clinical-scale organs and spatially delivering them to the regionally distinct proximal and distal lung. For these reasons, we hypothesize that recellularization with multipotent progenitor cells at the stage of lung specification would facilitate ex vivo expansion and localized differentiation after scaffold seeding. The Nkx2.1-expressing population can then be directed toward mature epithelial cell phenotypes by exogenous growth factors and intrinsic matrix-derived cues. We demonstrate that induction of the Nkx2.1-expressing progenitor population is enhanced in cell–matrix culture compared with differentiation in traditional culture.
Advancement toward a transplantable, fully regenerated organ would likely require seeding of purified progenitor cells or improving in situ control of cell fate to exclude off-target lineages. Gene modification of the source iPSCs may be required for lineage selection, which has been reported for embryonic stem cell lines [32]. Yet, these modifications also present the risk of introducing additional genetic mutations, and alternate techniques may be required.

In the advancing field of organ engineering, a requirement for small-scale models remains. The high-throughput matrix slice model and rodent-scale whole-organ scaffolds allow for testing of specific cell populations, variable seeding techniques, and biomimetic culture conditions in a reproducible fashion. Matrix reseeding efficacy at various stages of differentiation can also be tested to assess expansion requirements. An additional benefit of whole-organ seeding is the recapitulation of endothelial and epithelial cross-talk across their respective physiologic niches—a critical phenomenon in lung development [33, 34] and repair [35]. The efficient differentiation of human iPSCs toward lung cell populations is also beneficial for patient-specific disease modelling [36], facilitating the study of cell biology and the testing of therapeutics in many diseases that currently lack strong animal models.

Successful transplantation and reperfusion of the dual epithelial and endothelial recellularized construct is an important proof of principle. Although these experimental results do not directly reflect normal lung graft physiology, they provide foundational evidence to support the aim of clinical application. After ex vivo cell delivery and culture, an “in vivo culture” period may be required for further regeneration. Complete recapitulation of the cellular components of the airways and alveoli will likely be aided by the recipient’s endogenous environment and repair program [37]. At minimum, the implanted construct must permit adequate perfusion, with or without ventilation, but could otherwise be “cultured” in vivo for an extended period before the reacquisition of full function. These concepts outline the critical next steps in the development of a regenerated, transplantable lung for therapeutic use.

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References

DISCUSSION

DR THOMAS K. WADDELL (Toronto, Ontario, Canada): So two questions, one about proliferation and one about differentiation. You said that the cells are proliferating, but do you actually have quantitative data about what fold-expansion occurred in the slices or in the ex vivo perfused organs? And 1B, how confident are you that they will stop proliferating when they need to when you have got full recellularization?

The differentiation question is about what you showed us that they express, all the good things. You did not show us, I think, very much proof that they did not express pancreas markers, for example, or thyroid markers or some of the wrong turns on the developmental pathway that they might be very prone to.

DR GILPIN: In terms of proliferation, we have Ki67 staining to demonstrate proliferation in vitro, on slices, and on whole scaffolds. We're in the process of 5-bromo-2-deoxyuridine (BrdU) labeling in the cultures to get a time course of proliferative capacity throughout differentiation. We also have basic cell counts at different time points in cell expansion prior to recellularization that also indicate proliferation.

Whether they would stop proliferating? I cannot definitively say when they would stop proliferating. I assume, or I hope, that as they reach their committed, mature phenotype, they would become more quiescent. But that is really an assumption and not something that I can say will happen for sure. There may be overproliferation within the scaffold, and that might be an issue that we will need to address.

In terms of off-target differentiation, that is an absolutely correct statement. I showed you the thyroid transcription factor-1 (TTF-1) induction. It is not 100%. It is a mixed population. It is a heterogeneous population. The endothelial differentiation is not 100%. It is a mixed population. It is a heterogeneous population. The endothelial differentiation is much more specific, with greater than 90% endothelial phenotype. The percentage of TTF-1 expression would be 40% to 60% on average, so the rest of that is everything else. And I would not argue otherwise.

The goals at this point were to assess whether these cells were capable of whole-scaffold recellularization. Moving forward, I think we are going to have to engineer a selection process to have a pure TTF-1 population for recellularization if we are going to exclude any off-target differentiation, but that is not what we have done here.

DR SAVERIO LA FRANCESCA (Houston, TX): Very interesting data. Just as a surgeon, I am trying to wrap my head around your lung transplant model because I am not quite sure that at the capillary level you still have an intact endothelium. You cannot put a lung back in unless you have that, and that, of course, is sort of the Holy Grail of the whole recell process.

Another point is that you also in your paper showed oxygenation, but you really should do arterial blood gas measurements from the left cuff, because if you get a mixed sample, it is kind of nice, but it really does not tell you that the recell lung, in this case, the left, is really oxygenating, which I do not think it is. So it is kind of premature. It is certainly the right direction, and the work is interesting, but just kind of a word of caution, if you will.

DR GILPIN: Yes. I agree that the mixed blood sample is not fully representative of the regenerated organ function, but really, what we wanted to know is whether we can move towards keeping these animals alive, so systemically, if they are being oxygenated to a sufficient level. That was really the goal of sampling the mixed population.

But I agree. By occluding the other vein and sampling only the regenerated organ, I do not think we would get adequate oxygenation. It is not a fully regenerated organ, and that is not the statement I am trying to make. It is a work in progress, and I think the body is the place that regeneration is going to work best—in vivo regeneration—and one of our goals of the transplant model was to demonstrate that this would be possible.

In terms of construct perfusability, we have scanning electron microscopy and transmission electron microscopy to show the vasculature network of the scaffold is indeed intact. We have also performed perfusion-leak assays to demonstrate a lack of damage down to the capillary level. Using other cell types, we have also previously published the extended survival after transplantation of regenerated lung constructs (Song JF, Ann Thorac Surg, 2011), further demonstrating the feasibility of this approach.