Embryonic and Induced Pluripotent Stem Cells for Lung Regeneration

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Abstract

The discovery of embryonic and induced pluripotent stem cells (ESCs and iPSCs) has ushered in an exciting new era of regenerative medicine. Human pluripotent stem cells can be "directed" *in vitro* toward lung epithelium by applying specific stepwise combinations of growth factors that recapitulate the molecular mechanisms of respiratory development in animal models. In a relatively short time, there has been significant progress in deriving lung epithelium from ESCs/iPSCs. These directed differentiation protocols include high concentrations of activin A to induce definitive endoderm followed by dual inhibition of bone morphogenic protein and TGF-β signaling pathways to produce anterior foregut endoderm. Subsequent stimulation of Wnt, bone morphogenic protein, and fibroblast growth factor signaling leads to lung epithelial lineage specification, identified by the expression of Nkx2.1. These cells subsequently express other markers of the developing lung and a variety of lung epithelial subtypes. The major limitation in the field currently is deriving and characterizing mature, functional lung epithelium. The generation of iPSCs is now well established, and researchers have generated iPSCs from patients with acquired and inherited lung diseases. This platform offers unparalleled opportunities to model lung development and disease using human cells.

Keywords: embryonic stem cells; induced pluripotent stem cells; lung regeneration; lung development

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Pluripotency is the ability of a cell to differentiate into the various cell lineages of the three primary germ layers. Cells of the inner cell mass within the blastocyst embryo are the best studied pluripotent cells in mammals, and their potential to give rise to all cells of the developing organism, including all cells of the lung, has captured the interest of lung researchers for many decades. In 1981 investigators published methods for the stable capture in culture of these pluripotent cells (1, 2), which were able to self-renew *in vitro*, or differentiate on exposure to various culture media. These specially engineered cells were named embryonic stem cells (ESCs), the gold standard pluripotent stem cell population.

In 2004 our group and several others began our shared missions of trying to generate lung epithelial lineages from ESCs *in vitro*. Because mouse ESCs resembled the phenotype of the inner cell mass in the embryo, we elected to pursue a strategy termed "directed differentiation," described by Gordon Keller and others (3–6), whereby the investigator uses a series of growth factor–supplemented media with the goal of replicating *in vitro* the sequence of developmental stages known to occur *in vivo* in the developing embryo. Because the entire lung epithelium is known to originate from the anterior foregut endodermal gut tube, the first step was optimization of the differentiation of ESCs into definitive endoderm through the induction of Nodal signaling, using Activin A–supplemented medium (Figure 1), a method discovered by Kubo and colleagues (5). During the course of these studies, Shinya Yamanaka's Nobel Prize–winning discovery was published, describing how to induce pluripotency in somatic cells via the overexpression of Oct4, Klf4, Sox2, and cMyc (7). The reprogrammed cells, induced pluripotent stem cells (iPSCs), are remarkably similar to ESCs (8). We compared ESCs with iPSCs in terms of their functional potential to form definitive endoderm *in vitro* and found that Nodal signaling induced functionally similar definitive endoderm from either ESCs or iPSCs, despite very subtle differences in the global transcriptomes of the endodermal cells derived from these two types of pluripotent stem cells (PSCs) (8, 9). To this day we and others have continued to find that the same recipes that differentiate ESCs into endodermal lineages work similarly to differentiate iPSCs.

Having demonstrated the efficient and reproducible derivation of definitive

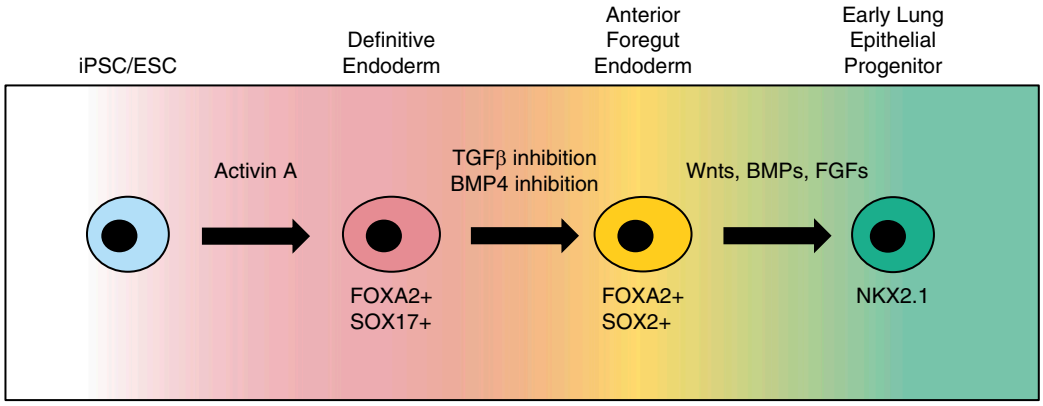

Figure 1. Schematic of the directed differentiation of induced pluripotent stem cells (iPSCs)/embryonic stem cells (ESCs) to NKX2.1 lung progenitors. A high, sustained concentration of Activin A induces definitive endoderm (FOXA2+/SOX17+). Subsequent transforming growth factor (TGF)-β and bone morphogenic protein (BMP) inhibition suppresses CDX2, maintains FOXA2, and up-regulates SOX2 expression, reminiscent of anterior foregut endoderm. Exposure to Wnts, BMPs, and fibroblast growth factors (FGFs) leads to induction of NKX2.1.

endoderm from ESCs or iPSCs, the next goal was developing medium recipes that would induce the transcription factor, Nkx2.1, within endoderm (Figure 1) (10). Nkx2.1 is a master regulator of lung development, and all lung epithelia are thought to derive via an Nkx2.1^{+} endodermal progenitor. Nkx2.1 is also the first known gene marker within endoderm that distinguishes cells fated to be lung, a fate decision referred to as "lung lineage specification." Green and colleagues found that dual inhibition of bone morphogenic protein (BMP) and transforming growth factor (TGF)-β signaling pathways produced anterior foregut endoderm from human ESCs and iPSCs, providing cells that were competent to specify to a lung cell fate (11). Our group engineered an Nkx2.1^{GFP} knock-in reporter mouse and ESC line, and used this reagent to optimize a protocol able to generate 160 Nkx2.1^{+} endodermal progenitors in 14 days from a single starting ESC (10). Rajagopal and colleagues simultaneously published a similar protocol for the derivation of Nkx2.1^{+} lung epithelia from both mouse and human pluripotent stem cells (12).

Purified Nkx2.1^{+} endodermal progenitors derived from ESCs or iPSCs have the capacity to undergo further directed differentiation into cells expressing transcripts characteristic of thyroid epithelial cells as well as the various proximal and distal lung lineages, including the distal markers Sox9, surfactant protein C (SPC) and SPB, as well as the proximal markers Sox2, CC10, p63, and Foxj1 (10, 12–14). A major hurdle now facing researchers in our field is increasing the efficiency of the proximal and distal patterning of these cells and clarifying what degree of maturation, if any, is occurring in the cells being produced. Given the experience of investigators who have optimized the differentiation protocols for producing ectodermal and mesodermal lineages from ESCs or iPSCs, using the developing embryo as a roadmap for how to derive mature lung lineages from ESC/iPSC precursors will likely hold the key to success (15). Unfortunately, little is known about the constellation of signaling pathways that regulate proximal and distal lung epithelial maturation during development; hence intense study of lung development will be necessary to reveal the growth factors and signaling pathways that are likely to be required for producing mature bona fide lung epithelial cells *in vitro* from stem cells (10–12). Even in the current early state of our field of research, investigators have convincingly demonstrated the derivation from human ESCs and iPSCs of cells expressing markers indicative of a variety of lung epithelial subtypes (14).

A particular challenge is the characterization of these *in vitro* stem cell–derived candidate lung lineages, given that most established definitions of lung epithelia are based on their *in vivo* structure and function, beyond mere expression of mRNA or protein marker genes (15). Without an available orthotopic/*in situ* grafting assay a major hurdle is to test and compare the regenerative capacity of ESC/iPSC-derived putative lung progenitors versus their *in vivo* counterparts. Because structure and function are intimately linked in the lung, it may be useful to try to engineer three-dimensional structures reminiscent of *in vivo* lung tissue if we are to properly interrogate and characterize the newly engineered cells being derived from PSCs. Three approaches published to date to try to produce more recognizable structures or polarized epithelia from ESCs or iPSCs include the following: (*1*) using purified Nkx2.1^{+} endodermal progenitors to reseed decellularized rodent lung constructs (10), (*2*) implantation of heterogeneous mixtures of endodermal and nonendodermal differentiating PSCs into the subdermal or kidney capsule compartments of immunodeficient mice to allow spontaneous sphere or lumen formation of differentiating cells (12, 14), and (*3*) air–liquid interface cultures to stimulate ciliation, polarization, and barrier function of the candidate airway epithelia being produced (16, 17).

Despite the challenges described above, the application of iPSC technology has been a seminal step for lung researchers, because it has provided an unlimited supply of patient-specific cells from individuals with acquired and genetic lung disease (18). In 2010 pluripotent reprogramming was used to derive the first 100 lung disease–specific iPSC lines including lines from individuals with cystic fibrosis and α_1-antitrypsin deficiency, the two most common monogenic lung diseases (19). Using patient-specific and disease-specific iPSCs, fundamental disease biology has been recapitulated *in vitro* by differentiating iPSCs to the cell type of interest. Important examples include the

characteristic intracellular accumulation of misfolded, mutant α_1-antitrypsin protein in iPSC-derived hepatocytes (20, 21), aberrant intracellular trafficking of mutant cystic fibrosis transmembrane conductance regulator (CFTR) (16) in iPSC-derived ciliated cells from patients with cystic fibrosis, and impaired granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling and GM-CSF–dependent gene expression in iPSC-derived macrophages from patients with hereditary pulmonary alveolar proteinosis (22, 23). Researchers yearn for better, more accessible and human models to further our understanding of lung disease. iPSCs should provide novel opportunities to model the person-to-person differences of human lung disease, identify and screen drugs for efficacy or toxicity *in vitro*, and enable the development of exciting personalized therapeutic approaches.

Generating cells or even whole organs for orthotopic transplantation is one of the most ambitious, albeit futuristic, applications of iPSCs. Solid organ transplantation is limited by both organ availability and the complications that arise from either immune rejection or the immunosuppression required to attenuate rejection. iPSCs represent an inexhaustible supply of cells genetically identical to the patient donor (18). An autologous and transplantable human lung engineered entirely from patient-specific iPSCs, with the full repertoire of cell types and three-dimensional architecture to allow normal lung function, remains years if not decades away. Despite this, important proof-of-concept studies have demonstrated the feasibility of transplanting a bioartificial lung generated by reseeding a decellularized native rat lung scaffold with primary rodent cells, and the engineering of alveolar epithelium from pluripotent stem cells (10, 24, 25). In the case of monogenic lung diseases, technologies including zinc finger nucleases (ZFNs), transcription activator–like effector nucleases (TALENs), and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas have ushered in a new era of precise genome editing, making possible the correction of genetic mutations responsible for inherited lung diseases (26, 27). For example, ZFNs were designed to target and correct the mutant Z α_1-antitrypsin allele in iPSCs from an individual with α_1-antitrypsin deficiency (21). The gene-corrected iPSCs produced normal α_1-antitrypsin protein after differentiation into human hepatocyte-like cells and demonstrated secretion of human α_1-antitrypsin *in vivo* after transplantation into mice.

What are the hurdles facing the broad application of iPSC technology to lung disease–related research? The routine generation of iPSCs from individuals and the use of gene-editing techniques in these cells are now well established and no longer a limiting factor. The current focus is on the challenging task of generating the full repertoire of functional epithelial, endothelial, and interstitial cells that compose the lung. This step is crucial to leveraging this platform to model disease, screen and test drug therapies, and eventually consider *in vivo* therapy. In this era of hype and "stem cell clinics," researchers (and their funders) would do well to avoid overexaggerating prior accomplishments achieved with these cells to prevent the potentially harmful repercussions of premature clinical translation of iPSC-derived therapies. Instead, we should continue to promote the rigorous and reproducible experimentation required to gain a detailed understanding of the hurdles we must overcome before treating patients with iPSC-derived cells (18). ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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