COMMENTARY

Induction of multiciliated cells from induced pluripotent stem cells

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The differentiation of induced pluripotent stem cells (iPSCs) to mature cell types—and ultimately to functional tissues and organs—holds great promise for personalized disease modeling, drug screening, and the development of cell-based therapies (1). Since Shinya Yamanaka described this Nobel Prize winning technology in 2006, there have been a number of advances in the field, especially in the differentiation of neural, cardiac and hepatic cell types (1). However, the differentiation of iPSCs and embryonic stem cells (ESCs) to lung cell types has lagged somewhat, and this is largely because the lungs are complex structures with many different cell types (2). Another challenge for the field is the differentiation of iPSCs to cells that are functionally competent. Anatomically and functionally, the lungs consist of distinct regions: the proximal airways that function in mucociliary clearance for host defense, the conducting airways, and the distal alveoli for gas exchange. There have been a series of papers published over the last 3 y (detailed below) that have developed protocols for iPSC and ESC differentiation to several lung cell types. In PNAS, Firth et al. (3) present another advance in the generation of proximal airway epithelium from iPSCs. Using the air-liquid interface (ALI) culture system, the authors demonstrate that putative lung progenitors obtained from iPSC-directed differentiation respond to inhibition of Notch signaling by promoting ciliogenesis and express the functional cystic fibrosis transmembrane regulator (CFTR) chloride channel. Studies in differentiation of iPSCs to functional airway epithelium are critical because the field holds great promise for developing personalized therapies for patients with respiratory diseases.

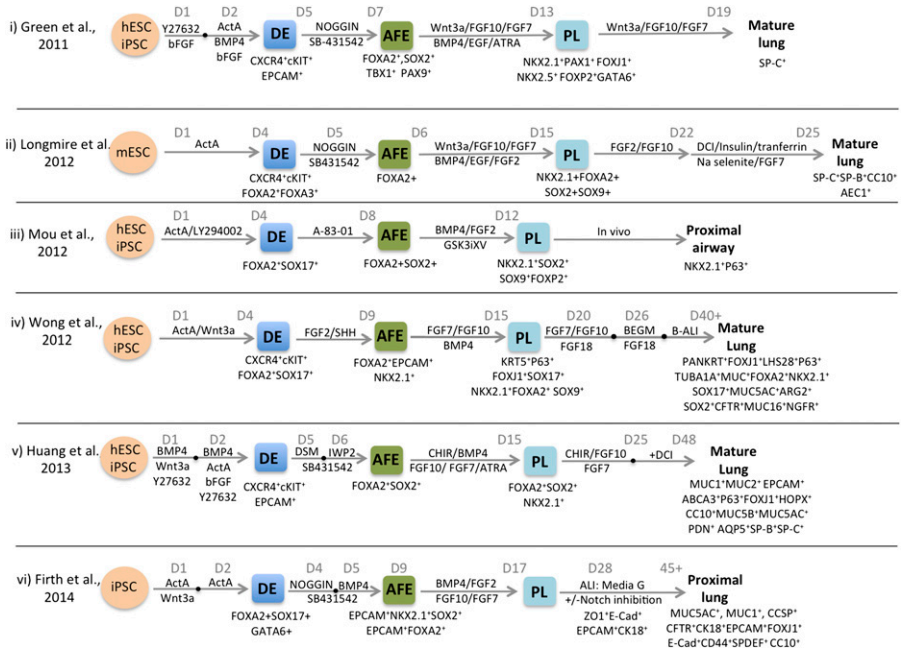

Fig. 1. (*i–vi*) Schematic of published protocols (3–8) for directed differentiation to lung epithelium iPSCs and ESCs in vitro. ActA, Activin A; DSM, Dorsomorphin; PL, progenitor lung.

Directed differentiation of human ESC and iPSC, along known lung developmental pathways, was first described by Green et al. (4) to transition definitive endoderm (DE) to anterior foregut epithelium (AFE) and then to lung progenitor cells. Activin A was used to induce DE, which was then exposed to dual transforming growth factor beta (TGF-β) and bone morphogenic protein (BMP) inhibition to generate AFE characterized by high levels of SRY-related HMG-box 2 (SOX2), maintenance of forkhead box A2 (FOXA2) expression, and low CDX2, high PAX9, and high TBX1 levels (Fig. 1*i*). Nk2 homeobox 1 (NKX2.1) is the earliest marker that has been identified to date that suggests differentiation of AFE to a lung cell fate. Subsequent treatment of the AFE stage cultures with WNT3a, keratinocyte growth factor (KGF), fibroblast growth factor (FGF)10, BMP4, and EGF (WFKBE), together with all-transretinoic acid, resulted in presumed ventralization of the epithelium with expression of NKX2.1, NKX2.5, and PAX1, suggesting that the epithelium had acquired a lung cell fate. However, the frequency of $NKX2.1^+$ $FOXA2^+$ cells was low and specific mature markers of airway epithelium were scarce.

A follow-up study by Longmire et al. (5) used Nkx2.1-GFP reporter mouse ESCs for purification of $Nkx2.1^+$ cell populations derived from AFE. Mouse ESCs recapitulated the Green et al. (4) human ESC studies and differentiated into AFE with the WFKBE mixture, although FGF2 was also required (Fig. 1*ii*). In the day 15-sorted $Nkx2.1^+$ AFE cells that were replated and cultured for 9 d, there was sporadic expression of proximal and distal lung cell markers. Differentiation to cells with thyroid markers was also seen. Treatment at day 22 of the cultures with dexamethasone, 8-bromo-cAMP, and isobutylmethylxanthine (DCI), and KGF, transferrin, and sodium selenite improved distal differentiation with increased expression of surfactant proteins (SP) and Clara cell 10kD protein. Instillation of day 15-sorted

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cells into decellularized mouse lungs resulted in sporadic expression of markers found in type I alveolar epithelial cells. Day 15-unsorted cells only resulted in some ciliated cells seen by microscopy. The transcriptome from sorted Nkx2.1 GFP^+ cells from these differentiated cultures was found to have some overlap with the transcriptome from developing mouse lung.

Dual TGF-β and BMP4 inhibition was again seen to promote the generation of AFE from DE with the greatest likelihood of developing lung progenitors and suppressing neural progenitors, in an article from Mou et al. (6) (Fig. 1*iii*). However, a very low percentage of proximal ($Nkx2.1^+Sox2^+$) and distal ($Nkx2.1^+Sox9^+$ or $FoxP2^+$) lung progenitors were seen with these conditions. To generate proximal epithelium KGF, BMP7, Wnt inhibition and MAPK/ERK inhibition was used and sporadic $p63^+Nkx2.1^+$ proximal airway epithelial cells were seen in vivo. This group also generated iPSCs derived from cystic fibrosis (CF) patients. The efficiency of $Nkx2.1^+$ cells here was only 10–30%, and again with only sporadic $P63^+/NKX2.1^+$ cells.

An alternative strategy for generating AFE was described by Wong et al. (7) by exposing DE to sonic hedgehog (SHH) and FGF2 to induce AFE (Fig. 1*iv*). The authors reported 78% of the cells in the culture expressed NKX2.1. They also found markers of other endodermal derived organs. However, the high efficiency of $NKX2.1^+$ cells could not be replicated by Huang et al. (8). Wong et al. (7) then added FGF10, KGF, FGF18, and a low concentration of BMP4, followed by ALI culture for proximal lung differentiation (Fig. 1*iv*). The authors found up-regulation of gene and protein expression for basal, ciliated, and mucus cells, but not Clara cells. No distal lung cell markers were detected. Functional expression of the CFTR was found in some iPSC lines, and CF patient iPSC-derived epithelial cells were treated with CF corrector compounds, and there was a trend to improved CFTR function.

Another advance for the field came with the refinement of the AFE induction protocol by sequential TGF-β and BMP signaling inhibition, followed by Wnt inhibition to recapitulate anteriorization and ventralization developmental steps (8) (Fig. 1*v*). This process resulted in increased efficiency of lung field induction with 86% $FOXA2^+NKX2.1^+$ cells. Continued differentiation of the day 25 lung epithelial progenitors with DCI until day 48 resulted in more than 50% of the cells expressing SPB with <5% SPC expression. Dissociated human fetal lung cultured under the same conditions had similar expression patterns to these cultured iPSCs, suggesting that this differentiation protocol produces lung epithelial cells that more closely resemble fetal rather than adult lung. Electron microscopy demonstrated lamellar bodies and functionally the cells were shown to take up surfactant protein.

Firth et al. present another advance in the generation of proximal airway epithelium from iPSCs.

Fig. 1 provides an overview of some protocols for differentiating AFE and lung progenitors, but this is not an exhaustive review of the literature. There are some consistent themes across the protocols that suggest key pathways in the DE and AFE differentiation processes, and there is the development of new protocols that more highly efficiently generate NKX2.1-expressing iPSCs. However, reproducibility of the NKX2.1-expressing cell efficiency between laboratories and different cell lines still remains an issue. Fig. 1 also describes the cell markers found under different differentiation conditions and at different time points in the directed differentiation process. Here again, a large variation is seen in the cell types identified, and functional studies of cells have so far only been performed for CFTR and SP uptake, with most differentiated cells being closer to fetal rather than adult cells.

The PNAS Firth et al. (3) article provides an advance for the field with the differentiation to a mature ciliated epithelium. The authors used similar directed differentiation steps to Green et al. (4) (Fig. 1*vi*), followed by ALI cultures where the cells are cultured with the basal surface in contact with the medium and the apical surface exposed to the air. This process produced a polarized epithelium with a basal layer of cells that expressed mesenchymal markers and appeared essential for differentiation.

There are two types of cilia: motile and primary cilia (9, 10). Airway epithelial cells that are destined to develop motile cilia first generate primary cilia, and therefore the presence of primary cilia represents an early stage of multiciliated cells. Foxj1 expression is not required for primary cilia formation, but is turned on as motile cilia develop (9). The iPSC-derived epithelium described by Firth et al. (3) demonstrates acetylated α-tubulin expression on the apical surface of the cells, and is suggestive of primary cilia. Notch pathway inhibition is associated with enhanced ciliogenesis during lung development and repair (11, 12), and the differentiating iPSC were responsive to inhibition of Notch signaling with an increase in microtubular structures on the apical epithelium. Electron microscopy is needed to determine whether these are motile or primary cilia, and video microscopy studies are needed to assess whether these cilia beat normally. Functional CFTR expression on the ciliated cells also suggests maturation of this iPSC-derived epithelium. The differentiation of functional airway epithelium is critical for developing patient-specific, iPSC-derived proximal airway epithelial cell cultures for disease modeling, drug discovery, gene correction, and cell/tissue-based therapies, and the study by Firth et al. (3) provides some clues as to how this may be achieved in the future.

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