Derivation of Patient-Specific Alveolar Type II Cells from Surfactant Protein-B Deficient Induced Pluripotent Stem Cells

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Surfactant protein B (SFTPB) deficiency is a fatal disease affecting newborn babies. A mutation in the surfactant protein B gene results in the inability to reduce surface tension and ultimately, death. This thesis shows that induced pluripotent stem (iPS) cells derived from patient specific SFTPB deficient fibroblasts can be differentiated into alveolar type II cells using a variety of methods including 2D and 3D culture. The iPS cells are also a target for gene therapy with a lentivirus vector containing the wild type sequence of the SFTPB gene, inserted into both wild type and SFTPB deficient iPS cells. These cells were differentiated into lamellar body containing, surfactant expressing alveolar type II cells and the transfected SFTPB deficient cells showed gene and protein expression of surfactant protein B. These findings suggest that a lethal disease can be targeted and reversed, the first step towards a potential cure for a vulnerable population.
Acknowledgements

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I would like to acknowledge Daochun Luo for creating the lentiviral vector and infecting my various cell lines with the wild type SFTPB sequence, Irene Tseu for taking care of my stem cells when I was on maternity leave, Jinxia Wang for helping me perform dozens of rounds of RT-PCR, Behzad Yeganeh for his Western blots of my samples and Sheri Shojaie for teaching me valuable skills such as flow cytometry, 3D tissue embedding and staining and all of the social parties she planned. I would also like to thank Drs. F.S. Cole and Aaron Hamvas for providing me with the fibroblasts from a patient with the SFTPB deficiency.

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<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
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<td>AA</td>
<td>amino acid</td>
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<tr>
<td>ABCA3</td>
<td>ATP-binding cassette transporter A3</td>
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<td>ACTB</td>
<td>actin</td>
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<tr>
<td>AFE</td>
<td>anterior foregut endoderm</td>
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<td>AM</td>
<td>alveolar macrophage</td>
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<tr>
<td>AQP5</td>
<td>aquaporin 5</td>
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<td>alveolar type I</td>
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<td>adenosine triphosphate</td>
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<td>alveolar macrophage</td>
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<td>bovine serum albumin</td>
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<td>CaCl</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>CRISPR associated protein 9</td>
</tr>
<tr>
<td>CCRM</td>
<td>Center for Commercialization of Regenerative Medicine</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CDX2</td>
<td>caudal type homeobox 2</td>
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<td>CHIR</td>
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<tr>
<td>cKit</td>
<td>Mast/stem cell growth factor receptor</td>
</tr>
<tr>
<td>CL</td>
<td>chloride</td>
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<tr>
<td>cm</td>
<td>centimeter</td>
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<td>cytomegalovirus</td>
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<tr>
<td>CO2</td>
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</tr>
<tr>
<td>CPM</td>
<td>carboxypeptidase M</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
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<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
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<tr>
<td>DCI</td>
<td>dexamethasone, cAMP, isobutylmethylxanthine</td>
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<td>definitive endoderm</td>
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<td>DME</td>
<td>dulbecco’s modified eagle’s medium</td>
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<td>differentially methylated regions</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DPPC</td>
<td>dipalmitoyl phosphatidylcholine</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>FBM</td>
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<td>FBS</td>
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<td>FOXA2</td>
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<td>G</td>
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GFP  green fluorescent protein
GM-CSF  granulocyte macrophage colony-stimulating factor
GPR116  G-protein coupled receptor 116
H&E  haematoxylin and eosin
HBSS  hank’s balanced salt solution
HEPES  N-2-Hydroxyethylpiperazine-N’-2-Ethanesulfonic Acid
hESC  human embryonic stem cell
hiPSC  human induced pluripotent stem cell
HIV  human immunodeficiency virus
HMD  hyaline membrane disease
HOPX  HOP homeobox
IBMX  3-Isobutyl-1-methylxanthine
ICC  immunocytochemistry
IF  immunofluorescence
IGF  insulin-like growth factor
IMDM  Iscove's Modified Dulbecco's Media
IRES  internal ribosome entry site
ITS  insulin, human transferrin and selenous acid
K  potassium
kDA  kilodalton
KGF  keratinocyte growth factor
KLF4  Kruppel like factor 4
LB  lamellar body
MEF  mouse embryonic fibroblast feeder cell
miRNA  micro ribonucleic acid
Mm  millimeter
MVB  multivesicular body
MYC  avian myelocytomatosis viral oncogene homolog
N2  nitrogen
Na  sodium
NaB  sodium butyrate
NEAA  nonessential amino acids
NGS  normal goat serum
NKX2-1  Nkx2 homeobox 1
O2  oxygen
OCT4  octamer-binding transcription factor 4
ORF  open reading frame
P  probability of error
PBS  phosphate buffered saline
PC  phosphatidylcholine
PEG  Polyethylene glycol
PFA  paraformaldehyde
Q-PCR  quantitative polymerase chain reaction
RA  retinoic acid
RDS  respiratory distress syndrome
RNA  ribonucleic acid
<table>
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<td>rho-associated kinase</td>
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<tr>
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<td>RT</td>
<td>room temperature</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>SFM</td>
<td>serum free media</td>
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<td>SFTPA</td>
<td>surfactant protein A</td>
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<td>SFTPB</td>
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<td>TU</td>
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<td>ug</td>
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<td>ventralized anterior foregut endoderm</td>
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<td>vascular endothelial growth factor</td>
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<td>wingless type MTV integration site</td>
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<td>ROCK inhibitor</td>
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<tr>
<td>7-AAD</td>
<td>7 aminoactinomycin D</td>
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Chapter 1

Introduction
1.1 Embryonic Lung Development

The lung undergoes a long and complicated process to reach its role as an organ of gas exchange. There are 5 distinct stages in both human (see Figure 1) and mouse lung development although the final stage differs in the timing [1]. In the embryonic phase, anterior endoderm gives rise to the lung, which begins with the formation of a groove in the ventral lower pharynx, budding off to form the true lung primordium [2]. Lung progenitors emerge from anterior foregut endoderm (AFE) in the developing embryo and invade the splanchnic mesenchyme to undergo branching morphogenesis in the pseudoglandular stage. During this period, the developing epithelium begins to secrete fluid into the budding airways, which is important for the further growth of the primordial lung [3]. Branching occurs within a preprogrammed set of rules and is governed by the balance of attraction and inhibition of fibroblast growth factor (FGF) 10 and other signals from the mesenchyme [4]. The canalicular stage comprises the branching of the respiratory portion of the lung from the terminal bronchioli. These air spaces form an acinus comprising respiratory bronchioles and the alveolar ducts. In this stage, the capillaries invade the mesenchyme and begin to surround the acini. The saccular stage is defined by type I and II pneumocytes divided by primary septa from the developing capillary bed. The interstitial space or matrix becomes rich with a variety of cell types as well as collagen and elastic fibers. Bipotential alveolar progenitors in the mouse develop into ATII and alveolar type I (ATI) [5]. The final embryonic stage in humans is defined by the alveolarization of the lung. Secondary septa begin to form, and the basement membrane of the capillary endothelium and the saccular epithelium merge to form a thin barrier. A large number of small protrusions form along the primary septa, becoming larger and subdivide the sacculi into smaller subunits, the alveoli [6]. This phenomenon continues well into extra uterine life.
Pulmonary epithelial cell types and mesenchymal progenitors are established before the formation of the lung bud. [7] In murine models, the following signals have been elucidated in early and late lung development. Fibroblast growth factor (FGF), sonic hedgehog (SHH), bone morphogenetic protein (BMP) and wingless type MTV integration site (WNT) signal foregut endodermal cells to initiate and maintain branching morphogenesis. [8] The primordial lung bud expresses Nkx2 homeobox 1 (NKX2-1) which is a very important transcription factor in lung development. [9] The conducting airways are marked by sex determining region Y box 2 (SOX2) [10] while the peripheral respiratory cells express sex determining region Y box 9 (SOX9) and NKX2-1. SOX9 in mice is expressed at the distal tips of the branching epithelium in a highly dynamic manner as branching occurs and is down-regulated, concurrent with the onset of terminal differentiation of type 1 and type 2 alveolar cells. [11]
Figure 1. Schematic diagram of the complexity of the lung structure during human lung development.
1.2 Alveolar type II cells and the alveolus

As septation progressively increases the surface area of the peripheral lung in preparation for gas exchange after birth, alveolarization results in the maturation of respiratory epithelial cells into ATI and ATII cells.

ATI cells are extremely thin and cover 95% of the alveolus while comprising only 8% of the total cells in the normal adult human lung [12]. They are closely approximated to a capillary network surrounding the alveolus with only a basement membrane separating the two, facilitating gas exchange.

ATII cells are responsible for pulmonary surfactant biosynthesis, contribute to barrier function, and participate in lung repair as a progenitor population for the maintenance of alveolar integrity. [13] They only cover 3-5 % of the alveolar surface area but constitute 60% of alveolar epithelial cells. [14] Both human and murine ATII cells help keep the alveolar space free of fluid and transport sodium through well-described apical sodium channels and the basolateral sodium/potassium (NA+/K+ ATPase [15]. Another important role this cell plays is host defense. In addition to producing surfactant proteins A and D (SFTPA and SFTPD) which are opsonins and regulate inflammatory cell function, ATII cells produce cytokines and growth factors that also affect immune cells [16].

The cells synthesize, secrete and recycle all components of surfactant. This is in part regulated by the transcription factor NKX2-1, which is important in transcriptional activation of surfactant proteins (SFTPA, SFTPB, SFTPC, SFTPD) and ABCA3. [17] SFTPC is the only surfactant protein exclusively synthesized by ATII cells.

ATII cells transdifferentiate over time in vivo and in culture to ATI cells but there also exists an intermediary cell which expresses a combination of ATII and ATI cell markers, including
NKX2-1 and HOP homeobox (HOPX) or aquaporin 5 (AQP5). [18] Other progenitor population of ATII cells have been isolated from human lungs which expressed the surface markers pro-SFTPC and CD90. [19]

Although the mouse model has revealed many mysteries of the ATII cell, human cells are more difficult to study. Their location makes it difficult to obtain cell samples and once a specimen is collected, it is difficult to isolate the cells and maintain them in culture due to poor regeneration and transdifferentiation into ATI cells. There is also a rapid loss of expression of surfactant proteins. [20] Some researchers have used human fetal lung epithelial cells treated with a cocktail of dexamethasone, cyclic adenosine monophosphate (cAMP) and isobutylmethylxanthine, DCI, to push them toward an ATII cell phenotype and examine their gene expression patterns during alveolarization. [21] New culture techniques have permitted isolation and purification of human ATII cells along with improvement of maintenance in culture, but the samples needed for isolation usually come from resected lung tissue in severely diseased lungs. [22]

Interstitial fibroblasts play an important, but sometimes detrimental role in the homeostasis of the alveolus. The spindle-shaped interstitial fibroblast comprises 30–40% of the cells in the normal adult human lung and secretes the extra-cellular matrix (ECM) scaffold for the alveolus [12]. The ECM has profound effects on epithelial biology as seen in experiments where a variety of matrices were used to grow and maintain ATII cells in tissue culture conditions [23] including collagen, fibronectin and laminin-5. Interactions between fibroblasts and ATII cells are required for induction of surfactant synthesis and are mediated by a glucocorticoid receptor. The fibroblasts derive from splanchnic mesenchyme and prompt the respiratory epithelium to enhance lung maturity. [24] But they can also be detrimental to the lung as seen by activated
myofibroblasts, which are major contributors to fibrotic lung disease through matrix production, α-smooth muscle actin (α-SMA)–mediated contractile phenotype and transforming growth factor (TGF)-β [25].

Lung disease caused by aberrations in surfactant metabolism is a great burden on humans, and an understanding of the various mechanisms is an important step to develop symptomatic therapy and a cure.

1.3 Pulmonary Surfactant Physiology

The discovery of surfactant deficiency by Mead & Avery was seminal in the field of neonatology. Before the 1970’s, hyaline membrane disease (HMD) or respiratory distress syndrome (RDS) was a lethal disease that caused respiratory failure in preterm infants. Lung tissue from these patients showed a deficiency of surface-active lipid-rich material for reducing surface tension. [26] This discovery led to the biochemical, physiological and molecular advances involved in surfactant homeostasis. This included the important role of dipalmitoyl-phosphatidylcholine (DPPC) in surface tension lowering [27], the presence of proteins in surfactant [28], and finally, the use of exogenous surfactant in the treatment of prematurely born infants with RDS [29].

Surfactant is composed of approximately 90% lipids and 10% proteins. It is mainly made up of phosphatidylcholine (PC), and it, along with other surfactant lipids are routed from the endoplasmic reticulum (ER) to the multivesicular body (MVB) and then the lamellar body (LB) via the adenosine triphosphate (ATP)-binding cassette transporter A3 (ABCA3), located on the limiting membrane of the LB, where it is stored. These are combined with 4 surfactant proteins – SFTPA, SFTPB, SFTPC, SFTPD - each contributing to homeostasis depending on their specific structures and activities. [30] The hydrophilic surfactant proteins SFTPA and SFTPD are
members of the collectin family consisting of oligomers of trimeric subunits. They are required for surface film dynamics as well as for innate immune defense [31]. They are not lung specific, and are found throughout the body. SFTPB and SFTPC are small, hydrophobic proteins, specific to the lung. They are synthesized as large precursor molecules that are cleaved into their mature forms and are the most important proteins in surfactant spreading and stability during the respiratory cycle.

Surfactant precursor proteins pro-SFTPB and pro-SFTPC are processed proteolytically during their movement to MVBs and LBs. The active proteins SFTPB and SFTPC are assembled with the phospholipids into large surfactant pools and stored in LBs. The contents are secreted into the airway via stimulation by catecholamines, purino receptor agonists and cell stretch. The LBs unwind and interact with SFTPA and SFTPD to produce tubular myelin and multilayered surface films that spread over the alveolus and reduce surface tension. SFTPB promotes adsorption of lipid molecules into the expanding surface film at the air-liquid interface, re-spreading of films from collapse, membrane fusion and lysis, formation of tubular myelin and surfactant reuptake by type II cells. [32] Pulmonary surfactant is recycled, catabolized or reutilized actively by the ATII cells. Alveolar macrophages (AM) help in surfactant uptake and degradation via signaling of the granulocyte-macrophage colony-stimulating factor (GM-CSF) [33]. (Figure 2)

The surfactant protein B gene is located on chromosome 2 and is genomically made up of 11 exons. The preproprotein is 381 amino acids (aa) and is translocated into the lumen of the ER. It is cleaved into the precursor pro-SFTPB molecule which is 42 kDa (358 aa), by cleavage of the N- and C-terminal arms. Experiments have shown that deletion of the N-terminal side leads to the accumulation of SFTPB in the ER [34]. Dimerization of the 8 kDa (79 aa) monomeric
SFTPB protein occurs once the cleavage of the proprotein is complete and is required for optimal activity in vivo [32]. It is required for the formation of lamellar bodies, processing of SFTPC, formation of tubular myelin and surface tension lowering in the peripheral lung. Homozygous SFTPB knock-out mice died of respiratory failure after birth [35], and blocking SFTPB with monoclonal antibodies in rabbits led to respiratory failure and loss of surfactant activity [36]. Neonates with SFTPB deficiency died from respiratory failure [37]. A recent study testing commercially available therapeutic surfactant found that film formation in vitro differed among therapeutic surfactants and was highly dependent on SFTPB content. The results supported a critical role of SFTPB for promoting surface film formation [38].

Human SFTPC is located on chromosome 8 and contains six exons. The 179 amino acid (aa) proprotein is partially translocated through the ER membrane. Processing of pro-SFTPC requires the presence of SFTPB. The mature SFTPC protein is 4.2 kDa (35 aa) and forms an α-helix capable of spanning a membrane bilayer. It is only found in ATII cells and its sequence is highly conserved among animal species. Many of its activities overlap those of SFTPB including monolayer film stability [39]. SFTPC deletion is non-lethal but tied to the optimal functioning of the surfactant film. SFTPC-null mutant (SFTPC^{−/−}) mice have disturbed surfactant stability and severe lung disease but did not have respiratory failure at birth. Although lamellar body formation occurred in SFTPC^{+/−} mice, inflammation, remodeling, and abnormal lipid accumulations were noted within the lungs of SFTPC-deficient mice [40]. Likewise, SFTPC deficiency was associated with severe interstitial lung disease in a family lacking mature (or active) SFTPC [41]. While deficiency of SFTPC has been associated with severe lung disease in infancy, most mutations in the SFTPC gene are inherited or caused by de novo mutations that are inherited as a dominant gene [42]. Mutated pro-SFTPC proteins have been identified in infants...
with a wide range of pulmonary diseases, thought to be caused by the production of a misfolded pro-SFTPC, abnormal trafficking through the ATII cell, accumulation in the Golgi and ER resulting in toxicity due to activation of intracellular stress signaling causing injury with apoptosis, and clinically, interstitial lung disease [43].

A novel receptor, GPR116, may sense the size or composition of the alveolar pool and is important in alveolar homeostasis, but the signaling pathways are not well understood. [44]

Clinically, the understanding of surfactant composition and biology has allowed extracts from animal lungs, including pigs and cows, to be delivered intratracheally in humans to reverse atelectasis. [45] This transformed the care of the preterm infant, allowing survival and reducing morbidities.[46] Once the surfactant proteins SFTPB and SFTPC were discovered and cloned in the 1980s, their genetic sequence opened the door to diagnosing mutations in the genes of surfactant associated proteins causing respiratory failure in newborns. The most common genes affected are SFTPB, SFTPC, ABCA3 and NKX2-1.
Figure 2: Schematic of surfactant metabolism in the ATII cell. SFTPB and SFTPC traffic through the golgi and late endosome/multivesicular body to the lamellar body via ABCA3. The contents of the lamellar body, including surfactant proteins and lipids, are secreted into the alveoli as tubular myelin, where they form a phospholipid-rich film that is essential for preventing alveolar collapse. Approximately half of the alveolar surfactant pool is cleared through a GM-CSF dependent alveolar macrophage pathway or secreted out to the environment through the airway. Most of the remaining surfactant is taken up by the ATII cell and recycled to the lamellar body, via the multivesicular body/late endosome (MVB/LE), for re-secretion, while a portion is degraded in lysosomes.
1.4 Surfactant protein B associated disease

Surfactant protein B is synthesized by type II alveolar epithelial cells and bronchiolar epithelial (Clara) cells and was the first gene mutation associated with respiratory failure in term infants of unexplained etiology and unresponsive to therapy. [37]

Mice with a single mutated SFTPB allele (+/-) have been shown to be unaffected, whereas the homozygous SFTPB/- offspring died of respiratory failure immediately after birth [47]. Lungs of SFTPB/- mice developed normally but remained atelectatic in spite of postnatal respiratory efforts, and babies with this mutation didn’t respond to exogenous surfactant [48]. SFTPB protein and mRNA were undetectable and tubular myelin was lacking. Type II cells of SFTPB/- mice contained no fully formed lamellar bodies. An aberrant form of pro-SFTPC was detected, and fully processed SFTPC peptide was markedly decreased in lung homogenates of SFTPB/- mice.[49] A number of mutations have been found in the SFTPB gene but the most common mutation, a GAA substitution for C at genomic position g.1549 in codon 121, the 121ins2 mutation, is associated with approximately 70% of the cases of SFTPB deficiency. [50] This is inherited in an autosomal recessive fashion and causes a frameshift mutation resulting in an unstable transcript with the absence of pro- and mature SFTPB protein. [51] This results in abnormal surfactant composition and function, impaired SFTPC processing, increased surface tension and end expiratory collapse. Clinically, babies with this mutation usually present with unexplained respiratory failure which is refractory to surfactant replacement and usually causes death during the first months of life, despite intensive ventilator support.[52] Lung transplantation is the only available treatment option.[53] But problems remain with this option including immune suppression, risk of rejection and a general decreased quality of life. The discovery of the ability to reverse somatic cells into pluripotent cells in patient specific diseases,
may be the key to reduce the burden of whole organ transplantation and improve the lives of those with fatal diseases.

1.5 Induced Pluripotent Stem Cells

The generation of human embryonic stem cell (ESC) lines from blastocysts by Thompson et al. opened the door to studying embryonic differentiation in vitro. These cells were able to proliferate undifferentiated in culture for many months, and maintained the potential to form all three embryonic germ layers, including endoderm, mesoderm and ectoderm. [54] This led the way to the discovery of 4 factors, Oct3/4, Sox2, c-Myc and Klf4 that reverted fibroblasts to a pluripotent stem cell state by Dr. Yamanaka et al. [55] Pluripotency was first achieved in mouse embryonic and tail-tip fibroblasts by the four Yamanaka factors, although other methods have been derived since, using different factors. Similar reprogramming experiments involving selection for Nanog or Oct4 expression yielded germline-competent iPS cell lines from mouse embryonic fibroblasts [56]. It has been shown that secondary iPS cells (generated from mouse fibroblasts harbouring doxycycline-inducible vectors and obtained from chimeric mice produced from primary iPS cells) can be generated with efficiency roughly 50 times greater than primary iPS cells [57]. Strategies to introduce these factors range from integrating retroviral vectors to transient synthetic mRNA delivery [58] and current active research is ongoing to maximize the efficiency and safety of reprogramming.

Induced pluripotent stem (iPS) cells share the potential of differentiation as ESCs, including comparable gene expression levels of key pluripotency factors such as Nanog, Oct4, and Rex1, and similar hypomethylation patterns at the promoters of Nanog and Oct4 [59]. Both types of cells generate cells of the endodermal, mesodermal, and ectodermal lineages in vitro and form teratomas containing cells derived from all three of the germ layers in vivo. However, it has
recently been shown that iPS and ES cells have many differences in DNA methylation patterns, and that reprogramming is associated with differentially methylated regions (DMRs), some of which reflect the epigenetic memory of the cell of origin [60]. Epigenetic memory refers to the similarities observed in the gene expression patterns of reprogrammed cells and cells of the somatic tissue of origin, which does not result from changes in DNA sequence, but from epigenetic modifications such as DNA methylation and acetylation. Epigenetic memory affects the differentiation potential of iPS cells, since iPS cells harbouring residual epigenetic marks reflecting the somatic cell type of origin differentiate more readily along lineages related to the tissue of origin, but have restricted differentiation potential to other lineages [61]. Furthermore, some of the DMRs observed in iPS cells represent iPS cell specific methylation patterns and are produced as a result of reprogramming, suggesting that iPS cells harbor an epigenome that is not equivalent to that of ES cells. These are some of the problems with reprogramming somatic cells that have restricted clinical application of this technology.

However, iPS cells remain an ideal cell for tissue regeneration and replacement. They can be derived from patient-specific skin cells then transformed into a variety of cell types which can be transplanted to the site of injury, reducing the chance of immune rejection that results with whole organ transplantation [62]. iPS technology has also been used in disease modeling in vitro and gene therapy [63].

The generation of lung tissue from iPS cells has lagged behind other tissue organs due to its complexity and number of cell types. Differentiation following the pattern of embryonic development has been a successful tactic but results in a heterogeneous population of airway and peripheral lung cells. The next section will focus on the various protocols used to achieve lung cells from pluripotent cells.
1.6 Directed Differentiation to early lung progenitors and beyond

The pattern of embryonic lung development begins with definitive endoderm (DE). In vitro, this can be induced through Nodal signaling via the addition of high levels of activin A.[64] DE is characterized by the presence of Sox17 as well as the surface markers Cxcr4 and c-Kit. [65, 66]. Lung primordial cells arise within the anterior foregut endoderm (AFE) and it was discovered that inhibition of BMP, Wnt and TGF-β signaling pathways induced differentiation of DE cells into AFE. In vitro this was achieved using a variety of small molecule inhibitors including NOGGIN, IWP2 and SB431452. [67] AFE generation is marked by the up-regulation of SOX2, TBX1 and FOXA2 and down-regulation of CDX2 (a hindgut marker). Using a SOX2-GFP reporter line, cell surface markers CD56 and CD271 were discovered and used to sort pure populations of AFE. [68] AFE cells then undergo dorsoventral patterning, giving rise to ventral lung bud progenitors. Signaling from Wnt, BMP4, FGF10, KGF and RA were found to be required for lung field induction as confirmed by the expression of NKX2-1 and FOXA2 [69]. Recently, the surface marker carboxypeptide M (CPM) was found to be upregulated along with NKX2-1 allowing the purification of the lung progenitor population. [70] Co-culturing of sorted CPM+ cells with pulmonary fibroblasts in a 3D transwell resulted in formation of spheroids with lamellar-body-like structures and an increased expression of surfactant proteins compared with 2D differentiation.

Combinations of known molecules involved in the differentiation of respiratory epithelium include a cocktail of BMP4, FGF10, KGF, Wnt and RA. In the mouse, RA signaling inhibits distal lung formation and favours proximal differentiation [71] while removal of BMP4 from the differentiation cocktails resulted in increased expression of SFTPC mRNA. Many differentiation protocols have attempted to make ATII (SFTPC+) cells but the efficiency has
been less than 5%. [69, 70]. Addition of DCI which stimulated distal epithelial differentiation in human fetal lung explants, dramatically increased expression of SFTPB, but not SFTPC. Fetal rat lung explants cultures have shown that SFTPC expression was more responsive to mechanical stretch, which may be what is missing in static, 2D culture systems. [72]

1.7 Thesis Rationale, Hypothesis and Specific Aims

Neonatal respiratory distress syndrome is caused by a quantitative lack of surfactant or genetic deficiencies in the genes of the pulmonary surfactant metabolic pathway. [73] Characterization of these mutations using adult and fetal alveolar type II (ATII) cells have provided insight into the regulation of surfactant production, but are limited due to lack of access to patient specific cells and the inability to maintain in long term cell culture. [21] A rare, recessive, lethal mutation in the surfactant protein-B (SFTPB) gene (also known as 121ins2) causes lethal neonatal respiratory distress syndrome and the only options for affected infants include evaluation for lung transplantation and comfort care [53]. With the discovery of the reprogramming of skin fibroblasts into induced pluripotent stem cells (iPS) and further differentiation into organ specific cells such as pulmonary cells, diseases of the distal lung can be managed with patient specific cell transplants, bypassing the need for an organ donor and intensive immunosuppression. Multiple labs have focused on the proximal lung cells in their differentiation protocols with minimum focus on peripheral lung cells [67, 74], and the groups that attempted differentiation to ATII cells had an efficiency of <5% of SFTPC+ cells [69, 70]. An improvement in the protocol and ability to sort for a specific cell type will allow the study of diseases of the ATII cell and the surfactant system, and gene therapy through the use of a lentiviral vector, the SFTPB gene mutation can be corrected and provide a platform to study potential treatment options for an otherwise fatal disease. My hypothesis is that alveolar type II
(ATII) cells can be derived from iPS cells derived from fibroblasts of individuals genetically
deficient in pulmonary surfactant B through directed differentiation and that introduction of a
wild type SFTPB gene can result in reversal of the disease phenotype.

The specific aims were:

1) to reverse the SFTPB deficient phenotype of 121ins2 iPS cells by introducing a
lentivirus carrying the correct wild type SFTPB sequence,

2) to transform wt, 121ins2 and lentiviral transfected 121ins2+SFTPB-GFP cells into
ATII cells via directed differentiation.
Chapter 2

Materials and Methods
2.1 Obtaining patient specific 121ins2 skin biopsy

Consent for biopsy was obtained from the parents from a patient with 121ins2 deficiency post lung transplant. The skin was cleansed with povidone-iodine solution and anesthetized with 2 percent lidocaine with epinephrine. The 5mm punch biopsy was performed and the skin was placed in a tube of sterile Dulbecco’s minimal essential medium (DMEM). The wound was closed with 5–0 nylon suture.

2.2 Growing Fibroblasts from skin biopsy

A 6-well plate was coated with 0.1% gelatin and 800 μl of DMEM plus 20% (v/v) FBS media was added to each well. The skin biopsy piece was placed in a lid of a 10cm plate filled with media and transferred to a dissecting microscope. Dissection of the skin biopsy was done by removing the fat and connective tissue, and slicing the keratin layer into 12-15 evenly sized pieces. Biopsy pieces were placed into each well of the prepared 6-well plate with fibroblast medium, which was then placed in a 37°C incubator. Fibroblasts were grown until confluence then passaged or frozen.

2.3 Derivation of iPS cells from fibroblasts

Fibroblasts from a patient with 121ins2 SFTPB deficiency (generously donated by Drs. Cole and Hamvas) were given to the Center for Commercialization of Regenerative Medicine (CCRM) for iPS cell derivation as well as fibroblasts from an individual with a normal surfactant profile. Reprogramming factors hOct4, hSox2, hKlf4 and hMyc (CytoTune iPS Sendai Reprogramming Kit) were added to the fibroblasts and incubated for 24 hours. After 24 hours, the viral solution was removed and replaced with fresh fibroblast expansion medium and changed daily for a week. After day 7, cells were re-seeded onto 10-cm tissue culture dishes
coated with human foreskin fibroblasts. Human ESC complete media made up of DMEM/F12, 20% (v/v) knockout serum replacement (Gibco (Life Technologies)), 0.1 mM β-mercaptoethanol (Sigma-Aldrich) and 10 ng/ml FGF-2 (R&D) was exchanged daily until approximately day 28, or until colonies were ready for picking. Authentic colonies were identified, cleaned and expanded on human foreskin fibroblast-coated dishes in hESC complete media. The iPS cells were characterized through karyotyping, immunostaining and FACS for OCT4, NANOG, SSEA4, TRA160, TRA181, and RT-PCR for pluripotency markers and cell line authentication (STR analysis) to confirm parental origin (figure 3).

### 2.4 Lentiviral infection of iPS cells with SFTPB\textsubscript{wt}-GFP insert

GeneCopoeia’s EX-M0587-Lv201 lenti-vector was used. The vector contained a CMV promoter driving the open reading frame (ORF) of homo sapiens surfactant protein B (Accession: BC032785.1) followed by SV40-eGFP-IREs-puromycin. To generate the lentivirus, the HIV-based EX-M0587-Lv201 lenti-vector, in conjunction with GeneCopoeia’s Lenti-Pac\textsuperscript{TM} HIV Expression Packaging vectors were co-transfected into HEK293T cells using GeneCopoeia’s EndoFectin\textsuperscript{TM} Lenti Transfection Reagent. Cells were incubated in the presence of 5% CO\textsubscript{2} at 37°C overnight. Growth medium was changed to Opti-MEM containing 3% FBS with the addition of GeneCopoeia’s Titerboost. Conditioned medium was collected after 24, 48 and 72 hours of incubation and centrifuged at 2000 x g for 30 minutes. The supernatant was transferred to a new tube and PEG 6000 solution was then added to make the final PEG 6000 concentration to be 8.5% (w/v) and the final NaCl concentration to be 0.3 M. The mixture was incubated on ice for 3 to 6 hours and then centrifuged at 2000 x g for 30 minutes. The viral particle containing pellet was resuspended by pipetting in 1/20 of the original harvest volume of Opti-MEM. Infection of the lentivirus was performed on 24-well or 6-well plates. Wt iPS and
121ins2 iPS cells were plated one day before infection, at a cell density about 60-70% confluence. Five million transduction units (TU) per milliliter of infectious particle was used to infect 10^6 cells together with polybrene (final concentration 80 µg per milliliter). Growth medium was changed one day after infection, with the addition of puromycin (0.25 µg per milliliter). Cells were selected under puromycin for more than two weeks and positive cells were verified by GFP expression in iPS cells using fluorescence microscopy. For the verification of SFTPB expression in vitro, total RNA was extracted from iPS cells using the RNeasy Mini Kit (Qiagen). Purified total RNA was reverse transcribed to cDNA using the miRNA reverse transcription kit with SFTPB specific primers (Life Technologies). qRT-PCR was performed and analyzed with StepOne Software (Applied Biosystems). For sequences of primers, see supplementary table 1.

2.5 Maintenance of iPSC

Sendai generated human dermal fibroblasts iPSC lines (wt, 121ins2 and 121ins2+SFTPB-GFP cells) were cultured on mouse embryonic fibroblasts as previously described [67]. Mouse embryonic fibroblasts (GlobalStem) were plated at a density of ~25,000 cells/cm². The human iPSCs were cultured in a medium of DMEM/F12, 20% (v/v) knockout serum replacement (Life Technologies)), 1% (v/v) nonessential amino acids (NEAA) (Life Technologies), 1% (v/v) L-glutamine (Life Technologies), 1% (v/v) penicillin/streptomycin (Life Technologies) 0.1 mM β-mercaptoethanol (Sigma-Aldrich) and 10 ng/ml FGF-2 (R&D Systems). Medium was changed daily and cells were passaged using collagenase IV solution (STEMCELL Tech) every 5-7 days at 1:10 dilution. Cultures were maintained in an undifferentiated state in a 5% CO2/air environment. Human iPSC differentiations were carried out in a 5% CO2/5% O2/90% N2 environment.
2.6 Directed Differentiation of iPS cells to distal lung cells (2D culture)

When the human iPSCs reached 70% confluence (Day 0), the cells were incubated in 10 μM of Y-27632 (Wako) for one hour and then in accutase (Innovative Cell Technologies) for 20 minutes at 37°C [70]. The detached iPSCs were then dissociated into single cells via pipetting, incubated on a 10 cm non-coated plate for 30 minutes at 37°C to remove MEFs and then seeded on matrigel-coated plates (BD Biosciences) at a density of 1.1 x10⁵ cells/cm². Step 1 medium: RPMI1640 medium (Life Technologies), 1x B27 supplement and 50 U/ml of penicillin/streptomycin as the basal medium contained 100 ng/ml of human activin A (R&D systems), 1 μM of CHIR99021 (Stemgent), 10 μM of Y-27632 (Day 0) and 0.25mM (Day 1) and 0.125mM (Day 2-6) of sodium butyrate. On Day 6, the medium was changed to Step 2 medium. From Step 2 to Step 4, the basal medium consisted of DMEM/F12 plus Glutamax (Life Technologies), 1x B27 and N2 supplements (Life Technologies), 50 U/ml of penicillin/streptomycin, 0.05 mg/ml of L-ascorbic acid (Sigma-Aldrich), and 0.4 mM of monothioglycerol (Wako) [67]. This medium was supplemented with 100 ng/ml of human recombinant NOGGIN (R&D systems) and 10 μM of SB-431542 (R&D systems). On day 8, NOGGIN was changed to 1 μM of IWP2 (Tocris).

On Day 10, the medium was changed to Step 3 medium, containing the basal Step 2 medium with 20 ng/ml of human recombinant BMP4 (R&D Systems), 0.5 μM of all-trans retinoic acid (RA) (Sigma-Aldrich) and 3 μM of CHIR99021.

On Day 14, the medium was changed to Step 4 medium in each protocol. For FGF10-based differentiation on culture plates (protocol 4A, Figure 5a), the medium was changed to basal medium with 100 ng/ml of human recombinant FGF10 (Wako) for 8 days. For CHIR99021/KGF/FGF10/EGF-based differentiation on culture plates (protocol 4B, Figure 5a)
the medium was changed on Day 14 to Step 2 basal medium containing 3 μM of CHIR99021, 10 ng/ml of FGF10 (R&D Systems), 10 ng/ml of KGF (R&D Systems) and 10 ng/ml of EGF (R&D Systems) for 8 days. Each medium was replaced every two days throughout the differentiation process.

On Day 22, the medium was changed to Step 5 medium consisting of IMDM, 10\% (v/v) FBS (Life Technologies), 2 mM l-glutamine, 1 mM nonessential amino acids, 1% (v/v) penicillin/streptomycin supplemented with 50 nM of dexamethasone (Sigma-Aldrich), 0.1 mM of 8-Br-cAMP (Sigma-Aldrich), 0.1 mM of 3-Isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), 25 ng/ml of KGF, 10 ng/ml of EGF, 10 ng/ml of FGF10 (protocol 5a, Figure 5b) and 10 ng/ml of VEGF (protocol 5C, Figure 5b).

### 2.7 3D culture of VAFE cells to lung spheroids

The protocol for the 3D culture was modified from a previous report [70]. As in the 2D protocol, AFE cells were bathed in Step 3 medium on day 10, containing the basal Step 2 medium with 20 ng/ml of human recombinant BMP4 (R&D Systems), 0.5 μM of all-trans retinoic acid (RA) (Sigma-Aldrich) and 3 μM of CHIR99021. After 4 days, the VAFE cells were stained for the primary antibody CPM and sorted. A total of 2.0 x 10^6 CPM+ cells isolated were mixed with 1.0 x 10^6 of fetal human lung fibroblasts (17.5 weeks of gestation, DV Biologics) in 1:1 Matrigel/Step 5 medium, and a total volume of 400 μl was seeded onto a 12-well cell culture insert (BD Biosciences) with 10 μM of Y-27632. Step 5 media consisted of Ham’s F12 (Life Technologies), 50 nM of dexamethasone, 0.1 mM of 8-Br-cAMP, 0.1 mM of 3-Isobutyl-1-methylxanthine (IBMX), 25 ng/ml of KGF, 25 ng/ml of FGF10 (protocol 3D5A, Figure 5c) and/or 10 ng/ml of VEGF (protocol 3D5C, Figure 5c), 0.25% of 15 mM of HEPES (Life technologies), 0.8 mM of CaCl₂ (Sigma-Aldrich), 0.1% (v/v) ITS premix (BD Biosciences), and
50 U/ml of penicillin/streptomycin. One ml of Step 5 medium was placed in the lower chamber and changed every other day for 21 days.

### 2.8 Flow cytometry

The cells grown in culture plates (2D system) were dissociated with enzyme-free gentle cell dissociating reagent (STEMCELL) for 10 minutes at 37°C. The detached cells were diluted in DMEM/F12 (Life Technologies) with 2% (v/v) FBS and centrifuged at 300 g at room temperature. The cell pellets were immersed with sorting buffer 2% (v/v) FBS in PBS, cell clumps were removed using a cell strainer with a 40-µm pore size (BD falcon) and single cells were collected by centrifuged. The cells were incubated with primary antibodies for 30 minutes with gentle shaking, washed twice with 2% (v/v) FBS/PBS, and if necessary, incubated with the secondary antibodies for 30 minutes. After rinsing with 2% (v/v) FBS/PBS twice, cells were analyzed using a BD FACS Aria II flow cytometer (BD Biosciences) or were sorted using a MoFloXDP BRV/UV or AriaII-SC BRV (BD Biosciences). Unstained controls were used for gating CXCR4⁺, c-KIT⁺ and CPM⁺ cells whereas negative control cells that do not express GFP were used for gating GFP⁺ cells. The list of antibodies used for FACS is shown in supplemental table 2.

### 2.9 Immunofluorescence/H&E

The cells grown on cover slips (2D culture) were fixed with 4% (v/v) paraformaldehyde (PFA) in PBS (R&D Systems) for 15 minutes at RT. After washing three times with PBS, the cells were immersed in 0.5% (v/v) Triton X-100 in PBS for 10 minutes at RT, followed by incubation with blocking solution consisting of 5% (v/v) normal donkey serum (Millipore), 1% (w/v) BSA (Sigma-Aldrich) in PBS for 60 minutes at RT. The cells were then incubated in the primary antibody solution for 30 minutes at RT followed by washing three times with 0.1% (v/v)
Tween in PBS. The cells were incubated in the secondary antibody solution for 60 minutes at RT and washed three times with 0.1% (v/v) Tween in PBS. All primary and secondary antibodies used in the present study were diluted in blocking solution as indicated in Supplementary Table S2. Nuclei were counterstained with Hoechst-33342 (Thermo Fisher).

For the 3D cultures, after fixation with PFA for one hour, the 3D matrigel disc in the transwell containing the spheroids and the pulmonary fibroblasts was embedded in paraffin, sectioned at 5 μm thickness, dewaxed, rehydrated then stained with hematoxylin and eosin (H&E) to visualize the spheroids under a light microscope. For immunofluorescence of the 3D cultures, antigen retrieval on the fixed sections was performed with 10 mM sodium citrate. The sections were then blocked and stained with antibodies as described above.

2.10 RNA isolation, cDNA preparation and Q-PCR

Total RNA was isolated using the Arcturus PicoPure RNA isolation Kit (Invitrogen) according to the manufacturer’s manual. First-strand cDNA was synthesized from 80 ng of total RNA using the SuperScript IV First-Strand Synthesis System (Invitrogen). The cDNA samples were amplified using 2X SYBRSelect Master Mix (Invitrogen) with ABI7300 Real-Time PCR System (Life Technologies). All reactions were started at a cycle of 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. The PCR reactions were performed in triplicate for each sample. The level of expression of each gene was calibrated to that of the housekeeping gene, β-actin (ACTB), and compared to the level of the expression of each gene in the fetal human lungs. All primer sets are shown in Supplementary Table S1.
2.11 Electron microscopy

Samples were fixed in a solution containing 4% (v/v) formaldehyde and 1% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH7.4) and then post fixed in 1% (w/v) osmium tetroxide. The specimens were then dehydrated in a graded series of acetone from 50% to 100% and subsequently infiltrated and embedded in Epon-Araldite epoxy resin. Ultrathin sections were cut with a diamond knife on the Reichert Ultracut E (Leica Inc). Sections were stained with uranyl acetate (2-3%) and lead citrate (0.1%-0.4%) before being examined in a JEM-1011 (JEOL USA Corp) microscope.

2.12 Statistical analysis

Statistics were calculated using 2-way ANOVA, one-way ANOVA and Bonferroni posttests. Error bars represent the standard error of the mean (sem) of three or more replicates. Single asterisks (*) indicate statistical significance, with p-values reported in figure legends.
Chapter 3

Results
3.1 Derivation of patient specific 121ins2 and wild type iPS cells from fibroblasts

Fibroblasts derived from the skin biopsy of a 121ins2 SFTPB patient were sent to the CCRM for transformation into iPS cells using the sendai virus as a vector for the reprogramming factors hOct4, hSox2, hKlf4 and hMyc. Two clones each of wt iPS cells derived from cord blood using episomes in a female patient and 121ins2 hiPS cells were successfully derived and expressed the markers of pluripotency OCT4 and TRA-1-60 (Figure 3). The karyotype was normal (Supplemental Figure S1).
Figure 3. Human iPS cells derived from 121ins2 dermal fibroblasts express pluripotency markers. (a) Positive immunofluorescence for OCT4, TRA-1-60, NANOG and SSEA4 (b) flow cytometry for SSEA4, TRA1-60 and OCT4.
3.2 Successful infection of 121ins2 iPS cells with SFTPB-GFP lentivirus

The lentivirus containing Ex-M0587-Lv205:SFTPB expression vector with pSMPUW-EF-1-GFP_Puro-Neo was transfected with the HPK-LvTR-20 packaging kit (Figure 4a). Two weeks after transfection of the wt and 121ins2 hiPS cells, expression of GFP was observed under a live cell immunofluorescence microscope (Figure 4b). Expression levels of SFTPB were measured by qRT-PCR and normalized to the housekeeping gene ACTB (actin). Values were averaged and normalized to non-transfected hiPS cells Figure 4c. SFTPB expression levels were statistically higher in the SFTPB-GFP infected iPS cells.
Figure 4. Human 121ins2 iPS cells were successful infected by the lentivirus carrying the unprocessed pre-form SFTP B protein sequence. (a) A schematic of the lentiviral vector containing a CMV promoter driving SFTP B followed by SV40-eGFP-IRES-puromycin (SFTP B-GFP) (b) A phase contrast (left) and GFP live cell (right) images of 121ins2 iPS cells infected with the SFTP B-GFP lentivirus (10x magnification). (c) Gene expression of SFTP B in a 121ins2+SFTP B-GFP iPS cell line compared to an untransfected 121ins2 iPS cell line (n=3).
3.3 hiPS cells differentiate into lung progenitor cells via definitive endoderm, anterior foregut endoderm and ventralized anterior foregut endoderm

Using directed differentiation, hiPS cells (wt, 121ins2 and 121ins2+SFTPB-GFP cells) were successfully differentiated to definitive (DE), anterior foregut endoderm (AFE) and ventral anterior foregut endoderm (VAFE) cells using a combination of growth factors and small molecules that mimic fetal lung development. Figure 5 a-c depicts the timeline of the differentiation, the cell type and its identifying markers and the combination of growth factors and small molecules used for induction of the differentiation at every step. Expression of the pluripotent marker NOGGIN was downregulated at all steps in the differentiation (Figure 5d).
**a**

hESC & hiPSC → Definitive Endoderm → Anterior Foregut Progenitors → Ventralized Anterior Foregut Progenitors

- D0: Activin A, CHIR, NaB
- D6: NOGGIN, SB-431542, IWP2
- D10: BMP4, ATRA, CHIR99021
- D13: 

**b**

Lung specific progenitors

- NKX2-1
- CPM

- D14: 4A: FGF10
- 4B: CHIR/KGF/FGF10/EGF
- 4C: FGF10/KGF

- D22: 5A: DCI/KGF/FGF10/EGF
- 5B: DCI/KGF/FGF10/EGF/VEGF

- D35: ATII cell

**c**

Ventralized Anterior Foregut Progenitors

- NKX2-1
- CPM

- D14: Human Fetal Pulmonary Fibroblasts

- D35: 3D Lung Spheroids

- 5A: DCI/KGF/FGF10
- 5C: DCI/KGF/FGF10/VEGF
Figure 5. Timeline of differentiation of iPSC to ATII in 2D and 3D culture over 35 days. (a) Timeline showing 2D culture steps from pluripotent stem cell to definitive endoderm (DE), anterior foregut endoderm (AFE) and ventral anterior foregut endoderm (VAFE) cells, the markers expressed by desired cell type and the combination of growth factors and small molecules used for the differentiation. (b) Timeline showing 2D culture of VAFE cells to alveolar type II cells (ATII), markers expressed by desired cell type and the combination of growth factors and small molecules used for the differentiation. (c) Timeline for 3D culture after sorting CPM positive VAFE cells at day 14 of culture. Sorted VAFE cells were then mixed with human fetal pulmonary fibroblasts and matrigel and cultured in a transwell insert. Lung spheroids were examined after 21 days after sorting in defined lung differentiation media. (d) NOGGIN expression in wt iPS cells was lost during directed differentiation.
3.3.1 hiPSc differentiate into definitive endoderm using activin A, CHIR99021 and Sodium Butyrate (NaB)

High concentration of activin A coupled with CHIR99021 and NaB for 5 days was used to induce hiPS cells into definitive endoderm cells (Figure 6). The flow cytometric analysis and immunofluorescence for endodermal surface markers show the majority of the cells were double positive for CXCR/cKIT. The efficiency of differentiation of wt iPS to DE cells was 74% ± 2.8, 121ins2 iPS to DE cells was 78% ± 3.5 and 121ins2+SFTPB-GFP iPS to DE cells was 81% ± 4.6.
Figure 6. Marker analysis of cells induced to definitive endoderm (DE) cells. (a) Immunofluorescence for DE markers CXCR4 and SOX17 in wt iPS cell (20x magnification). (b) Flow cytometry of wt iPS and 121ins2 iPS cells for CXCR4 and CKIT. DE cells are double positive for CXCR4/Ckit negative for TRA-1-80 (representative of 5 separate experiments).
3.3.2 iPSc derived DE cells differentiate into anterior foregut endoderm using NOGGIN, IWP2 and SB431452

The DE cells were induced to AFE cells using a combination of small molecules (NOGGIN, IWP2, SB-431542) that inhibit the BMP, Wnt and TGF-β signaling pathways for 4 days. Inhibition of BMP and TGF-β signaling for 48 h is followed by a 48-h inhibition of TGF-β and Wnt signaling. This significantly increased the fraction of FOXA2+ cells [69], confirming induction to AFE cells. AFE cell differentiation was validated through gene and protein expression of transcription factors FOX2 and SOX2 (Figure 7).
Figure 7. Marker analysis of 121ins2 iPS cells induced to anterior foregut endoderm (AFE) cells. (a) Real-time analysis of AFE cells validated the expression of FOXA2 and SOX2. (b) Immunofluorescence confirmed that the cells co-expressed FOXA2 (red) and SOX2 (green). DAPI was used for nuclear staining (20x magnification).
3.3.3 iPSc derived AFE cells differentiate into ventral anterior foregut endoderm using BMP4, ATRA and CHIR99021

The AFE cells were induced to VAFE cells using a combination of growth factors (BMP4) and hormones (retinoic acid (RA)), and CHIR99021, a glycogen synthase kinase-3β inhibitor, and an activator of canonical Wnt signaling. Expression of VAFE was determined by the appearance of the transcription factor NKX2-1 as well as the surface marker CPM. Figure 8 shows (a) the sorting efficiency for CPM+ cells derived from iPS wt cells (61%±5.6) and 121ins2 iPS cells (91%±3.6). The FACS efficiency for 121ins2+SFTPB-GFP iPS derived VAFE cells for CPM was 83%±4.6. Figure 8 b and c show the co-expression of NKX2-1 and CPM antigens in CPM +sorted cells. Figure 8d shows the mRNA expression profile for NKX2-1 during the directed differentiation of 121ins2+SFTPB-GFP iPS cells from stem to VAFE cell.
Figure 8. Marker analysis of wt, 121ins2 and 121ins2+SFTPB-GFP iPS cells induced to ventral anterior foregut endoderm cells (VAFE) cells. (a) Flow cytometry of wt iPS and 121ins2 iPS derived VAFE cells for carboxy peptidase M (CPM). (Top panels) Primary antibody was anti-human CPM and secondary antibody was conjugated with Alexa488. (bottom panel) FACS sorting of 121ins2+SFTPB-GFP iPS derived VAFE cells using primary anti-human CPM antibody and secondary antibody conjugated with fluororof 657. Cells in red insert were collected. (b,c) Immunofluorescence of sorted CPM positive VAFE cells derived from wt iPS (b) and 121ins2 iPS (c) cells co-express NKX2-1 (green) and CPM (red). (d) Real-time PCR for expression of NKX2-1 during directed differentiation of 121ins2+SFTPB-GFP cells into VAFE cells at various stages of development. Expression is observed at the VAFE/lung progenitor stage, while no transcripts were detected at the pluripotent stem cell, DE and AFE cell stages. The genes were normalized to the house keeping gene GAPDH and expressed relative to undifferentiated iPS cells.
3.4 2D distal differentiation of VAFE cells using variations of growth factors and small molecules

VAFE cells were sorted for CPM then plated onto matrigel coated plates in distal lung cell induction media. A combination of growth factors was used to determine the best efficiency of SFTPC expression, which is a singular marker for ATII cells (Figure 9). Branching morphogenesis and the growth of the endoderm into mesoderm is an extremely important phase in lung development, and is marked by cross talk between the two tissue types. One important growth factor is FGF10 which was used in all combinations. When removed from the cocktail, there was no SFTPC expression (data not shown). Figure 9 shows SFTPB and SFTPC expression in iPSc derived ATII cells normalized to ACTB and relative to a non-differentiated iPS cell. The cell types were differently affected by the growth hormones, with SFTPB being most highly expressed in the iPS 121ins2+SFTPB-GFP cells no matter which combination was used. The 121ins2 cells lacked SFTPB expression, while wt iPS derived ATII cells had highest SFTPB expression in the 4B5A combination of growth factors (i.e. CHIR99021/KGF/FGF10/EGF for 7 days and subsequently DCI/KGF/FGF10/EGF for 14 days). For SFTPC expression, the 121ins2 derived ATII cells responded best to the 4B5C combination (CHIR99021/KGF/FGF10/EGF for 7 days followed by DCI/KGF/FGF10/EGF/VEGF for another 14 days), while the other cell types showed no statistically significant difference in the other combinations. SFTPC and SFTPB were seen in the cytoplasm of differentiated cells. Induction efficiency of ATII cells was analyzed by scoring the number of SFTPB+ and SFTPC+ cells relative to the total number of nuclei in an average of five randomly selected images from one out of 3 different experiments. The average score was 10% for the wt and 121ins2+SFTPB-GFP derived ATII cells.
Figure 9: Expression of surfactant proteins B and C in iPS wt, 121ins2 and 121ins2+SFTPB-GFP derived ATII cells in 2D culture. (a) Gene expression data comparing various growth factor combinations in wt, 121ins2 and 121ins2+SFTPB-GFP iPS cells. iPSC wt was the undifferentiated stem cell acting as the negative control. SFTPB expression (top panel) was increased in all 121ins2+SFTPB-GFP iPS derived ATII cells independent of growth factor cocktail compared to other cell types. SFTPC expression (bottom panel) was increased in all cell types compared to control. * indicates p <0.05. n=2-5 repeats depending on cell type. 4A: FGF10; 4B: CHIR/KGF/FGF10/EGF; 4C: FGF10/KGF; 5A: DCI/KGF/FGF10/EGF; 5C: DCI/KGF/FGF10/EGF/VEGF (b) Immunofluorescence of iPS 121ins2-SFTPB-GFP derived ATII cells after directed differentiation using 4B5A cocktail. Cells co-express SP-B (green) and SP-C (red). 20x magnification. (4B5A = CHIR99021/KGF/FGF10/EGF for 7 days and subsequently DCI/KGF/FGF10/EGF for 14 days)
3.5 3D distal differentiation towards alveolar type II (ATII) cells from VAFE cells sorted for CPM and co-cultured with human pulmonary fibroblasts in matrigel

VAFE cells were sorted for CPM then seeded onto a transwell with fetal pulmonary fibroblasts and matrigel in 3D distal lung cell induction media. A combination of growth factors was used to determine the best efficiency of SFTPC expression. Figure 10 shows SFTPB and SFTPC expression in iPSc derived ATII cells normalized to ACTB and relative to a non-differentiated iPS cell. The cell types were differently affected by the growth factors, with SFTPB being most highly expressed in the iPS 121ins2+SFTPB-GFP derived ATII cells no matter which combination was used. The 121ins2 cells lacked SFTPB expression and the wt iPS derived ATII cells had highest expression in the 3D5C combination of growth factors (DCI/KGF/FGF10/VEGF for 21 days) although this did not reach statistical significance. For SFTPC expression, the iPS 121ins2+SFTPB-GFP derived ATII cells displayed statistically significant levels of SFTPC expression in both combinations 3D5A and 3D5C (5A: DCI/KGF/FGF10; 5C: DCI/KGF/FGF10/VEGF, both for 21 days). The other cell types showed similar levels of SFPTPC expression, although not statistically significant. SFTPC and SFTPB immunopositivity were seen in some of the spheroids of the 3D samples. Induction efficiency of ATII cells was analyzed by scoring the number of SFTPB+ and SFTPC+ cells relative to the total number of nuclei in an average of five randomly selected images from one experiment out of three. The average was 15% for the iPS wt derived ATII cells and 20% for the 121ins2+SFTPB-GFP derived ATII cells.
Figure 10: Expression of surfactant proteins B and C in iPS wt, 121ins2 and 121ins2+SFTPB-GFP derived ATII cells in 3D culture. (a) Gene expression data comparing various growth factor combinations in wt, 121ins2 and 121ins2+SFTPB-GFP iPS cells. iPSc wt was the undifferentiated stem cell acting as the negative control. SFTPB expression (top panel) was increased in all 121ins2+SFTPB-GFP iPS derived ATII cells independent of growth factor cocktail compared to other cell types. SFTPC expression (bottom panel) was also increased in all 121ins2+SFTPB-GFP iPS derived ATII cells independent of growth factor cocktail compared to other cell types. * indicates p <0.05 (N=3 separate experiments). 5A: DCI/KGF/FGF10; 5C: DCI/KGF/FGF10/VEGF (b) Immunofluorescence of iPS 121ins2-SFTPB-GFP derived ATII cells after directed differentiation using 3D5A cocktail. Cells co-express SP-B (green) and SP-C (red). 20x magnification.
3.6 Lamellar bodies in wild type and 121ins2 corrected SFTPB-GFP cells

After differentiation, the cells were fixed and evaluated using transmission electron microscopy. Both cell types had completed their differentiations in the 4B5A cocktail (CHIR99021/KGF/FGF10/EGF for 7 days and DCI/KGF/FGF10/EGF for subsequent 14 days) of growth factors on 2D matrigel. Figure 11 shows lamellar bodies that are pathognomonic for ATII cells in both iPS wt and iPS 121ins2+SFTPB-GFP derived ATII cells. Figure 11a also shows the microvilli and tubular myelin of the iPS wt derived ATII cells. The 3D differentiations did not have enough sample to be evaluated with TEM.
Figure 11: TEM images of ATII cells derived from wt iPS cells and iPS 121ins2+SFTPB-GFP cells. (a) iPS cells were differentiated in 2D culture using the 4B5A (CHIR99021/KGF/FGF10/EGF for 7 days and subsequently DCI/KGF/FGF10/EGF for 14 days) combination of growth factors. First panel (4000x magnification) showing ATII cells with microvilli (arrow). Second panel shows structures (arrow) that resemble lamellar bodies (20,000x magnification). Third panel depicts cell full of lamellar bodies and possible tubular myelin (arrow). (b) TEM images of ATII cells derived from iPS 121ins2+SFTPB-GFP cells using same culture conditions as wt iPS cells. Cells contain lamellar structures (arrow) that resemble lamellar bodies (25,000 x magnification).
Chapter 4

Discussion, Conclusion and Future Directions
4.1 Discussion

This project has shown a proof-of-principle that human induced pluripotent cells derived from fibroblasts deficient in surfactant protein B, can be infected with a lentivirus carrying the wild type allele, and after directed differentiation towards an alveolar type II phenotype, express surfactant protein B. The alveolar type II cells also express surfactant protein C, confirming their cell type, and show gene as well as protein expression of surfactant protein B which were not present in the 121ins2 iPS cells. The iPS derived ATII cells also showed pathognomonic lamellar bodies on electron microscopy. The directed differentiation was more efficient than what is currently published in the literature, but still less than 10-15% depending on cell type. This may be due to the type of iPS cell and its state of pluripotency versus differential capability prior to differentiation, the timing and concentration of the growth factors, and the type of matrix that the cells were grown on. Future studies will be required to understand the optimal mix of growth factors to increase the efficiency of differentiation towards ATII cells as well as the optimum matrix type and concentration to aid in this transformation. We also explored the best possible dimensional shapes of the matrix and showed that 3D matrices in combination with mesenchymal lung fibroblasts did increase the efficiency of iPS differentiation into ATII cells.

The rationale for this project has been to create a platform to study the devastating disease of surfactant protein B deficiency. By creating patient specific iPS cells from dermal fibroblasts, and then differentiate them into ATII cells, the disease can be studied in a human cell type that is difficult to obtain clinically. This platform permits the application of gene therapy and other techniques to correct the genetic mutation and give babies a chance at survival without the need for a lung transplantation.
Gene therapy is an attractive concept for correcting simple base pair mutations, and is making a comeback in various disease models such as cystic fibrosis [75]. There are various methods that can be used to correct the mutation including zinc finger nucleases, viral infection and CRISPR/Cas9. The lentivirus is a retrovirus, with a ss RNA genome with a reverse transcriptase enzyme. Once in the host cell cytoplasm, it transcribes viral DNA which is sent into the nucleus and incorporates itself into the genome with the enzyme integrase. When the host cell divides, it replicates the viral DNA along with its own to make viral proteins. Biologically, a lentivirus vector can be used to insert or delete a gene in order to study or correct a specific disease. A risk of using this vector is that the viral DNA integrates itself into the hosts DNA randomly which can affect the expression of native genes by turning on or permanently increasing expression of promoters or transcription factors.[76] Our decision to use the lentivirus method was because large viral titers can be produced in order to ensure infection, and the integrase of the virus targets active transcription units randomly throughout the entire genome quickly, thus ensuring successful replication of the gene that is absent in the host cell. This process takes a couple of weeks to months depending on successful infection and integration. A recent group used zinc finger nucleases to correct the SFTPB mutation in the mouse model. [47] They applied nuclease-encoding, chemically modified mRNA to deliver site-specific nucleases in a well-established transgenic mouse model of SFTPB deficiency in which SFTPB cDNA is under the control of a tetracycline-inducible promoter. Administration of doxycycline drives SFTPB expression at levels similar to those in wild-type mice, whereas cessation of doxycycline leads to phenotypic changes similar to those of the human disease. The investigators inserted a constitutive CAG promoter immediately upstream of the SPTPB cDNA to allow doxycycline-independent expression using an adenovirus vector to transfect lung cells with the corrected
mRNA intratracheally into the lungs and prolonged life in treated mice. This treatment was transient and limitations included the need for co-transfection of an AAV-DNA donor template in conjunction with mRNA, the short duration of the cure in vivo, probably owing to the natural turnover of the transfected lung cell populations, and the use of a transgenic mouse model in which an artificial cassette is targeted rather than a humanized model. Future goals for my project would be to use CRISPR/Cas9 to permanently correct the 121ins2 mutation in the iPS cells, and deliver the differentiated ATII cells without a vector into the lungs of SFTPB deficient mice.

Reprogramming the 121ins2 iPS cells in parallel with the wt iPS and the corrected 121ins2 iPS cell has shown that every starting cell population will have various differentiation potentials and efficiencies at each stage. At the first step of the derivation of DE cells, the cells showed consistent but different capabilities of becoming endoderm. The wt iPS cells had the poorest differentiation capability while those with the 121ins2 SFTPB mutation were easily derived into the various stages of endoderm. This may be due to the type of reprogramming the fibroblasts went through to become iPS cells, their state of pluripotency, and their responsiveness to the matrix and growth factors. All cell types were created by the Center for Commercialization of Regenerative Medicine using the Sendai viral method, but the fibroblasts came from different human beings, and therefore, may have been epigenetically quite different. iPS cell diversity can also be due to the level of the reprogramming, and those cells in a more transitional phase, may be more difficult to differentiate.[77] Molecular markers are currently being pursued to evaluate the quality of iPS cells.

Reprogramming AFE to VAFE cells also showed a difference between the starting cell types, with the wt iPS cells having the least amount of VAFE markers as determined by flow...
cytometry and 121ins2 iPS cells exhibiting the highest expression. Studies have shown that increasing the time that DE cells are exposed to AFE promoting factors, may improve their derivation into VAFE cells. [69] It’s important to get the timing of the growth factors and small molecules perfected in order to induce the most efficient generation of ATII cells from iPS cells. Gotoh et al also showed that the growth factor concentration had to be altered for each iPS cell type in their differentiation protocol to achieve the highest yield of VAFE cells. [70] At the VAFE stage, published reports have used a stepwise protocol of cocktails to generate both proximal and distal cell types. Some have shown the appearance of proximal markers first and distal markers later in time, but lineage studies have shown that at the progenitor lung cell stage, there are specific cell types that are predetermined to become distal or proximal cells. [7]

Understanding the signals and transcription factors important in determining and directing lung progenitor cells to the distal ATII phenotype will help create a pure population, decreasing the heterogeneity of lung cells in the final product. Factors such as a 3D matrix and supportive pulmonary fibroblasts are just the first step in attaining the desired ATII cells.

The 3D matrix has been studied in various organ systems and has been shown to improve differentiation of iPS cells into specific organoids. [78] The 3D system allows the cell to interact at every pole with important signaling molecules, not just the area that is in close approximation like on the 2D system. Organoids can be used in disease modeling in vitro as they enable iPS cell derived cells to self-organize in a 3D structure akin to their normal tissue morphology. Under these conditions, a cellular micro-niche directs appropriate cellular differentiation. The makeup of the matrix is important [79]. We compared various matrices, including matrigel, fibronectin, ECM protein and collagen IV. The ECM protein matrix is made up of collagens, laminin, fibronectin, tenascin, elastin, and a number of proteoglycans and glycosaminoglycans from
Sigma. Our 2D monolayer culture data showed that each matrix had an impact on the efficiency of ATII cells derivation from iPS cells (Supplementary Figure 3). These matrices were not tested in our 3D culture system so it will be important for further optimization of differentiation efficiency to test different matrices in our 3D culture system. Hydrogel is another matrix that has shown good efficacy in derivation of various organoids because the elastic moduli can be altered to make the matrix more or less stiff.[80] The stiffness of the matrix is something that needs to be explored to determine the optimal flexibility for directed lung differentiation.

Although the lung is made up of endoderm, the interaction with the mesoderm is absolutely necessary for normal growth and development [81]. Fetal pulmonary fibroblasts were used in the 3D culture system to replicate the stage of lung development involving the cross talk between developing endoderm and mesoderm. During this stage, the endoderm invaginates into mesoderm and begins highly regulated branching morphogenesis through cell receptor signaling to advance and repress cell growth in an organized fashion. [82]. The pulmonary fibroblasts represent the mesodermal portion of the fetal lung and were shown to improve differentiation towards a small airway phenotype when mixed in with the CPM+ VAFE cells within the 3D matrigel of the transwell as compared to cultured alone in a monolayer in a culture dish below the transwell insert (data not shown). Future goals would be to elucidate the exact signals secreted between the fibroblasts and the differentiating cells during directed lung differentiation. This would allow the use of the signals themselves without the use of a secondary cell type.

During development, although the fetal lungs are producing fluid, and blood is shunted away from the lungs, the fetus still breathes. These fetal breathing movements (FBM) are felt to be important in lung growth and development and prominent distinctions of FBM include its episodic nature and apnea-sensitivity to hypoxia. [83] Animal studies have shown that stretch
induces ATII differentiation as well as increasing the secretion of surfactant [72, 84]. We tested this phenomenon in our 2D system, and applied stretch to CPM positive lung progenitor cells seeded onto matrigel-coated stretch plates. We used a flexible-bottomed culture plate with the Flexcell® FX-5000™ Tension System to provide a mechanical load regimen to cells in monolayer (Supplementary Figure 4). The cells were stretched for 15 minutes, and then allowed to rest for 45 minutes, mimicking the in utero breathing movements of the fetus. Our preliminary results showed an increase in SFTPC expression of wt iPS derived ATII cells compared to the nonstretched control cells. (Supplementary Figure 5) A future goal will be to stretch VAFE cells enrobed in a 3D matrix together with pulmonary fibroblasts.

4.2 Conclusion

This thesis reports the directed differentiation of wild type and 121ins2 deficient induced pluripotent stem cells into alveolar type II cells as well as the introduction of a wild type SFTPB gene via a lentiviral vector into the mutated iPS cells to induce expression of SFTPB. Both PCR and IF showed the expression of surfactant protein B in previous deficient cells, and putative structures resembling lamellar bodies were seen in the corrected ATII cells. The differentiation process from iPS to ATII was guided by a combination of various growth factors and small molecules that mimicked lung development and both 2D and 3D models of differentiation successfully created ATII cells with the 3D model being more efficient.

4.3 Future Directions

Correction of 121ins2 mutation

The insertion of the wild type SFTPB gene by a lentivirus into the mutated 121ins2 iPS cell produced SFTPB successfully at the gene and protein level. Limitations included constitutive expression at all levels of the differentiation timeline, random integration into the
host cell’s DNA and use of a virus. New technology that can correct the mutation without altering the composition of the host cell’s DNA without the use of a viral vector would be best. The recently developed CRISPR/Cas 9 system could be the ideal approach.

**Directed differentiation to ATII cells**

Mimicking embryonic lung development has allowed the elucidation of signals required to coax stem cells into ATII cells. The differentiation efficiency is still very limited and the timing and concentration of growth factors and small molecules must be adjusted and optimized for each cell type. The 3D model of differentiation can be improved upon through the use of hydrogel with the correct stiffness and applying stretch mimicking fetal breathing movements to the differentiating lung cells. Once a pure population of iPS cell derived ATII cells can be created and purified, clinical application will be more feasible. With the ability to correct the 121ins2 mutation without a viral vector, and subsequently differentiate into ATII cells, these functioning surfactant secreting cells can then be applied to newborn babies burdened by this fatal disease, restore lung function and reduce morbidity and mortality.

**Surfactant protein B deficiency mouse model**

Once the ATII cells are derived from corrected 121ins2 iPS cells, their function can be tested in a 121ins2 mouse model of SFTPB deficiency. The corrected ATII cells will be purified and then instilled into the trachea of newborn 121ins2 SFTPB deficient mice. We have created a vector composed of SFTPC linked to GFP-Puromycin which turns on once SFTPC is activated. This then turns on the puromycin resistance gene and allows SFTPC expressing cells to be resistant to the addition of antibiotic to the culture media, thus purifying the iPS derived ATII cells. After instillation, the mortality and morbidity of the SFTPB deficient mice will be compared to controls and their lungs will be evaluated for SFTPB expression. The instilled cells
should remain in the lung [85] and produce surfactant protein B to reduce surface tension in the lungs and increase survival. Ideally, differentiation towards a bipotential cell that can produce both ATII and ATI cells would allow the engraftment of a cell type that can replicate itself and continue to create ATII cells \textit{in vivo}. After testing in the mouse model, this method can then be applied clinically, to a human newborn, whose only other option is to wait for lung transplantation and the morbidities inherent to organ recipients, or die.
References


Supplementary Materials
Supplementary Figures

Figure S1: Karyotype of iPS cells derived from fibroblasts.
Figure S2: Genomic sequence of the wt and 121ins2 SFTPB gene. Top panel shows the wt sequence and bottom panel shows the substitution of the base C for GAA, resulting in a frameshift mutation (arrow).

Figure S3: Gene expression of SFTPC of wt iPSc differentiated into ATII on different matrices in 2D monolayer cultures. N=2 separate experiments in triplicate. ECM is made up of collagens, laminin, fibronectin, tenasin, elastin, and a number of proteoglycans and glycosaminoglycans.
Figure S4: Schematic of equibiaxial strain application to cells plated in the well of a BioFlex® culture plate. From [http://www.flexcellint.com/BioFlex.htm](http://www.flexcellint.com/BioFlex.htm).

Figure S5: Gene expression of SFTPC in iPS wt cells differentiated to ATII cells using stretch vs. no stretch in the 4B5A cocktail. (CHIR99021/KGF/FGF10/EGF for 7 days and DCI/KGF/FGF10/EGF for subsequent 14 days) Negative control was iPS wt stem cell. N=1.
## Supplementary Tables

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### Table S1: Primer sets used in RT-PCR.

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**Table S2: Antibodies used in FACS and Immunofluorescence**

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