Extracellular Matrix-Mediated Lung Epithelial Cell Differentiation

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Physiology
University of Toronto

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Abstract

Differentiation of functional lung epithelial cells from pluripotent stem cells holds the potential for applications in regenerative medicine. However, efficient differentiation to proximal and distal lung epithelial cell populations remains a challenging task. The three-dimensional extracellular matrix scaffold is a key component that regulates the interaction of secreted factors with cells during development by often binding to and limiting their diffusion within local gradients. The development of matrices that can recapitulate the in vivo environment is key for directing lung lineage-specific differentiation. Here we examined the role of the lung ECM in differentiation of pluripotent cells in vitro and demonstrate the robust inductive capacity of the native matrix alone using decellularized adult lung scaffolds. The decellularization procedure was optimized and the scaffolds generated were carefully characterized to ensure complete removal of resident cells and preservation of the ECM. Lung scaffolds were recellularized with mouse and rat embryonic stem cell-derived endoderm and maintained for up to three weeks of culture at air liquid interface, in defined, serum-free medium conditions. Recellularization of lung scaffolds with endodermal cells resulted in differentiation to early NKX2-1+/SOX2+ proximal lung progenitor cells and a heterogeneous basal epithelial cell
population, within seven days of culture. Extended culture resulted in robust differentiation to mature airway epithelia, complete with FOXJ1+/TUBB4A+ ciliated cells and SCGB1A1+ secretory club cells, with morphological and functional similarities to native airways. Differentiated day 21 cells contained beating ciliated cells in culture and exhibited functional CFTR protein expression. Heparitinase I, but not chondroitinase ABC, treatment of scaffolds revealed that the differentiation achieved is dependent on heparan sulfate proteoglycans (HSPG) and its bound factors on the ECM. This work demonstrates the importance of a 3-dimensional matrix environment and the role of site-specific cues for directing differentiation of pluripotent stem cells to lung epithelial cells. This is a valuable step towards uncovering ECM-mediated signaling during lung specification and offers a platform for modeling lung development and airway-related diseases using pluripotent stem cells.
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<tr>
<td>3D</td>
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<td>AKT</td>
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<td>Albumin</td>
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<td>Air liquid interface</td>
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Chapter 1

Introduction
1. Introduction

The ability to extract sufficient amounts of oxygen from the environment has had a huge impact on the evolution of the respiratory system. In particular, breathing amphibians and mammals with their high metabolic rates have been forced to evolve a sophisticated organ to meet their oxygen requirements. To accomplish this, the lung consists of two highly branched tubular systems that have developed in a coordinated way to carry air and blood. Uncovering the precise signaling pathways in lung branching morphogenesis and lung cell fate has been challenging. Fundamental research in developmental biology using increasingly sophisticated techniques for disease modeling, genetic engineering and regenerative medicine bring us closer to understanding the underlying mechanisms in lung development and discovering novel therapeutic strategies that could improve outcomes associated with chronic, idiopathic and congenital respiratory problems.

1.1 Lung development

1.1.1 Developmental stages

Lung development can be subdivided into six distinct stages (Table 1-1). The early stages of lung formation comprise the embryonic and pseudoglandular periods, after which the prospective conductive airways have been formed and the acinar limits can be recognized. During the pseudoglandular period the primitive airway epithelium starts to differentiate, and neuroendocrine, ciliated, and goblet cells appear while mesenchymal cells have begun to form cartilage and smooth muscle cells. In the subsequent canalicular period, the airway branching pattern is completed, and the prospective gas exchange region starts to develop. During this period respiratory bronchioli appear, interstitial tissue decreases, vascularization of peripheral mesenchyme increases, and distal cuboidal epithelium differentiates into type I (AT1) and type II (AT2) cells. During the saccular period, the growth of the pulmonary parenchyma, the thinning of the connective tissue between the air spaces, and maturation of the surfactant system are the most important steps toward ex utero life. Although capable of function, the lung is structurally in an immature condition at birth. The structures present are smooth-walled transitory ducts and saccules with thick primitive septa containing a double capillary network. The final two stages of lung development are alveolarization and microvascular maturation. Human lung development progresses further in utero compared to mouse lungs. Completion of the saccular stage of development and continuation into alveolar and microvascular maturation occur after birth in
mice, while alveolarization begins at 36 weeks gestation in humans (Table 1-1). The final two stages of development occur concurrently and are characterized by an increase in the gas exchange surface area through septation of the alveoli, and by fusion of the dual-layer capillaries into a single-layer network. One of the important hallmarks of lung development is the signaling crosstalk between the epithelial and mesenchymal tissue layers. The combination, concentration, and spatial-temporal localization of a multitude of molecular signaling factors all working in harmony determine the fate of branching, proliferation, and cellular differentiation of the developing lung.

1.1.2 Lung bud formation

Lung development starts as an endodermal outgrowth of the ventral foregut around the fourth week of human development. Genetic studies have implicated several transcription factors, morphogens, peptide growth factors, and their cognate receptors in specifying the morphogenetic progenitor field of early and late lung development along the foregut axis, listed in Figure 1-1. Tightly regulated reciprocal communication between the developing lung endodermal and mesenchymal components is necessary to orchestrate precise temporal and spatial signaling.

The crucial event during the embryonic stage is the initiation of lung formation at the right place along the anterior-posterior axis of the foregut. One important transcription factor in this process is forkhead box A2 (FOXA2)\textsuperscript{3,4}. Foxa2 is expressed in the ventral foregut endoderm before and immediately at the start of lung bud formation\textsuperscript{5-7}. Targeted ablation of Foxa2 in mice has led to embryonic death between E6.5 and E9.5 before the onset of lung formation\textsuperscript{8,9}; however, chimeras rescued for the embryonic-extraembryonic constriction showed that Foxa2 was essential for foregut and lung formation\textsuperscript{10}. The foregut rapidly elongates into a single tube dividing into a dorsal esophagus and a ventral trachea, the latter of which bifurcates into the left and right primary lung buds. In a similar process, the mouse respiratory system develops from a pair of endodermal buds in the ventral half of the primitive foregut, just anterior to the developing stomach at 9.5 days of gestation. At this stage, the earliest known lung and thyroid-specific endoderm marker, homeodomain transcription factor NKK2-1, appears and forms a localized domain of expression in the anterior foregut\textsuperscript{11-13}. Initial branching starts by division into the right and left lung buds, invasion into the surrounding mesenchyme, and deposition of a network of extracellular matrix (ECM) proteins.
Lung bud development and subsequent branching is dependent on fibroblast growth factor (FGF) 10 expression in the local mesoderm surrounding the buds and FGF10 receptor (FGFR2IIIb), an FGFR2 splice variant, in the endoderm. FGF10 is a member of the large family of FGFs essential to many processes during embryonic development. In the murine lung, Fgf10 messenger RNA (mRNA) is dynamically expressed in the distal mesenchyme adjacent to the primitive lung buds. The importance of FGF10 for lung development was shown in Fgf10-deficient mice that die at birth because of respiratory failure. Transgenic mice overexpressing a dominant-negative FGFR2IIIb in the distal lung epithelium show a severe pulmonary defect, forming only a trachea and two main bronchi, without any lateral branches. Taken together, these data indicate that FGF10 signaling through FGFR2IIIb plays a crucial role in the initiation of lung bud formation. Once a localized source of FGF10 in the mesoderm surrounding branching tips has been established, this acts as a distal signaling center. There is evidence that Sonic hedgehog (SHH) and Sprouty (SPRY) are negative regulators, and WNT and Bone morphogenetic protein (BMP) signaling are positive regulators of this process.

Following elongation of the two buds, in humans the left lung bud will give rise to two main stem bronchi, and the right lung bud gives rise to three main stem bronchi. In the mouse, the right lung bud will form four stem bronchi whereas the left lung consists of one stem bronchus. The secondary bronchi will then extend into the surrounding mesenchyme and start to branch and rebranch in a process termed branching morphogenesis. This branching is initially highly stereotyped, while less so in the later stages of lung development.

Transforming Growth Factor-β1 (TGFβ) belongs to a superfamily that includes activin, BMP, and TGFβ1, 2, and 3. These peptides can exert a variety of biologic effects including regulation of cell growth, differentiation, and expression of a variety of proteins. In the lung, Tgfβ1 mRNA and protein are found in the mesenchyme and epithelium, respectively. Addition of exogenous TGFβ1 to cultured embryonic mouse lung explants and in vivo overexpression of Tgfβ1 in distal lung epithelial cells resulted in decreased branching morphogenesis, distal epithelial cell differentiation and formation of the pulmonary vasculature, indicating an inhibitory role for TGFβ1 during branching morphogenesis. Most, if not all, biologic activities of TGFβ are transmitted via type I and type II TGFβ receptors. Signal transduction requires the formation of a heteromeric complex of type I (TGFβR1) and type II (TGFβR2) receptors. Inhibition of TGFβR2 signaling stimulated lung morphogenesis in whole lung explant.
cultures, underlining again the negative effects of TGFβ signaling on branching morphogenesis\textsuperscript{36}. In vivo studies show that Tgfβ1-null mutants have no gross developmental abnormalities, however 50\% of the null mutants die before E11.5 as a result of defects in yolk sac vascularization\textsuperscript{37-39}. Tgfβ3-null mutants die after birth with cleft palate and delayed pulmonary development, and Tgfβ2-deficient mice die after birth and display a lung phenotype with postnatal collapse of alveoli and terminal airways\textsuperscript{40}. SMAD proteins are downstream effector proteins in the TGFβ signaling pathway\textsuperscript{41}. SMAD1-3 proteins are expressed in distal lung epithelium, and SMAD4 is expressed in both distal lung epithelium and mesenchyme\textsuperscript{37,42}. Down-regulation of Smad2/3 and Smad4 expression increased branching morphogenesis in cultured lung explants. Exogenous TGFβ1 did not reverse this inhibitory effect, which is consistent with TGFβ1 being upstream of SMAD proteins\textsuperscript{37}.

Two additional transcription factors that have been implicated in lung branching morphogenesis include GATA binding protein 6 (GATA6) and V-Myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN). GATA6 belongs to the GATA family of zinc finger–containing transcription factors and is expressed in epithelial and mesenchymal cells of the developing lung bud\textsuperscript{43,44}. GATA6 is essential for endoderm formation as its targeted deletion resulted in embryonic death due to failure of visceral endoderm formation\textsuperscript{45,46}. In the lung, GATA6 appears to be important for branching morphogenesis as inhibition of expression in the lungs results in decreased branching\textsuperscript{46,47}. Expression of a GATA6 engrailed dominant negative fusion protein in the distal lung epithelium resulted in a lack of alveolar type I cells and a perturbation in alveolar type II cells together with a reduction in the number of proximal airway tubules\textsuperscript{48}. On the other hand, overexpression of Gata6 using the Sftpc promoter resulted in disrupted branching morphogenesis and a lack of distal epithelial cell differentiation\textsuperscript{49}. This suggests that a balanced Gata6 expression level is important for branching during the pseudoglandular period of lung development and also during later stages of lung development in alveolar type I and II cell differentiation. MYCN is a member of the MYC family of proto-oncogenes, which includes MYCN, MYC, and MYCL, all belonging to the basic helix-loop-helix (bHLH) class of transcription factors. In the lung, MYCN is expressed in pulmonary epithelium\textsuperscript{50,51} and Mycn-null mutant mice die at midgestation\textsuperscript{51,52}. Leaky null mutants for Mycn survive until the onset of lung development; however, branching morphogenesis is dramatically reduced, resulting in severe lung hypoplasia\textsuperscript{50,53}. More recently, mice containing a lung-specific
Mycn-null mutation showed its critical role during lung development as these mice had abnormal lungs with a loss of proliferation, increased apoptosis, and reduced branching\textsuperscript{54}.

It is apparent that no one master regulator exists for lung development, rather together they control downstream effectors that drive and direct lung development\textsuperscript{55-57}. New players in this dynamic regulatory network are being identified, including micro-RNAs that will undoubtedly continue to expand our understanding of molecular mechanisms involved in branching morphogenesis and lung tissue maturation.

1.1.3 Contributors to proximal-distal patterning

As branching proceeds, endoderm-derived epithelial cells will line the airways while the surrounding mesenchyme will provide the elastic tissue, smooth muscles, cartilage, vascular system, and other connective tissues. NKX2-1\textsuperscript{+} primary lung buds give rise to all epithelial cell phenotypes along the proximo-distal axis, each with different morphologies and patterns of gene expression\textsuperscript{58,59} (Figure 1-2). Lineage restriction in the epithelium starts as the lung progresses from the pseudoglandular to cannalicular phase of lung development.

During mouse lung specification, differentiated cell types begin to appear, with proximal cells exhibiting mature phenotypes prior to the distal lineage cells. Epithelial cell types of the proximal lung in the tracheobronchial and bronchiolar lineages are columnar and pseudostratified. Early basal cells expressing transcription factor Transformation related protein 63 (TRP63) can be found in the mouse tracheal epithelium as early as E10.5. By E15.5, multiciliated cells expressing Forkhead box protein J1 (FOXJ1) and Tubulin Beta 4A Class IVa (TUBB4A) appear to line the airways and by E17.5 secretory club cells expressing Secretoglobin family 1A1 (SCGB1A1) (also known as Clara/club cell secretory protein), can be detected, predominantly in the more distal bronchiolar region of the airways\textsuperscript{60,61}. Basal cells function as progenitor cells that can self-renew and give rise to both secretory cells and ciliated cells\textsuperscript{60,62}. At E13 in the mouse, subsets of epithelial cells begin to express Achaete-scute homolog 1 (ASCL1), which can be turned off by NOTCH signaling\textsuperscript{63,64}. Cells that retain ASCL1 expression give rise to pulmonary neuroendocrine (NE) cells, while cells that downregulate its expression, give rise to secretory club cells. Mucus cells are also a proximal airway epithelial lineage that play an important role in airway remodeling during lung disease. SAM pointed domain containing ETS transcription
factor (SPDEF), an ETS domain transcription factor, is expressed in these mucous cells, also known as goblet cells, in the respiratory system and the gut\textsuperscript{65,66}.

The distal lung differentiates after proximal epithelial cell types have been established. Early developmental studies in murine lungs have shown that between E16-18 in the mouse, the distal lung is composed of glycogen-rich cuboidal AT2 cells, expressing surfactant proteins (SFTP)\textsuperscript{67}. Surfactant containing lamellar bodies can be found by E18 in AT2 cells, and Podoplanin (PDPN)- and Aquaporin5 (AQP5)-positive AT1 cells can be detected shortly after that\textsuperscript{60}. A recent model shows evidence for a bi-potential alveolar progenitor cell that can directly give rise to both AT1 and AT2 cells during development\textsuperscript{68}. Over the last two decades regulatory molecules involved in epithelial morphogenic patterning of the lung have been identified; however, the lineage relationship among these lung epithelial cells is not entirely understood.

Epithelial transcription factors such as FOXA2, NKK2-1 and GATA6 have been shown to influence lung epithelial specification. All lung endoderm initially expresses NKK2-1 and it continues to be expressed in adult bronchiolar and alveolar epithelial type II cells, where it plays an important role in the regulation of SCGB1A1 and surfactant protein synthesis\textsuperscript{69}. Targeted disruption of NKK2-1 results in severe hypoplasia of the lung lacking separation of trachea from the esophagus, arrest of branching morphogenesis, and epithelial cell differentiation at the pseudoglandular stage\textsuperscript{13,70}. FOXJ1 is a transcription factor of the winged helix–forkhead family, expressed in various tissues during development. In the developing and adult lung, FOXJ1 expression is restricted to ciliated cells of the bronchial and bronchiolar epithelium\textsuperscript{71,72}. The role of FOXJ1 in ciliated cell differentiation was clearly demonstrated when it was overexpressed in distal pulmonary epithelial cells. High levels of Foxj1 expression inhibited branching morphogenesis and development of distal epithelial cells; however, it enhanced the development of ciliated cells\textsuperscript{71} while Foxj1-null mutant mice completely lack respiratory ciliated cells\textsuperscript{73,74}.

SHH does not appear to be involved in regulating proximo-distal epithelial specification since SFTPC and SCGB1A1 are expressed in Shh-deficient mice\textsuperscript{75}. BMP4 is implicated in lung epithelial specification; it is expressed in early distal lung tips and at lower levels in the mesenchyme adjacent to the distal lung buds\textsuperscript{23,76}. Overexpression of BMP4 in the distal epithelium in vivo resulted in hypoplastic lungs with grossly dilated terminal lung buds separated by abundant mesenchyme\textsuperscript{23}. In vitro, exogenous BMP4 clearly enhanced peripheral lung epithelial branching morphogenesis and SFTPC expression\textsuperscript{77}. The secreted BMP antagonist
Noggin is expressed in distal mouse lung mesenchyme early in development. Its overexpression or that of the dominant-negative BMP receptor dnAlk6 in distal pulmonary epithelium, resulted in a proximal pulmonary epithelial phenotype. Similar results were obtained when Gremlin, another BMP antagonist, was overexpressed in the distal lung epithelium. These studies clearly indicate a role for BMP4 in proximo-distal epithelial differentiation during lung development.

### 1.1.4 Current model of epithelial type lineaging

The current model of lung epithelial differentiation depicts a multipotent progenitor population located at the tip of the growing lung buds. These tip cells are likely maintained by FGF10 and WNT signaling and lineage tracing experiments suggest that they have the ability to directly give rise to both proximal and distal epithelial lineages. As the lung buds extend and continue to branch specific gene expression patterns emerge in the stalk epithelial cells versus the tips, giving rise to SRY-related HMG-box(SOX)2⁺ proximal airway progenitors and SOX9⁺ distal alveolar progenitors, respectively. SOX2⁺ cells in the elongating stalk proliferate and give rise to all epithelial cells of the future bronchi and bronchioles: NE cells, ciliated cells, club cells and goblet cells. There is also evidence that NOTCH signaling plays a role in specification of bronchial epithelia. NOTCH2 receptor mediates the club and ciliated cell fate, while three NOTCH receptors (NOTCH 1, 2, 3) contribute in an additive way to regulate the abundance of NE cells.

Using an *Id2-CreEr* knock-in mouse strain to lineage trace the bud tip cells, one study suggests that Inhibitor of DNA binding 2 (ID2) expression, which encodes a bHLH transcription factor, identifies cells that can give rise to both proximal and distal lineages during the pseudoglandular stage, and become restricted to generating distal epithelial cells during the canalicular stage. The classic model of alveolar epithelial differentiation suggest that AT1 cells arise from AT2 cells. However, recent lineage tracing studies using an inducible *Shh-CreEr* allele demonstrate that during lung development both AT1 and AT2 cells can arise directly from a common progenitor, supporting the idea of a multipotent tip progenitor population. Many questions remain regarding the fate of these progenitor cells and whether they truly represent a single progenitor population or a mixture of subpopulations with restricted differentiation potential.
Table 1-1 Stages of lung development

<table>
<thead>
<tr>
<th>Stage</th>
<th>Gestational Age</th>
<th>Main Events</th>
<th>Epithelial Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic</td>
<td>3.5-8 wk</td>
<td>Formation of lung bud, trachea, left and right primary bronchus, and major airways</td>
<td>Undifferentiated columnar epithelium</td>
</tr>
<tr>
<td></td>
<td>9.5-14.5 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudoglandular</td>
<td>5-17 wk</td>
<td>Establishment of the bronchial tree; all preacinar bronchi are formed</td>
<td>Proximal: columnar epithelium; ciliated, nonciliated, basal, neuroendocrine cells</td>
</tr>
<tr>
<td></td>
<td>14.5-16.5 days</td>
<td></td>
<td>Distal: cuboidal epithelium; precursor type II cells</td>
</tr>
<tr>
<td>Canalicular</td>
<td>16-26 wk</td>
<td>Formation of the prospective pulmonary acinus by narrowing of terminal buds, and increase of capillary bed</td>
<td>Proximal: columnar epithelium; ciliated, nonciliated, basal, neuroendocrine cells</td>
</tr>
<tr>
<td></td>
<td>16.5-17.5 days</td>
<td></td>
<td>Distal: differentiation of cuboid type II to squamous type I cells</td>
</tr>
<tr>
<td>Saccular</td>
<td>24-38 wk</td>
<td>Formation of alveoli precursors: saccules, alveolar ducts, and alveolar air sacs</td>
<td>Proximal: ciliated, nonciliated club, basal, neuroendocrine cells</td>
</tr>
<tr>
<td></td>
<td>17.5-5 dpn</td>
<td></td>
<td>Distal: type I cells flatten and II cells mature</td>
</tr>
<tr>
<td>Alveolar</td>
<td>36-2 ypn</td>
<td>Formation of alveoli by septation of alveolar air sacs, thinning of interalveolar septa</td>
<td>Proximal: columnar epithelium; ciliated, nonciliated, basal, neuroendocrine cells</td>
</tr>
<tr>
<td></td>
<td>5-30 dpn</td>
<td></td>
<td>Distal: mature type I and II cells mature</td>
</tr>
<tr>
<td>Microvascular Maturation</td>
<td>Birth-3 ypn</td>
<td>Fusion of the capillary bed to a single layered network</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14-21 dpn</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

dpn: days postnatally, ypn: years postnatally
Figure 1-1 Transcription and growth factors known to regulate lung development

Figure 1-1 The anterior foregut begins as a single epithelial tube surrounded by mesoderm. Early specification of the future trachea and lungs is regulated by the dose and timing of several transcription and growth factors. Key factors that play a role in early foregut to late proximal and distal lung lineage specification have been listed under their corresponding stage. Certain transcription factors such as NKX2-1 and growth factors such as FGF10, SHH and BMP have been implicated in both early and lung epithelial specification.

Figure 1-2 The main epithelial cell populations of the lung are depicted in this schematic, including the corresponding protein markers used for their identification. The proximal airway lineage includes FOXJ1 and TUBB4A positive ciliated cells, ASCL1 and HES1 positive pulmonary NE cells, TRP63 and KRT5 positive basal cells, and finally SCGB1A1 positive secretory club cells. Submucosal glands are also found in the upper airways and harbour mucus-producing goblet cells, not depicted in this schematic. The gas exchange distal region of the lung is comprised of two types of epithelial cells, AT1 and AT2. Surfactant protein positive AT2 cells are cuboidal in morphology, and PDPN and AQP5 positive AT1 cells are thin-walled and provide the gas exchange surface of the alveoli.

1.2 Extracellular Matrix

1.2.1 Structural diversity of the ECM

During embryonic patterning individual cells divide, migrate, differentiate, and respond to environmental cues. The ECM has been implicated in these dynamic processes during development, maintenance and disease. Cells are continuously connected to the matrix, a latticework of glycoproteins that in addition to providing structural support, directs tissue morphogenesis. The ECM provides a specialized microenvironment and regulates cell behavior by interacting with cell surface receptors. The largest family of receptors which mediate cell adhesion to matrix proteins are known as integrins. There are several additional cellular receptors that bind to different matrix components including the elastin receptor and other non-integrin receptors that bind laminin and elastin. This allows regulation of extracellular and intracellular signaling emanating from extrinsic factors, including growth factors, hormones, and biomechanical forces. Proteolysis of the ECM also generates neoepitopes that confer functions on cells and tissues distinct from those specified by their nonproteolyzed counterparts. To decipher how the ECM confers numerous different functions, an appreciation of its complex structure is essential. The ECM is an oligomeric, 3D network composed of four major protein components: collagens, structural glycoproteins (e.g., fibronectin, laminin, tenascin-C), proteoglycans (e.g., heparan sulfate (HS), chondroitin sulfate (CS), syndecans), and elastic fibers (e.g., elastin, microfibrillar proteins). Matrix proteins are secreted by the epithelial and stromal cells it surrounds. Developmental processes including the response of unpatterned tissue to morphogen gradients are regulated by glycosaminoglycans (GAGs), where the surface of most cells and the ECM are decorated by HS proteoglycans. These are multifunctional proteins that engage in numerous cell-matrix interactions and function by binding and regulating local concentrations of growth factors and morphogens. Not every ECM network contains all of these components, however, nor does the composition of the ECM remain constant within any particular tissue. The distribution and organization of the ECM are both dynamic and tissue-specific.

The distinct composition of the lung ECM gives the lungs their unique porous and elastic properties. The basement membrane of endoderm-derived organs including the lung contain a specialized ECM that is composed predominantly of laminin, type IV collagen, and heparan sulfate proteoglycans. Laminin is the first intercellular protein produced and has been shown to
play a role in numerous aspects of lung morphogenesis by mediating cell adhesion, proliferation, and epithelial polarization during tubular formation\textsuperscript{95,96}. There are 15 different laminin isoforms and the embryonic lung has been shown to contain at least four different isoforms, each potentially serving different functions in during development\textsuperscript{97,98}. Varied laminin isoforms confer heterogeneity and generate tissue-specific basement membranes, mainly due to variability in the laminin $\alpha$ chains\textsuperscript{99}. Fibronectin expression peaks during branching and plays a role in cleft formation between daughter bud tips\textsuperscript{100}, while elastin precursor, tropoelastin, expression is highest at the peak of septation and is suggested to be a driving force in the maturation of the alveolar gas exchange regions.

Within the ECM, additional structural and functional diversity is generated through the use of alternative gene promoters and RNA splicing, and by posttranslational modifications, including glycosylation and sulfation of newly synthesized matrix proteins\textsuperscript{101,102}. Once secreted into the extracellular space, ECM proteins require integration into a functional network. Identifying binding partners for a specific ECM protein is therefore a prerequisite to ascertaining its biochemical and cell-signaling properties. Accordingly, understanding the biology of a single ECM component requires an appreciation of the structure and functions of numerous other affiliated proteins. Due to the number of steps involved in coordinating ECM expression, secretion, and assembly, deciphering how individual ECM proteins contribute to structural morphogenesis during developmental processes has been a challenging task.

### 1.2.2 Multifunctionality of the ECM

Normal development requires precise temporal and spatial coordination of cellular proliferation, migration, differentiation and programmed cell death (eg. apoptosis). Deciding which of these programs a cell will ultimately elect is determined, to a large extent, by the ECM. Promotion or suppression of cellular proliferation by the ECM results in either activation or silencing of genes involved in the regulation of the cell cycle\textsuperscript{103-106}. To counteract uncontrolled cellular proliferation and to sculpt or refine developing tissue structures, select cells must be eliminated from developing tissues. To this end, loss of cell contact with the ECM leads to apoptosis during development and cellular differentiation\textsuperscript{107,108}. Tissue-specific ECM components also regulate the transcription of genes associated with specialized differentiated functions, including alkaline phosphatase expression in osteoblasts, albumin production in hepatocytes, intermediate filament
protein expression in keratinocytes and cleft formation during branching of the developing lung.

Both mapping and identifying gene mutations that lead to heritable connective tissue disorders and the generation of animal models in which ECM genes have been mutated or ablated have been successfully used to ascertain the functions of individual ECM proteins within specific tissues. Many of the diseases resulting from ECM gene mutations are due to the defective structural integrity of specific tissues. There are numerous human diseases related to ECM protein mutations. Mutations in collagen type VII, collagen type XVII and laminin 332 genes can cause the skin-blistering disease Epidermolysis Bullosa. Mutations in type I collagen genes cause Osteogenesis Imperfecta, and mutations in both collagen I and tenascin-X genes can cause Ehlers-Danlos syndrome. In addition to gene defects that alter mechanical properties of the ECM, mutations in the fibrillin-1 gene that cause Marfan syndrome appear to increase TGF-β signaling, leading to a cellular disease phenotype.

Although mutations in ECM genes can produce heritable disorders, animal studies suggest that many structural ECM glycoproteins are essential for embryonic or fetal development. As a result, mutations in these genes often cause lethality early in development, complicating the study of gene function in vivo. Inactivation of the fibronectin gene in mice, for example, results in embryonic death due to mesodermal, neural tube and vascular developmental defects, and has been shown to be required for normal gastrulation. More targeted studies have provided information on the role of fibronectin in several developmental processes. Injection of inhibitory peptides or antibodies into postgastrulation embryos prevents fibronectin-cellular interactions and disrupts neural crest migration.

An alternative approach to genetic manipulation for elucidating the role of matrix proteins during various cellular processes is exposure to agents that perturb protein-cell interactions. Such agents can include small molecules or protein-specific antibodies that will interfere with the matrix protein function. Fibronectin-binding antibody or synthetic peptides have demonstrated the importance of fibronectin-cell interactions during cell migration and normal heart development in the chick heart.

Genetic studies have also been useful in revealing unexpected functions for certain matrix proteins. For instance, ablation of the elastin gene was predicted to cause structural defects in the
3D structure of blood vessels. Elastin-null animals, however, die within days of birth as a result of obstructive arterial disease characterized by proliferation of the subendothelial smooth muscle\textsuperscript{120}. Thus, elastin exerts an unexpected growth inhibitory role during normal vascular morphogenesis. Adult elastin haplo-insufficient mice are hypertensive, also as a result of abnormal vascular development and remodeling\textsuperscript{121}. Results from ECM-mutant animals therefore demonstrate the important roles ECM components have in both development and in response to injury.

1.2.3 Cell-matrix interactions via integrin receptors

Integrins are transmembrane receptors composed of 24 αβ heterodimeric members that link the external ECM environment to the internal cell milieu. Integrin receptors respond to the molecular composition and physical properties of the ECM and integrate both mechanical and chemical signals through direct association with the cytoskeleton. The heterodimers are composed of non-covalently associated 18 α and 8 β subunits\textsuperscript{122}, with distinct protein functions. The α subunit determines integrin ligand specificity and 9 of the integrin α chains contain an I domain with a metal ion dependent adhesive site (MIDAS), which comprises the ligand-binding site. The β subunit connects to the cytoskeleton and affects multiple signaling pathways.

Activation of integrins may stimulate the cell cycle, inhibit apoptosis and change the shape, polarity and motility of the cell\textsuperscript{122}. The extracellular domain binds to ECM ligands while the cytoplasmic domain binds to adaptor proteins, mediating “outside-in” and “inside-out” signaling\textsuperscript{123}. During outside-in signalling, ligand binding leads to separation of the two legs, allowing the β subunit cytoplasmic domain to bind intracellular proteins such as talin and kindlins. An example of inside-out signaling is the intracellular activation of talin, leading to its binding the β subunit and triggering the transition of the integrin heterodimer to a state with high affinity for extracellular ligands\textsuperscript{124}.

A number of integrins play central roles during fetal and embryonic development. Knockout mice have been used extensively to elicit the role of integrins during the development of numerous tissues. During vascular development, α5β1 integrins that recognise the Arg-Gly-Asp (RGD) peptide motifs in fibronectin play a primary role\textsuperscript{125}. Mutation of α5 leads to early embryonic lethality due to mesodermal defects and poor vascularization of both the yolk sac and the
embryo, while a $\beta_1$ mutation manifests as gastrulation defects and pre-implantation mortality.

The role of integrins is slowly being uncovered during lung development. The $\alpha v \beta 6$ integrin recognises the latency associated peptide (LAP) that non-covalently binds TGF$\beta$, keeping it from binding to its receptor. Binding of $\alpha v \beta 6$ to TGF$\beta$-LAP, results in the dissociation of the complex and activation of TGF$\beta$ receptors on epithelial cells and alveolar macrophages, leading to suppression of inflammation. $\beta 6$ deficient mice develop progressive pulmonary inflammation, resulting in emphysema. Re-expression of $\beta 6$ rescues the pulmonary inflammation pathology. Integrin $\alpha 8 \beta 1$ is another integrin shown to play a role in lung development. The integrin $\alpha 8$ deficient mice develop fusion of the medial and caudal lobes of the lung as well as abnormalities in airway division. Mice lacking the $\alpha 3$ subunit manifests in lung and kidney malformations due to aberrant branching morphogenesis.

The cytoplasmic domain of the integrin does not possess catalytic activity, and therefore specific adaptors are recruited to the plasma membrane and contribute to signaling events. This is termed the integrin adhesome and consists of over 232 components that are divided into intrinsic and transiently associated components. Some of the adhesome molecules are involved in the physical linking of integrins to the actin cytoskeleton, whereas others are involved in adhesion-mediated signalling, which affects multiple cellular downstream targets (Figure 1-3). This complex promotes the recruitment and activation of several protein kinases such as focal adhesion kinase (FAK) and SRC, leading to the activation pathways involving ERK, Jun N-terminal kinase (JNK) or RHO-family small GTPases. These signalling events are crucial for cellular migration, proliferation, survival and gene expression.

There is specificity in the interaction of distinct integrins with regards to their ECM ligands. For example, certain integrins recognize specific ECM proteins, including fibronectin, vitronectin, and tenascin-C, which contain a small tripeptide sequence designated RGD. By contrast, $\beta 4$ integrins interacting with $\alpha 3$-, $\alpha 6$-, and $\alpha 7$-subunits recognize laminins, whereas integrins composed of the $\beta 1$-integrin subunit and the $\alpha 1$, $\alpha 2$, $\alpha 10$, or $\alpha 11$ subunit bind collagen. This apparent redundancy in ligand binding specificity suggests that integrins might have overlapping functions.
The ECM can function as an organizing centre for signaling complexes comprised of matrix proteins, secreted or matrix-bound growth factors and their receptors on the cell surface. Integrins activate several signaling pathways independently, but can also act synergistically with growth factor receptors. They can activate a latent growth factor by inducing conformational changes or by presenting it to a protease. Growth factor stimulation can activate FAK, indicating that integrin and growth factor signaling pathways intersect at focal adhesions \(^{(\text{Figure 1-3})}\)\(^{134}\). Various extracellular growth factors regulate cell migration and dynamics by means of integrin-mediated signaling. Consistent with this notion, integrin clustering promotes recruitment and activation of growth-factor receptors within focal adhesion complexes.

### 1.2.4 Matrix Metalloproteinases

Matrix metalloproteinases (MMP) are a family of 24 proteins, where 6 are associated with cell membranes or protein transmembrane domains and the remaining are secreted\(^{135}\). Cell-associated MMPs are responsible for the majority of ECM degradation activity. They are highly regulated to preserve the integrity of tissues\(^{136}\). ECM remodelling is an important mechanism whereby cell differentiation can be regulated, including processes such as the establishment and maintenance of branching morphogenesis and stem cell niches. In the mouse, MMP2, MMP14, and an MMP inducer, CD147, are constitutively expressed in all five distinct stages of lung development\(^{137}\). In one model of branching morphogenesis, cleft formation is suggested to be driven by accumulation of TGFβ, which stimulates ECM deposition and directs branching to either side of the accumulated ECM. This process is facilitated by TGFβ-mediated inhibition of MMPs. In support of this hypothesis, TGFβ1 and TGFβ3 inhibit expression of MMP1 and upregulate expression of TIMP1 in fibroblasts\(^{89}\).

### 1.2.5 Biomechanical forces and the ECM

The developing embryo is exposed to mechanical forces that maintain and modify cell behavior. Integrins can serve as mechanoreceptors that transmit forces between the cytoskeleton and the ECM to maintain structural integrity of tissues\(^{138}\). The majority of integrin-mediated attachments between ECM fibers and resting cells are to a bundle of actin filaments in the focal adhesion (FA)\(^{139}\). FAs modulate cellular responses to control proliferation, cytoskeletal remodelling and migration of cells\(^{140}\).
Cells respond to force on integrin-mediated adhesions by remodelling the ECM. For example, cyclic stretching of fibroblasts and other cell types activates expression of genes for collagens, fibronectin, and metalloproteinases, and stretched cells assemble a dense ECM that is enriched in collagen\textsuperscript{141}. Matrix assembly usually occurs in a directional manner according to the applied force\textsuperscript{142}. Application of force to integrin α5β1 is required for conversion to a state that can be chemically cross-linked to the fibronectin beneath the cell. Inhibition of cell contractility blocks cross-linking but can be rescued by application of force from fluid shear stress\textsuperscript{143}. In early lymphangiogenesis, interstitial fluid pressure stretches lymphatic endothelial cells which stimulates integrin-dependent proliferation, expanding the lymphatics\textsuperscript{144}.

The idea that integrins detect biomechanical signals is further supported by the finding that FAK is involved in mechanosensing during cell migration\textsuperscript{145}. Biomechanical force also modulates the expression and activities of ECM components and proteases including tenascin-C and MMP2, positive regulators of angiogenesis\textsuperscript{146}. Collectively, these studies indicate that not only do biomechanical signals influence the ECM and its receptors, but also downstream signals generated by mechanical force modulate cell adhesion components. Additional studies are clearly needed to determine how local force differentials modulate cell behavior within the developing embryo.

### 1.2.6 Stem cell-matrix interactions

Stem cell maintenance, self-renewal, and cell fate determination in stem cell populations depend on the ECM\textsuperscript{147-150}. Matrix-mediated changes in cell adhesion of hematopoietic stem cells (HSC) in their microenvironment, for example, allows for the self-renewal and subsequent differentiation of these multipotent progenitors into blood and other cell types\textsuperscript{151}. Therefore precise cell-matrix interactions act as an important biological switch that dictates stem cell differentiation or mobilization at specific tissue sites during development and maintenance of early and adult stem cell populations. Despite overwhelming evidence for the importance of cell-matrix interactions during development, the molecular mechanisms underlying the cross-talk between cells and the surrounding matrix and the role each component plays in specification along the proximal-distal axis of the lung remains largely unknown.
Tissue stiffness and matrix composition initiate specific signaling pathways that regulate cell behaviour. The selection of integrins expressed on the cell surface specifies the signaling pathway due to the differential binding affinity of ECM ligands for integrin receptors. Integrins, via their cytoplasmic domain, recruit specific adaptor proteins to the plasma membrane. This in turn can regulate GF receptor signaling. Integrins colocalize at focal adhesion sites with growth factor receptors and their associated signaling molecules, in response to GF stimulation. Associated signaling molecules include Src and FAK, as well as cytoskeletal molecules such as paxillin, talin, and vinculin. ECM, extracellular matrix; GF, growth factor.

1.3 Stem Cells

1.3.1 Stem cell characteristics

Stem cells are undifferentiated cell types that have the capability to self-renew and differentiate into multiple lineages. During homeostasis, stem cells are typically slow-cycling or quiescent. However, with environmental stimulation, such as tissue damage, stem cells can proliferate rapidly and give rise to daughter cells referred to as transient amplifying cells. These daughter cells will continue to proliferate and generate the necessary specialized progenies for tissue repair and maintenance. Stem cells can undergo symmetric or asymmetric cell division. Symmetric division gives rise to two identical daughter cells, while in asymmetric division one daughter cell loses some parental stem cell characteristics and becomes more committed to a specific cell lineage. A progenitor cell refers to a pluripotent cell that has more limited differentiation potential and unlike stem cells do not have unlimited self-renewal capabilities.

Historically, stem cells were believed to receive regulatory cues from other cells in the local environment, defined as the stem cell niche\(^\text{205}\). Recent studies have shown evidence that some differentiated progeny of stem cells can themselves contribute to the niche and provide regulatory feedback to their stem cell parents\(^\text{206}\). For example, differentiated macrophages in the hematopoietic system, can home back to the bone marrow and regulate the maintenance of hematopoietic stem cells in the niche by promoting retention or enhancing their egress into the bloodstream\(^\text{207}\).

There is a hierarchy to stem cell differentiation potential. Totipotent cells have the ability to give rise to all cell types in the body, including the placenta and germ cells. These are the most primitive cells and exist only in the initial cell divisions after fertilization. The descendents of totipotent cells are pluripotent cells found in the inner cell mass of the blastocyst. These cells give rise to embryonic stem cells that can differentiate to every cell type of all germ layers. Multipotent cells are stem cells with yet more restricted differentiation ability than pluripotent cells. As stem cell progeny become more committed, the potential to give rise to various cell lineages becomes more restricted. Eventually leading to unipotency, where a unipotent progenitor cell can differentiate into one other cell type.

Despite the classical belief that unipotent cells can only give rise to a particular lineage, there is a growing body of evidence supporting the concept that cells committed to one lineage can in
fact give rise to cells outside of their lineage tree. For example, bone marrow stromal cells have been successfully differentiated to endodermal hepatocytes, mesodermal cardiomyocytes and ectodermal neuronal cells\textsuperscript{208-210}.

### 1.3.2 Types of stem cells

#### 1.3.2.1 Embryonic stem cells

Embryonic stem cells are pluripotent cells that can give rise to the three germ layers endoderm, mesoderm and ectoderm. They are derived from the inner cell mass of a blastocyst within the first 5-7 days after fertilization\textsuperscript{211}. ESCs transiently exist in the embryo and can be isolated and maintained in an undifferentiated state indefinitely under pluripotent culture conditions\textsuperscript{212}. There are several pluripotency markers associated with ESCs including POU domain, class 5 transcription factor 1 (POU5F1) (also known as OCT4), SSEA-1 and NANOG homeobox\textsuperscript{213}.

#### 1.3.2.2 Adult stem cells

Adult stem cells are a diverse group of multipotent cells that reside in stem cell niches across various tissue types. They have been found in many tissue types including the brain, heart and lungs, spurring a lot of excitement for their potential use in transplants and regenerative cell therapy. Most populations have limited differentiation potential to some or all cells of their origin tissue.

The bone marrow is an abundant source of adult stem cell types with three distinct populations characterized to date: HSCs, MSCs, and bone marrow-derived tissue committed stem cells. HSCs are found in the bone marrow and to a lesser extent in peripheral blood and express an array of markers such as FLK2, SCA1 and c-KIT\textsuperscript{214}. The second bone marrow stem cell population is the MSC population which can be isolated and maintained \textit{in vitro} to divide indefinitely. MSCs express three distinct surface markers (CD73, CD90, CD105) and subpopulations of MSCs have been described with differing proliferative and morphological characteristics\textsuperscript{215,216}. The third group of HSC refers to tissue-specific progenitor cells that have been identified in the bone marrow, often expressing the chemokine (C-X-C motif) receptor 4 (CXCR4). Accounts of bone marrow cells expressing other tissue-specific markers have been reported\textsuperscript{217-220}.
Apart from the bone marrow, there are many accounts of tissue-specific progenitor cell types, including the lungs. These cells are thought to reside in specific sites (the stem cell niche) and can remain quiescent until needed for tissue maintenance and response to disease or injury.

1.3.2.3 **Perinatal tissue stem cells**

The placenta and cord blood are both rich sources of hematopoietic progenitor and HSCs, capable of differentiating to all blood cell types\(^ {221} \). In addition to HSCs, placental tissue is enriched in MSCs, with multi-lineage differentiation capability\(^ {222} \). Human placental MSCs have been isolated, amplified and differentiated successfully \textit{in vitro} to different cell types. Other pluripotent populations successfully isolated from perinatal tissue include amniotic fluid and fetal membrane stem cells. With successful \textit{ex vivo} expansion, these populations offer a renewable stem cell source for regenerative medicine therapeutics.

1.3.2.4 **Induced pluripotent stem cells**

Adult somatic cells can be genetically reprogrammed to an embryonic stem cell-like state and are referred to as iPSC. Both mouse and human iPSC have demonstrated pluripotent stem cell characteristics including the expression of stem cell markers and the ability to give rise to all three germ layers. This discovery originally demonstrated that fibroblasts could become pluripotent by viral overexpression of four transcription factors, OCT4, SOX2, cMYC, and Kruppel-like factor 4 (KLF4) using retroviral transduction\(^ {223,224} \).

Since then, numerous methods have been developed to generate iPSCs with increased efficiency and footprint-free of any viral vector integration. Advances in reprogramming somatic cells have employed the use of single cassette reprogramming vectors with Cre-Lox mediated transgene excision\(^ {225,226} \), and nonintegrating adenoviruses, although with lower efficiencies\(^ {227,228} \). A collection of nonviral reprogramming techniques have also emerged that use mRNA transfection, episomal plasmids, and PiggyBac transposon-mediated gene transfer to induce pluripotency\(^ {229-232} \). Selecting the best reprogramming method for producing iPSCs is contingent on the adult cells being reprogrammed and their downstream application. The use of iPSCs for long-term translational medicine requires complete footprint-free techniques, regardless of the induced cell type. Beyond personalized medicine aspirations, iPSC technology is a valuable resource for using patient cells in disease modeling and drug screening techniques.
1.3.3 Adult lung stem cells

Despite low cell turnover in the adult lung, resident epithelial stem cell populations have been identified that can undergo long-term self-renewal and give rise to different cell types during homeostatic turnover or injury. Lung stem cell populations range from morphologically naïve (basal cells) to fully differentiated cells with specialized functions (club cells and AT2 cells). Lineage tracing techniques have allowed for the investigation of cell phenotype plasticity and identified several epithelial stem and progenitor lineages in different regions of the adult lung.

1.3.3.1 Proximal airway epithelium

Basal cells in the pseudostratified mucociliary epithelium are morphologically simple cells that characteristically express TRP63, cytokeratin 5 (KRT5) and cytokeratin 14 (KRT14). In vivo lineage tracing experiments and injury models have shown that basal cells can undergo long-term self-renewal and give rise to ciliated and secretory luminal cells during development, homeostasis and repair. Basal cells are a heterogeneous cell population with variable expression profiles for KRT5, KRT14, PDPN, and nerve growth factor receptor (NGFR), leading to many questions regarding their full regenerative capacity. For instance, it is unclear whether TRP63/KRT5 positive basal cells are all multipotent or if there are subsets with this capability. This was sparked by the observation that KRT14 expression is upregulated in most KRT5 positive basal cells during repair, suggesting that KRT14 negative basal cells could remain quiescent.

A recent model of airway basal cell progenitor activity demonstrates that basal cells relay a forward signal to their progeny, necessary for maintaining their phenotype. Using a series of cell ablation and lineage tracing techniques, it was shown that airway basal cells supply a NOTCH ligand to their daughter cells to maintain a secretory cells phenotype. Without this forward signal, secretory cells execute a terminal differentiation program and convert into ciliated cells. Interestingly, one group has suggested the ability of TRP63/KRT5 positive basal cells to replace alveolar epithelia following H1N1 influenza infection. Additional clonal lineage-tracing experiments under both steady-state and reparative conditions are required to understand the differentiation capability and precise mechanism by which basal cells differentiate.
1.3.3.2 Distal airway epithelium

Using injury models to deplete select epithelial populations, a subset of secretory cells with progenitor properties were identified and referred to as variant club cells. This subset of the club cell population expresses SCGB1A1 but not cytochrome p450 (CYP2F2) and were unharmed from naphthalene-induced injury to the airways. Lineage tracing studies have shown that SCGB1A1 positive, CYP2F2 negative club cells self-renew and differentiate to secretory and ciliated cell progeny under both homeostatic turnover and following injury. Variant club cells are known to cluster at transition points between the bronchiolar and alveolar regions, referred to as the bronchoalveolar duct junction (BADJ). This region considered to be a lung stem cell niche, as another stem cell population referred to as the bronchioalveolar stem cell (BASC) has been found to reside in this region. BASC cells coexpress markers of both the secretory cells and AT2 cells, SCGB1A1 and SFTPC respectively. The ability of this population to give rise to both bronchiolar and alveolar epithelium has been demonstrated both in vivo following bleomycin-induced injury and in vitro after clonal expansion.

1.3.3.3 Distal alveolar epithelium

AT2 cells are the main stem cell population in adult alveoli. There is overwhelming evidence that during late development and following injury AT2 cells have the ability to proliferate and differentiate into AT1 cells. This has been demonstrated using thymidine labeling studies, immunostaining for proliferative markers and transgenic lineage tagging. Recent evidence has emerged for the existence of additional alveolar stem cells beyond AT2 cells. One example is the KRT14/TRP63 positive basal cells, typically not found in the alveoli, which have been shown to differentiate to AT1 cells expressing PDPN, but not AT2 cells, in response to severe H1N1-induced injury. However, this study did not induce lineage tracing of the KRT14 positive cells until after initiation of injury, limiting interpretation of the in vivo differentiation potential of the cells. Another example of an alternative alveolar stem cell population was identified using flow cytometry to isolate epithelial cells coexpressing the α6 and β4 integrins without expression of SCGB1A1 or SFTPC. This progenitor population expands rapidly in response to lung injury and shows notable multipotent differentiation potential to airway and alveolar epithelial lineages. Numerous methods for ex vivo clonal expansion of AT2 and other adult lung stem cell types are being developed and their success holds great promise for cell-based regenerative medicine therapies.
1.4 Tissue decellularization

1.4.1 Overview

Decellularization refers to the complete removal of cells and debris from tissue and organs, whilst preserving the 3D structure, biochemical composition, and biological activity of the tissue ECM. The goal of decellularization is to maintain the native ECM structural proteins as well as the GAGs and proteoglycan composition to preserve the cues necessary for cells to thrive following repopulation of scaffolds in culture. The earliest attempts of decellularizing organs began in the 1970s using various techniques to isolate the ECM from livers, lungs, and kidneys\textsuperscript{177-179}. Both physical and chemical treatments were used to remove the donor cells and to study the impact of the remaining matrix proteins on primary cell behaviour in culture. These early studies demonstrate that basement membrane from different organs exert varying influences on the morphology and function of seeded cell types.

Over the last decade, interest in using decellularized organs has grown as the innate capacity of the ECM to better mimic the \textit{in vivo} environment of different organs is being realized. Natural scaffolds present numerous advantages over traditional culture conditions including 3D growth surface, tissue and site-specific cues, better cell survival and adherence, and biocompatibility for transplantation. By removing immunogenic components, minimizing immune rejection and the need for immunosuppressive drugs, if done successfully the use of decellularized scaffolds for tissue engineering presents several advantages over the use of allogeneic and xenogeneic transplant options\textsuperscript{180}.

1.4.2 Decellularization methodologies

Many approaches exist to tissue decellularization from treatments using physical manipulation, to enzymatic agents, to chemical agents. Most techniques use a combination of treatments with varying intensities and delivery methods, depending on the mechanical and biological properties of the tissue. Therefore no ideal decellularization agent exists for all tissue types and protocols may vary for the same tissue depending on the intended use following ECM isolation.

1.4.2.1 Physical agents

Physical methods aim to lyse and remove cells from the matrix through the use of temperature, force and pressure. Freeze-thaw cycles disrupt cellular integrity, show limited structural
disruption to the ECM, and can minimize the use of chemical treatments. Cytoprotectants such as trehalose have been used to limit damage to tissue architecture. Other physical treatments use pressure, sonication and electroporation, although sonication and electroporation have been found to cause disruption to the ECM of aortic tissues.

1.4.2.2 Enzymatic agents

Enzymatic agents commonly used for decellularization include proteases (eg. trypsin, dispases), esterases (eg. phospholipase A2), and nucleases (eg. DNase and RNase). The use of biologic agents is advantageous due to their specificity for biologic substrates. Trypsin has been shown to effectively cleave cell adherent proteins in treated tissue, however prolonged exposure resulted in damage to ECM collagen fibers. Endonucleases such as DNase and RNase are often used with or after other decellularization techniques to remove residual DNA from lysed cells. Enzyme deactivation due to the presence of natural protease inhibitors released from lysed cells can be ameliorated by refreshing enzyme solutions periodically or by using protease inhibitors such as aprotinin.

1.4.2.3 Chemical agents

Chemical treatments using alcohols, acids, alkalis, detergents and non-isotonic solutions are common candidates used for tissue decellularization. Although treatment with alcohol such as methanol and ethanol will remove cellular components by replacing intracellular water and lysing the cells by dehydration, alcohol alters matrix protein structure and elasticity by crosslinking the ECM. Similarly, acids and alkali effectively solubilize cytoplasmic components and remove nuclear material, however not without the catalysis of biomolecules and depletion of matrix proteins, GAGs and growth factors. Therefore, of the chemical agents used in decellularization, limited success has been reported with alcohols, acids and alkalis, and recent protocols have shifted towards the use of detergents.

Ionic, non-ionic and zwitterionic detergents have been used across all tissue types for decellularization. Ionic detergents such as sodium dodecyl sulfate (SDS), sodium deoxycholate, and Triton X-200 are commonly used decellularization agents due to their effective ability to solubilize cytoplasmic membranes, lipids, and nuclear components. Ionic detergents are strong and frequently used to denature and unravel proteins for polyacrylamide gel electrophoresis, and therefore will disrupt protein covalent bonds and deplete certain ECM
proteins during decellularization. SDS has been found to denature collagen, reduce GAGs by 50%, and significantly reduce ECM growth factor content\textsuperscript{172,191}. Ionic detergents can also be difficult to remove from the remaining matrix and if left behind can affect cytocompatibility. Therefore protocols using ionic detergents tend to limit the exposure to multiple washes at low concentration, low durations and reduced temperatures.

Non-ionic detergents (eg. Triton X-100, Tween 20, octyl glucoside) are considered to be gentle detergents that can solubilize proteins without major disruption to the native protein structure and enzymatic activity. Success of non-ionic detergents in decellularization protocols has been variable across tissue types, likely due to the difference in tissue density and cellularity\textsuperscript{173,194,195}. Contradicting results have been reported for decellularization of porcine versus rat aortic tissue using Triton X-100, highlighting the variability seen across tissue sources and delivery methods\textsuperscript{196,197}. Several studies have identified non-ionic detergents as the preferred method for preserving the basement membrane components following decellularization of ligament and porcine urinary bladder tissue\textsuperscript{198,199}.

Zwitterionic detergents have a net zero electrical charge and therefore do not disrupt the native state of proteins during treatments. Two commonly used zwitterionic detergents used in tissue decellularization are 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and sulfobetaine-10 (SB-10). Multiple groups have shown that CHAPS preserves GAGs and elastin during decellularization of pulmonary tissue while still removing 95% of nuclear material\textsuperscript{174,200}. One study has surprisingly reported collagen disruption in bladder tissue following CHAPS treatment, while non-ionic detergents Triton X-100 and ionic detergent sodium deoxycholate showed less disruption\textsuperscript{199}.

Ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA) are chelating agents that bind divalent metal cations at cell-adhesion sites of the ECM and effectively cause cell dissociation. These agents are very commonly used with other enzymatic agents or detergents to enhance decellularization and ensure complete removal of cellular components\textsuperscript{175,194,201}.

1.4.3 Cytocompatibility and immunogenicity

To ensure viability of the remaining tissue following decellularization, scaffolds will need to be cytocompatible for repopulation and biocompatible for transplantation. The presence of residual
decellularizing agents, particularly ionic detergents, has been shown to cause severe cellular toxicity and prevent optimal, if any, cellular adherence and growth on scaffolds\textsuperscript{194,202}. Optimal washing of decellularized scaffolds will need to be verified to ensure that remaining scaffolds do not contain high concentrations of toxic decellularizing agents. Biocompatibility is dependent on removal of all antigenic material including residual DNA from lysed donor cells and the alpha-Gal epitope expressed on most xenogeneic mammalian tissues\textsuperscript{203,204}. Complete decellularization can be assessed using quantitative assays for DNA and alpha-Gal removal, and certify biocompatibility.

1.5 Regenerative Medicine

Regenerative medicine refers to the study and application of innovative approaches in tissue engineering and molecular biology to repair and replace cells, tissues and organs. Despite advances in critical care medicine and interventional therapies, there is an immediate need for alternative options for the repair and replacement of lung tissue apart from lung transplantation. Two major aims of research in regenerative medicine are to understand the underlying repair mechanisms within tissues to better target and promote healthy regeneration, and secondly to develop cell therapy techniques that can harness the repair and replacement potential of exogenous stem cells. Regenerative medicine holds huge potential for the development of such novel therapies using directed differentiation techniques to generate functional proximal airway and distal alveolar epithelial cells.

1.5.1 Stem cells in regenerative medicine

Stem cells continue to receive a lot of attention in regenerative medicine due to their ability to directly contribute to different cell lineages or to create a pro-regenerative milieu for local progenitor cells to start the repair process\textsuperscript{152,153} (see section 1.5). As our understanding of stem cell biology and tissue repair mechanisms improves, so does the ability to create a microenvironment that recapitulates developmental processes and lineage specification. Stem cell niches are tissue-specific environments where stem cells reside. Recreating these niche environments allows us to regulate proliferation and differentiation into specific daughter cells\textsuperscript{154}. A key strategy is to transplant stem cells or their differentiated derivatives as therapy. An alternative to cell transplantation is to promote tissue-resident stem cells to regenerate by manipulating their microenvironment \textit{in vivo}. Targeted activation of endogenous stem cells
requires an understanding of the molecular pathways that normally control stem cell function and how those signals might have changed in the setting to injury or disease. Regenerative medicine is a highly multi-discipline field and requires efforts from stem cell biologists, developmental biologist, tissue repair experts and clinicians to name a few. Despite challenges, headways have been made in creating functional tissue using stem cells. Some examples outside of the lung include insulin producing islet cells for treatment of type I diabetes\textsuperscript{155}, repair of cardiac tissue following myocardial infarction\textsuperscript{156}, and oligodendrocyte precursor cell regeneration for developing remyelination therapies in multiple sclerosis\textsuperscript{157}.

1.5.2 Cell therapy

Cell therapy refers to the use of cells as treatment in the clinic and is typically administered to a patient through an injection. In addition to the use of HSC transplantation for the treatment of cancers of the blood or bone marrow, currently the main candidates for cell therapy applications involve bone marrow cells (BMC) and bone marrow stromal cells, also referred to as mesenchymal stem cells (MSC). BMCs and MSCs are readily harvested from donors and patients for use in allogeneic and autologous therapies. So far, both cell types have shown good safety profiles in human clinical trials. There is a substantial amount of evidence showing the ability of MSCs to suppress inflammation and activate endogenous repair mechanisms through indirect effects on immune cells and tissue-specific stem cells\textsuperscript{158,159}. Other studies however have shown that administered MSCs can only persist for short periods in the body\textsuperscript{160,161}.

Recent success with stem cell research has opened the door to the potential use of stem cells as therapy. The use of embryonic stem cells (ESCs) and somatic cells that are induced to a pluripotent state (iPSC) continues to be explored. This is due to in vitro scalability and multi-differentiation abilities of pluripotent stem cells. ESC-derived cell therapy applications include clinical trials for Parkinson disease, spinal cord injury, macular degeneration, diabetes, and heart disease\textsuperscript{162-164}, while iPSCs have been used in one human experimental procedure for macular degeneration in Japan. Cell-based gene therapy is another feature to cell therapy that promises the use of a host’s own cells, after genetic manipulations, to deliver a therapeutic protein or DNA to the patient\textsuperscript{165}. This can be achieved by replacing a malfunctioning gene within the cells affected by the condition. Due to the host’s own cells being used as the vehicle for gene delivery, this technique circumvents immunological barriers often associated with viral-based vectors. Recent
milestones reached in HIV treatment using *ex vivo* gene therapy approaches is an example of the promise this technique holds for the future\textsuperscript{166}.

Although the promise of using stem cells seems favourable for the near future, many questions remain as to the feasibility of such techniques today. Cell distribution, survival, function and overall clinical benefit in each unique disease setting must be understood. Due to outstanding questions, safety precautions, and regulatory barriers widespread availability of stem cell-based cell therapies are at least a few years away.

### 1.5.3 Stem cell tissue engineering

Tissue engineering is a branch of regenerative medicine that combines cells with scaffolds and biologically active molecules to generate functional tissues. The goal of tissue engineering is to assemble tissue constructs that can restore, maintain, or improve impaired tissue or whole organs. Artificial skin and cartilage are examples of engineered tissue that have been approved by the FDA, although with limited use in human patients today\textsuperscript{167}. Success in tissue engineering relies on our understanding of how individual cells respond to signals, other cell types and surrounding matrix to organize into tissues and organisms. As discussed in section 1.2, in addition to structural support, the molecular components of ECM have a profound effect on the biology of stem cells in regulating quiescence, proliferation, and cell fate. Therefore, a key step in creating artificial tissue is selecting a scaffold that can best create the environment required for the target cell types to thrive. Substrate stiffness and elasticity are two ECM parameters that have been used to demonstrate this importance on cell behavior and stem cell fate. Collagen coated substrates that are relatively stiff promote maximal cell spreading and drive enrichment of focal adhesions and in turn assembly of a cytoskeleton with stress fiber components, while culture of cells on soft gels promotes formation of a diffuse and less organized cytoskeleton\textsuperscript{168}.

Scaffolds can be generated using a wide array of sources including proteins, plastics, and natural organs. Novel approaches to tissue engineering using three-dimensional (3D) printing to print scaffolds with high-precision deposition of proteins show promise in generating custom tissue-specific scaffolds. Printed scaffolds lack the complexity of natural ECM, but show promise in generating simple scaffold models of liver, kidney, and bone\textsuperscript{169,170}.

Tissue decellularization is the process of removing donor cells from organs and maintaining the ECM as scaffold for repopulation with cells for tissue engineering (see section 1.4). It is not
surprising that complex 3D matrices are increasingly used in vitro to replace traditional two-dimensional cultures for recapitulation of tissue-specific microenvironments and directing differentiation of stem cells. Scaffolds are often supplemented with cocktails of cytokines and growth factors to promote tissue development. The use of existing scaffolds by decellularizing donor organs has been reported for bioengineering of numerous mesenchymal and endoderm-derived organs such as heart, liver, kidney and lung tissues. This approach holds great promise for using scaffolds from large animal donors such as porcine and sheep or cadaveric/discarded human tissue and combining it with a patient’s own cells to make customized tissue constructs or organs. If successful, these bioengineered organs would be available in abundance compared to viable donor organs for transplantation, and would not be rejected by the patient’s immune system.
1.6 Hypothesis and Objectives

Introduction

This thesis describes the role of the lung extracellular matrix in stem cell-derived endoderm lung epithelial differentiation. The major goals of the research were: (1) To establish an in vitro scaffold culture system using decellularized lung, suited for the study of lung cell-matrix interactions, and (2) To characterize the potential of the lung ECM to promote differentiation of seeded stem cell-derived endoderm to mature, functional airway epithelia.

The signaling components required for lung specification of progenitor cells requires, in addition to growth factors, a 3D matrix setting that can recapitulate the right microenvironment and mediate the interaction of the cells with the surrounding milieu. Human and animal studies have shown that pluripotent stem cells respond to environmental cues and can differentiate into the lung lineage. However, the reported efficiencies have been low and differentiated cell functionality has been limited. Furthermore, the ECM has rarely been incorporated as a key differentiation factor in promoting lung epithelial cell fate.

Hypothesis

Decellularized lung scaffolds support lung lineage differentiation of embryonic stem cell-derived endodermal cells.

Objectives

1. To develop a decellularization protocol that isolates an intact 3-dimensional extracellular matrix lung scaffold to support epithelial cell culture

2. To determine whether decellularized lung scaffolds can support the differentiation of stem cell-derived endodermal cells to lung progenitor cells

3. To characterize the multi-differentiation potential of seeded endodermal cells driven by decellularized lung scaffolds
Chapter 2

Generation and characterization of decellularized lung scaffolds for embryonic stem cell differentiation
2.1 Rationale

Cell adherence, proliferation, gene expression and differentiation are strongly influenced by matrix composition and structure. The development of matrices that can recapitulate the in vivo environment is key for directing lineage-specific differentiation. Synthetic and simple scaffold substitutes cannot recapitulate native tissue-specific ECM, while decellularized tissues have been shown to better capture this environment. We attempted to generate decellularized lung and characterize its ability to support extended culture of primary lung epithelial cells, and potentially provide a platform for lung-lineage stem cell differentiation.

**Objective of the study:** To develop a decellularization protocol that generates lung scaffolds capable of supporting lung epithelial cell culture.

**Summary of Results:** Decellularization was carried out using three different agents: enzymatic treatment with trypsin, chemical treatments with CHAPS or SDS detergents. Large variability was found in the extent of cell removal and structural changes of the lung ECM. CHAPS was selected as the best decellularizing agent and the solution was further optimized with the addition of hypertonicity and chelating agent EDTA. Removal of all resident cells was confirmed by tissue staining, electron microscopy (EM) and DNA analysis. Immunofluorescent (IF) confocal analysis, Western blot (WB) analysis, EM, and tensile testing confirmed preservation of ECM ultrastructure and protein composition. Recellularization of scaffolds with primary fetal rat lung epithelial cells resulted in cell adherence and formation of structures reminiscent of epithelial cells during early development. After 21 days of culture, recellularization of scaffolds led to re-establishment of epithelial structures positive for distal cells expressing cytokeratin 18 (KRT18) and pSFTPC and proximal cells expressing SCGB1A1 and TUBB4A.

**Conclusions:** The present study identifies an efficient and effective protocol to generate decellularized lung scaffolds that can support lung epithelial cell culture and could potentially be used to study stem cell differentiation towards the lung lineage.
2.2 Introduction

Regenerative medicine has become one of the major frontiers in the treatment of diseased tissues, and its progress is in large due to the rapid recognition of the potential for stem cells as therapeutic options. The use of stem cells in lung engineering has generated wide enthusiasm, however the actual development of functional lung tissue with pluripotent cells has been limited. Growth factor and matrix requirements for driving stem cell populations to differentiate into lung cell lineages with high efficiency are not well understood, and this is one of the major challenges of engineering lung tissue. The interaction of stem cells with the surrounding matrix environment is crucial for cell fate. Cell adherence, proliferation, migration, gene expression and differentiation are strongly influenced by matrix composition and structure\textsuperscript{148,247}. The development of matrices that can recapitulate the \textit{in vivo} environment is a key component for directing stem cell differentiation.

One of the most challenging aspects of lung tissue engineering is creating an artificial matrix that has similar biological composition and physiological functions to native lung tissue. Recapitulating an ECM that supports immunity, the mechanical function of breathing and the physiology of gas exchange certainly make this a complex task. The lung ECM is comprised of various collagen proteins that are associated with matrix components such as laminin, elastin, fibronectin, and proteoglycans, within a 3D ultrastructure that give the lung its distinct elastic and porous properties\textsuperscript{248}. Polymers and biomaterials such as Matrigel, Gelfoam, and collagen have been used as matrix substitutes for cell culture, however none have been successful in reproducing the complexity of the lung ECM\textsuperscript{180,249,250}.

There is a substantial amount of evidence suggesting that decellularized donor organs can be used as the ideal scaffolds for tissue engineering. These scaffolds can provide the appropriate 3D microenvironment for organ-specific differentiation\textsuperscript{251-253}. The use of scaffolds developed from decellularized tissue has been reported for the trachea in humans, and for the heart, liver, kidney, and lungs in rodents\textsuperscript{171,172,175,176,254}. In the case of lungs, decellularized rat tissue was successfully repopulated with primary lung epithelial and endothelial cells and the engineered lobe was capable of gas exchange for seven hours following orthotopic transplantation\textsuperscript{175,176}. This demonstrates the feasibility of this approach for \textit{ex vivo} lung tissue development using acellular lung scaffolds repopulated with stem cell-derived lung progenitor cells or fully differentiated lung epithelial cells. The ability to scale up lung scaffold production using human cadaveric or
xenogeneic sources could expand the therapeutic options available for lung tissue replacement. Such scaffolds can be seeded with cells derived from the patient’s own autologous cells to generate new segments of functional lung tissue or for processing and repopulating lungs marginally unsuitable for transplantation.

The decellularization process must effectively remove all immunogenic cellular components while preserving the natural composition and ultrastructure of the ECM. No universal decellularization protocol is available that results in the complete removal of cellular components, preserves the ECM and also can support stem cell culture for lung lineage specification. This chapter compares the efficacy of three decellularizing agents on rodent lung tissue and the optimal decellularization technique is identified. The resultant scaffold is characterized and demonstrated to support extended culture of primary lung epithelial cells.

2.3 Materials and Methods

Decellularization

All animal experiments were approved and carried out in accordance with the animal care committee guidelines of the Hospital for Sick Children Research Institute. To start decellularization, the lungs were accessed by opening the thoracic cavity using a median sternotomy. A small incision was made through diaphragm to cause the lungs to retract, reducing the chance of damaging the tissue. The inferior vena cava was ligated with a suture and a small incision was made in the left atrium to commence perfusion. The lungs were perfused through the right ventricle using heparinized Hank’s balanced salt solution (HBSS)-solution to facilitate removal of blood. Following perfusion, the trachea was exposed, separated from the esophagus, and cannulated with a plastic catheter near the thyroid cartilage. Using a gravity perfusion setup at 20 cm of H2O, lavages were carried out with different decellularization solutions. The lungs were filled to total lung capacity for one minute followed by removal of the catheter from the trachea to allow the fluid to flow out of the lungs. This was repeated for eight cycles, followed by 10 rinses with PBS.

Decellularization was attempted using six variations of decellularization solutions (Table 2-1). The lungs were decellularized with sequential tracheal lavages with decellularization solution, followed by extensive rinsing with phosphate buffered saline (PBS) (10-15 lavages). Lungs were
then removed from the animal and exposed to 90U/ml Benzonase endonuclease (Novagen, 70664-3) for 12 hours while on a rotator at room temperature to remove cellular components. Scaffolds were then treated with antimicrobial agents, 200U/mL penicillin streptomycin (Pen/Strep) (Gibco, 15140) and 25μg/mL amphotericin B (Gibco, 15290) for 6 hours at room temperature to prepare for recellularization. Thick vibratome sections (Leica) of acellular lung were generated at approximately 350μm and conditioned with culture media prior to recellularization.

**Isolation of primary fetal rat lung epithelial cells**

Time pregnant Wistar rats were euthanized by an excess of carbon dioxide on day 19 of gestation. Fetal lungs were dissected and excess adherent tissues were removed. The lungs were rinsed twice in cold HBSS and transferred to a glass petridish. The lungs were minced into very fine pieces, washed twice with HBSS, and centrifuged at 100xg for one minute. The lung tissue was trypsinized with 0.125% Trypsin (v/v) (GIBCO), 0.002% DNAse (w/v) (Worthington) in HBSS, in a trypsinizing flask under constant stirring for 20 minutes in a 37°C water bath. After trypsinization, the cells were neutralized in Minimum essential medium (MEM) + 5% (v/v) fetal bovine serum (FBS) (with 50μg/mL gentamycin) and then centrifuged in a 50 mL centrifuge tube at 450g for 5 minutes. The cell pellet was resuspended in 30 mL of MEM + 0.1% (w/v) collagenase and incubated for 15 minutes in a 37°C water bath. An equal amount of MEM + 5% FBS was added to the cell suspension and centrifuged at 450g for 5 minutes. The cell pellet was resuspended in MEM + 5% FBS and seeded into 75-cm² tissue culture flasks (15mL per flask) and incubated for one hr in 5% CO₂ incubator at 37°C to allow fibroblast cells to adhere. After this incubation period, the floating cells from the flasks were transferred to a 50mL tube and the flasks were washed with MEM to collect all floating cells. The collected cells were centrifuged at 450g for 5 minutes, and then reseeded into a second set of 75-cm² tissue culture flasks (15mL per flask) and incubated for one hr in 5% CO₂ incubator at 37°C to allow the remaining fibroblast cells to adhere. The floating cells were collected as before and centrifuged at 450g for 5 minutes. The collected cells were resuspended in 35mL MEM and washed 4 times by centrifugation (200g for 3 minutes) and resuspension. After the final wash, cells were resuspended in MEM + 5% FBS and seeded into 75-cm² tissue culture flasks (approximately 10mL per flask) and placed in the 5% CO₂ incubator at 37°C to allow the epithelial cells to adhere overnight. After 24 hrs of isolation, the epithelial cells were trypsinized and seeded onto decellularized lung scaffolds.
Recellularization of thick scaffold sections

Thick sections of decellularized lung were transferred to Nucleopore hydrophobic floating membranes (8μm pore size, Whatman, 110614) and seeded with 100,000 cells/section primary rat E19 epithelial cells and maintained for up to 21 days using MEM+10% FBS (v/v). Media was changed every 48 hours for up to 21 days.

DNA Assay

DNA was extracted from decellularized lung scaffolds using a chloroform/phenol extraction method. DNA was measured by ultraviolet (UV) absorbance at 260nm in microplates using a SpectraMax Absorbance Microplate Reader equipped with SoftMax Pro Data Acquisition & Analysis software.

Tensile Testing

Natural and decellularized sections of lung underwent tensile testing using Mach-1 Motion setup and software (Biosyntech). Sample sections (1-2cm² cross-sectional area and 500μm thick) were stretched until break point to assess the ultimate tensile strength, represented by stress-strain curves.

Histological Analysis

Cell-scaffold cultures were fixed in 4% (w/v) paraformaldehyde (PFA) (w/v) at 4°C for 8 hours. Samples were paraffin embedded and sectioned at 5μm. Sections were rehydrated and standard hematoxylin and eosin staining and Hart’s elastin stain was performed.

Immunostaining

Cell-scaffold cultures were fixed in 4% (w/v) PFA at 4°C for 8 hours. Samples were paraffin embedded and sectioned at 5μm. Sections were rehydrated and heat-induced epitope retrieval with citrate buffer (pH 6.0) was performed. Slides were then blocked for one hour with 5% (v/v) normal donkey serum and 1% (w/v) bovine serum albumin (BSA) (w/v) in PBS for 1 hour at room temperature. Samples were then incubated with primary antibodies at 4°C overnight in humidified chamber, and detected using Alexa Fluor conjugated secondary antibodies (Invitrogen) for one hour at room temperature. Nuclei were counterstained using 4’,6-diamidino-
2-phenylindole (DAPI) (Invitrogen). Details of antibodies are provided in the Table 2-1. Images were captured with Leica CTRMIC 6000 confocal microscope and Hamamatsu C910013 spinning disc camera (Leica Microsystems Inc.), and analyzed with Volocity software (PerkinElmer).

**Immunoblot Analysis**

Fifty μg of solubilized extracellular matrix supernatant in radioimmunoprecipitation assay (RIPA) buffer, and 1μg of purified laminin protein as positive control, were separated on a 5% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE) gel. Proteins were transferred to nitrocellulose paper (Millipore) and blocked with 3% (v/v) milk in PBS plus 0.1% (v/v) Tween-20. The membranes were then incubated overnight at 4°C with anti-laminin antibody (Table 2-2) at 1:1000 dilution in 1% (v/v) PBS-milk containing 0.2% (v/v) Tween-20. Blots were then incubated for 1 hour with secondary antibody conjugated with peroxidase, and were developed using chemiluminescent substrates (PerkinElmer) according to the manufacturer’s instructions and imaged on X-ray film.

**Electron Microscope Analysis**

Samples were fixed in 2.5% (v/v) glutaraldehyde in 0.1M phosphate buffer pH7.4. Transmission EM specimens were post-fixed in 1% (w/v) osmium tetroxide, dehydrated in an ascending series of acetone, infiltrated and embedded in Epon Araldite prior to polymerization at 60°C overnight. Ultrathin sections were then cut and mounted on grids, and stained with uranyl acetate and lead citrate prior to microscopy (JEOL, JEM1011) and image acquisition was completed using a CCD camera (AMT corp.). Scanning EM samples were post-fixed in osmium tetroxide, dehydrated in an ascending series of ethanol and critical point dried. Samples were then mounted on aluminum stubs using double-sided carbon tape. Samples were rendered conductive with a thin coat of gold palladium using a sputter coater and examined and photographed in a field emission scanning EM (JEOL, JSM 6700F).

**Statistical Analysis**

Statistical comparisons were performed using unpaired t-tests. P-value of 0.05 or less was considered significant.
Table 2-1 Decellularization Solutions

<table>
<thead>
<tr>
<th>Decellularization Solution</th>
<th>Components</th>
<th>Buffer</th>
<th>Total Lavages</th>
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<tr>
<td>1 Trypsin</td>
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<td>PBS</td>
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</tr>
<tr>
<td>2 SDS</td>
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<td>PBS</td>
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<td>10</td>
</tr>
<tr>
<td>4 CHAPS with EDTA</td>
<td>8mM (w/v) CHAPS 25mM (w/v) EDTA</td>
<td>PBS with 1M NaCl</td>
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<tr>
<td>5 CHAPS with EDTA</td>
<td>16mM (w/v) CHAPS 25mM (w/v) EDTA</td>
<td>PBS with 1M NaCl</td>
<td>8</td>
</tr>
<tr>
<td>6 CHAPS with EDTA</td>
<td>16mM (w/v) CHAPS 50mM (w/v) EDTA</td>
<td>PBS with 1M NaCl</td>
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Table 2-2 Antibody List

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<th>Company</th>
<th>Catalogue Number</th>
<th>Application, Dilution</th>
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</tr>
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<td>A-11006</td>
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2.4 Results

Hypertonic CHAPS-based solution is the best candidate for rodent lung decellularization

To evaluate the decellularization efficacy of commonly used agents on lung tissue we subjected adult mouse and rat lungs to three different treatments: (1) enzymatic treatment with trypsin, (2) chemical treatment with zwitterionic detergent CHAPS, and (3) chemical treatment with ionic detergent SDS. Decellularization was administered using a three step approach by first perfusing the lungs to remove blood, followed by treatment with the decellularizing agent through sequential airway lavages, and finally by extensive rinsing of tissue with PBS.

Following decellularization, the lungs were dissected from the neck and chest cavity and processed to examine the extent of decellularization. Hematoxylin and eosin (H&E) staining and DAPI fluorescent staining of tissue sections was used to analyze removal of resident cells and gross structural integrity of the remaining ECM (Figure 2-1). All decellularizing agents were prepared in PBS solution and PBS-only treated lungs were used as control for comparison. Treatment with 0.1% (v/v) trypsin had a modest disruptive effect on cell lysis and removal from the tissue, as intact nuclei were observed both with the H&E stain and DAPI stain on histological sections. Treatment with 8mM (w/v) CHAPS or 0.1% (w/v) SDS was more effective at removing resident cells, although with varying consequence to the tissue structure. Despite effective removal of cells, large cavities and tear-like structures were found following SDS treatment, while treatment with CHAPS was consistently best in preserving the gross structure of the remaining ECM. Based on these results, CHAPS detergent was selected as the base for further optimization of lung decellularization.

Although treatment of lung tissue with CHAPS alone was effective at removing most resident cells, to achieve complete decellularization with tissue devoid of all cellular components several variations to the CHAPS solution were tested for optimization. Due to the ability of hypertonic solutions to aid in removal of cellular components with minimal disruption to the ECM\(^{188}\), the decellularization solutions were prepared and tested using a hypertonic PBS buffer containing 1M NaCl. Chelating agent EDTA was also added at 25mM or 50mM concentration and CHAPS was tested at 8mM or 16mM concentration (Figure 2-2).
Based on histological analysis, the optimal decellularization solution for adult mouse and rat lung tissue was identified as 8mM CHAPS, 25mM EDTA and 1M NaCl. In the interest of matrix preservation, the higher concentration 16mM CHAPS with 50mM EDTA was not selected as the lower solution was sufficient for achieving complete decellularization. Following decellularization the lung scaffolds were treated with Benzonase nuclease to remove remnant cellular components from the lysed cells. Scaffolds were then treated with an antimicrobial cocktail for decontamination prior to use in any cell culture experiments (Figure 2-3).
Figure 2-1  Adult rat lungs are best decellularized using zwitterionic detergent CHAPS in comparison to treatment with trypsin and ionic detergent SDS

![Image showing tissue sections from adult rat lungs following decellularization. H&E staining (left panel) and DAPI fluorescent staining (right panel) are used to visualize the extend of cellular removal and gross tissue integrity. Decellularized tissues were compared to PBS-treated controls (top panel). Treatment with 0.1% (v/v) trypsin did not achieve sufficient decellularization as cell nuclei were still intact, while both 8mM (w/v) CHAPS and 0.1% (w/v) SDS were more effective at removing resident cells. Trypsin and SDS treatment both caused damage to overall tissue structure (arrow heads), while CHAPS treatment best preserved tissue structure. All images are at 200x magnification.]

Figure 2-1  Tissue sections from adult rat lungs following decellularization. H&E staining (left panel) and DAPI fluorescent staining (right panel) are used to visualize the extend of cellular removal and gross tissue integrity. Decellularized tissues were compared to PBS-treated controls (top panel). Treatment with 0.1% (v/v) trypsin did not achieve sufficient decellularization as cell nuclei were still intact, while both 8mM (w/v) CHAPS and 0.1% (w/v) SDS were more effective at removing resident cells. Trypsin and SDS treatment both caused damage to overall tissue structure (arrow heads), while CHAPS treatment best preserved tissue structure. All images are at 200x magnification.
Figure 2-2 Hypertonic CHAPS-based decellularization solution with the addition of EDTA effectively removes all donor cells while preserving the ECM.

Figure 2-2 Tissue sections from adult rat lungs following decellularization compared to PBS-treated controls (top panel). H&E staining (left panel) and DAPI fluorescent staining (right panel) are used to visualize the extent of cellular removal and gross tissue integrity. All combinations of the CHAPS and EDTA decellularization solution were effective at removing cellular components as apparent by the lack of intact nuclei in the stained tissue sections. Treatment with the lowest concentration combination of 8mM CHAPS and 25mM EDTA was sufficient at removing resident cells (middle panel). All images are at 400x magnification.
Decellularized lung scaffolds undergo endonuclease and antimicrobial agent treatment prior to recellularization.

Figure 2-3 Photographic representation of a natural lung (left panel) and an acellular lung (right panel) following decellularization with CHAPS-based solution. Two additional treatment steps for cell culture preparation are specified: Benzonase nuclease treatment, followed by antimicrobial agents.
Decellularization protocol preserves the lung ultrastructure and matrix protein composition

The lung scaffolds were characterized further for their structure and composition following decellularization. Benzonase treatment was highly effective at removing remnant nuclear components. Histological analysis showed no positive DAPI staining, while DNA analysis showed less than 20ng/mL of DNA remaining on the decellularized scaffolds (Figure 2-4). Electron microscopy, tensile testing and tissue staining for matrix proteins were carried out to confirm ECM integrity.

High-magnification scanning EM analysis of scaffolds confirmed the absence of host cells on treated scaffolds (Figure 2-5A). EM micrographs also revealed that the ultrastructure of the alveolar septae and the delicate microvessels surrounding the alveoli were maintained. Tensile testing analysis was used to compare the mechanical properties of decellularized scaffolds with natural lung. The stress-strain plot from the analysis showed a similar tensile strength profile between the two tissues, although as expected decellularized scaffolds had lower peak strength (Figure 2-5B). Preserved matrix protein composition was confirmed using IF staining and confocal microscopy for collagen I, collagen IV, laminin, HS proteoglycans, and fibronectin (Figure 2-6A). Elastin protein was visualized using Hart’s elastin stain and found intact at the alveolar septa and tips of decellularized scaffolds (Figure 2-6B).

Laminin is a large trimeric glycoprotein that is a major component of the basement membrane. It is important for branching morphogenesis, cell adherence and epithelial cell function. Close characterization of laminin protein on decellularized scaffolds was carried out using Western blot analysis and immunogold labelling for TEM analysis (Figure 2-7). Laminin was found preserved on decellularized scaffolds after 10 rinses and was detected by gold staining lining the remaining basement membrane on scaffolds.
Figure 2-4 Decellularization procedure with endonuclease treatment completely removes all cellular components

(A) Corresponding H&E and DAPI staining of natural and acellular lung tissue sections show the absence of cellular material including nuclei following decellularization. Scale bars=50μm. (B) Removal of DNA following Benzonase treatment of decellularized scaffolds was confirmed with DNA measurement. Mean ± SEM, n=3, *p<0.01.
Figure 2-5 Lung scaffolds maintain their architectural structure and strength following decellularization

(A) Scanning electron microscope analysis following decellularization show the surface of acellular lung scaffolds with intact matrix architecture. Scale bar=100μm, scale bar of inset=10μm. (B) Tensile testing represented by stress versus strain plots of natural and decellularized scaffolds characterizes mechanical properties of scaffolds. Acellular scaffolds have a similar tensile strength curve profile, although with a lower overall peak strength, to natural lungs.
Figure 2-6 Major ECM proteins including basement membrane components and elastin are detected by immuno-staining on decellularized scaffolds

Figure 2-6 (A) Natural (top panel) and acellular lungs (lower panel) were examined for ECM protein composition. Immunostaining shows presence of intact collagen I (Col-I), collagen IV (Col-IV), laminin (Lmn), heparan sulfate proteoglycans (HSPG), and fibronectin (Fn), while DAPI staining is absent from decellularized tissue. Scale bars=25μm. (B) Hart’s elastin stain shows intact elastin fibers in scaffolds following decellularization. Scale bar=25μm.
**Figure 2-7** Basement membrane protein laminin is maintained on decellularized scaffolds

(A) Immunoblot analysis for basement membrane-protein laminin following 5, 10, and 20 washes after decellularization shows that scaffolds maintain laminin protein composition for up to 10 rinses. Ponceau stain was used as loading control; presented Ponceau positive band is 50kDa. (B) Transmission EM analysis shows decellularized scaffolds with positive immunogold staining for laminin (black arrowheads). Scale bar=500nm.
Decellularized scaffolds support primary lung epithelial cell adherence and phenotype

To determine whether decellularized lung scaffolds could be used for cell culture studies, primary fetal rat lung epithelial cells were isolated and used to assess cell adherence, organization and phenotype on the scaffolds. To setup cell-matrix cultures, thick sections of rat lung scaffolds were generated and seeded directly with freshly isolated primary lung epithelial cells. To better recapitulate the lung microenvironment, cultures were seeded and kept at air liquid interface (ALI) for up to three weeks in supportive media (Figure 2-8A).

Decellularized lungs were kept in cold PBS for a maximum of one week following antimicrobial treatment. To generate thick sections of scaffolds, individual lobes were embedded in low melting point agarose and sectioned completely with a vibratome to generate 350μm thick segments of decellularized tissue. Each lung section was placed on a hydrophobic floating membrane to create ALI. Lung epithelial cells were spun down and resuspended in a small volume of culture media and seeded directly onto each section.

Seeded primary cells successfully repopulated the scaffolds. Several cell densities ranging from $10^4$ to $10^6$ cells per scaffold culture were tested. The optimal cell density that resulted in significant recellularization with optimal organization was identified as 100,000 cells per scaffold segment. Tissue analysis of day 21 cultures showed pKRT$^+$ epithelial cells that had organized into tubular structures (Figure 2-8B). Despite enriching for epithelial cells by selectively removing fibroblasts during isolation and plating, cells expressing mesenchymal marker vimentin (VIM)$^+$ were found in cell-matrix cultures. Epithelial structures expressing either distal or proximal markers were identified (Figure 2-9). Distal lung epithelial cell markers KRT18 and proSFTPC were detected, while other regions expressed proximal lung epithelial cell markers SCGB1A1 and TUBB4A. Primary cells isolated from their in vivo environment often lose their phenotype after a short period in culture, however seeded cells were able to maintain their differentiated state following 21 days of in vitro culture on lung scaffolds.
Figure 2-8 Lung scaffolds support primary rat embryonic epithelial cell adherence and organization

Figure 2-8 (A) Schematic representation of recellularization setup. Isolated primary rat fetal (E19) epithelial cells are seeded on thick sections (350μm) of acellular lung scaffold and cultured on a floating hydrophobic polycarbonate membrane to achieve an air-liquid interface. Cultures were maintained up to 21 days. (B) Tissue sections of day 21 cell-matrix cultures with H&E staining (left image) and IF staining (right image) for pKRT, and mesenchymal cell marker VIM. Tissue sections show recellularization of scaffolds with primary cells and organization of pKRT+ epithelial cells into tubular structures. Both images at 200x magnification.
**Figure 2-9** Primary embryonic rat epithelial cells maintain lung airway and alveolar phenotype on scaffolds

**Figure 2-9** Immunofluorescent staining and confocal analysis of tissue sections from day 21 cell-matrix cultures. Epithelial structures express markers of both distal alveolar lineage and proximal airway lineage. The two left images show KRT18$^+$ and pSFTPC$^+$ alveolar epithelial cells, while the two right images show SCGB1A1$^+$ and TUBB4A$^+$ airway epithelial cells.
2.5 Discussion

Cell therapy and tissue engineering applications in the lung have been limited compared to other organs. Efforts in lung regeneration have focused on the use of artificial scaffolds and simple two-dimensional culture systems. Such approaches can capture certain microscopic features of the airway and alveolar architecture, but have not produced matrices that can support lung lineage differentiation and tissue that is capable of lung function. Use of decellularized scaffolds for tissue replacement in the respiratory system was first described in 2008 using a decellularized human donor trachea, seeded with epithelial cells and MSCs derived from the recipient. This and other proof of concept studies demonstrate the feasibility of using acellular lungs for tissue regeneration and replacement.

The present study assessed the feasibility of generating decellularized lung scaffolds with the ability to support lung epithelial cell culture, and potentially provide a platform for lung-lineage stem cell differentiation. Decellularization was carried out using three different agents: enzymatic treatment with trypsin, chemical treatments with CHAPS, and SDS detergents. Large variability was found in the extent of cell removal and structural changes to the lung ECM.

Although trypsin has shown promising results in decellularization of heart tissue for aortic valve tissue engineering, it had limited success in this setting with modest removal of resident lung cells and a disruptive effect to the ECM. Ionic detergent SDS is one the most widely used decellularizing agents, however it proved to be too harsh and therefore inferior to the milder zwitterionic detergent CHAPS for achieving optimal lung decellularization. These observations highlight the importance of identifying and optimizing a tissue-specific protocol. Due to tissue differences in cell density and matrix composition, there is no universal method for decellularization of all organs. Although removal of resident cells from different types of tissues can be a relatively easy task, generating viable tissue scaffolds notably requires the more complex task of preserving the extracellular matrix. The ultrastructure of the matrix, specific ECM proteins and matrix bound growth factors may provide site-specific cues for progenitor cells, therefore it is important to develop a protocol that best preserves this environment. The end-goal of generating decellularized lung tissue is to obtain a native-like scaffold for repopulation with mature lung cells or their precursors. Partial cell removal, significant disruption to the ECM or incomplete removal of decellularizing agents after treatment will undoubtedly hinder any further application of scaffolds for cell culture and tissue engineering.
Following histological analysis of treated lungs, CHAPS was selected as the best decellularizing agent and the solution was further optimized with the addition of hypertonicity and chelating agent EDTA. Removal of all resident cells was confirmed by tissue staining and DNA analysis. Scanning EM micrographs confirmed that despite complete decellularization, the ECM ultrastructure of the large airways, vessels and alveolar regions were kept intact. Immunostaining for major matrix proteins confirmed that collagen I, collagen IV, laminin, fibronectin and HS proteoglycans were intact on scaffolds. The number of lavages during decellularization, however, was found to affect the amount of protein left intact. Basement membrane protein laminin was found to decrease with increased number of lavages, therefore 10 was set as the limit in the protocol to minimize ECM disruption. Biomechanical integrity of the matrix was assessed by tensile testing, and surprisingly despite removal of cellular components the tensile strength curves of natural and decellularized lung had comparable curve profiles, although an average of 22% reduction in peak tensile strength was measured for the acellular tissue.

Other published work have also identified CHAPS to be the best reagent for generating decellularized lung scaffolds. In previous studies, lungs were decellularized after complete removal from the animal, using a bioreactor to continuously circulate large volumes of decellularizing agents for several hours. In this study, rapid decellularization was achieved in approximately 20 minutes using short tracheal lavages with the lungs remaining in situ.

To test biocompatibility of the scaffolds generated using this protocol, we attempted to recellularize lung scaffolds with primary rat fetal lung epithelial cells. Thick sections of scaffolds were generated and seeded directly with freshly-isolated primary cells. Cells were seeded and maintained at air liquid interface to better mimic the lung environment. This approach allowed us to setup cell-matrix cultures consistently between batches and monitor cell adherence, survival and phenotype on scaffolds with varying cell densities and culture durations. We observed that within seven days of culture, seeded cells engrafted on scaffolds and started to form structures reminiscent of lung epithelial cells during early development. Most importantly after 21 days of culture, recellularization of scaffolds led to reestablishment of both proximal and distal epithelial cell populations. Interestingly, epithelial structures were formed with either distal cells expressing KRT18 and pSFTPC, or proximal cells expressing SCGB1A1 and TUBB4A. No tubular structures were identified that contained both airway and alveolar epithelial cells types. This suggests that although lung scaffolds support both populations, region-specific differences
are created during cell-matrix culture favoring one population over the other. Despite the
difficulty of culturing such cells in standard conditions in vitro, seeded pulmonary epithelial cells
thrived on scaffolds.258

We have demonstrated that lung scaffolds generated by this protocol can be used in an in vitro
culture system that supports primary rat fetal lung epithelial cells. Decellularized scaffolds are
suitable for lung epithelial cell culture, suggesting that this environment may be poised for
promoting lung lineage specification of progenitor cells.
Chapter 3

Early differentiation of stem cell-derived endoderm to basal cells on decellularized lung scaffolds
3.1 Rationale

In Chapter 2, we found that decellularized lung scaffolds can support primary lung epithelial cells phenotypes for extended periods in culture. This generated the hypothesis that there may be site-specific cues on lung scaffolds that can support the adherence of stem cell-derived endodermal cells and create an environment that better mimics the lung micro-environment during development in comparison to two-dimensional in vitro cultures.

Objective of study: To determine whether decellularized lung scaffolds can support and promote the culture and differentiation of stem cell-derived endodermal cells.

Results: Recellularization of lung scaffolds with sorted mouse ESC-derived endoderm resulted in engraftment and broad repopulation of scaffolds. Seeded cells organized and differentiated to NKX2.1+/SOX2+ proximal airway progenitor cells. Heterogeneous basal cell populations expressing an array of basal cell markers (TRP63, KRT5, KRT14, PDPN, NGFR) emerge as early as 4 days of culture on scaffolds. The basal cell population drops gradually with longer culture durations. This occurs in parallel with an increase in apoptotic activity and a decrease in proliferation. Individual matrix proteins, matrix substitute Matrigel, and decellularized kidney scaffolds did not support differentiation of seeded endoderm to the lung lineage.

Conclusions: Decellularized lung scaffolds support ESC-derived endoderm and promote differentiation to airway progenitor cells. The initial abundance of heterogeneous basal cells followed by a gradual reduction in this population suggests that basal cells may be precursors to other emerging lung epithelial cells on the scaffold.
3.2 Introduction

Recellularization of lung scaffolds has been attempted with numerous cell types including epithelial cell lines (A549 or C10), endothelial cells, MSCs, and lung cell digests\textsuperscript{175,176,259,260}. These studies have achieved success in creating rudimentary “lung equivalents” with partial function and are bringing us closer to generating viable grafts for tissue replacements. Some studies have engineered lungs using decellularized scaffolds seeded with primary epithelial and endothelial cells for transplantation. These engineered lungs were grafted into pneumonectomized adult rats and showed partial gas exchange capability for short periods \textit{in vivo}\textsuperscript{175,176}.

Although these proof-of-concept studies show promise for lung tissue regeneration, future application of a decellularization strategy requires a renewable, autologous source of pulmonary epithelium for repopulation of scaffolds. Resident lung stem cells or iPSCs are candidates that could be used for this purpose. Lineage restriction of pluripotent stem cells is a dynamic process mediated by many environmental components that include growth factors, cell-matrix interactions, cell-cell signaling, and mechanical forces\textsuperscript{88,148}. The interaction of the ECM with stem cell populations and the role of the ECM in promoting tissue-specific lineage restriction is not entirely understood. Several studies have attempted to promote differentiation of embryonic and induced pluripotent stem cells to lung epithelial cells with the addition of soluble growth factors in monolayer cultures\textsuperscript{261-267}. Repopulation of decellularized scaffolds has been used as an end-point assay in some of these studies, to assess the regenerative potential of the pre-differentiated cells\textsuperscript{261,263-265}. No reports have assessed the inductive capacity of the lung ECM alone during early lung specification. The capacity of decellularized lung scaffolds to promote differentiation to a lung epithelial phenotype and the feasibility to use this as a natural scaffold for the development of functional lung tissue using pluripotent cells are important questions that remain unanswered.

Chapter 2 demonstrated the capacity of lung scaffolds for supporting and maintaining primary lung epithelial phenotypes. In this study, we examine the consequence of culturing ESC-derived endoderm on decellularized scaffolds in supportive media, without the addition of inductive growth factors. Early differentiation of seeded cells is characterized for formation of epithelial structures and upregulation of lung progenitor markers.
Differentiation of endoderm on lung scaffolds is also compared to culture on other matrix protein substitutes.

### 3.3 Materials and Methods

**ESC culture and endoderm induction**

Mouse ESC lines (R1 (Nkx2-1mCherry)\textsuperscript{268}, G4 (dsRed-MST), and 129/Ola (Bry-GFP/Foxa2-hCD4) were maintained below passage 40, in the pluripotent state under feeder-free, serum-free culture using two-inhibitor (2i) conditions\textsuperscript{269}. Endoderm induction was achieved using a four day embryoid body (EB) culture method with activin A treatment using serum-free differentiation medium (SFDM)\textsuperscript{270}. Single cells were seeded at a 20,000 cells/ml density in low adherent plates (Nunc, Roskilde, Denmark) to allow for EB formation for 48 hours. Day 2 EBs were collected and reseeded at 1:2 density in SFDM supplemented with 50ng/ml activin A for an additional 48 hours. Day 4 EBs were harvested and sorted by fluorescence activated cell sorting for Kit oncogene (cKIT\textsuperscript{+}) and CXCR4\textsuperscript{+} cells representing definitive endoderm.

Anterior endoderm was generated for use in real-time (RT) polymerase chain reaction (PCR) as a control. Anteriorization was achieved by seeding sorted definitive endoderm cells as a monolayer on gelatin coated 6-well plates in SFDM supplemented with 10μM SB431542 (Sigma, 431542) and 100ng/ml NOGGIN (R&D Systems, 1967-NV/CF) for 24hours. Anteriorization was confirmed using RT-PCR analysis showing a reduction in Forkhead box A3 (FOXA3) expression (posterior endoderm).

**Decellularization and recellularization**

For a detailed description of decellularized scaffold generation for stem cell culture, please refer to section 2.3. In summary: rat lungs were decellularized using a CHAPS-based decellularization solution followed by a rapid wash and decontamination protocol to remove cellular material. Thick sections (350μM) of lung scaffolds were generated using a vibratome and placed on hydrophobic floating membranes in culture (Whatman #110614) to achieve ALI culture conditions. Scaffolds were seeded with 100,000 sorted definitive endoderm cells (cKIT\textsuperscript{+}/CXCR4\textsuperscript{+}) and maintained for up to 21 days using SFDM, with no additional growth factor supplementation.
Flow cytometry and cell sorting

For definitive endoderm, day 4 EBs were dissociated using TrypLE express (Gibco), for 3 minutes at 37°C. Single cell suspensions were labeled with cKIT and CXCR4 antibodies (Table 3-2) in sort buffer (HBSS- supplemented with 2% (v/v) fetal bovine serum and 10mM 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid (HEPES) buffer) for 20 minutes on ice. Cells were sorted using AriaII-GC (BD Biosciences) and data analysis was carried out using Diva (BD Biosciences) and FlowJo (TreeStar) software.

For identification of mcherry positive cells differentiated on scaffolds, day 7, 14, and 21 cultures were dissociated to isolate seeded cells for flow cytometric quantification. Cell-scaffold tissue cultures were incubated in an elastase solution (1mg/mL elastase in SFDM with 2% FBS, at 37°C for 30 minutes. Tissue was spun to remove elastase, and placed in FBS with 2.5% (v/v) DNAse and minced for one minute at 4°C, followed by vigorous shaking for 2 minutes to aid in releasing of cells. SFDM was added to mixture, and minced tissue was filtered using a pre-wetted 20μm nylon mesh filter. Cellular recovery using this method were as follows, day 7 cultures: 3.63x10⁵ ± 1.1x10⁴ (n=3), day 14 cultures: 4.55x10⁵ ± 5.3x10⁴ (n=3), day 21 cultures: 9.43x10⁵ ± 1.9x10⁵ (n=3). Cells were collected into Sort Buffer and analyzed for mcherry expression using a MoFlo (Beckman Coulter) apparatus and Diva (BD Biosciences) software.

Kidney scaffold decellularization

Adult mouse kidneys were sectioned to 1000μm using a vibratome (Leica). Sections were soaked in 0.1% (w/v) SDS using a peristaltic pump supplying fresh SDS at a flow rate range of 0.4-0.8 rpm for 72 hours. Decellularized kidney scaffolds were soaked in 100x pen/strep for 1 hour to remove any contaminates. Scaffolds were then washed with PBS and SFDM prior to seeding with sorted cKIT⁺/CXCR4⁺ endodermal cells.

Endoderm culture on isolated matrix proteins

Sorted endodermal cells were seeded onto isolate matrix proteins and cultured under the same conditions as with seeded lung scaffolds in SFDM, at air liquid interface using the hydrophobic floating membranes (Whatman, 110614). Matrix proteins used include mouse laminin (BD, 354232), human fibronectin (BD, 356008), rat-tail collagen I (BD, 356236), and mouse collagen
IV (BD, 354233). Matrix proteins were diluted to 10μg/mL and applied to the floating membranes prior to seeding with endoderm. For Matrigel (BD, 356230), a thick coat (200μl) was placed on floating membranes and allowed to gel prior to seeding with endoderm.

**Real time quantitative PCR**

RNA was extracted from cell-scaffold cultures using PicoPure RNA Isolation Kit (Life Technologies), and cDNA synthesis was carried out with 1μg of RNA using SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer’s protocol. Ten micrograms of template cDNA was used for real-time PCR (40 cycles of amplification) using SYBR GreenER quantitative PCR (qPCR) SuperMix with murine specific primer sets (*Table 3-1*). Analysis was performed using StepOnePlus qPCR (Applied Biosystems). Gene expression was normalized to RNA Polymerase II and expressed relative to selected positive (adult tissue, E13 lung) or negative controls (definitive endoderm).

**Histological analysis**

Cell-scaffold cultures were fixed in 4% (w/v) PFA at 4°C for 8 hours. Samples were paraffin embedded and sectioned at 5μm. Slides were rehydrated and standard hematoxylin and eosin staining was performed.

**Immunostaining**

Cell-scaffold cultures were fixed in 4% (w/v) PFA at 4°C for 8 hours. Samples were paraffin embedded and sectioned at 5μm. Sample sections were rehydrated and heat-induced epitope retrieval with citrate buffer was performed. Slides were then blocked for one hour with 5% (v/v) normal donkey serum and 1% (w/v) BSA in PBS for 1 hour at room temperature. Samples were then incubated with primary antibodies at 4°C overnight in humidified chamber, and detected using Alexa Fluor conjugated secondary antibodies (Invitrogen) for one hour at room temperature. Nuclei were counterstained using DAPI (Invitrogen). Details of antibodies are provided in *Table 3-2*. Images were captured with Leica CTRMIC 6000 confocal microscope and Hamamatsu C910013 spinning disc camera (Leica Microsystems Inc.), and analyzed with Volocity software (PerkinElmer).
For immunohistochemical staining for Ki67, sections were rehydrated and heat-induced antigen retrieval with citrate buffer was performed. Sections were immunostained for Ki67 expression using a standard procedure with a Ki67 primary antibody diluted in blocking buffer and incubated overnight at 4°C in humidified chamber. Slides were washed thoroughly with PBS and primary antibody was detected using biotinylated rabbit anti-rat secondary antibody (Jackson ImmunoResearch) diluted 1:200, followed by ABC kit (from Vector). The color was developed by addition of diaminobenzidine (DAB) (Sigma) and counterstained with Mayer’s hematoxylin (Sigma, H9627).

For transferase (TdT)-mediated dUTP nick end labeling (TUNEL) of apoptotic cells, tissue slides were rehydrated and antigen retrieval was completed as stated above. Assay was completed as per manufacturer’s protocol using TUNEL enzyme (Roche, 11767305001) and TUNEL Label Mix (Roche, 11767291910).

Electron microscope analysis

Samples were fixed in 2.5% (v/v) glutaraldehyde in 0.1M phosphate buffer pH7.4. Transmission EM specimens were post-fixed in osmium tetroxide, dehydrated in an ascending series of acetone, infiltrated and embedded in Epon Araldite prior to polymerization at 60°C overnight. Ultrathin sections were then cut and mounted on grids, and stained with uranyl acetate and lead citrate prior to microscopy (JEOL, JEM1011) and image acquisition was completed using a CCD camera (AMT corp.). Scanning EM samples were post-fixed in osmium tetroxide, dehydrated in an ascending series of ethanols and critical point dried. Samples were then mounted on aluminum stubs using double-sided carbon tape. Samples were rendered conductive with a thin coat of gold palladium using a sputter coater and examined and photographed in a field emission scanning EM (JEOL, JSM 6700F).

Statistical analysis

Statistical comparisons were performed using unpaired t-tests. For multiple comparisons of more than two groups, one-way ANOVA was used with Dunnett’s test for significance. P-value of 0.05 or less was considered significant.
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FACS: Fluorescence-activated cell sorting; IF: Immunofluorescent staining
3.4 Results

Endodermal cells organize into epithelial structures and express early lung-lineage markers on decellularized scaffolds

During embryonic development, lung-specific endoderm progenitors originate from definitive anterior endoderm found in the developing foregut\textsuperscript{271,272}. Therefore, definitive endoderm was generated from mouse ESCs using a 4-day embryoid body induction protocol with activin A treatment\textsuperscript{270,273} (Figure 3-1A). Flow cytometric analysis showed that over 50\% of ESCs were induced to definitive endoderm as apparent by the coexpression of cKIT and CXCR4 (Figure 3-1B). Transmission EM analysis of sorted and unsorted endoderm cultures reflected the heterogeneity of unsorted cells (Figure 3-1C).

Both unsorted and endoderm-sorted cells were seeded onto thick sections of decellularized scaffolds. Cell-scaffold cultures were setup in a similar manner to primary epithelia recellularization experiments, and maintained under air liquid interface conditions (Figure 2-8A). Tissue analysis of day 7 cultures showed organization of seeded endodermal cells into tubular structures. Sorted cells formed single-cell layered epithelial-like structures surrounding a lumen, while unsorted cultures showed limited organization and contained an abundance of apoptotic cells (Figure 3-1C). Furthermore, unsorted cell cultures showed several POU5F1\textsuperscript{+} (OCT4) regions of pluripotent cells (Figure 3-1C) in day 7 cultures. Based on these early recellularization studies, subsequent experiments were carried out with sorted definitive endoderm.

Seeded endoderm cells presented a pattern of organization reminiscent of the developing lung, lined by basement membrane proteins collagen IV and laminin (Figure 3-2A). Day 21 scaffold cultures were negative for pluripotency marker POU5F1 and maintained definitive endoderm transcription factor FOXA2 expression (Figure 3-2B). Tubule structures were formed, and over half of the seeded population co-expressed pan-epithelial cell markers Cadherin 1 (CDH1) and panKRT. In contrast to seeded scaffolds, endodermal cells cultured on membranes alone at ALI did not form epithelial structures, nor express any lung epithelial cell markers (Figure 3-2B).

RT-PCR analysis showed maintenance of endoderm transcription factor Foxa2 expression for the duration of culture on scaffolds (Figure 3-3A). Nkx2-1 is an important transcriptional...
regulator of the lung and is considered one of the earliest markers for emergence of lung-specific endodermal cells\textsuperscript{11,13}. There was upregulation of \textit{Nkx2-1} after 7 days of culture on scaffolds, and this expression was maintained for up to 21 days in culture. Proximal (\textit{Sox2}) and distal (\textit{Sox9}) epithelial progenitor markers were both detected at the mRNA level, although \textit{Sox2} levels were greater (Figure 3-3A).

\textit{NXK2-1} is not specific to lung development as it is also a transcriptional regulator during thyroid and forebrain formation\textsuperscript{274}. However, relative to expression of adult tissue, thyroid lineage markers Thyroglobulin (\textit{Tg}) and Paired box 8 (\textit{Pax8}) and neuroectoderm marker Oligodendrocyte transcription factor 2 (\textit{Olig2}) were hardly detected in seeded scaffold cultures (Figure 3-3B). Expression of posterior endoderm lineage markers such as Albumin (\textit{Alb}) (liver), Pancreatic and duodenal homeobox 1 (\textit{Pdx1}) (pancreas), and \textit{Foxa3} (posterior endoderm) was also not noted in lung scaffold cultures (Figure 3-3B).
Decellularized lung scaffolds are repopulated with ES-derived definitive endoderm cells and cultured at air liquid interface.

Figure 3-1 (A) Definitive endoderm induction (FOXA2+, cKIT+ & CXCR4+) is achieved with 48hr treatment of ES-derived embryoid bodies with 50ng/ml of Activin A. (B) Endodermal cells are sorted for cKIT and CXCR4 using fluorescence-activated cell sorting to obtain an enriched population. (C-E) both sorted and unsorted endoderm population was seeded onto scaffolds. (C) Transmission EM of definitive endoderm. Sorted cells appear more uniform compared to unsorted cells which include apoptotic cells and debris. (D) H&E staining of scaffold cultures reveals organization of sorted endodermal cells into tubular structures, superior to seeded unsorted population (day 7). Scale bar=50μm. (E) Confocal micrograph of immunostained scaffold cultures reveals POU5F1+ (OCT4) pluripotent cells in unsorted population after 7 days.
Figure 3-2 Seeded ES-derived endoderm cells form epithelial structures along the basement membrane

\[\text{Figure 3-2} (A) \text{ Seeded endodermal cells repopulate scaffolds and organize into tubular structures. Immunostaining for collagen IV and laminin shows that these scaffold proteins line the developing cellular (DAPI positive) structures. (B) Immunostaining analysis demonstrates that endodermal cells seeded on lung scaffolds maintain definitive endoderm marker FOXA2 and do not express pluripotency marker POU5F1. Structures are positive for epithelial cell markers CDH1 and panKRT. In contrast to cells seeded on scaffolds, endodermal cells cultured at ALI without the support of the lung matrix do not form organized structures and do not express epithelial cell markers. Scale bar represents 25μm.}\]
**Figure 3-3** Definitive endoderm upregulates early lung markers with culture on lung scaffolds

(A) Real-time PCR analysis reveals an upregulation of *Foxa2* and lung progenitor marker *Nkx2-1* expression. Expression is presented relative to E13.5 mouse lungs, mean ± SEM, n=4 experiments. Proximal airway progenitor marker *Sox2* and distal alveolar progenitor marker *Sox9* were both detected, n=4 experiments; with extended culture (day 21), *Sox2* expression is upregulated.

(B) Real-time PCR analysis shows that relative to expression of adult tissue (mean ± SEM, n=4 experiments), thyroid lineage makers *Tg* and *Pax8*, and neuroectoderm marker *Olig2* are hardly detected in seeded scaffolds after 7, 14, and 21 days of culture. Expression of additional endoderm lineage markers including *Pdx1* (pancreas), *Foxa3* (posterior endoderm), and *Alb* (liver) are also marginally detected.
Seeded endodermal cells differentiate to NKX2-1+/SOX2+ early proximal lung progenitors

Using an Nkx2-1mCherry ESC line with a nondisruptive mCherry reporter gene knockin, we were able to capture the lung epithelial progenitor population as it emerged with culture on the scaffolds. IF confocal analysis showed the presence of NKX2-1+/SOX2+/TRP63+ airway basal stem cells in day 7 cultures (Figure 3-4A). A minor population of distal lineage NKX2-1+/SOX9+ progenitor cells was also detected. By day 21, majority of the emerging NKX2-1+ cells co-expressed SOX2, while staining of SOX9 was not detected, indicating lineage restriction to the proximal airway275,276 (Figure 3-4B).

Flow cytometric quantification revealed that 9.8% of cells expressed mCherry at day 7, and this percentage increased to 46.4% with extended culture on scaffolds at day 21 (Figure 3-5). Elastase treatment was used to retrieve cells from scaffolds for flow cytometric analysis. Approximately $9.43 \times 10^5$ cells (n=3) were recovered from each day 21-scaffold culture using this method, and each seeded scaffold culture generated approximately $4.38 \times 10^5$ NKX2-1+ cells (n=3) at day 21. We conclude that decellularized lung scaffolds are able to support the adherence and organization of seeded endodermal cells and promote the upregulation of lung progenitor marker NKX2-1, with preference for the proximal airway lineage.

To determine whether individual matrix proteins can promote lung-lineage differentiation with ALI culture, we seeded endodermal cells on floating membranes coated with either collagen I, collagen IV, fibronectin, laminin, or matrix substitute Matrigel (Figure 3-6A). RT-PCR analysis showed the upregulation of all endodermal lineage markers Pax8, Foxa3, Pdx1, and neuroectoderm marker Olig2 on single matrix proteins compared with decellularized lung scaffolds (Figure 3-6B).

Analysis of day 21 cultures by tissue staining showed that no individual matrix protein or Matrigel was able to promote organization into epithelial structures comparable to that achieved with lung scaffolds (Figure 3-7A). High-magnification transmission EM analysis confirmed this observation, showing limited organization and lung lineage specification of endoderm on individual matrix proteins (Figure 3-7B).

To examine the ability of scaffolds derived from other organs in promoting lung lineage specification, we seeded decellularized mouse kidney scaffolds (mesoderm germ layer origin)

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with definitive endoderm. Kidney scaffolds were seeded in the same culture setup under ALI as lung scaffolds. Numerous attempts of kidney scaffold recellularization with definitive endoderm at varied densities did not repopulate the scaffolds (Figure 3-7C). Definitive endoderm did not adhere and proliferate, suggesting that kidney-derived ECM is distinct from lung-derived ECM in its ability to support and promote endoderm-lineage specification.
Figure 3-4 Seeded Endodermal Cells Differentiate to NKX2-1+/SOX2+ Proximal Lung Progenitors with Culture on Decellularized Scaffolds

(A-B) Immunostaining of day 7 cultures shows the presence of NKX2-1+/SOX2+/TRP63+ co-expressing airway basal stem cells. A small population of distal lineage NKX2-1+/SOX9+ progenitor cells is also present at this time point. Top panel scale bar represents 13μm; bottom panel scale bar represents 25μm. (B) Immunostaining of day 21 cultures for NKX2-1, SOX2, and SOX9 reveals that the majority of NKX2-1+ cells co-express SOX2, while SOX9 protein expression is rare. Scale bar represents 50μm.
**Figure 3-5** NKX2-1 progenitor cells increase with longer culture on lung scaffolds

![Flow cytometry bar chart showing increase in NKX2-1 mcherry+ cells with extended culture](image)

**Figure 3-5** NKX2-1\textsuperscript{mcherry+} cells are quantified with flow cytometry. The proportion of NKX2-1\textsuperscript{+} cells increases with extended culture up to 46.42% ± 3.6% at day 21, n=3 experiments.
Figure 3-6 Individual matrix proteins do not support lung lineage commitment of seeded endodermal cells

(A) Schematic representation of alternate scaffold recellularization. Sorted endodermal cells are seeded on floating membranes coated with specific matrix proteins or Matrigel and cultured at air-liquid interface in base supportive media. (B) Real-time PCR analysis reveals upregulation of other endodermal lineage (*Pax8*, *Foxa3*, *Pdx1*) and neuroectoderm (*Olig2*) markers with culture of definitive endoderm on individual matrix proteins compared with lung scaffolds. Gene expression is presented relative to definitive endoderm; mean ± SEM, n=3 experiments.
Figure 3-7 Endoderm culture without lung scaffold does not promote differentiation to a lung phenotype

(A-B) H&E staining and transmission EM of seeded endodermal cells reveal the inability of individual matrix proteins and Matrigel to promote differentiation to lung epithelial cells. H&E scale bar=50μm, EM scale bar=2μm. (C) Decellularized kidney scaffolds seeded with definitive endoderm and cultured at air liquid interface cannot support the adherence, proliferation, and differentiation of sorted endodermal cells. Scale bar=50μm.
Early endoderm-scaffold culture promotes the emergence of a heterogeneous basal cell population

Basal cells found in the mouse trachea and human airways are considered to be the adult lung stem cell population. They can undergo rapid expansion and differentiate to more specialized progeny when needed during homeostasis and in response to injury. Basal cells expressing TRP63, KRT5 and KRT14 have been shown give rise to ciliated and club cell lineages both in vivo and in vitro culture systems. To determine whether basal cells are an early progenitor population emerging from seeded endodermal cells on lung scaffolds, cell-scaffold cultures at various time points were stained for a panel of known basal cell markers: TRP63, KRT5, KRT14, PDPN, and NGFR.

IF confocal analysis showed that many epithelial structures positive for basal cell marker TRP63 co-expressed KRT5, PDPN, NGFR or KRT14 after 7 days of culture. Basal cell populations appeared to be heterogeneous, with some expressing only TRP63, while others were positive for both TRP63 and KRT5. A rarer population of TRP63+/KRT5+ cells was also identified on scaffolds. The emergence of different basal cell populations during early (day 4-7) and late (day 14-21) time points in culture are summarized in Figure 3-9B. Transmission EM micrographs of day 7 cultures show an abundance of unspecialized cells lining epithelial structures, likely representative of basal cell morphology at this time point (Figure 3-9C).

Real-time PCR analysis of day 4, 7, 14, and 21 scaffold cultures showed upregulation Trp63 and Krt5 expression at day 7 of culture (Figure 3-10A). This expression remained high compared to endodermal cells, but dropped gradually with longer culture durations. This drop in gene expression was paralleled in IF tissue staining for TRP63+/KRT5+ basal cells in cultured tissue segments (Figure 3-10B). With longer duration, the number of basal cells decreased and the morphology of the epithelial structures changed. In early day 4 and day 7 cultures, smaller sporadic structures were found and with extended culture the structures appeared to elongate and started to resemble mature mouse airways lined with TRP63+/KRT5+ basal cells (Figure 3-10B). These results were consistent with previously published work on the progenitor activity of airway basal cells, suggesting that multipotent TRP63+ basal stem cells in early cultures may be differentiating to specialized secretory and ciliated cells lining the developing airway structures.

Longer culture on scaffolds also resulted in a decline in cell proliferation, as apparent with a reduction in Ki67+ cells (Figure 3-11A). Day 7 cultures resembled highly proliferative mouse embryonic lungs, while day 21 cultures resembled adult lung tissue with completely
differentiated epithelial cells. This decrease in proliferation occurred in parallel with an increase in the number of apoptotic cells at day 21 compared to day 7 (Figure 3-11B). Apoptosis was detected using TUNEL staining of tissue.
**Figure 3-8** Epithelial structures positive for basal cell markers are present on early scaffold cultures

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<tr>
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**Figure 3-8** Immunofluorescent confocal images of day 7 seeded scaffold cultures. Epithelial structures positive for at least two early basal cell markers are identified (arrow heads). Basal cell populations identified include: TRP63+/KRT5+, PDPN+/TRP63+, NGFR+/TRP63+ and KRT14+/NGFR+. Scale bar=25μm.
Figure 3-9 Basal cells identified on early and mature scaffold cultures are a heterogeneous population

(A) Immunofluorescent confocal images of day 7 and 14 scaffold cultures for TRP63 and KRT5 basal cell markers. Basal cells positive for one or both markers can be identified, KRT5+/TRP63− (arrowhead), KRT5+/TRP63+ (arrow), KRT5−/TRP63+ (double arrowhead). Scale bar=50 μm.

(B) Summary of basal cell populations at different time points of scaffold culture, “+”: found in culture, “−”: not found in culture.

(C) Transmission electron micrograph of day 7 epithelial cells on scaffold cultures. Structures lack specialized cell characteristics at this time point. Left scale bar=4 μm, right scale bar=2 μm.
**Figure 3-10** Basal cell population drops with longer culture duration as epithelial structures mature on scaffolds

(A) Real-time PCR analysis reveals upregulation of basal cell markers *Trp63* and *Krt5* at seven days of scaffold culture. Expression of both genes drops with longer culture duration; mean ± SEM, n=3 experiments.

(B) Immunofluorescent confocal images for TRP63⁺ and KRT5⁺ expression at different time points on scaffolds. Basal cells become more organized with longer culture duration and start to resemble mature airway structures lined with TRP63⁺/KRT5⁺ basal cells.
Figure 3-11 Extended culture on scaffolds shows a reduction in Ki67⁺ proliferative cells, while the proportion of apoptotic cells stained with TUNEL increase.

<table>
<thead>
<tr>
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<th>Natural Mouse Lung</th>
<th>Scaffold Culture</th>
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<tbody>
<tr>
<td>A</td>
<td>E16</td>
<td>Day 7, Day 21</td>
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</table>

Immunostaining for Ki67 shows a decline in proliferation with progressive differentiation of seeded cells to airway epithelia from day 7 to day 21 (scale bar represents 25µm); mean ± SEM, n=3 experiments. (B) TUNEL staining for identification of apoptotic cells on tissue sections was completed for different time points. Cell counts of immunostained sections shows the percentage of apoptotic (TUNEL⁺) cells at days 7, 14, and 21 cultures. Scale bars represent 25µm. Mean ± SEM, n=3 experiments.
3.5 Discussion

Recent reports have had success with promoting differentiation of pluripotent stem cells to NKX2-1+ lung progenitor cells using monolayer cultures and supplementation with inductive factors. However, there have been no reports of the differentiation potential of natural lung ECM alone in promoting lung lineage restriction, without the use of exogenous factors. In this study we showed that decellularized scaffolds alone directed differentiation of definitive endoderm to NKX2-1+/SOX2+ proximal lung progenitor cells and showed the emergence of a heterogeneous population of mainly TRP63+ basal cells (Figure 3-12). Lung progenitors were detected at day 7 of culture and were in abundance by day 21, with approximately 46% of all cells expressing NKX2-1. This rise could be due to the preferential expansion of NKX2-1+ cells on scaffolds or apoptosis of cells that are not committed to the lung lineage. Although few NKX2-1+/SOX9+ cells were also detected after at 7 days, they disappeared with longer duration of culture, suggesting that differentiation to the distal lineage perhaps requires additional inductive factors after NKX2-1+ specification on scaffolds.

Interestingly, this distinct ability of decellularized lung scaffolds to differentiate endoderm into airway progenitor cells with limited contamination from other endoderm lineages was further restated by endoderm culture on individual matrix proteins (fibronectin, laminin, collagen I, collagen IV) and ECM substitute Matrigel. Unlike lung scaffold cultures, an upregulation of thyroid, pancreas and liver epithelial cell markers was noted in these cultures. Furthermore, decellularized kidney scaffolds were not successful in supporting endoderm culture and consequently tissue-specific differentiation. This suggests that a mesoderm-derived organ like the kidneys cannot readily support adherence and differentiation of cells from a different germ layer, such as endoderm.

Lineage tracing experiments have shown that basal cells can undergo long-term self-renewal and give rise to ciliated and secretory luminal cells when required. In this study, endoderm-derived basal cells were found in abundance in early proliferative day 7 cultures. However, the basal cell population declined steadily with extended culture, parallel with a decline in proliferative activity on scaffolds. Basal cells are a heterogeneous cell population with diverse expression profiles of TRP63, KRT5, KRT14, PDPN, and NGFR. This heterogeneity was also found on scaffold cultures, leading to questions regarding the regenerative capacity and differentiation ability of different basal cell subpopulations. The initial abundance followed by a
fall in cell numbers with extended culture on scaffolds, suggests that basal cells could be progenitor cells that with extended culture undergo a terminal differentiation program and give rise to specialized lung epithelial cells.

This culture setup provides a means to examine early endoderm specification to NKX2.1\(^+\) lung-specific progenitor cells. Lineage restriction to SOX2\(^+\) proximal airway-progenitor cells was observed with the emergence of a heterogeneous basal stem cell population. We conclude that decellularized lung scaffold cultures provide a 3D \textit{in vitro} platform to better understand early cell-matrix signaling during proximal lineage lung specification.
**Figure 3-12** Definitive endoderm differentiates to proximal airway progenitors and basal epithelial cells after 7 days of scaffold culture

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**Figure 3-12** Schematic representation summarizing this chapter’s characterization of early endoderm differentiation on lung scaffolds. Seven day of endoderm culture on decellularized lung without the addition of exogenous growth factors resulted in differentiation to NKX2-1⁻/SOX2⁺ airway progenitors and a heterogeneous TRP63⁺ basal cell population.
Decellularized lung scaffolds direct differentiation of endoderm to functional airway epithelial cells, with the requirement of matrix-bound heparan sulfate proteoglycans
4.1 Rationale

Three-dimensional cultures supplemented with various morphogens and growth factors have shown some success in promoting differentiation of pluripotent cells to the lung lineage. In Chapter 3 we found that decellularized lung scaffolds promote the differentiation of stem cell-derived endodermal cells to proximal airway progenitor cells as early as seven days of ALI culture, without the need for exogenous inductive factors. We believe that decellularized lung scaffolds could drive further differentiation of proximal progenitors to mature airway epithelial cells and that growth factor-binding ECM proteins such as chondroitin sulfate and heparan sulfate proteoglycans may be involved in mediating this matrix-guided differentiation.

**Objective of study:** To characterize the multi-differentiation potential of seeded endodermal cells driven by decellularized lung scaffolds.

**Results:** Extended culture of ESC-derived endoderm on decellularized scaffolds resulted in the formation of fully differentiated luminal airway structures with TRP63+/KRT5+ basal cells, SCGB1A1+ club cells, and FOXJ1+/TUBB4A+ beating ciliated cells. This was achieved with notable morphological and functional resemblance to native airway epithelia. Differentiated day 21 cells exhibited functional cystic fibrosis transmembrane conductance regulator (CFTR) protein expression. Disruption of HS proteoglycans on lung scaffolds using heparitinase-I enzymatic treatment resulted in a complete loss of organization and differentiation.

**Conclusions:** Here we report the robust differentiation of embryonic stem cell-derived endoderm to mature, functional airway epithelial cells using defined, serum-free culture on decellularized lung scaffolds at air liquid interface. We demonstrate the importance of a 3D matrix environment with site-specific cues that are bound to heparan-sulfate proteoglycans, necessary for achieving differentiation to airway epithelial cells on lung scaffolds.
4.2 Introduction

Directed differentiation of pluripotent cells to the lung lineage is dependent on precise signaling events in the microenvironment. Due to the dynamic nature of this process it has been challenging to mimic the precise events of lung organogenesis in vitro to generate mature functional lung epithelial cells. Recent reports have used step-wise lineage restriction strategies with soluble growth factor supplementation of two-dimensional cultures to achieve lung differentiation. In these reports, pluripotent cells, whether embryonic or induced pluripotent stem cells, were first differentiated to the definitive endoderm germ layer. Endodermal cells were subsequently pushed to an anterior endoderm fate and thereafter to lung progenitor cells, as identified by the expression of NKX2-1. These lung progenitors were further differentiated to proximal airway or distal alveolar lung epithelial cells with continued growth factor supplementation. Such two-dimensional strategies have had some success in generating lung epithelial cells, however there are several limitations including low efficiencies, possible contamination from other endodermal lineages, lack of a 3D structure, and in some instances use of undefined culture conditions with serum supplementation.

Repopulation of decellularized scaffolds has been used as an end-point assay to assess regenerative potential of pre-differentiated cells. A recent report has demonstrated the importance of the matrix environment for maintaining lung progenitor identity, but again using pre-differentiated NKX2-1+ lung progenitor cells and growth factor-supplemented culture media, precluding assessment of the scaffolds alone on differentiation. No reports have assessed the inductive capacity of the ECM alone during lung specification.

Lung development involves the division, migration, gene expression and differentiation of individual cells in response to environmental cues. The ECM is a complex and dynamic structure that in addition to providing mechanical support can guide tissue morphogenesis by integrating and regulating these processes. The lung ECM can be used as a natural platform for endoderm culture to better mimic the in vivo lung developmental milieu. In chapter 3 we demonstrated the ability of decellularized lung scaffolds to promote differentiation of seeded endoderm to NKX2-1+ lung progenitor cells and to a heterogeneous population of basal stem cells. In this study we examine the potential of these scaffolds to direct differentiation of seeded cells to mature, fully functional lung epithelial cell populations. We uncover an important role...
for matrix-bound heparan sulfate proteoglycans in supporting endoderm culture on scaffolds and for achieving differentiation to airway epithelial cells.

4.3 Materials and Methods

ESC culture and endoderm induction

Mouse ESC lines (R1 (Nkx2-1mCherry), G4 (dsRed-MST), and 129/Ola (Bry-GFP/Foxa2-hCD4) were maintained below passage 40, in the pluripotent state under feeder-free, serum-free culture using 2i conditions\textsuperscript{268,269}. Endoderm induction was achieved using a four day EB culture method with activin A treatment using serum-free differentiation medium SFDM\textsuperscript{270}. Single cells were seeded at a 20,000 cells/ml density in low adherent plates (Nunc, Roskilde, Denmark) to allow for EB formation for 48 hours. Day 2 EBs were collected and reseeded at 1:2 density in SFDM supplemented with 50ng/ml activin A for an additional 48 hours. Day 4 EBs were harvested and sorted by fluorescence activated cell sorting for cKIT\textsuperscript{+}/CXCR4\textsuperscript{+} cells representing definitive endoderm.

Anterior endoderm was generated for use in RT-PCR analysis as a control. Anteriorization was achieved by seeding sorted definitive endoderm cells as a monolayer on gelatin coated 6-well plates in SFDM supplemented with 10μM SB431542 (Sigma, 431542) and 100ng/ml NOGGIN (R&D Systems, 1967-NV/CF) for 24hours. Anteriorization was confirmed using RT-PCR analysis showing a reduction in Foxa3 expression (posterior endoderm).

Decellularization and recellularization

For a detailed description of decellularized scaffold generation for stem cell culture, please refer to section 2.3. Briefly: rat lungs were decellularized using a CHAPS-based decellularization solution followed by a rapid wash and decontamination protocol to remove cellular material. Thick sections (350μM) of lung scaffolds were generated using a vibratome and placed on hydrophobic floating membranes in culture (Whatman #110614) to achieve ALI culture conditions. Scaffolds were seeded with 100,000 sorted definitive endoderm cells (cKIT\textsuperscript{+}/CXCR4\textsuperscript{+}) and maintained for up to 21 days using SFDM, with no additional growth factor supplementation.

Fluorescence activated cell sorting
For definitive endoderm, day 4 EBs were dissociated using TrypLE express (Gibco), for 3 minutes at 37°C. Single cell suspensions were labeled with cKIT and CXCR4 antibodies in sort buffer (HBSS- supplemented with 2% (v/v) fetal bovine serum and 10mM HEPES buffer) for 20 minutes on ice. Cells were sorted using an AriaII-GC cell sorter (BD Biosciences) and data analysis was carried out using Diva (BD Biosciences) and FlowJo (TreeStar) software.

**Histological analysis**

Cell-scaffold cultures were fixed in 4% (w/v) PFA at 4°C for 8 hours. Samples were paraffin embedded and sectioned at 5μm. Slides were rehydrated and standard hematoxylin and eosin and periodic acid-Schiff (PAS) stainings were performed.

**Real time quantitative PCR**

RNA was extracted from cell-scaffold cultures using PicoPure RNA Isolation Kit (Life Technologies) and cDNA synthesis was carried out with 1μg of RNA using SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer’s protocol. Ten micrograms of template cDNA was used for real-time PCR (40 cycles of amplification) using SYBR GreenER qPCR SuperMix with murine specific primer sets (*Table 4-1*). Analysis was performed using StepOnePlus qPCR (Applied Biosystems). Gene expression was normalized to RNA Polymerase II and expressed relative to selected positive (adult tissue, E13 lung) or negative controls (definitive and anterior endoderm).

**Immunostaining**

Cell-scaffold cultures were fixed in 4% (w/v) PFA at 4°C for 8 hours. Samples were paraffin embedded and sectioned at 5μm. Sample sections were rehydrated and heat-induced epitope retrieval with citrate buffer (pH 6.0) was performed. Slides were then blocked for one hour with 5% (v/v) normal donkey serum and 1% (w/v) BSA in PBS for 1 hour at room temperature. Samples were then incubated with primary antibodies at 4°C overnight in humidified chamber, and detected using Alexa Fluor conjugated secondary antibodies (Invitrogen) for one hour at room temperature. Nuclei were counterstained using DAPI (Invitrogen). Details of antibodies are provided in *Table 4-2*. Images were captured with Leica CTRMIC 6000 confocal microscope.
and Hamamatsu C910013 spinning disc camera (Leica Microsystems Inc.), and analyzed with Volocity software (PerkinElmer).

**Immunoblot analysis**

For SCGB1A1 detection, 50μg of day 21 cell-scaffold culture media, and adult rat lung lysate as positive control, were separated on a 3%–12% (w/v) gradient SDS-PAGE gel (Invitrogen). Proteins were transferred to nitrocellulose paper (Millipore) and blocked with 3% (v/v) milk in PBS plus 0.1% (v/v) Tween-20. The membranes were then incubated overnight at 4°C with anti-SCGB1A1 antibody (Table 4-2) at 1:1000 dilution in 1% (v/v) PBS-milk containing 0.2% (v/v) Tween-20. Blots were then incubated for 1 hour with secondary antibody conjugated with peroxidase, and were developed using chemiluminescent substrates (PerkinElmer) according to the manufacturer’s instructions and imaged on X-ray film.

**Electron microscope analysis**

Samples were fixed in 2.5% (v/v) glutaraldehyde in 0.1M phosphate buffer pH7.4. Transmission EM specimens were post-fixed in 1% (w/v) osmium tetroxide, dehydrated in an ascending series of acetone, infiltrated and embedded in Epon Araldite prior to polymerization at 60°C overnight. Ultrathin sections were then cut and mounted on grids, and stained with uranyl acetate and lead citrate prior to microscopy (JEOL, JEM1011) and image acquisition was completed using a CCD camera (AMT corp.). Scanning EM samples were post-fixed in osmium tetroxide, dehydrated in an ascending series of ethanol and critical point dried. Samples were then mounted on aluminum stubs using double-sided carbon tape. Samples were rendered conductive with a thin coat of gold palladium using a sputter coater and examined and photographed in a field emission scanning EM (JEOL, JSM 6700F).

**Efflux assay**

Iodide efflux from differentiated cultures following cyclic adenosine monophosphate (cAMP) agonist stimulation was measured periodically to assess CFTR channel activity. Day 21 scaffold cultures were loaded with sodium iodide solution (3.0mM KNO₃, 2.0mM Ca(NO₃)₂, 11mM glucose, 20mM HEPES, 136mM NaI) in a 6-well plate for 1h at 37°C, for iodide uptake (three cell-scaffold cultures were pooled for each assay) measurements. Samples were then washed 10
times with wash solution (3.0mM KNO₃, 2.0mM Ca(NO₃)₂, 11mM glucose, 20mM HEPES, 136mM NaNO₃) and epithelium sodium channel (ENaC)-specific inhibitor amiloride (100μM). The last wash was collected as a blank reading, followed by eight sequential readings of cAMP-stimulated halide flux using 300μl of wash solution containing FIG (10μM forskolin, 100μM 3-isobutyl-1-methylxanthine, and 50μM genistein). Each reading represented 1 minute of exposure to FIG wash solution. Vehicle dimethyl sulfoxide (DMSO) was used as a negative control reading. Solutions from each one-minute time point were collected into a 96 well plate and absolute iodide electrode potential value (mV) was measured using a halide-selective microelectrode (Lazar Research Laboratories). Readings were recorded using Digidata 1320A Data Acquisition System and Clampex 8.1 software. Using a calibration curve, recorded mV values were converted to iodide concentration in μM.

**Enzymatic treatment of scaffolds**

Decellularized scaffolds were treated with Heparitinase I solution (0.1M sodium acetate, 10mM calcium acetate, 10mU heparitinase I (Amsbio, 100704)) for 4 hours at 37°C. Enzymatic activity was confirmed by UV spectral analysis of treated scaffolds at 232nm for HS disaccharides in the wash supernatant, as well as Tomato-lectin staining (Vector Laboratories, B-1175 1:500 dilution; detection with PE-conjugated Streptavidin, Biolegend, 405203 1:200 dilution) of the untreated and treated scaffolds. Alternatively, scaffolds were treated with chondroitinase ABC solution (0.4M Tris-HCl buffer pH8.0, 0.4M sodium acetate, 0.1% (w/v) BSA, 5mU chondroitinase ABC (Amsbio, 100330-1A) for 1 hour at 37°C. Enzymatic activity was confirmed by immunoblot analysis for removal of CS proteoglycans from treated scaffolds. Following enzymatic treatment, scaffolds were rinsed first with PBS (containing penicillin-streptomycin and amphotericin B) for one hour, followed by SFDM media. Sorted endodermal cells were seeded on scaffolds and cultured at air liquid interface as previously described.

**Lung matrix conditioned media preparation**

Decellularized lung scaffolds were treated with Heparitinase I (Amsbio 100704) as described above. Following treatment, scaffolds were rinsed with PBS five times (10mL of solution total). The post-rinse PBS was collected, filtered through a 0.2μM filter to remove debris, and added at a 1:10 (PBS:media) ratio to SFDM base differentiation media for “rescue” culture experiments with Heparitinase-treated scaffolds.
**Protein profiler array**

A protein antibody array (R&D Systems, ARY015) was used to identify candidate analytes remaining on decellularized scaffolds, as per manufacturer’s instructions. Briefly, following decellularization, scaffolds treated with or without heparitinase I were homogenized in PBS with protease inhibitors. After homogenization, 1% (v/v) triton X-100 was added to samples. Samples underwent one freeze-thaw cycle and were then centrifuged at 10,000g for 5 minutes to remove cellular debris. Protein concentration was determined using Bradford protein assay (Bio-rad). Sample lysates were diluted (300μg of protein per sample), mixed with a cocktail of biotinylated detection antibodies, and incubated overnight with the protein array membrane. Streptavidin-HRP and chemiluminescent detection reagents were used for detection. A signal was generated at each capture spot on membrane, corresponding to the amount of protein bound. Pixel density for each spot was detected and quantified using ImageJ open access software.

**Statistical analysis**

Statistical comparisons were performed using unpaired t-tests. For multiple comparisons of more than two groups, one-way ANOVA was used with Dunnett’s test for significance. P-value of 0.05 or less was considered significant.
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FACS: Fluorescence-activated cell sorting; IF: Immunofluorescent staining; WB: Western blot
4.4 Results

Prolonged culture of endodermal cells on lung scaffolds promotes differentiation to multiciliated cells and secretory club epithelial Cells

To determine whether NKX2-1+/SOX2+ cells cultured on lung scaffolds alone differentiate further to mature epithelia, seeded cells were examined for expression of proximal and distal lung epithelial cell markers. RT-PCR analysis showed upregulated gene expression of proximal airway epithelia Foxj1 (ciliated cell), Scgb1a1 (club cell), Trp63 (basal cell), and Cftr (Figure 4-1A), while expression of distal lung epithelial cell markers Aqp5 (type I alveolar cell marker), Sftpb, and Sftpc (type II alveolar cell markers) were much lower (Figure 4-1B).

Tissue staining of day 21 culture sections obtained from various tissue depths showed that decellularized scaffolds were completely repopulated after seeding, and formed structures resembling mouse airways in the adult lung (Figure 4-2). IF confocal microscopy of day 21 scaffold cultures revealed ciliated cells (FOXJ1+, TUBB4A+), club cells (Claudin10 (CLDN10)+, SCGB1A1+), and basal cells (TRP63+, KRT5+) (Figure 4-3). Quantification of cells with positive immunostaining for lung lineage markers revealed 24.3% TUBB4A+, 21.1% SCGB1A1+, and 21.7% TRP63+ cells at 21 days of culture on scaffolds. Compared to day 7 cultures, there was a gradual reduction in the proportion of basal cells in day 14 and day 21 cultures. This observation parallels temporal Trp63 and Krt5 mRNA expression levels in scaffold cultures, observed in the previous chapter (Figure 3-10A). With extended culture, proportion of ciliated cell and club cell populations rise as the basal cell population drops (Figure 4-3A).

Decellularized scaffolds were generated from both upper airway and alveolar lung regions (Figure 4-4). Comparable cellular organization and differentiation to proximal lung epithelial cells was achieved on lung scaffolds generated from both regions. With the exception of a small NKX2-1+/SOX9+ distal progenitor population detected only at day 7, no distal epithelial cells were found in culture at day 14 and day 21 of scaffold culture.

EM analysis was used for high-magnification morphological analysis of differentiated epithelia (Figure 4-5A-C). Seeded cells resembled that of native mouse airways (Figure 4-5D-E), where mature multiciliated cells were present among nonciliated secretory cells with rounded apical surfaces. Both scanning and transmission EM analyses revealed tight junction-coupled
pseudostratified columnar epithelial sheets reminiscent of adult airway epithelial structures. Close examination of mature cultures also revealed the presence of pit structures reminiscent of emerging submucosal glands that function to adjust the flow of fluid and mucus secretions in the proximal segments of the respiratory tracts\textsuperscript{283-285}. Scaffold cultures showed an upregulation of mucin-producing cell marker mucin 5, subtypes AC (\textit{Muc5ac}) with prolonged culture (Figure 4-6A). Numerous gland-like orifices were discovered with scanning EM analysis, and a strong PAS-positive stain was apparent in the developed submucosal tubules (Figure 4-6B-C).

Decellularized scaffolds derived from mouse lungs seeded with definitive endodermal cells produced similar results to that obtained with rat lung scaffolds. Differentiation of definitive endoderm to airway epithelia using rat lung scaffolds was reproducible with two additional mouse ESC lines: G4 and 129/Ola, as well as a rat ESC line (DAc8). Overall, decellularized adult lung scaffolds promote efficient differentiation of stem cell-derived definitive endoderm to airway epithelial cells.
Figure 4-1 Endoderm culture on lung scaffolds promotes upregulation of airway epithelial gene expression

(A) RT-PCR analysis reveals the expression of airway epithelial genes Foxj1, Scgb1a1, Trp63, and Cfr. (B) Alveolar epithelial markers Aqp5, Sftpb, and Sftpc were detected at much lower levels, although Aqp5 and Sftpb increased with extended culture. Gene expression is presented relative to adult lungs, mean ± SEM, n=4 experiments, *p < 0.001.
**Figure 4-2 Seeded endoderm repopulates entire lung scaffold**

**Figure 4-2** Hematoxylin and eosin staining of day 21 tissue sections generated from various depths shows repopulation of entire decellularized scaffolds. Seeded cells show structures typical of adult mouse airway epithelia.
Figure 4-3 Immunofluorescent and confocal analysis of differentiation to airway epithelial on scaffold cultures

A

![Immunofluorescent images showing differentiation to airway epithelial on scaffold cultures](image)

B

![Additional immunofluorescent images](image)
Figure 4-3 Immunofluorescent and confocal analysis of differentiation to airway epithelial on decellularized lung scaffolds

IF staining and confocal microscopic analysis of day 21 cell-matrix cultures showed mature airway epithelial populations. (A) IF tissue stain showing TUBB4A⁺ ciliated cells (green), SCGB1A1⁺ club cells (red) and KRT5⁺ basal cells (yellow). Scale bar represents 50μm. Cell proportions were quantified as percent of total cell population by cell counts from three experiments at days 7, 14, and 21. A reduction in basal cell population is seen with extended culture, while ciliated and club cells expand with longer culture duration. Mean ± SEM, n = 3 experiments. (B) Immunostaining of day 21 cultures for additional airway lineage markers shows the presence of ciliated cells and club cells positive for markers: TUBB4A⁺,CLDN10⁺ (left image), and FOXJ1⁺, SCGB1A1⁺ (right image). Scale bars=25μm. CLDN10⁺ represents secretory club cells, and FOXJ1⁺ represents ciliated cells.
Figure 4-4 Proximal and Distal Lung Scaffolds Both Promote Airway Differentiation

Figure 4-4 Decellularized sections from both the proximal (A) and distal (B) regions of the lungs were generated and seeded with sorted endodermal cells. Tissue and IF staining show that both scaffold sources promoted differentiation to airway ciliated (TUBB4A) and club (SCGB1A1) cells. Scale bars represent 25μm.
Figure 4-5 Scanning and transmission electron microscopy show mature ciliated cell and secretory cell morphology on scaffold cultures

**Day 21 Scaffold Culture**

(A) Scanning EM image of the surface epithelia of day 21 cultures displays mature tight junction-coupled (white arrowhead) ciliated and nonciliated cells with similar morphology to native mouse airways (D). (B-C) Transmission EM analysis of day 21 tissue sections confirms the presence of tight junction-coupled (white arrowhead) ciliated cells (ci), club cells (cc), and basal cells (bc), secretory cells (se), comparable to native mouse airways (E).

Scale bars: (A) 2.5μm, (B) 4μm, (C) 2μm, (D) 5μm, (E) 2μm.

**Natural Adult Mouse Airway**

Figure 4-5 (A) Scanning EM image of the surface epithelia of day 21 cultures displays mature tight junction-coupled (white arrowhead) ciliated and nonciliated cells with similar morphology to native mouse airways (D). (B-C) Transmission EM analysis of day 21 tissue sections confirms the presence of tight junction-coupled (white arrowhead) ciliated cells (ci), club cells (cc), and basal cells (bc), secretory cells (se), comparable to native mouse airways (E).

Scale bars: (A) 2.5μm, (B) 4μm, (C) 2μm, (D) 5μm, (E) 2μm.
Figure 4-6 Extended culture on lung scaffolds shows emergence of PAS-positive submucosal gland-like structures

**Figure 4-6** (A) RT-PCR analysis reveals upregulation of secretory cell marker Muc5ac in seeded cells with extended culture. Gene expression is presented relative to adult lung; mean ± SEM, n=3 experiments. (B) Scanning EM analysis reveals the presence of pit structures with ciliated and secretory cells lining the orifice. Scale bar=5μm. (C) Pit structures present on epithelial surfaces show a strong PAS positive stain suggestive of mucin-producing cells lining these structures. Scale bar=50μm.
Morphological progression of scaffold-seeded endoderm to airway epithelial cells resembles natural lung differentiation

The progression of epithelial cell differentiation in developing mouse airways was examined using electron microscopy at different time points, spanning from the embryonic (E13), to pseudoglandular (E15), to canalicular (E17) stages of lung development (Figure 4-7A). Morphological hallmarks were identified and compared to progressive differentiation of endodermal cells to mature airway epithelial cells on decellularized scaffolds (Figure 4-7B). At embryonic day 13 of lung development, epithelial structures contain undifferentiated columnar cells without any features of ciliated or secretory cells. At the pseudoglandular stage, there was an emergence of monociliated cells that disappeared by the canalicular stage. These single ciliated cells were found in day 7 of endoderm-derived scaffold cultures and the monociliated projections could be visualized by scanning and transmission EM micrographs (Figure 4-7B). As development progressed into the canalicular period in the mouse, short cilia were visible by SEM on single cells surrounded by round cells lacking the microvilli and apical projections characteristic of maturing nonciliated cells\textsuperscript{286,287}. Differentiation of airway secretory cells was also initiated at this stage, where rounded cells containing characteristic intracellular granules were visualized by TEM\textsuperscript{288}. Differentiation of endoderm to early secretory cells was observed at 14 days of culture on lung scaffolds (Figure 4-8A). By 21 days of scaffold culture, differentiated club cells were identified containing mature secretory vesicles and microvilli lining the cell surface (Figure 4-8B). SCGB1A1 (club/Clara cell secretory protein) was detected by Western blot analysis in the culture media suggesting that mature club cells were synthesizing and secreting SCGB1A1 into the airway structures (Figure 4-8C).

Using a combination of SEM, TEM and IF confocal microscopy, the progressive lung lineage specification of ESC-derived endodermal cells to fully differentiated airway epithelial cells was captured from recellularization at day 0 to day 21 of culture on decellularized scaffolds (Figure 4-9). This temporal analysis captures the development of tight junction-coupled lung progenitor cells expressing basal cell marker TRP63 at day 4, to mature TUBB4AT\textsuperscript{+} multiciliated and SCGB1A1\textsuperscript{+} secretory club cells lining differentiated airway structures at day 21 of culture. Ultrastructural morphologic evaluation was used to designate similar embryonic dating to endoderm differentiation on lung scaffold cultures (Table 4-3).
<table>
<thead>
<tr>
<th>Endoderm Scaffold Culture</th>
<th>Mouse Lung Developmental Stage (days)</th>
<th>Airway Epithelium Characteristics</th>
</tr>
</thead>
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<tr>
<td>Day 4</td>
<td>Embryonic (9.5-14.5)</td>
<td>Undifferentiated columnar epithelium</td>
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<tr>
<td>Day 7</td>
<td>Pseudoglandular (14.5-16.5)</td>
<td>Emergence of monociliated cells, surrounded by undifferentiated cells</td>
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<tr>
<td>Day 14</td>
<td>Canalicular (16.5-17.5)</td>
<td>Short multiciliated cells, surrounded by maturing non-ciliated cells. Early secretory cells containing intracellular granules and apical microvilli</td>
</tr>
<tr>
<td>Day 21</td>
<td>Postnatal to adult</td>
<td>Established airway structures containing multiciliated cells and club cells with mature secretory vesicles</td>
</tr>
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</table>
Figure 4-7 Monociliated cells are found during early differentiation on decellularized lung scaffolds, resembling pseudoglandular stage of lung development.

**Figure 4-7** (A) Progression of lung development at embryonic (E13), pseudoglandular (E15) and canalicular stages is visualized with scanning (top panel) and transmission (bottom panel) microscopy. Monociliated cells are present (arrow heads) during the pseudoglandular stage and disappear by the end of the canalicular stage, where multiciliated cells are starting to emerge. (B) Monociliated cells are present at day 7 of endoderm-scaffold cultures (arrow head). Early epithelial cells have formed tight junctions (double arrow head). Lu, lumen; ci, ciliated cell; se, secretory cell.
Figure 4-8  Endoderm-derived secretory cells on decellularized lung scaffolds generate and secrete SCGB1A1 protein

Figure 4-8  (A) Transmission EM of day 14 cultures shows early secretory cells on scaffold cultures. (B) Day 21 cultures show characteristic secretory vesicles inside differentiated Club cells with microvilli lining the cell surface. (C) Western blot of d21 culture media shows secreted SCGB1A1 that is not detected in media of endodermal cells cultured without decellularized lung scaffold. Coomassie stain is used as loading control. ba, basal cell; ci, ciliated cell; cl, club cell.
**Figure 4-9** Visualized progression of endoderm to airway epithelial cells on decellularized lung scaffolds

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 14</th>
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Figure 4-9 Visualized progression of endoderm to airway epithelial cells on decellularized lung scaffolds

(A-B) Electron micrographs of the progression of definitive endoderm cells to differentiated airway epithelial cells. (C-D) Immunofluorescent confocal images of definitive endoderm cells to differentiated airway epithelial cells; staining for basal cell markers TRP63 (left panel, green) and KRT5 (right panel, red), club cell marker SCGB1A1 (left panel, red), and ciliated cell marker TUBB4A (right panel, green).

Seeded cells appear to organize into structures and form tight junctions with four days of culture on lung scaffolds, with some populations expressing basal cell marker TRP63. Day 7 cultures mark the appearance of monociliated cells and many TRP63+ epithelial cell structures. Day 14 cultures show the emergence of TUBB4A+ multiciliated cells and by 21 days, mature airway epithelial structures are present with the addition of SCGB1A1+ secretory club cells.
Differentiated airway epithelial cultures consist of mature cells with CFTR function

To assess functionality of differentiated cells, we examined day 21 cultures for ciliary activity and functional expression of CFTR protein. Fundamental to airway mucociliary function, differentiated ciliated epithelial cells contained the appropriate 9+2 dynein arms of respiratory cilia (Figure 4-10). Furthermore, these epithelial cells displayed coordinated beating in culture (Movie 4-1).

Mature differentiated epithelial sheets had established junctional complexes that were clearly identifiable with EM analysis (Figure 4-5A) and IF staining for tight junction-associated protein, TJP1 (Figure 4-11A). Stacked confocal images showed polarized CFTR expression on the apical cell membranes, a characteristic feature of functional airway epithelial cells required for chloride and water transport in the airways (Figure 4-11B). To test CFTR channel function on mature scaffold cultures, we used a cAMP-stimulated halide flux assay. Iodide efflux from scaffold cultures after cAMP agonist stimulation was measured periodically to assess channel activity. Differentiated day 21 cells showed peak iodide efflux within the first minute of CFTR channel stimulation, demonstrating robust expression of functional CFTR protein in mature scaffold cultures (Figure 4-11C).
**Figure 4-10** Mature endoderm-derived ciliated cells are motile and beat in culture

(A) H&E staining of day 21 scaffold cultures shows a sheet of ciliated cells lining a luminal structure. Scale bar represents 25μm. (B) Transmission EM image of cilia shows formation of the appropriate 9+2 dynein arms of motile respiratory cilia. Scale bar represents 100nm. (C) Light microscope still image of mature beating cilia in culture. Dashed line represents the apical side of ciliated cells facing the *lumen* (Lu) side of scaffold culture. Ciliary activity causes a swirl effect on the culture media immediately above the apical surface of the epithelium.
**Figure 4-11** Differentiated airway epithelial cultures have Functional CFTR Protein expression

(A) IF staining of cell cultures shows mature epithelial sheets with established tight junctions, represented by positive TJP1 staining of CDH1+ epithelial cells. Scale bar represents 50μm. (B) Stacked confocal images (X-Z plane) show polarized CFTR expression on the apical cell membranes. Scale bar represents 25μm. (C) Iodide flux was measured after cAMP agonist-induced CFTR activity in day 21 scaffold cultures. cAMP agonists, forskolin and 3-isobutyl-1-methylxanthine, and a CFTR potentiator, genistein (together as forskolin, 3-isobutyl-1-methylxanthine (IBMX), and genistein) (FIG), were added (0 min, red arrow) and replaced periodically for 8 minutes while iodide flux was measured every minute. Peak efflux, 70.6 ± 7.7μM, was reached immediately with addition of FIG at 1 min. No efflux was detected with added vehicle control (DMSO). n=3 experiments; mean ± SEM.
Organization and differentiation of endodermal cells is dependent on scaffold heparan sulfate proteoglycans and its bound factors

To better understand the ECM inductive signals present in decellularized lungs, we selectively cleaved and removed two major growth factor-binding matrix proteins, HS and CS. Decellularized scaffolds were subjected to enzymatic degradation with heparitinase-I or chondroitinase ABC to selectively cleave HS or CS and release any bound factors. Immunostaining of scaffolds and spectral UV analysis of incubation solution after heparitinase or chondroitinase ABC-treatment confirmed HS or CS cleavage on scaffolds, respectively (Figure 4-12A-B). Presence of basement membrane proteins laminin and collagen IV was evaluated by IF microscopy to ensure their preservation following enzymatic treatments (Figure 4-12C-D). Enzyme-treated scaffolds were seeded with definitive endoderm and cultured under ALI conditions as before for up to 21 days.

Tissue staining of day 21 enzyme-treated scaffold cultures showed that CS-cleavage did not hinder lung-lineage differentiation of endodermal cells, while HS-cleavage on scaffolds resulted in a complete loss of organization and differentiation (Figure 4-13A). SEM analysis showed a lack of epithelial morphology and tight junction coupling of seeded cells in heparitinase I-treated scaffolds, while chondroitinase ABC-treated cultures resembled untreated controls (Figure 4-13B). This suggested that the essential cues in decellularized scaffolds that promote lung lineage differentiation are bound to HS proteoglycans.

Interestingly, endodermal cells seeded onto heparitinase-treated scaffolds and cultured in a media supplemented with the supernatant of treated scaffolds, referred to as ‘scaffold conditioned media’ (CM), resulted in airway differentiation of seeded cells, indistinguishable from untreated control scaffolds (Figure 4-14A). IF analysis of CM differentiated cultures showed the presence of TUBB4A+/FOXJ1+ ciliated cells and CLDN10+/SCGB1A1+ (Figure 4-14B). This suggested that scaffold CM contains the factors that are bound to HS proteoglycans on decellularized scaffolds and have the remarkable ability to promote airway-lineage differentiation. This further validated the notion that the ability of decellularized scaffolds to promote differentiation to an airway phenotype is dependent on HS proteoglycans and the factors that are bound to these matrix proteins.
In an attempt to identify candidate factors that are bound to HS proteoglycans and are cleaved after enzyme treatment, we used a proteome profiler antibody array to characterize heparitinase I-treated decellularized lungs. Using a commercially available array, we detected 31 proteins that remained on lung scaffolds after decellularization (Figure 4-15). As an initial screen, a comparison of the protein profile from scaffolds treated with or without heparitinase I identified several candidate proteins that were removed and found in the wash supernatant after enzyme treatment: CXCL12, serpinE1, PDGF-AB, HGF, MMP8, FGF2, proliferin, IL10, and CCL3. The identified candidate proteins may play a role in modulating cell-matrix interactions or lung endoderm specification, however the array profile is by no means an exhaustive list and a more in-depth analysis to parse out the HS-bound inductive factors is required.
Figure 4-12 Enzymatic Treatment of Acellular Scaffolds with Heparitinase I Cleaves Heparan Sulfate Proteoglycans

(A) Natural, acellular and heparitinase I-treated acellular scaffolds were stained for heparan sulfate using biotinylated tomato lectin. Immunostaining reveals the effective cleavage of heparan sulfate proteoglycans following heparitinase I treatment. Scale bar=25μm. (B) Cleaved heparan sulfate from scaffolds is detected in the remaining supernatant following heparitinase I treatment using UV spectral analysis for optical density at 232nm. This confirms the efficacy of the enzymatic treatment and removal of heparan sulfate proteoglycans from scaffolds. N=3 experiments; mean ± SEM, *p<0.01. (C-D) Immunostaining for laminin and collagen IV reveals that the basement membrane remains intact following enzymatic treatment with heparitinase-I and chondroitinase ABC. Scale bar=50μm.
**Figure 4-13** Organization and differentiation of endodermal cells is dependent on HS proteoglycans on decellularized lung scaffolds

<table>
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<th>Heparitinase I⁺</th>
<th>Chondroitinase ABC⁻</th>
<th>Chondroitinase ABC⁺</th>
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**Figure 4-13** (A) Acellular scaffolds were recellularized after heparitinase-I or chondroitinase ABC treatment. H&E staining of day 21 seeded scaffold cultures show limited organization and differentiation in the heparitinase I-treated group, while chondroitinase ABC-treated cultures resemble control groups. Scale bar represents 50μm. (B) Scanning EM analysis of cultures show a lack of epithelial morphology and tight junction coupling of seeded cells in heparitinase I-treated scaffolds, where cells appear rounded with no resemblance to a lung phenotype. Scale bar represents 10μm.
Figure 4-14 Seeded endoderm differentiates to airway epithelia on heparitinase-treated scaffolds with addition of scaffold conditioned media

<table>
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<tr>
<th>A</th>
<th>Heparitinase-treated</th>
<th>Heparitinase-treated scaffold CM</th>
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(A) H&E of decellularized scaffold cultures treated with heparitinase I. Left image shows lack of organization and differentiation of seeded endoderm after 21 days of ALI culture on treated scaffolds. Seeded cells differentiate to airway epithelia with the addition of scaffold conditioned media on heparitinase-treated scaffolds.

(B) Immunofluorescent confocal microscopy shows TUBB4A⁺/FOXJ1⁺ ciliated cells and CLDN10⁺/SCGB1A1⁺ club cells in day 21 cultures with scaffold conditioned media on heparitinase-treated scaffolds.
Antibody assay identifies HS-bound matrix proteins removed from decellularized scaffolds after heparitinase I treatment

Proteome profiler antibody array detects 31 proteins from the array profile that are remaining on lung scaffolds (black). Comparison of the protein profile from decellularized scaffolds treated with or without heparitinase I revealed several HS-bound proteins that are removed from scaffolds and found in the wash supernatant after enzyme treatment (red rectangles): CXCL12, serpinE1, PDGF-AB, HGF, MMP8, FGF2, proliferin, IL10, and CCL3. Data presented are average of two arrays from separate experiments.
4.5 Discussion

Lineage restriction of pluripotent cells is dependent on the interaction of niche components in the microenvironment, where growth-factor signaling, cell-cell contact, and cell-matrix interactions combine and control stem cell fate. Growth factors are a salient component of directing cellular identity, and the ECM plays an important role in controlling growth factor availability by often binding and regulating local concentrations in space and time. Because of the complex nature of these interactions during lung development, in vitro differentiation of pluripotent stem cells to functional airway epithelial cell populations remains a challenging task.

Here we report the robust differentiation of embryonic stem cell-derived endoderm to mature, functional airway epithelial cells using defined, serum-free culture on decellularized lung scaffolds at air liquid interface. In chapter 3 it was demonstrated that decellularized scaffolds alone directed differentiation of definitive endoderm to NKX2-1+/SOX2+/TRP63+ lung progenitor cells as early as 7 days of culture. This progenitor population expanded with longer duration and formed fully differentiated airway structures with basal cells, club cells, and beating ciliated cells. This was achieved with notable morphological and functional resemblance to native airway epithelia and mouse lung developmental stages. Thus, decellularized lung scaffold cultures provide a 3D in vitro platform to better understand cell-matrix signaling during lung development and an efficient approach for generating mature airway epithelia from pluripotent stem cells.

Recent reports have had success with promoting differentiation of stem cell-derived NKX2-1+ lung progenitor cells to airway or alveolar epithelial cells by supplementing monolayer cultures with inductive factors such as FGF2, WNT3a, and BMP4. However, monolayer protocols have achieved lung differentiation to a limited repertoire of functional epithelial cells lacking relevant 3D tissue structure, with low efficiencies, potential contamination from other endodermal lineages, and in some cases with undefined culture conditions using fetal bovine serum. This study is the first to demonstrate the efficient generation of stem cell-derived mature airway structures with CFTR function differentiated with extended culture on decellularized lung scaffolds alone.

Matrix-associated HS and CS proteoglycans are major modulators of growth factor binding and signaling on the ECM surface and function by stabilizing FGF/FGFR complexes, increasing local
gradients, and promoting FGF internalization and processing\textsuperscript{94,274,289,292}. Specification to the airway lineage in our model was found to be dependent on HS proteoglycans and bound factors remaining on scaffolds. Using a proteome profiler antibody array as an initial screen, we showed that numerous proteins remain on scaffolds after decellularization. A list of potential candidates implicated in lung specification was identified using this method, although the array profile is not an exhaustive list and a more in-depth analysis is required to parse out the HS-bound proteins present on scaffolds that are essential for differentiation.

Given the role of the ECM in integrating and mediating inductive signals in a 3D setting and our success with the efficient differentiation of embryonic stem cell-derived endodermal cells to functional airway epithelia, decellularized lung scaffolds are an attractive \textit{in vitro} platform for lung airway engineering.
Chapter 5

Summary and Future Directions
5.1 Summary and Concluding Remarks

While tissue engineering is a highly desirable long-term goal for lung regeneration, it remains burgeoning. One of the most challenging aspects of lung tissue generation is creating an artificial matrix that has similar biological composition and physiological functions to native lung tissue. As reviewed in Chapter 1, it is clear that no master regulator exists for lung development, rather numerous transcription factors and morphogens together control downstream effectors that drive and direct this process. Normal development requires precise temporal and spatial coordination of cellular processes and deciding which program a cell will ultimately elect is determined, to a large extent, by the ECM.

Recapitulating an ECM that supports immunity, the mechanical function of breathing and the physiology of gas exchange certainly makes for a complex task. Many reports have demonstrated the use of natural scaffolds generated from decellularized organs in tissue regeneration proof-of-concept studies. Despite the growing acceptance of using decellularized tissue in regenerative medicine approaches, there are limited studies that examine the direct role of the ECM in tissue-specific differentiation of pluripotent stem cells. The studies presented in this thesis focus on generating viable lung scaffolds and characterizing their subsequent contribution to lineage restriction of definitive endoderm germ cells following recellularization. This work demonstrates the capacity of decellularized lung scaffolds to direct differentiation of endoderm to mature functional airway epithelia, with the requirement of matrix-bound heparan sulfate proteoglycans (Figure 5-1).

In chapter 2, decellularization of adult mouse and rat lungs was carried out using three different approaches: enzymatic treatment with trypsin, chemical treatments with CHAPS, and SDS detergents. We demonstrated that milder zwitterionic CHAPS detergent, with the addition of hypertonicity and chelating agent EDTA, best achieved decellularization. Tissue staining, immunofluorescent confocal analysis, electron microscopy, DNA analysis, WB analysis and tensile testing confirmed cellular removal and preservation of the ECM following lung decellularization. Despite optimization of the protocol with CHAPS, it was noted that preservation of the matrix was dependent on the frequency of lavages. With more lavages, there was a gradual decrease in basement membrane protein laminin detected by immunoblot. This demonstrated that even exposure to milder decellularizing agents may disrupt the ECM protein composition and careful characterization is necessary to ensure the isolated scaffold can
nonetheless support cell culture. CHAPS has also been identified by other groups as the best reagent for lung decellularization, however, our technique and delivery is distinct from other published work. Previous studies have performed decellularization by removing the lungs and using a bioreactor to circulate large volumes of decellularizing agents for several hours\textsuperscript{175,176}. In this study, complete decellularization was achieved rapidly and economically in approximately 20 minutes using short tracheal lavages with the lungs remaining \textit{in situ}.

Biocompatibility of the scaffolds was confirmed using an \textit{in vitro} ALI culture system. Thick sections of decellularized lung were seeded with primary rat fetal lung epithelial cells and maintained in culture for 21 days. Despite difficulty in culturing primary lung epithelial cells in standard conditions \textit{in vitro}, seeded pulmonary epithelial cells thrived on scaffolds\textsuperscript{258}. Recellularization led to reestablishment of both proximal and distal epithelial cell populations. Epithelial structures formed with distal cells expressing KRT18 and pSFTPC, or proximal cells expressing SCGB1A1 and TUBB4A. No tubular structures were identified that contained both airway and alveolar epithelial cells types. This suggests that although lung scaffolds support both populations, region-specific differences are created during cell-matrix culture favoring one population over the other. We concluded that this optimized decellularization process and ALI culture system can support lung epithelial cell culture and could be suitable as a platform for stem cell differentiation towards the lung lineage.

Repopulation of decellularized scaffolds is increasingly used as an end-point assay to assess regenerative potential of pre-differentiated cells\textsuperscript{261,263-265}. A recent report has demonstrated the importance of the matrix environment for maintaining lung identity using pre-differentiated NKX2-1\textsuperscript{+} lung progenitor cells and growth factor-supplemented culture media. Although these reports demonstrate the feasibility of using lung scaffolds in tissue engineering applications, none have assessed the inductive capacity of the ECM alone during early lung specification. In chapter 3, we revealed the exciting ability of lung decellularized scaffolds to promote differentiation of definitive endoderm germ cells to NKX2-1\textsuperscript{+}/SOX2\textsuperscript{+} proximal lung progenitor cells. Lung progenitors were detected at day 7 of culture and were in abundance by day 21, with approximately 46\% of all cells expressing NKX2-1. It is unclear whether the rise in lung progenitors with extended culture is the result of preferential expansion of Nkx2-1\textsuperscript{+} cells or due to increased apoptosis of cells that are not committed to the lung lineage. Although limited NKX2-1\textsuperscript{+}/SOX9\textsuperscript{+} alveolar progenitor cells were found on scaffolds at 7 days, they disappeared...
with longer duration of culture suggesting that differentiation to the distal lineage perhaps requires additional inductive factors after NKX2-1+ specification on scaffolds.

This distinct ability of decellularized lung to differentiate endoderm into airway progenitors was further restated by the lack of differentiation achieved with seeded individual matrix proteins (fibronectin, laminin, collagen I, collagen IV) and ECM substitute Matrigel. Interestingly, decellularized kidney scaffolds did not support cell adherence or tissue-specific differentiation. To generate kidney scaffolds, due to its composition and higher density, an SDS-based solution was used to achieve decellularization, in contrast to lung decellularization that was achieved using a CHAPS-based solution (data not shown). This was a limitation in the direct comparison of decellularized lung versus kidney scaffolds for endoderm culture. However, despite failure to support endodermal cell culture, these kidney scaffolds have been shown to support both ESC-derived mesodermal cells and also nephron progenitor cells in culture and differentiation (data not shown). Therefore, the inability of kidney scaffolds to support endoderm was likely due to the kidney matrix microenvironment, and not due to SDS exposure during the decellularization process. These observations, suggested that mesoderm-derived kidney scaffolds biologically differ from lung scaffolds in their ability to support endoderm lineage differentiation.

Decellularized scaffolds derived from mouse lungs seeded with mouse definitive endodermal cells produced similar results to that obtained with rat lung scaffolds. Furthermore, differentiation of definitive endoderm to airway epithelia using rat lung scaffolds was reproducible with a rat ESC line (DAc8). This demonstrated the conserved nature of the interaction between lung scaffolds and cell lines derived from both species.

Recent reports have had success with promoting differentiation of pluripotent stem cells to NKX2-1+ lung progenitor cells using monolayer cultures and supplementation with inductive factors. Stem cell-derived lung progenitors have also been further differentiated towards airway or alveolar epithelial cells using additional supplementation with high concentrations of inductive factors such as FGF2, WNT3a, and BMP4. These studies demonstrate the feasibility of using different stem cell sources in lung tissue differentiation. Although promising, monolayer differentiation protocols using different cocktails of growth factors have achieved lung differentiation to a limited repertoire of functional epithelial cells lacking relevant 3D tissue structure. Some protocols have reported low efficiencies with potential contamination from other endodermal lineages such as Alb+ liver cells,
while other studies have used undefined culture conditions with FBS supplementation\textsuperscript{264,273}. In chapter 4, we further investigated the potential of decellularized scaffolds without supplementation of exogenous factors, to promote differentiation of NKX2-1\(^+\) lung progenitors into mature airway epithelia.

Extended culture of seeded cells resulted in expansion of progenitor populations and formed fully differentiated airway structures by 21 days of culture complete with TRP63\(^+\)/KRT5\(^+\) basal cells, SCGB1A1\(^+\) club cells, and FOXJ1\(^+\)/TUBB4A\(^+\) beating ciliated cells. This was achieved with notable morphological and functional resemblance to native airway epithelia and mouse lung developmental stages. Importantly, mature epithelial cells had established junctional complexes and expressed functional CFTR protein, demonstrated using a modified iodide efflux assay.

Interestingly, scaffolds generated from both the upper airway and alveolar lung regions directed differentiation to airway epithelial cells, with comparable cellular organization and differentiation. With the exception of a small NKX2-1\(^+\)/SOX9\(^+\) distal progenitor population detected at day 7, no traces of mature AT1 and AT2 epithelial cells were found in culture at day 14 and day 21 on scaffolds derived from the alveolar regions. This suggested that seeded endodermal cells similarly adhere to basement membrane proteins found in both regions of the lungs. The basement membrane throughout the adult lung is comprised of collagen IV, laminin, HSPG and CS\textsuperscript{87, 88}. Following cell adherence, newly synthesized matrix proteins are likely secreted and deposited by seeded cells, though this was not confirmed in this study. While seeded endodermal cells were restricted to the lung lineage, the lack of differentiation from NKX2-1\(^+\)/SOX9\(^+\) distal progenitors to mature alveolar epithelia is likely due to the requirement of additional inductive factors, not present on decellularized scaffolds.

A key finding of this work was identifying basal stem cells as the likely progenitors giving rise to the other airway epithelial cell populations on early scaffold cultures. Lineage tracing experiments have shown that basal cells can undergo long-term self-renewal and give rise to ciliated and secretory luminal cells both \textit{in vivo} and \textit{in vitro} cultures\textsuperscript{62,233,234}. In this study, cells expressing TRP63 were found as early as 4 days of endoderm culture on scaffolds. Basal cells are a heterogeneous cell population with diverse expression profiles of TRP63, KRT5, KRT14, PDPN, and NGFR\textsuperscript{235,236}. This heterogeneity was found on scaffold cultures, leading to questions regarding the regenerative capacity and differentiation ability of identified subpopulations. Basal cells were found in abundance in early proliferative day 7 cultures, while their presence declined
steadily with extended culture. This fall occurred in parallel with a decline in proliferative cells and with the rise of differentiation to ciliated and club cells. The initial abundance followed by a fall in cell numbers, suggests that basal cells could be the precursor cells that undergo a terminal differentiation program and give rise to mature airway epithelia with extended culture. We concluded that decellularized lung scaffold cultures provide a 3D in vitro platform to better understand cell-matrix signaling during lung development and an efficient approach for generating mature airway epithelia from pluripotent stem cells.

In the 3D scaffold culture model, endoderm was maintained at air-liquid interface for the entire duration (from seeding to day 21) of culture. Although air-liquid interface has been demonstrated to support lung epithelial differentiation in vitro, especially mucociliary epithelium, the majority of lung development and airway epithelial cell differentiation occurs in utero, prior to any exposure to air flow. Therefore, our 3D culture model is limited in its ability to mimic the developmental milieu of lung development, since the bulk of lung lineage specification in situ occurs under a submerged setting. Future work could examine the effect of different oxygen levels, air-flow exposure, and mechanical fetal breathing simulation on lineage specification of endoderm on scaffolds. It is important to note that our differentiation approach is also limited by the absence of any mesodermal cells such as endothelial cells, pericytes, and interstitial fibroblasts. As described in Chapter 1, the cross-talk between the lung epithelium and mesenchymal components mediates branching morphogenesis and establishes proximal-distal patterning. It is interesting, however, that the environment recapitulated by lung scaffolds alone was sufficient to drive differentiation into mature airway epithelium in vitro. This suggests that some inductive factors secreted by non-epithelial layers including growth factors may be present on adult lung scaffolds following decellularization.

The ECM plays an important role in controlling growth factor availability during development and homeostasis by often binding and regulating local concentrations in space and time. A significant finding of this work was the importance of matrix heparan sulfate proteoglycans and their bound factors in promoting lung differentiation. Specification to the airway lineage in our model was found to be dependent on HS proteoglycans and bound factors remaining on scaffolds. Using the proteome profiler array, 31 proteins were identified on lung scaffolds following decellularization. From this list, nine proteins were found to have been depleted with exposure to heparitinase I. FGF2 and FGF7 (HGF) were among those
identified and are known to have a role in natural lung development and lineage restriction in vitro\textsuperscript{265, 267, 268}. CXCR4 ligand, CXCL12 (also known as SDF1), was also found among the proteins bound to HSPG on lung scaffolds. CXCR4 is expressed in embryonic endoderm and was used to identify ESC-derived definitive endoderm. Interactions between CXCR4\textsuperscript{+} endodermal cells and its ligand found on lung scaffolds may have a role in cell-matrix crosstalk in our culture setup. Other candidates including matrix metalloproteinase MMP8, together with cytokines involved in chemotactic and anti-inflammatory activity, CCL3 and IL10, were also identified. These cytokines are known to affect the microenvironment during repair and tissue remodeling. To determine whether these candidates are important for lung differentiation in our culture setup, their interactions can be inhibited using antibodies or selective inhibitors in future work. This array profile was by no means an exhaustive list and a more in-depth analysis to parse out the HS-bound inductive factors is required. High-resolution mass spectrometric analysis and/or larger proteome profiler arrays containing over 500 proteins can be used to reveal other HS-bound differentiation factors essential to lung differentiation. Given the role of the ECM in integrating and mediating inductive signals in a 3D setting and our success with the efficient differentiation of embryonic stem cell-derived endodermal cells to functional airway epithelia, decellularized lung scaffolds are an attractive in vitro platform for the study of cell-matrix interactions and for lung airway engineering. It is unclear whether the differentiation activity characterized on decellularized scaffolds follows the natural progression of endoderm specification in lung development or whether it represents a reparative pathway activated during lung tissue injury. We have noted elements of both in the lung scaffold cultures. Step-wise specification of definitive endoderm to early NKX2-1\textsuperscript{+} lung progenitors and thereafter to SOX2\textsuperscript{+} airway progenitors is reminiscent of developmental processes that have been described in the lung\textsuperscript{81, 276}. However, the upsurge of a heterogeneous basal cell population and high proliferative activity noted early on scaffold cultures, followed by a drop in TRP63\textsuperscript{+} basal cells with the emergence of mucociliary epithelium, is indicative of lung tissue repair in response to injury\textsuperscript{62, 235}. Therefore the differentiation undertaken by endodermal cells on lung scaffolds could encompass two distinct pathways of lung development and repair, or perhaps the fundamental interactions that occur during development and repair are not divergent in nature; at least not in this culture setup using decellularized lung scaffolds.

This differentiation strategy generates a renewable source of functional airway epithelial cells in a 3D matrix setting that can be further examined for regenerative potential in vivo. Seeded
decellularized lung scaffolds from human or xenogeneic sources can be used for generation of lung epithelia that may be valuable for airway repair and regeneration and can serve as a platform for drug discovery in human airway-related diseases such as cystic fibrosis.
Figure 5-1 Summary schematic

Section of Decellularized Lung

Definitive Endoderm

ESC

- Heparitinase I

day 0

Airway Epithelium

day 21

Undifferentiated Cells

+ Heparitinase I

Heparan Sulfate Proteoglycans
5.2 Future Directions

5.2.1 Identifying the role of key matrix proteins in lung specification

As reviewed in Chapter 1 and the work presented in this thesis, cell-matrix interactions play a fundamental role in cell fate, yet many questions remain. To investigate the role of different matrix proteins in differentiation to early lung precursors and airway epithelial cells, inhibition of cell-matrix interactions could be done by targeting and inhibiting select integrin receptor signaling. This inhibition can be achieved using cell adhesive peptides or monoclonal antibodies specific to integrin receptors and their protein ligands\textsuperscript{293,294}. Targeted integrin receptors could include α6β4 (laminin), α1β1 and α2β1 (collagen), and α5β1 (fibronectin). Compromised scaffolds should be characterized for overall structure using tissue staining, electron microscopy, and mechanical integrity using elastic measurements prior to recellularization. Following ALI culture of seeded compromised scaffolds, adherence, proliferation, and differentiation could be characterized to identify key matrix proteins involved in lung-lineage specification and to better understanding these interactions during development.

5.2.2 Characterize basal cell subpopulations and their differentiation potential

\textit{In vivo} lineage tracing experiments and injury models have shown that basal cells can undergo long-term self-renewal and give rise to ciliated and secretory cells during development, homeostasis and repair. Due to their heterogeneity with variable expression profiles for KRT5, KRT14, PDPN, and NGFR, questions regarding the regenerative capacity and expandability of basal cell subpopulations remain. Our findings suggest that early TRP63\textsuperscript{+} basal cells are the progenitor cells that give rise to fully differentiated ciliated and club cell populations on scaffolds. Transcription factor \textit{Trp63} is required for basal cells identity during cellular specification, and future work could employ CRISPR technology to target its expression and in turn disrupt the emergence of basal cells on scaffolds. Subsequent recellularization experiments would determine whether an initial basal cell identity is required for generation of mature airway epithelial cell populations on decellularized scaffolds, and help elucidate the differentiation pathway taken by seeded endodermal cells to become functional airway epithelial cells.

5.2.3 Direct differentiation to the alveolar lineage on decellularized lung scaffolds

This work has demonstrated robust endoderm differentiation into mature airway epithelial cell populations using lung scaffold cultures, however no distal alveolar cell characteristics and
protein expression was detected, beyond NXX2-1 expression. This suggested that perhaps differentiation to the distal lineage requires additional inductive factors following NXX2-1 specification on scaffolds. We have preliminary work that demonstrates a shift to distal identity with continuous supplementation of scaffold cultures using select growth factors that have been implicated in alveologenesis. Growth factors added to culture media at distinct intervals included FGF2, FGF7, FGF10, Wnt3a, retinoid acid, and dexamethasone. Under such conditions, we observed an increase in Sftpc mRNA and positive proSFTPC immunostaining of tissue sections, following three weeks of scaffold culture. Large glycogen deposits and structures reminiscent of multivesicular bodies typically found in type II epithelial cells were visualized with TEM. Future work will need to focus on optimizing the dosage and duration of exposure to specific growth factors to promote differentiation to a more mature alveolar epithelial population. Presence of mature lamellar bodies and quantification of surfactant production in culture will be used to assess differentiation and cellular function. Furthermore, future work could examine the effect of different oxygen levels, submerged culture, air-flow exposure, and mechanical fetal breathing simulation on lineage specification of endoderm on lung scaffolds.

5.2.4 Determine the regenerative potential of scaffold cultures for in vivo engraftment

Assessing the capacity of differentiated scaffold constructs to incorporate and revascularize in vivo is an important next step for the use of decellularized scaffold applications in regenerative medicine. Initial experiments could be performed by implanting the scaffold culture into the greater omentum fat pad of young postnatal or adult mice. Cultures could be generated from sex-mismatched autologous ESC lines for easy identification of cells after implantation. For example endoderm generated from the male-derived mouse C2 ESC line can be seeded and differentiated on lung scaffolds, and implanted into the lungs of female C57BL/6 stain mice. Transplantation can be done with scaffolds alone, early day 7 cultures, or mature day 21 cultures containing differentiated airway epithelia. These proof-of-concept experiments will look for any signs of rejection or incorporation and revascularization of transplants.

Future transplantation experiments could implant scaffold cultures directly into the distal lung parenchyma, as described previously in adult mice. Briefly, a thoracotomy is performed and the left lobe of the lung is mobilized. An injection of PBS is given into the lung parenchyma to create a reservoir for the scaffold. The scaffold is then injected into the site using an angiocatheter. Transplants can be removed and examined 7 days and 21 days after surgery.
Tissue could be analyzed for signs of inflammation, proliferation, revascularization, and lung differentiation. If the scaffolds are tolerated well in healthy mice studies, the capacity of these cells to improve function in an injury model could be examined thereafter.
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Chapter 1 is modified from published works (1) and (2).
Chapter 2 is modified from published works (3) and (4).
Chapters 3 and 4 are modified from published work (3).

Published papers


