In Vitro Development of Engineered Lung Tissue

by

Thomas H Petersen

Department of Biomedical Engineering
Duke University

Date: ____________________________
Approved:

Laura E Niklason, MD, PhD, Co-Chair

George A Truskey, PhD, Co-Chair

Nelson J Chao, MD, MBA

William M Reichert, PhD

Barry R Stripp, PhD

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

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ABSTRACT

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Abstract

Lung disease is the third most common cause of death in the United States, resulting in 400,000 deaths annually [6]. For many diseases, lung transplantation is the only definitive treatment, yet only 2700 lung transplants are performed each year, limited primarily by a lack of donor organs [41]. The development of engineered lung tissue, which could be created using a patient’s own cells, could have a significant impact on the treatment of end-stage lung disease.

In this dissertation, we describe the first work intended to engineer whole lung tissue. We utilize a decellularized rodent lung scaffold that is then seeded with pulmonary cells in order to create engineered lung tissue. We will first describe the production of a decellularized lung matrix, which retains several key characteristics of normal lung matrix and is superior to other matrix options for lung tissue engineering. We will analyze the mechanical integrity of the scaffolds and compare the collagen, elastin, fibronectin, and proteoglycan content of the scaffolds to native tissue. We also use a variety of imaging modalities to study the architecture, microstructure and ultrastructure of the scaffolds.

We will then document the design and validation of a bioreactor that is capable of the long-term culture of whole lung tissue in the laboratory. To validate the bioreactor design, we culture native lung tissue for up to 7 days and demonstrate that the bioreactor enables the maintenance of cell viability, cellular differentiation state, and lung morphology.

We then demonstrate that the decellularized scaffolds, when cultured in the bioreactor with a suitable cell source, can support the adherence and proliferation of a wide range of cell types, including pulmonary endothelial, epithelial, and mesenchymal cells. We examine the effects of bioreactor conditions, including medium type,
ventilation and perfusion, and an air-liquid interface, on the growth and differentiation of epithelial and endothelial lung tissue. Finally, we evaluate several key functional aspects of the engineered tissues, including surfactant production and the formation of a barrier between the vascular and airway compartments.

In summary, this work demonstrates the development of tools, techniques and platforms for the growth of engineered lung tissues, and provides a foundation for the continued development of whole, functional engineered lung tissue.
To Mom and Dad

and

To my wonderful wife Lisa
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<tbody>
<tr>
<td>acLDL</td>
<td>acetylated low-density lipoprotein</td>
</tr>
<tr>
<td>AFU</td>
<td>arbitrary fluorescence unit</td>
</tr>
<tr>
<td>ALI</td>
<td>air-liquid interface</td>
</tr>
<tr>
<td>Ang-1</td>
<td>angiopoietin-1</td>
</tr>
<tr>
<td>AQP/AQP-5</td>
<td>aquaporin-5</td>
</tr>
<tr>
<td>BGJb</td>
<td>Biggers, Gwatkin, Judah tissue culture medium for bone (Fitton-Jackson formulation)</td>
</tr>
<tr>
<td>CCSP</td>
<td>Clara cell secretory protein (also, CC10)</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>ECGS</td>
<td>endothelial cell growth supplement</td>
</tr>
<tr>
<td>ECIS</td>
<td>electric cell-substrate impedance sensing</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGM-2</td>
<td>endothelial growth medium 2 (Lonza)</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBM</td>
<td>fetal breathing movement</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
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</table>
FITC fluorescein isothiocyanate
FN fibronectin
H&E hematoxylin and eosin stain
HCl hydrogen chloride
MCDB-131 MCDB medium formulation 131
MDa megadalton
MHC major histocompatibility complex
MLE-12 mouse lung epithelial cell line 12
NaCl sodium chloride
NFDM non-fat dry milk
PBS phosphate-buffered saline
PCNA proliferating cell nuclear antigen
PECAM(-1) platelet-endothelial cell adhesion molecule 1
PLGA poly-L-lactic acid
PLLA poly(lactic-co-glycolic acid)
PMSF phenylmethanesulphonylfluoride
RBC red blood cell
RIPA radio-immunoprecipitation assay
RT room temperature
SDS sodium dodecylsulfate
SEM scanning electron microscopy
SNP sodium nitroprusside
SPA surfactant protein A
SPC surfactant protein C
TBS tris-buffered saline
TBS-T tris-buffered saline with tween-20
xxii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>UTS</td>
<td>ultimate tensile strength</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
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Acknowledgements

First, I would like to thank my advisor, Laura Niklason, for her continued guidance and support over the past several years.

I would especially like to thank my wonderful wife, Lisa. She is absolutely wonderful and amazing in so many ways.

I would like to acknowledge the support of the various members of the Niklason lab, especially Elizabeth Calle, Maegen Bradley and Liping Zhao. I am especially indebted to Liz, who has contributed ideas, assistance, and energy towards virtually every aspect of this project.

I would also like to acknowledge the support of several key people who contributed towards the development of this project. I particularly thank Erica Herzog, Rob Homer, and Barry Stripp for useful discussions and contributions. Additionally, I would like to thank the rest of my doctoral committee, Nelson Chao, Monty Reichert, and George Truskey, for their guidance. In addition, and importantly, I would like to acknowledge valuable assistance from the Yale Electron Microscopy facility and the Yale Histology facility; the staff of these facilities have been incredibly helpful and I am very grateful.

Finally, and most importantly, I would like to thank my parents. My mom has instilled in me a love of science, herself having obtained a PhD in chemistry, as well as a sense of stubbornness that is often useful in scientific endeavors. My dad has shown me how to lead a life of honor and respect, and how to persevere in the face of difficulty. They both have always given everything of themselves such that my brothers and I could have every possible opportunity to succeed. Their sacrifices have benefitted us in many ways, and our accomplishments in life would not have been possible without their love and dedication.
Chapter 1

Introduction

1.1 Motivation and Significance

Every year, 400,000 Americans die of lung disease. Of further concern, the death rate due to lung disease is increasing, while the death rates for the other major disease categories are decreasing (heart disease, cancer and stroke) [6]. For several lung diseases, including cystic fibrosis, emphysema/COPD, and idiopathic pulmonary fibrosis, lung transplantation remains the only definitive treatment. However, patient survival after lung transplant is only 50% at 5 years and 24% at 10 years [138]. There is therefore great demand for the development of engineered lung tissue that could be used for transplantation. One advantage of engineered lung tissue is that the tissue can be grown using a patient’s own cells, thereby avoiding the need for strong immunosuppression, as is required with current lung transplantation. Immunosuppression is necessary to prevent rejection of the transplanted organ, but can lead to a wide range of problems, including infection, malignancy, kidney impairment, cardiovascular problems, and neurologic disorders [159, 41]. In this dissertation, we utilize tissue engineering strategies to demonstrate initial steps towards the growth of engineered lung tissue.

Tissue engineering is a growing field that seeks to combine cellular, molecular, technological and medical advances to create replacement tissues suitable for implantation. Promising work has been done on a variety of tissues, including blood vessels, urinary bladder, heart valves, and cardiac tissue [144, 169, 15, 152]. However, lung is a difficult tissue to engineer in the laboratory. Lung requires a complex matrix that can withstand the mechanical pressures of breathing, that can support the growth
of endothelial, epithelial and mesenchymal cells, and that provides a means for gas exchange between two very different yet intimately juxtaposed compartments.

The primary motivation of pulmonary tissue engineering is to develop functional engineered lung tissue generated from a patient’s own cells that can be used in a clinical setting for lung transplantation. In this work, we demonstrate initial progress towards the development of functional engineered lung tissue using a rodent model. However, the primary motivation of this work is to demonstrate the development of tools that can be utilized to facilitate lung tissue engineering, and then utilize these tools to demonstrate that lung tissue engineering is a feasible, albeit difficult, goal to strive towards. As such, we describe strategies to produce suitable scaffolds for lung tissue engineering, to design and develop a bioreactor that can be used to culture lung tissue in the laboratory for long time periods, as well as the methodology for seeding cells onto the lung scaffolds, and the identification of conditions that are favorable for the growth of engineered lung tissue in the laboratory.

Besides potential patient use in clinical settings, engineered lung tissue can be used in the laboratory to study a wide variety of important aspects of pulmonary biology and physiology. There are very few in vitro 3-dimensional lung culture models [198]. Furthermore, pulmonary endothelial and epithelial cells are more difficult to culture in the laboratory than many other cell types [128, 161], and there has been relatively slow progress in the field of pulmonary progenitor and stem cell biology [25, 141]. The development of an in vitro lung tissue that replicates key features of the native pulmonary environment would enable the more controlled study of pulmonary diseases, lung biology, development and physiology.
1.2 Statement of Thesis Research

In this dissertation, we describe the first work on the growth of complete engineered lung tissue. The objectives of this work are to demonstrate that decellularized lung scaffolds are promising substrates for the growth of engineered lung tissue, and to demonstrate the development of tools, techniques, and platforms that can be utilized in the continued development of engineered lung tissue.

1.3 Overview of Our Approach to Lung Tissue Engineering

Tissue engineering strategies principally employ three key components: a scaffold, a cell source, and a bioreactor. We have chosen to obtain scaffolds for lung tissue regeneration by decellularizing native rodent lung. The scaffolds that we obtain are devoid of cells and almost all DNA, as well as immunogenic markers, yet retain several key extracellular matrix molecules that are important for cell attachment and proliferation. In addition, the scaffolds have a connecting trachea that leads to an alveolar network, as well as a pulmonary artery and pulmonary vein. Although it is not entirely clear the degree to which the connecting vasculature and capillary network are still intact, there is at least an outlining structure. This organized 3-dimensional structure is critically important if one hopes to design large pieces of engineered tissue that would be useful for transplantation.

A bioreactor is required for the in vitro culture of any 3-dimensional tissue. We have developed a bioreactor that can culture whole lungs in the laboratory for at least 1 week. The bioreactor is capable of ventilating lungs via negative pressure as well as providing perfusion and ventilation at physiologic rates and pressures. As a proof of principle of the bioreactor design, we have performed preliminary ex vivo cultures of
freshly explanted whole rat lungs in this bioreactor. As a second proof of principle, we have used this bioreactor to culture engineered lung tissue, demonstrating that the bioreactor can support the growth of engineered tissues, which contain a wide variety of key pulmonary cell types and that display preliminary evidence of functionality.

The final key issue in the creation of engineered lung tissue is the choice of a cell source. We have chosen syngeneic neonatal rat pulmonary cells, as this is a cell source that contains a wide variety of cell types, is relatively easily available in large numbers, and contains cells that are young and plastic. We have shown that the cells we isolate from neonatal rat lungs are diverse, containing many key cell types in the lung, are of high viability, and can adhere to and proliferate on decellularized lung matrices.

We have used these neonatal lung cells, as well as purified populations of lung microvascular endothelium, to culture engineered lung tissue by combining these cell sources, the decellularized scaffolds, and the bioreactor that we developed. We have shown adherence and proliferation of endothelial cells, mesenchymal cells, and several types of epithelial cells, including types I and II epithelium, Clara cells, bronchoalveolar stem cells, and basal cells. We then utilize a variety of tools and techniques to
evaluate the engineered lung tissue in order to better understand the factors that impact the development of this tissue and to identify ways to improve the growth of the engineered tissue.

1.4 Design Goals for Engineered Lung Tissue

In this section, we outline a series of objectives for the growth of engineered lung tissue. These design goals are intended to be a comprehensive set of objectives during the development of engineered lung tissue. Some of these objectives may take years or even decades to fulfill, and therefore we do not intend to achieve all of these objectives in this dissertation. However, we keep these objectives in mind during the evaluation of this work in order to guide our progress and assess our outcomes.

**Vasculature and airway.** There must be a patent, perfused vasculature and a patent airway tree that can be ventilated.

**Gas exchange.** The lung must be capable of exchanging sufficient gas between the airway and vascular compartments to meet the physiological needs of the animal. In particular, the partial pressure of oxygen in the pulmonary vein should be at least 50 mmHg, a minimal level that would be indicative of pulmonary function and compatible with survival [64, 153].

**Mechanics.** The tissue must be strong enough to withstand all needed movements, in particular breathing motions and vascular perfusion, as well as manipulation during surgical implantation. Therefore, the tissue should mimic the mechanical properties of normal lung in terms of strength and elasticity.
**Immunogenicity.** The tissue should not provoke an immune response when implanted into a host.

### 1.5 Outline of Chapters

#### 1.5.1 Chapter 2: Background

In chapter 2 we review relevant background information for this dissertation, including lung physiology, architecture, and cellular composition; prior work on pulmonary tissue engineering; the lung extracellular matrix; and the decellularization of tissues.

#### 1.5.2 Chapter 3: Decellularization of Rat Lung and Morphological Characterization of Decellularized Scaffolds

Chapter 3 presents our work on the creation of a decellularized lung matrix by decellularizing native rodent lung. We evaluate the hypothesis that native lung tissue can be decellularized to remove cellular components and antigenic molecules, yet retain key extracellular matrix molecules. We demonstrate histological analyses of native and decellularized tissues, and evaluate the potential immunogenicity of the decellularized scaffolds by evaluation of major histocompatibility antigens. We evaluate the composition of the decellularized scaffolds using immunofluorescence, and evaluate the permeability of the decellularized scaffolds to 5 µm particles, which mimic the size of red blood cells and provide a measure of the overall intactness of the airway-vascular barrier. Finally, we use electron microscopy and microCT imaging to evaluate the microstructure and ultrastructure of the scaffolds.
1.5.3 Chapter 4: Mechanical Properties of Decellularized Rat Lung

In this chapter, we evaluate the hypothesis that decellularized lung scaffolds retain salient mechanical features of native lung, due principally to contributions from collagen and elastin, and that these features can be evaluated using a simple mechanical model. We use histochemical staining and quantitative assays to evaluate the collagen, elastin and glycosaminoglycan content of the decellularized scaffolds. We demonstrate that collagen is retained at levels indistinguishable from native lung, elastin is retained at about 40% of native levels, while glycosaminoglycans are largely absent. We then evaluate the mechanical integrity of the scaffolds and correlate these findings to extracellular matrix components using modelling studies.

1.5.4 Chapter 5: Development and Validation of a Bioreactor for In Vitro Culture of Adult Rodent Lung

In chapter 5, we depart from the studies of decellularized lung matrices in chapters 3 and 4, and discuss the development of a bioreactor for the in vitro culture of whole rodent lungs. We first describe the overall bioreactor design, and then specifically evaluate the hypothesis that the developed bioreactor can support the in vitro culture of whole lobes of lung tissue, demonstrated by maintenance of cell viability, lung morphology, and cell differentiation state. We also present discussions of oxygen utilization, nutrient requirements, perfusion and ventilation conditions, and pressure profiles in the bioreactor. We then describe the impact of specific bioreactor conditions, principally vascular perfusion and ventilation of the airways with medium or air, on the survival of native lung tissues and maintenance of cell viability and differentiation. The overall goal of this chapter is to develop and validate a bioreactor for use in the culture of engineered lung tissue.
1.5.5 Chapter 6: Epithelial Development in Engineered Lung Tissues

In this chapter, we begin cultures of engineered tissue, utilizing the decellularized scaffolds and the bioreactor that we have developed. Overall, we evaluate the hypothesis that it is feasible to engineer 3-dimensional lung tissue by combining a decellularized lung scaffold with appropriate cell sources and a suitable bioreactor. We also evaluate the specific impacts of key bioreactor conditions, including medium type, perfusion, ventilation, and an air-liquid interface, on the development of engineered epithelial tissues. We describe the growth and differentiation of epithelial cells under these conditions as well as the production of surfactant.

1.5.6 Chapter 7: Endothelial Development in Engineered Lung Tissues

In this chapter, we evaluate the development of engineered lung endothelium. We demonstrate that the extracellular matrix, in particular fibronectin, plays a key role in aiding and affecting endothelial attachment to the decellularized scaffold. We then evaluate the hypothesis that the permeability of the endothelium can be reduced with angiopoietin-1 and sphingosine-1-phosphate, when EC are cultured on both tissue culture plastic and on decellularized matrices. Finally, we evaluate the impacts of perfusion and ventilation on endothelial development and evaluate these tissues for functional barrier formation using VE-cadherin expression, transmission electron microscopy and a functional permeability assay.
1.5.7 Chapter 8: Functional Evaluation of Engineered Lung Tissue, Conclusions, and Directions for Future Study

In this chapter, we describe the combined culture of engineered epithelial and endothelial tissue, and describe screening experiments used to identify conditions for the co-culture of these cell populations in an engineered tissue. We discuss and identify a strategy for the combined culture of endothelial and epithelial populations in an engineered lung tissue. We then summarize the functional characteristics of engineered lung tissues, including surfactant production, endothelial barrier formation, and mechanical integrity.

Finally, we review the conclusions of this dissertation, and discuss key future areas for the continued development of engineered lung tissues.
Chapter 2

Background

In this chapter, we review background material that is relevant to this thesis and lung tissue engineering. The information is not meant to be comprehensive but rather offer selective supportive and background information relevant to the work described in this thesis. Additional, more specific background material is provided in the introduction to each chapter.

2.1 Justification

There are several motivations for this work. The first is the development of engineered lung tissues that could eventually be used in a clinical setting for lung transplantation. In addition, and of equal importance, is the development of platforms to enable the study of lung biology and physiology in controlled laboratory settings.

2.1.1 Clinical relevance

Lung disease is a source of significant morbidity and mortality. It is the third most common cause of death in the United States, and kills one in every five people in the U.K. [112, 6]. Of greater concern, the death rate due to lung disease has been increasing, while the death rates for the other most common diseases are decreasing (heart disease, cancer and stroke) [6]. The economic price of lung disease is significant, costing the U.S. economy $154 billion per year in direct and indirect costs [6]. Lung transplantation remains the only definitive treatment for several lung diseases, including cystic fibrosis, emphysema/COPD, and idiopathic pulmonary fibrosis [191]. However, the survival after lung transplant is only 50% at 5 years and 24% at 10 years.
There is therefore great demand for the development of whole segments of engineered lung tissue that could be used for transplantation. Furthermore, such tissues could eventually be created using a patient’s own cells via the use of adult-derived stem cells, thereby producing autologous tissues that would greatly reduce the risk of complications from immune rejection of transplanted organs, a significant problem for lung transplantation. In addition, such tissue engineering strategies would address the serious shortage of suitable donor organs, as the average waiting time for a lung transplant is significant, with approximately 3000 patients on the waiting list at any time. The median wait time is currently 132 days, which is significantly shorter than the median waiting time in 1997 of 1053 days due to changes in the waiting list procedures and available donor allocation rules [138].

Clearly, the development of engineered lung tissue that could be utilized in a clinical patient setting is a long-term objective. Therefore, in the short term we focus more on the development of tools and technologies that can be used to advance lung tissue engineering, as well as understanding the factors that impact lung tissue growth and survival in vitro.

2.1.2 Development of a platform for in vitro study of lung biology & physiology

In addition to the direct clinical implications of the development of a tissue engineered lung, this work is also intended to demonstrate the production of a platform for the in vitro study of lung biology and physiology. Such a platform would allow researchers to study lung behavior in a more controlled environment than the various animal models currently used, an especially important objective as the mechanisms underlying many lung diseases are incompletely understood. An in vitro lung platform could also allow pharmacologic testing and investigation in human or animal
tissue before proceeding to time-consuming and costly human or animal trials, as well as allowing more rigorous control of experimental conditions. Currently, very few in vitro 3-dimensional lung culture models exist [198]. Therefore, in this work we demonstrate the development of an in vitro system that can be used to culture whole rodent lungs, enabling the detailed study of lung biology and physiology in controlled laboratory settings. Rodent pulmonary endothelial and epithelial cells are more difficult to culture in the laboratory than many other cell types [128, 161], making it more difficult to study lung cell behavior in isolated cell preparations. Thus, there are very few in vitro culture methods for rodent pulmonary cells, in particular pulmonary epithelium. In this work we also demonstrate the production of a scaffold that is suitable for the in vitro culture of pulmonary epithelium and endothelium, which would therefore facilitate the study of pulmonary cell behavior in the laboratory.

2.2 Lung Architecture and Cellular Composition

2.2.1 Overall architecture

The lung is organized much like a tree, with the trachea as the trunk, bronchi as the branches, and alveoli as the leaves. The human lung has approximately 23 levels of branching, yielding 30,000 terminal bronchioles, the region distal to each of which is a pulmonary acinus. Each acinus contains 10,000 alveoli, thus yielding approximately 300 million alveoli in the adult human, although numbers range from 200 to almost 800 million [120]. The lung is divided into the conducting zone and the respiratory zone, which largely function as the names imply. The conducting zone distributes air down to the level of the terminal bronchioles. Each terminal bronchiole becomes a respiratory bronchiole, with alveoli appearing along the wall, thus functioning in both air conduction and respiration. The bronchiole transitions to an alveolar duct, in which the walls consist entirely of alveoli, and the duct terminates after several
divisions at the alveolar sacs. Approximately half of alveoli arise from alveolar ducts and half are in alveolar sacs.

Figure 2.1: Schematic of the airway tree, showing epithelial cells of the lung. Differentiated cells are orange and stem cells for each compartment are green.

The large and small bronchi, down to approximately the 11th level of airway division, run in parallel with branches of the pulmonary artery and are kept open by cartilage in the airway walls. After the small bronchi, the bronchioles are embedded in the pulmonary parenchyma, and are thus kept patent by the elasticity of the lung. During these airway divisions, the total cross-sectional area is continuously increasing.

This branching structure is difficult to replicate from a tissue engineering standpoint. The scaffolds used in most tissue engineering applications are macroscopically heterogeneous, as it would be very difficult to construct both the appropriate microscopic structure (i.e. alveolar and capillary architecture) as well as macroscopic organization (i.e. airway tree and major arteries and veins).

The pulmonary vasculature follows a similar branching pattern, with the pulmonary arterial branches initially traveling together with the large airways and the returning pulmonary veins. The pulmonary vasculature branches extensively to yield
a very dense network of capillaries. The pulmonary capillary network is different than most capillary beds. In most capillary beds, capillaries are arranged largely as parallel tubules. However, in the lung the capillary bed is a dense, interconnecting network of capillaries. The typical length of a pulmonary capillary is only 8-12 µm, barely longer than the ∼5-7 µm diameter of a capillary [205, 204, 92]. This capillary network surrounds the alveolar sacs, and brings a large volume of blood in close proximity to the alveoli.

The alveolus is the site of gas exchange, and as such is the key functional location in the lung. Essentially, the remainder of the lung is designed to facilitate this sole function: alveolar gas exchange. Alveoli are sacs which, when inflated, are approximately 250 µm in diameter. Alveoli are surrounded by the capillary network, as shown in figure 2.2(a). Collagen and elastin fibers, and other ECM components, along with the capillaries, run through the alveolar septa, which are the shared walls between alveoli. Such ECM components tend to run along one side of the septa, thus creating one thicker side and one thinner side. The thinner side consists only of the thin capillary endothelial and epithelial cell wall, with a fused basement membrane between them. This thinner side is the principal site of gas exchange, with a thickness of only ∼ 0.3 µm (see figure 2.2(b)). The thicker side is 1-2 µm, due to the extracellular matrix components that reside here, and evidence indicates this side is also the principal site of fluid resorption from the airspaces.

The functionality of the lung is critically tied to its structure. The airway tree and the vasculature are highly branched and closely intertwined. The highly branched airway tree and vasculature enable an extremely large surface area of interaction between the air and blood compartments. This surface area is estimated to be as high as 50 to 70 square meters in humans [23], and this large surface area enables gas exchange to occur via diffusion across the very thin alveolar-capillary membrane. The
complex structure of the lung is critical, and is a feature that must be reproduced in engineered lung tissue. Using current technologies, it is not possible to recreate this highly complicated structure using a gel or polymer scaffold, therefore complicating most approaches to pulmonary tissue engineering. In this work, we utilize decellularized native lung as a scaffold for the growth of engineered lung tissue. Thus, the complex pulmonary structure is already in place, although we must take care to not damage the structure during decellularization. The decellularized lung scaffold is thus a key feature of current efforts at pulmonary tissue engineering.

2.2.2 Epithelium

The airways are lined with a variety of specialized epithelial cells. Airway epithelium serves several functions. The larger airways are principally conducting pathways to the respiratory zone where alveoli reside. However, the airway epithelium also serves to warm and humidify inspired air, and provide a defense against inhaled chemicals,
particles and pathogens. The mucous layer is principally responsible for many of these functions, and the epithelium carefully balances water and solute secretion and resorption to maintain a mucous with the proper consistency and water content [120]. In the distal tissue, alveoli are covered in flat (type I) epithelium that are specially designed to allow for efficient gas exchange while cuboidal (type II) epithelium secrete surfactant.

**Columnar (ciliated) epithelium.** Ciliated cells are found in both large and small airways, as diagrammed in figure 2.1. In the large airways, ciliated cells cover approximately half of the surface area. These cells beat in coordinated fashion at 12-16Hz to propel mucous up the airway tree, an important function in the defense against bacteria, viruses, and inhaled particles.

**Mucous cells.** Mucous-producing cells, also called Goblet cells, reside in the bronchi and bronchioles and are principally responsible for producing the mucous layer of the respiratory tract. Submucosal secretory cells also contribute, as well as a few other cell types in small roles. Mucin is the key component of mucous (mucin makes up about 1%; mucous is \( \sim 97\% \) water), and is secreted in response to many different stimuli in order to keep the respiratory tract moist and facilitate the trapping of inspired foreign bodies [120].

**Basal cells.** Basal cells reside beneath the columnar epithelium, in the proximal airways. They are a less differentiated, presumptive progenitor cell source and give rise to the columnar epithelium as well as Goblet cells [112, 93]. They are characterized by expression of cytokeratin-14 [30, 93], a heterodimeric member of the keratin family, which form networks that impart mechanical integrity to epithelial layers [95]
Clara cells. Clara cells are non-ciliated secretory epithelial cells that are found in the terminal bronchioles and are a presumptive progenitor cell for distal pulmonary epithelium [73]. They secrete a variety of proteins, including surfactants A, B and D, as well as the characteristic Clara cell secretory protein (CCSP), the most abundant secreted protein in pulmonary airways. The function of CCSP is not known, although it is thought to regulate the local inflammatory response [180] and is decreased in diseases such as asthma, COPD and lung cancer [21, 201]. Clara cells also function as a local progenitor cell, serving as the sole source of bronchiolar renewal [112].

Bronchoalveolar stem cells. These are a putative progenitor cell population that are defined by expression of both CCSP and the type II pneumocyte marker SPC. They are found at the broncho-alveolar duct junction, and can differentiate into both Clara cells and type II pneumocytes, and thus type I pneumocytes, thereby allowing them to repopulate most of the distal lung phenotypes [112, 107].

Type II epithelium. Type II epithelial cells are rounded cells that are found in the distal lung tissue. They are characterized by production of surfactant protein C (SPC), a small hydrophobic peptide that is the result of a series of post-translational modifications of the 21kDa pro-SPC molecule [20]. The pre-processed, pro-SPC peptide is the form of SPC used as a marker for type II epithelium in this document. In addition to producing surfactant, type II pneumocytes are a local progenitor cell, and can divide to give rise to type I epithelium.

Type I epithelium. Type I epithelial cells cover the vast majority, approximately 90%, of the airway surface of the lung. These cells are extremely thin (0.1 µm thick in some regions) in order to facilitate diffusion of gases between the alveolar and capillary networks. In addition, these cells assist in maintaining the water balance in the lung.
During breathing, water is lost due to evaporation in the airways, termed insensible water loss. This water must be replaced to avoid drying of the alveolar epithelium. In addition, immediately after birth, during the transition to air breathing, water must be resorbed. The main molecular effectors of this water movement are the family of water channel proteins termed aquaporins. The various members of this family are expressed in specific locations with permeabilities and selectivities tuned to their needed functions. Aquaporin-5 (AQP5) is found primarily in type I epithelial cells on their apical membrane [109, 143]. AQP5 is commonly used as a marker for type I epithelium, and will be used as such in these studies.

2.2.3 Vasculature

The pulmonary circulation operates at very low perfusion pressures, compared to the systemic arterial circulation. As a result, the media of pulmonary vessels is much thinner, and pulmonary arterioles contain virtually no muscular tissue, in contrast to systemic arterioles [120].

Capillary endothelium. The capillary endothelial cell principally provides a quiescent barrier between the capillary network and the alveoli. Although generally quiescent, capillary endothelium is not a static cell, but responds to shear stress, pulsatile flow, and transmural pressure [71]. Junctions between endothelial cells permit the passage of large molecules and macrophage through 5nm cell-cell junctions. The capillary endothelium is as thin as 0.1 µm in the capillary beds surrounding the alveoli (excepting the region where the nucleus resides), in order to facilitate gas exchange.
2.2.4 Interstitial cells

In addition to the endothelial and epithelial populations, a variety of supportive cell types are found in the lung. These cells include fibroblasts, myofibroblasts and smooth muscle cells, and they are important in lung remodeling and repair [184]. Smooth muscle cells are found in some of the larger vessels. Fibroblasts are scattered throughout the interstitium and secrete and maintain the extracellular matrix. In addition, macrophages are found throughout the lung, principally in the airways, where they scavenge for foreign bodies.

2.3 Extracellular Matrix

The extracellular matrix is a key component of any tissue, and the lung is no exception. The extracellular matrix permits cell communication with the surrounding environment, including with other cells. In addition, the matrix can affect the spatial and temporal patterns of cellular signals, as well as cells themselves [110]. The extracellular matrix includes both the basement membrane and the interstitial connective tissue, which interact with the cellular makeup of the lung to affect many aspects of lung function and physiology.

The extracellular matrix is composed principally of elastin and collagens, which are interwoven with fibronectin fibrils and proteoglycan molecules, which allows plastic deformation of the lung parenchyma [112]. In addition, other ECM components include laminins, heparan sulfate proteoglycans, entactin, hyaluronate, chondroitin sulfate, and glycosaminoglycans [59].

Collagen. The collagens are the most important structural component of the lung, as they are responsible for the overall mechanical strength of the lung. In addition, collagen affects development and is important for branching morphogenesis [38]. Col-
Collagens are a large family of extracellular proteins, which contain triple helical domains of three polypeptide α-chains of the amino acid sequence Gly-x-y, where x and y are typically proline or hydroxyproline but can be any amino acid. The mechanical strength of collagen is conferred by this triple helical conformation, in which each α-chain is coiled in a left-handed fashion, and then the three α-chains are coiled around each other to form a right-handed superhelix with the glycine residues closely packed in the interior of the molecule.

There are many subtypes of collagen, consisting of different combinations of α-chains, but the most prevalent in the lung are types I, IV and V [184]. Extensive posttranslational modifications alter the basic amino acid sequence to tailor the properties of the collagen subtypes. In the lung, type I as well as type III are the principal structural collagens [17, 184, 183], while type IV is a key basement membrane component [59]. Type IV collagen interacts with other components of the basement membrane, including laminins, nidogen and heparan sulfate proteoglycan, and can interact with cells by direct binding or indirectly through laminins [110].

The Young’s modulus of a single collagen fiber has been reported at 3-9GPa [166], while the modulus of collagen fibers are on the order of 100MPa [83].

Although collagen is important to pulmonary structure and function, oversecretion of collagen types I and III, principally by fibroblasts, leads to pulmonary fibrosis. This can lead to decreased gas exchange as a result of a thickened interstitium, and if observed in an engineered lung would be associated with poor outcome.

**Elastin.** Elastin is an important ECM component in tissues that require reversible distension, and elastin allows for the intrinsic recoil property of lung tissue. Elastin is rich in hydrophobic amino acids and scarce in acidic residues, with the hydrophobic regions believed to be important in conferring its elastic properties [59]. Elastin fibers combine with microfibrils to make up the functional elastic fibers of the lung, which
are very extensible and maintain a linear stress-strain relationship out to ~200% strain [110]. The Young’s modulus of single elastin fibers has been reported between 0.6-1.2MPa [66, 2], while the modulus of elastin measured in tissues is approximately 300-400kPa [2, 83].

Elastin deficiency is characteristic of emphysema, underscoring its clinical and physiological importance [65, 59]. However, insufficient elastin is not the sole cause of emphysema, as emphysema can occur with normal levels of elastin [185]. In addition, although elastin can be produced during in vitro culture [37], it is often difficult to induce elastin production in engineered tissues [47], underscoring the importance of retaining elastin during decellularization.

**Fibronectin.** Fibronectin is a dimeric cell-adhesive glycoprotein that aids cell attachment to the extracellular matrix via a repeated peptide sequence, the Arg-Gly-Asp (RGD) sequence [163]. Fibronectin is secreted by bronchial epithelial cells [59], type II epithelium [158], endothelium [101] and fibroblasts [116]. Fibronectin is important for the adherence of a variety of cell types to the extracellular matrix or to tissue culture plastic [193], and is frequently used for in vitro cell culture to promote cell attachment and growth [18]. Fibronectin interacts with cells to impact their morphology, movement and differentiation [110], and is also important in vascular and airway development and during wound healing, providing a “cell adhesion and guidance system” [59, 17].

**Proteoglycans.** Proteoglycans are proteins that are found on cell surfaces, within intradeellularized vesicles, and incorporated into the ECM [110]. Proteoglycans are defined by a core protein that has undergone post-translational modification with a glycosaminoglycan (GAG). GAGs are polysaccharides consisting of a simple linear polymer of monosaccharides, some of which are modified to provide heterogeneity
across the spectrum of GAGs. GAGs can impact a protein’s structure and function, and also help control macromolecular and cellular movement across the basal lamina [110]. The size and complexity of proteoglycans can vary greatly, and they can associate to form even more complex structures, and may play a role in the mechanical integrity of the lung [36].

**Laminins.** Laminins assist cell binding through integrins and other receptors. There are at least 11 different types of these large molecules, and they interact with cells in complex fashions, allowing laminins to affect cell shape and permeability [58].

### 2.4 Barrier function

The epithelium, endothelium, and extracellular matrix are all key components of preventing fluid translocation and pulmonary edema. Preventing fluid movement from the capillary network into the alveoli is critical to lung function; pulmonary edema is the clinical result of excessive fluid movement across the capillary-alveolar barrier [127]. The capillary endothelium [69] and alveolar epithelium [53] together prevent fluid movement across this barrier by creating a physical barrier. In addition, the type I alveolar epithelium aids fluid resorption from the alveoli [55]. The decellularized scaffold would not be expected to retain fluid movement or the movement of small molecules; in the absence of cells, this degree of retention would be extremely difficult.

However, the extracellular matrix provides a physical barrier to the movement of large macromolecules and red blood cells into the alveoli [27, 58], and also assists in preventing interstitial fluid accumulation [140]. This function would be expected to be retained in a decellularized matrix. We therefore aim to retain sufficient ECM components such that macromolecular transit is restricted such that at least 99% of
macromolecules cannot transit across the alveolar-capillary membrane.

2.5 Lung Physiology

As we attempt to develop engineered lung tissue, it is important to keep in mind many aspects of normal pulmonary physiology. In addition, many physiological variables are important in the design of a bioreactor for in vitro lung culture. We outline some key aspects of pulmonary physiology below.

**Breathing.** In the adult, breathing occurs via coordinated action of the diaphragm and intercostal muscles. During inspiration, these muscles contract, expanding the thoracic cavity. This creates a negative pressure in the thoracic cavity, causing the lung to inflate and relieving the pressure differential. When the pressures in the airspace of the lung and in the thoracic cavity equate, inspiration stops. Exhalation occurs mostly through passive relaxation of the lung and chest muscles, raising pressures in the distal airspaces and causing air to flow out of the lungs [120].

Airway pressures in the rat during mechanical ventilation are typically 15-22 mmHg [35, 99]. The adult rat has a tidal volume of 0.6-2.0ml and a vital lung capacity of 8.4 +/- 1.7ml, with a respiratory rate of 70-115 breaths per minute [171]. In utero, fetal breathing movements in the rodent begin at approximately embryonic day 12 at 0.5/min, with progressively higher frequency breathing movements occurring with development at rates up to 15/min [3, 98]. These breathing movements play important roles in lung development, as discussed in section 2.6.

**Surfactant.** The lung has a huge surface area (∼ 70m² in the adult human), which is necessary to allow sufficient gas exchange. However, this large surface, and the resulting air-liquid interface, exerts surface tension that resists lung distension. In
order to help alleviate this resistance to lung inflation, a variety of surfactants, which are phospholipid molecules that function as detergents or wetting agents, are present in the lungs. Surfactant decreases the pressures required to inflate the lung, and also critically helps prevent alveolar collapse at end-exhalation [51].

Surfactant consists mostly (~90%) of phospholipids, the most common of which is dipalmitoylphosphatidylcholine (DPPC). The remaining ~10% are proteins, the specific surfactant proteins types A, B, C and D. Surfactant is primarily secreted by type II epithelium, which also reabsorbs it continuously such that the half-life of DPPC, for example, is approximately 12 hours in many animals [24, 213].

Surfactant function is conferred principally by the unique structure of the phosphatidylcholines (PC, mostly DPPC), which consist of a 3-carbon glycerol backbone with 2 long saturated fatty acid chains and 1 phosphorylchlorine polar molecule. The polar phosphorylchlorine is soluble in the aqueous portion of the alveolar fluid, while the nonpolar fatty acid chains project into the air above the fluid layer. The PC molecules can thus line up at the interface of the air and liquid layers and disrupt the surface tension that is normally generated by intramolecular attractive forces of the liquid [32]. The surfactant proteins play important roles in greatly accelerating the spreading and adsorption of PCs [51], thus accelerating the formation of surface films of PCs at the air-liquid interface [24].

**Perfusion.** The right ventricle pumps blood to the lungs through the pulmonary artery, which is then returned via the pulmonary vein to the left side of the heart and then into the systemic circulation. Compared to the systemic circulation, the pulmonary circulation is a low pressure system. In the rat, the peak pulmonary artery pressure is typically 15mmHg, although it can rise to 30 mmHg during hypoxia or stress [114]. This compares to systemic arterial pressures of ~150 mmHg [176]. The pulmonary venous pressure is only ~0-5 mmHg [176]. At the capillary level, there is a
small net positive transendothelial pressure in the vasculature of \( \sim 5-8 \) mmHg, which, besides keeping the capillaries patent, is a driving force for fluid movement out of the vasculature [140]. This fluid movement is controlled by endothelial, epithelial and lymphatic means; however, if this fluid balance is disturbed, pulmonary edema can result and gas exchange severely impaired. The adult rat has a heart rate of 200-300 bpm with a total pulmonary blood flow of 40-80ml/min [171]. In contrast, during fetal development only 8-10\% of cardiac output flows through the lungs [91], due to an arterial-venous shunt and high pulmonary vascular resistance. These physiological values are relevant to the design of a bioreactor for the \textit{in vitro} culture of whole segments of lung tissue.

\section*{2.6 \textit{In Vitro} Culture of Whole Lung Tissue}

The \textit{in vitro} culture of whole rodent lungs has been demonstrated by several groups over the past few decades [218, 85]. However, this prior work has been done with fetal rodent lungs, which are extremely small and do not require active perfusion or ventilation. Perfusion is not required as diffusion supplies sufficient nutrient supply, and ventilation is not critical as fetal lungs do not require regular breathing. Such studies are generally performed by placing the tissue on a mesh at the air-liquid interface (ALI) in relatively simple media (without many growth factors). Funkhouser \textit{et al.} cultured fetal rat lungs on a mesh at the ALI and demonstrated lung development and surfactant production that were grossly similar to \textit{in vivo} studies after 14 days of culture [67]. However, the rate of development was slower \textit{in vitro} and organ growth slowed as lung size increased. This study also demonstrated that there are sufficient self-contained developmental signals to allow development to proceed in the \textit{ex vivo} environment. Similar studies have also been conducted in mice [102], demonstrating organ culture for up to 27 days and confirming that, after certain embryonic
stages, pulmonary embryonic development can proceed independent of exogenous in vivo signals.

These studies and others demonstrate that the in vitro culture of lung tissue is a feasible objective. However, the lessons learned from these studies do not easily apply to the growth of engineered lung tissue. First, the size of any useful engineered lung tissue will preclude relying on diffusion for nutrient delivery. The above approaches to in vitro organ culture are only successful in fetal rodent lungs. Thus, a means of perfusion is required, adding great complexity to the experimental design. In addition, breathing movements will almost certainly be required for the successful culture of a functional engineered lung tissue. Although breathing movements were not required for the fetal organ cultures discussed above, the importance of fetal breathing movements (FBMs) has been documented and is important for the advanced development of pulmonary tissue [97]. In the absence of FBMs, type I pneumocytes cannot undergo late differentiation and thus cannot flatten [98] and type II pneumocytes may not be able to achieve their final differentiated state [96]. The clinical result of a lack of FBMs is pulmonary hypoplasia, a common neonatal disorder that has been found in 15% of neonatal autopsies [209].

There have been published reports of whole rodent lung culture in vitro. However, culture times are kept very brief, less than a few hours and most last only 30-60 minutes. Most of these studies utilize a commercially available system, the isolated perfused lung system from Hugo Sacks Elektronik (Hugstetten, Germany) [211, 199, 86]. There is no evidence that this system, or a similar system that has been separately developed [156], have been utilized or validated for long-term cultures lasting longer than 24 hours.
2.7 Tissue Engineering of the Lung: Prior Work

There has been significant progress on developing tissue engineered substitutes for the trachea and mainstem bronchi. In addition, there has been some work on engineering replacement distal lung parenchyma [142]. However, there have been no reported attempts on the development of whole segments of engineered lung tissue.

**Tracheal tissue engineering.** Sato *et al.* used an entirely decellularized mainstem bronchial replacement, made of a collagen-coated polypropylene mesh cylinder, which was implanted successfully into 8 dogs [168]. Suzuki *et al.* [187] used a similar collagen/polypropylene mesh scaffold, embedded with adipose-derived stem cells, and successfully implanted this scaffold into 2.0 x 6.0mm tracheal defects in rats. They demonstrated increased thickness and improved angiogenesis in the stem cell containing grafts. These grafts were not full-circumference, although the combination of a stem cell source with an engineered matrix yielded promising results.

Using the same approach, Omori and coworkers [149] replaced the cricoid cartilage and/or cervical trachea (2-6cm graft length) in dogs with a collagen/polypropylene mesh. These decellularized grafts demonstrated similar mechanical properties to normal cricoid cartilage, and implants remained patent for up to 40 months.

Weidenbecher *et al.* [206] generated scaffold-free cartilage sheets that were allowed to mature for 10 weeks by *in vivo* abdominal implantation in rabbits. While the resultant “neotracea” appeared promising on histologic analysis and qualitative bending tests, more substantive measurements and assessment of the grafts is required, as well as orthotopic transplant.

Most recently, Macchiarini *et al.* were able to implant a tissue-engineered trachea into a human patient suffering from end-stage bronchomalacia. The group first took a donor trachea, and decellularized the tissue using repeated (25) cycles of treat-
ment with sodium chloride, sodium deoxycholate, and deoxyribonuclease I (DNase I). The researchers then harvested the recipient’s cells from bronchial and bone marrow biopsies and cultured them in vitro to yield epithelial cells and chondrocytes, respectively. These cells were then cultured on the decellularized donor trachea in a rotating bioreactor for 4 days, after which it was surgically implanted in place of the diseased mainstem bronchus. The clinical outcome was excellent, with no signs of graft rejection and a patent, functional graft at 12 months follow-up [122, 14].

The above reports of tissue engineering of the trachea, mainstem bronchi and cricoid cartilage demonstrate promising results regarding the ability to replace the large conducting airways down to the level of the mainstem bronchi. Evidence indicates that a purely decellularized graft is capable of providing sufficient mechanical and structural function while the graft is repopulated by autologous cells, at least in some species. However, further work is needed to improve cellular re-population of longer or even full-length tracheal/bronchial replacements.

**Tissue engineering of the pulmonary parenchyma.** There have been several reports of tissue engineered distal lung tissue, which generally combine a collagen or polymer scaffold with a pulmonary cell source [39, 135, 45, 10]. All of these approaches suffer from a lack of 3-dimensional organization, which is a critical deficiency. In order for engineered lung tissue to be useful, it must be able to connect to a vasculature and an airway. The airway must be continuous with alveoli, while the vascular connections must lead to a dense capillary network surrounding the alveoli. All work to date, while promising, has simply demonstrated the ability to induce the growth of alveoli in various 3-dimensional substrates such as gels or polymers. However, there is no organization to these alveoli; they are not connected to a larger airway or even to each other. Furthermore, there is no vascular network surrounding the alveoli, which is an absolutely critical feature of functional lung tissue in order to allow gas exchange.
Below, we outline in greater detail the approaches to lung tissue engineering to date.

Chen et al. demonstrated the growth of epithelial and mesenchymal cells on a collagen-glycosaminglycan scaffold that was seeded with pulmonary cells from 19-day fetal rats and cultured for up to 3 weeks. They found evidence of organized, ciliated, pseudo-stratified epithelium as well as presumptive developing alveoli [39].

Mondrinos et al. [135] cultured fetal mouse pulmonary cells on several different scaffolds, and in some cases demonstrated growth of type II epithelium and formation of primitive ring structures, possibly indicative of alveolar formation. The scaffolds utilized were Matrigel, a PLLA fiber matrix, and a PLGA foam. Further work by the same group used subcutaneous implants in mice of Matrigel plugs that were seeded with mouse fetal pulmonary cells [136]. They demonstrated the maintenance of lung epithelial cell phenotype via surfactant protein C (SPC) staining and also the formation of vasculature in the plugs both with and without FGF-2 supplementation.

Cortiella and colleagues described the culture of ovine pulmonary cells on both polyglycolic acid and Pluronic F-127 scaffolds, and demonstrated expression of Clara cell secretory protein (CCSP) and SPC after extended in vitro culture [45]. However, these polymer scaffolds induced a foreign body response when implanted into nude mice, underscoring the difficulties of using artificial scaffolds in lung regenerative approaches. Attempts to implant these constructs in the lung of adult sheep were not successful and demonstrated no pulmonary tissue formation [45].

In the first successful in vivo test of engineered distal lung tissue, Andrade et al. used Gelfoam sponges that were inosculated with fetal rat lung cells and injected into the lung parenchyma of adult rats [10]. They demonstrated evidence of staining for CCSP, SPC and von Willibrand factor (vWF), as well as the formation of vasculature structure and the possible appearance of lung alveolar structures.

In summary, while the above work is promising, much more must be done to
demonstrate the production of functional engineered lung tissue. There are no approaches that have the potential to create organized airway or vascular structures, which would be required for functional lung tissue. In addition, no tools have been demonstrated that enable the long-term culture of whole lobes of engineered lung tissue. In this work, we aim to address some of these key issues.

2.8 Decellularization

Decellularized tissues have been used to create scaffolds for a variety of tissue engineering applications, including pericardium [134], heart valves [16], trachea [43], liver [117] and heart [152], among others. A decellularized organ offers several advantages for use as a tissue engineering scaffold. First, the decellularized scaffold contains the appropriate 3-dimensional organization required for tissue function, including a vascular system and airway network in the case of lung. In addition, ECM components are widely conserved across species, thus reducing the likelihood of a decellularized scaffold inducing an immune response upon xenogeneic implantation [22]. Finally, native ECM offers the optimal substrate for cell attachment, spreading, growth and differentiation.

The goal of the decellularization process is to remove cellular and nuclear material while retaining key aspects of and minimizing any damage to the extracellular matrix (ECM) of the lung. There are many different types of decellularization protocols, which make use of physical, chemical and enzymatic methods. In this work, chemical methods are used; it was presumed that physical methods would cause excessive concomitant destruction of the delicate lung ECM, while enzymatic methods would not be cost-effective given the large volumes of liquid required for the treatment of an entire organ.

Physical methods of decellularization include freezing, pressure, sonication and
agitation [75], and have typically been used to decellularize tendons or ligaments. Due to the delicate nature of lung tissue and concern for the ultrastructure of the tissue, we did not explore the utility of physical methods for lung decellularization.

Enzymatic methods of decellularization include the use of proteases, nucleases, and calcium chelators. The most common enzymatic approach uses trypsin, although this can remove fibronectin, laminin, and elastin [75]. Due to the importance of these substances, we did not evaluate enzymatic methods of decellularization.

Chemical methods of decellularization are the most common, and include a wide variety of techniques, including alkaline or acidic solutions, detergents (including ionic, non-ionic, and zwitterionic), hypotonic or hypertonic solutions, and chelators [75]. The chemicals used in these studies were sodium chloride, CHAPS, and EDTA. A hypertonic sodium chloride solution can efficiently lyse cells, although it does not assist in removing cellular components from the tissue. CHAPS is a zwitterionic detergent, which allows efficient solubilization and thus removal of cellular material. EDTA is a chelating agent that binds key divalent ions (i.e. Ca$^{2+}$) that aids in disrupting cell attachment to the ECM. In addition, the solution is of high alkalinity, which helps solubilize cytoplasmic cellular components as well as GAGs which otherwise clog the matrix [75].
Chapter 3

Development and Characterization of a Decellularized Lung Scaffold

In this chapter, we describe the decellularization of native lung to create a decellularized lung scaffold. The motivation for this work is to create a decellularized lung scaffold that can be used for tissue engineering applications if combined with suitable cell populations. We evaluate the hypothesis that native lung tissue can be decellularized to remove cellular components and antigenic molecules, yet retain key extracellular matrix molecules and retain an intact barrier between the airway and vascular compartments.

In order to evaluate this hypothesis, we first describe the technique we have developed for decellularization, and then demonstrate that the decellularized scaffolds we produce are devoid of cells and immunogenic markers. We show that the DNA content of the decellularized scaffolds is \( \sim 1.2\% \) of native levels. We evaluate the remaining extracellular matrix to show that collagen types I and IV are present in appropriate locations but that fibronectin is lost. We then utilize both scanning and transmission electron microscopy to evaluate the ultrastructure of the scaffolds, and demonstrate that alveolar septae are intact along with an intact alveolar basement membrane that can significantly inhibit the translocation of 5 \( \mu m \) particles between the airway and vascular compartments. We demonstrate that vascular perfusion pressure has a significant impact on the retention of the scaffold ultrastructure, and demonstrate that with low perfusion pressures we retain at least some capillaries in the decellularized scaffold. Finally, we utilize micro-CT imaging to evaluate the patency of the vasculature and airway compartments.
In the next chapter, we continue our evaluation of the decellularized scaffolds but with a focus on mechanical integrity, including mechanical testing, quantitation of collagen and elastin content, and modelling studies of the observed mechanical behavior.

3.1 Introduction

Progress in the development of engineered lung tissues has been slower than for many other tissue types. This is in part due to the complexity of pulmonary tissue and the unique challenges it poses to tissue engineers, but also is a result of difficulty in culturing lung endothelial [128] and epithelial [161] cells in vitro and the slower progress in the field of pulmonary progenitor and stem cells [25, 141].

However, there has been significant progress in developing tissue engineered substitutes for the trachea and mainstem bronchi [122, 168, 149, 187]. Recent work by Macchiarini et al. demonstrates that using decellularized native lung as a scaffold for the creation of engineered lung tissues is a viable approach [122]. These researchers were able to implant a tissue-engineered trachea into a human patient suffering from end-stage bronchomalacia. The tissue engineered trachea was created by first decellularizing a cadaveric donor trachea, confirming that cell and antigen removal was complete, and seeding with the recipient’s cells cultured from bronchial and bone marrow biopsies. After culture in a rotating bioreactor for 4 days [14], the engineered trachea was surgically implanted in place of the diseased mainstem bronchus. The clinical outcome was excellent, with no signs of graft rejection and a patent, functional graft at 12 months follow-up [122, 14].

There have been several reports of tissue engineered distal lung tissue, which generally consist of a collagen or polymer scaffold combined with a pulmonary cell source. Chen et al. demonstrated the growth of alveolar-like structures after in vitro
culture of fetal rat pulmonary cells on a collagen-glycosaminoglycan scaffold [39]. Mondrinos et al. cultured fetal mouse pulmonary cells on several different scaffolds, and in some cases demonstrated growth of type II epithelium and formation of primitive ring structures, possibly indicative of alveolar formation [135]. Further work by the same group used subcutaneous implants in mice of Matrigel plugs that were seeded with mouse fetal pulmonary cells [136]. They demonstrated the maintenance of lung epithelial cell phenotype via surfactant protein C (SPC) staining and also the formation of vasculature in the plugs both with and without FGF-2 supplementation [136].

While tissue engineering approaches to lung regeneration are feasible, these efforts are still in their infancy. In this work, we utilize native lung tissue as a starting point to develop decellularized scaffolds of the entire lung, and strive to demonstrate their suitability as scaffolds for the future development of engineered lung tissues. A decellularized lung scaffold is superior to other scaffolds used in lung regeneration approaches for several reasons. First, a decellularized lung begins as native lung, with an ideal 3-dimensional architecture, including pre-formed alveoli, a complete airway tree leading from the trachea down to the alveoli, and arterial and venous networks that connect to a dense capillary network surrounding the alveoli. Second, connections are in place for the trachea, pulmonary artery and pulmonary vein, thus facilitating the growth of engineered tissue and eventually allowing the implantation of engineered lung tissue. Finally, the scaffold consists of principally the same extracellular matrix components present in native lung, located in the correct anatomical regions. This can facilitate cellular adherence to the scaffold as well as help direct the cellular development of the engineered tissue.
3.1.1 Scaffold design criteria

The goal of the decellularization process is to remove cellular and nuclear material while retaining key aspects of the extracellular matrix (ECM) of the lung. Furthermore, we aim to minimize any impact on the ECM by the decellularization process. Some disruption of the ECM is inevitable as it must be disturbed to some degree in order to allow exposure of the decellularization reagents to all cells in the tissue. The rationale for the use of a decellularized scaffold is that ECM components are widely conserved across species [195, 9], thus reducing the likelihood of a decellularized scaffold inducing an immune response upon xenogeneic implantation. Here, we outline proposed design goals for the creation of a decellularized scaffold for lung tissue engineering. We do not achieve all of these goals in this thesis, but by identifying our target objectives, we can assess progress during the course of development of a decellularized scaffold.

**Cellular toxicity.** The decellularized matrix must be fully compatible with cell culture. The decellularization process requires the use of harsh chemical treatments that are not compatible with cell survival [75]. Therefore, we must ensure that all cytotoxic remnants of decellularization are removed such that there is no detectable effects of remnant chemicals on cell culture.

**Removal of cellular material and DNA.** In order to use a decellularized scaffold for tissue engineering, we first must ensure that it is devoid of any viable native cells. If we intend to seed cells onto the decellularized scaffolds, we must be certain that the cells we observe are not remnants of the native lung. A scaffold absolutely must contain zero viable cells. DNA content should be below 0.1% of the dry weight of the scaffold, and preferably below 0.01%, based on levels found in currently available commercial decellularized scaffolds for skin grafts [74].
Removal of antigenic components. The scaffold must not retain immunogenic components of the native lung and must not provoke an immune response if the scaffolds are implanted into an animal model. We therefore evaluate the MHC Class I and Class II content of the decellularized scaffolds. MHC class I antigen is present on all cells, and the presence of this antigen will give an indication of the immunogenicity of the scaffold, while MHC class II antigen has been implicated in acute allograft rejection [157, 8]. For true assessment of immunogenicity, the scaffolds must be implanted into an animal model and the immune response assessed.

Extracellular matrix components. While we wish to remove all cellular and immunogenic material from the scaffolds, we do not want to damage the extracellular matrix, which is left behind after cellular removal. The extracellular matrix (ECM) of the lung serves several functions. It imparts mechanical strength and elasticity to the tissue, it provides a substrate for cell growth, it helps direct the development of embryonic lung tissue, and it aids in cellular communication in the lung via mechanical interactions with cells [75]. Because of these important functions, we must take care to minimize damage to the ECM during the decellularization process. We aim to retain 90% of the collagen, elastin and fibronectin of native lung tissue. Collagen and elastin are critical for the mechanical function of the lung, while fibronectin is a key substrate molecule that is important for the adherence and proliferation of both type II epithelium [158, 108] and endothelium (section 7.3.2).

Retention of vascular and airway structures. After decellularization, we must have an intact airway tree and vascular network. Thus, there must be a trachea that is continuous with the alveolar network, and an intact pulmonary artery and vein with an intervening capillary network. The trachea, pulmonary artery and pulmonary vein must be intact and of sufficient strength to survive manipulations during engineered
tissue culture and subsequent implantation into an animal model.

**Barrier function.** The epithelium, endothelium, and extracellular matrix are all key components of preventing fluid translocation and pulmonary edema. Preventing fluid movement from the capillary network into the alveoli is critical to lung function; pulmonary edema is the clinical result of excessive fluid movement across the capillary-alveolar barrier [127]. The capillary endothelium [69] and alveolar epithelium [53] together prevent fluid movement across this barrier by creating a physical barrier. In addition, the type I alveolar epithelium aids fluid resorption from the alveoli [55]. The decellularized scaffold would not be expected to retain fluid movement or the movement of small molecules; in the absence of cells, this degree of retention would be extremely difficult.

However, the extracellular matrix provides a physical barrier to the movement of large macromolecules and red blood cells into the alveoli [27, 58], and also assists in preventing interstitial fluid accumulation [140]. This function would be expected to be retained in a decellularized matrix. We therefore aim to retain sufficient ECM components such that macromolecular transit is restricted such that at least 99% of macromolecules (5 μm size) cannot transit across the alveolar-capillary membrane.

**Summary of design objectives.** In summary, these design objectives are intended to be a comprehensive outline of our goals for the production of a decellularized lung scaffold. We do not intend to be able to fulfill all of these objectives from the work in this dissertation, but the identification of these goals allows us to assess our progress towards the development of decellularized lung scaffolds.
3.2 Materials and Methods

3.2.1 Organ harvest

Lungs were harvested from young adult (3 month-old) male Fischer 344 rats. All animal experimental work was performed with approval from the Yale University Institutional Animal Care and Use Committee. Animals were anesthetized via intraperitoneal injection of sodium pentobarbital (Sigma, 40 mg/kg). After induction of anesthesia, the abdomen was entered via a transverse incision just below the costal margin. The diaphragm was punctured, and the rib cage was cut to reveal the lungs. The lungs were perfused via the right ventricle with PBS containing 50 U/ml heparin (Sigma). After perfusion was complete, the heart, lungs and trachea were dissected free and removed en bloc.

3.2.2 Bioreactor components

For decellularization, a simplified version of the bioreactor described in detail in section 5.2.2 was utilized. All bioreactor components were obtained from Cole-Parmer (Vernon Hills, IL). A silicone stopper and 500ml glass jar formed the basis of the bioreactor. PharMed tubing (Westlake, OH), sizes L/S 14 and L/S 16, was inserted through the silicone stopper to enable the necessary connections to the lung, including a perfusion loop and air ventilation. Pressure was monitored using a TruWave pressure transducer (Edwards Lifesciences, Irvine, CA) between the perfusion pump and the connection to the pulmonary artery. Perfusion was accomplished using a Masterflex L/S variable speed roller pump (Masterflex, Vernon Hills, IL).

3.2.3 Decellularization process

Fluid used for decellularization was 8mM CHAPS, 1M NaCl, 25mM EDTA in PBS. All chemicals were obtained from Sigma, and PBS was obtained from Gibco. The
bioreactor was filled with decellularization fluid, and the bioreactor was transferred to an incubator kept at 37°C. The perfusion pressure was monitored at the inflow to the pulmonary trunk and kept below 30 mmHg. The decellularization fluid was replaced with fresh fluid at the following time points: 30 min, 1 hour, 2 hours, 4 hours, 6 hours. For most conditions, decellularization was stopped after 4 or 6 hours.

3.2.4 DNA assay

DNA content of tissues was quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Eugene, OR), following manufacturer’s instructions. Briefly, tissue samples were weighed and lyophilized, diluted in TE buffer and mixed with the Quant-iT PicoGreen reagent. Fluorescence was measured at 535 nm with excitation at 485 nm, and DNA content was quantified using a standard curve. At least 4 samples were measured for both native and decellularized samples.

3.2.5 Western Blotting

Tissues for Western blotting were digested in cold RIPA buffer (Boston Bioproducts) with added protease inhibitors (Sigma) and homogenized at 15,000 rpm for 30 seconds. After incubation for 1 hour at 4°C, insoluble particles were removed by centrifugation at 14,000 g for 25 min. Protein concentration was quantified via Bradford assay [29], then boiled in Laemmli’s reducing buffer (Boston Bioproducts) for 25 min at 65°C. Samples were stored at -80°C until analysis.

Samples were run on variable percent polyacrylamide gels, using 25-30 µg of protein. After electrophoresis, protein was transferred to a nitrocellulose membrane. Membranes were rinsed in TBS, then blocked for 1 hour in 5% non-fat dry milk (NFDM) or 3% bovine serum albumin in TBS with 0.05% tween-20 (TBS-T). Primary antibodies were applied overnight in 2% NFDM or 3% BSA in TBS-T. Antibody
source and dilutions are given in table 3.1.

Secondary antibodies were from Santa Cruz and were raised in either donkey or goat, and were applied for 1 hour at room temperature at a dilution of 1:2000. Protein was detected using substrate from Supersignal West Pico, which was applied for 5 minutes before film development.

### 3.2.6 Immunofluorescence

Tissue blocks were fixed for 4 hours in 3.7% formaldehyde (Sigma), then transferred to 70% ethanol and embedded in paraffin. Thin (5 \( \mu \)m) sections were prepared by the Yale University Histology core facility. Tissue sections were deparaffinized in xylene, rehydrated through an ethanol gradient, and rinsed in buffer (PBS + 0.2% triton-X) for 15 minutes. Antigen retrieval was performed in 0.01M citric acid, pH 6.0, at \( \sim 70^\circ C \) for 20 minutes. After cooling to room temperature, sections were rinsed in buffer, then blocked in PBS with 5% bovine serum albumin (BSA) and 0.75% glycine for 1 hour at R.T. Primary antibodies were applied at the concentrations given in table 3.1 in blocking buffer overnight at 4\( ^\circ \)C. Slides were rinsed 3 times in buffer and then secondary antibodies were applied at 1:500 dilution in blocking buffer for 1 hour at R.T. Secondary antibodies were AlexFluor 555 donkey anti-goat or goat anti-rabbit and AlexaFluor 488 chicken anti-rabbit, obtained from Invitrogen. Slides were mounted using DAPI-containing mounting media (Vector Labs), and images acquired using a Zeiss Axiovert 200M inverted fluorescent microscope.

### 3.2.7 Scanning electron microscopy

Samples were fixed using 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1M cacodylate buffer (EMD Biosciences, Gibbstown, NJ) for 2 hours at room temper-
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Concentration</th>
<th>Western</th>
<th>IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-actin</td>
<td>Abcam</td>
<td>n/a</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Sigma</td>
<td>1:2000</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>AQP-5</td>
<td>Millipore</td>
<td>1:100</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>CCSP/CC10</td>
<td>B. Stripp (Duke)</td>
<td>1:10,000</td>
<td>1:20,000</td>
<td></td>
</tr>
<tr>
<td>cytokeratin-14</td>
<td>ThermoScientific</td>
<td>n/a</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>MHC Class-I</td>
<td>Abcam</td>
<td>1:1000</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>MHC Class-II</td>
<td>Abcam</td>
<td>1:1000</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Santa Cruz</td>
<td>1:1000</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>SP-A</td>
<td>Santa Cruz</td>
<td>1:100</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>SP-C</td>
<td>Millipore</td>
<td>1:500</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Santa Cruz</td>
<td>1:500</td>
<td>1:1000</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1: Antibody sources and concentrations for immunofluorescence (IF) and Western blotting. Abbreviations are given in text and the List of Abbreviations.

ature, then rinsed in cacodylate buffer, sliced, and dehydrated through an ethanol gradient. Samples were further dehydrated in hexamethyldisilizane for 10 min and dried overnight, then sputter coated with gold and analyzed using a JOEL JXA-8600 at the Yale University Geology and Geophysics facility.

3.2.8 Transmission electron microscopy

Samples were fixed using 4% paraformaldehyde in PBS and then placed in 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1M sodium cacodylate buffered fixative (pH 7.4) for 2 hours at room temperature. The samples were rinsed 3 times in 0.1M sodium cacodylate buffer and postfixed in 1% osmium tetroxide for 1 hour, then en bloc stained in 2% uranyl acetate in maleate buffer pH 5.2 for a further hour. Then, the samples were rinsed, dehydrated through a graded ethanol series and infiltrated with epon resin and baked overnight at 60°C. Hardened blocks were cut using a Leica UltraCut UCT and 60nm sections were collected on nickel grids and stained using 2% uranyl acetate and lead citrate. Samples were viewed on a FEI Tencai Biotwin
TEM at 80kV. Images were taken using a Morada CCD digital camera using iTEM (Olympus) software.

3.2.9 Microsphere retention

Decellularized or native lungs were attached to cannulae as described in section 3.2.1, and the lung was inflated via the trachea with PBS containing 5 µm microspheres. The vasculature was then flushed with 3 rinses of 10ml PBS. Microspheres were washed twice in dH₂O to remove debris and lyse any cells that would otherwise affect the native lung readings. Using a Coulter counter set to measure particles between 4.9 µm and 5.1 µm, the microsphere concentration in each sample was quantified and compared to a baseline reading taken before microsphere injection.

3.2.10 Micro CT Imaging

Native or decellularized lungs were fixed in 10% neutral buffered formalin (Sigma) and injected with contrast agent through either the airway or vasculature. Contrast agent was 20% bismuth and 5% gelatin (Sigma) in PBS. After injection of contrast, the lung was cooled in an ice bath to polymerize the gelatin.

Image acquisition

For the whole lung, the pulmonary vasculature was imaged with a micro-CT imaging system (GE eXplore Locus SP, GE Healthcare), set to a 0.029-mm effective detector pixel size. The micro-CT was operated at 60kV peak x-ray tube voltage, 80mA tube current, 1600millisecond per frame, 22 detector binning model, 720 views, and 0.5° increments per view. For the high resolution imaging of one lobe (right superior-lobe), samples were positioned on a computer-controlled rotation stage and scanned 360 around the vertical axis in rotation steps of 0.4°. The tube is operated at an 80
kV peak and 80 mA. The exposure time for each view was typically 3000 millisecond, with detector binning model set to 1x1 and resolution of 0.0065mm. Both acquisitions resulted in a set of contiguous axial VFF-formatted images through the lung or one lobe.

**Imaging reconstruction and Quantitative Image Analysis**

With the use of Microview Software (GE Healthcare), the raw data was corrected and reconstructed with voxels of dimensions 58 µm x 58 µm x 58 µm to visualize the whole vascular tree in the lung. For the high-quality of the vascular tree (one lobe), voxels of dimensions was set to 6.5 µm x 6.5 µm x 6.5 µm. This software was also used to reconstruct maximum intensity projection images from the raw data. A radiologist (Zhenwu Zhuang) with 20 years of experience performed image reconstruction and analyzed the micro-CT images [219]. Multiplanar reformation, spatial filtering, and volume rendering techniques allowed us to view the data set in transverse, sagittal, coronal, hybrid planes, and 3D format. Binarized images were used for object extraction and region-of-interest measurements. Three-dimensional volume images are reconstructed from the angular views by using a modified Feldkamp filtered back-projection algorithm. However, with this system, an entire rat lung (field of view, approximately 3.0 cm) may be studied, with images having typical cubic voxel dimensions as small as 58 µm. The opacity of each voxel is represented by a 16-bit gray-scale value.

**3.3 Results**

**3.3.1 Decellularization method**

We have identified a decellularization method that removes cellular material from complete lobes of intact rodent lungs. The method we identified was the result of
extensive experimentation and serial iterations using feedback from the techniques described in later sections (histology, mechanical testing, and collagen quantization). Some of the conditions that were explored are outlined in table 3.2. The decellularization conditions were initially chosen based on their prior use in the literature [16, 117, 75]. We determined that decellularization with 1M NaCl, 8mM CHAPS and 25mM EDTA was optimal to remove cellular material and yet did not appear to remove collagen or elastin fibers (based on histology) or damage the structural integrity of the matrix (based on mechanical testing). In comparison, decellularization with solutions containing SDS were found to damage the mechanical strength of the matrix, as shown in figure 4.7 and discussed in section 4. Other conditions were found to either not efficiently remove cellular material or cause significant declines in the matrix integrity via histology.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Conditions</th>
<th>Chosen Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decellularization solution</td>
<td>NaCl; CHAPS; EDTA; SDS; triton-X-100; DNase I; dH2O; NH4OH</td>
<td>1M NaCl, 25mM CHAPS, 2mM EDTA</td>
</tr>
<tr>
<td>Total decell. time</td>
<td>1, 2, 4, 6, 8hr</td>
<td>4hr</td>
</tr>
<tr>
<td>Vascular perfusion with decell. solution</td>
<td>1, 2, 3, 4, 6, 8hr</td>
<td>3hr</td>
</tr>
<tr>
<td>Ventilation with decell. solution</td>
<td>0, 1hr</td>
<td>1hr</td>
</tr>
<tr>
<td>Elastase inhibition</td>
<td>1mM PMSF</td>
<td>Without</td>
</tr>
<tr>
<td>Pre-decell. vasodilation</td>
<td>1uM SNP</td>
<td>1uM SNP</td>
</tr>
</tbody>
</table>

**Table 3.2:** Conditions that were evaluated for the decellularization of rat lung. Abbreviations are given in the List of Abbreviations.

### 3.3.2 Histological analysis

We have used histology to characterize many decellularized lung scaffolds. In total, we have decellularized over 200 lungs, and many of these have been analyzed via histology to document the removal of cellular material. Of note, in the examination
of histologic sections of these decellularized lungs, we have not observed a single intact
cell, based on H&E staining and DAPI-staining for nuclei and DNA. On occasion we
have noted unwound DNA or cellular antigen, but intact cells have not been observed.
Figure 3.1 demonstrates H&E staining of native and decellularized lung, while figure
3.2 shows DAPI-staining for remnant DNA. We also note apparent preservation of the
pulmonary structure; alveolar septae appear intact on standard histological sections,
as do the larger airways and blood vessels.

![Native lung](image1) ![Decellularized lung](image2)

**Figure 3.1:** H&E staining and quantitative DNA assay of native and decellularized
lung. Removal of cellular material yet retention of overall scaffold architecture is
noted via histology, while DNA is removed to ~1.2% of native levels. * indicates
p<0.01
3.3.3 DNA content

In order to document removal of cellular material, a quantitative DNA assay was performed. This demonstrated a drastic reduction in DNA content in decellularized scaffolds compared to native lung (figure 3.1(c)). Decellularized scaffolds contain approximately 1.2% of the DNA found in native lung, which corresponds to 1.83±0.29ng of DNA per mg dry weight. This compares to 38.7±5.8ng/mg for native lung. While extensive rinsing of the scaffolds is used to minimize remnant DNA, complete removal of all DNA is difficult and small amounts of DNA remain, as demonstrated by DAPI stains showing small clusters of unwound DNA in figure 3.2. The drastic reduction in DNA content is indicative of cellular removal, and together with the histological findings confirms that all viable cellular material is absent from the scaffolds. Furthermore, the decellularization conditions are extremely harsh, including high concentrations of salt and detergent and high alkalinity, and it is highly unlikely that any cell can survive such conditions.

![Figure 3.2](image)

**Figure 3.2:** Staining for remnant DNA in decellularized scaffolds. DNA is stained blue using DAPI, showing DNA in nuclei in native lung and weak occasional elongated DNA in decellularized scaffolds (at bottom center of image). Images were exposed for the same time to enable comparison.
3.3.4 Immunogenicity

We characterized the immunogenicity of the decellularized scaffolds by staining for MHC Class I and II antigens. Major histocompatibility complex (MHC) class I and II proteins are membrane glycoproteins that are important in the antigen-specific immune response. MHC Class I antigen is expressed on all nucleated cells, while MHC Class II antigen is found on specialized cells of the immune system. MHC Class I antigens allow an organism to recognize ‘self’ from ‘non-self’, and are thus important to remove from the decellularized scaffold in order to avoid immune problems upon future implantation of engineered lung tissue into an animal model. We demonstrate in figure 3.3 Western blotting results for MHC Class I and II antigen as well as $\beta$-actin. We show that both MHC Class I and II antigens are not detected in decellularized scaffolds by immunoblotting, confirming that the scaffolds would not be expected to provoke a significant immune response if implanted into a host. Note that $\beta$-actin is also lost, consistent with the absence of cellular material. The same amount of protein was loaded for all wells.

**Figure 3.3:** Western blot for MHC Class I and II antigen, demonstrating lack of MHC Class I or II antigen in decellularized scaffolds.
3.3.5 Extracellular matrix characterization

Collagen. Collagen is the most important structural component of the lung, being principally responsible for the overall mechanical strength of the tissue. Immunofluorescence was used to characterize the distribution of collagens I and IV in native and decellularized lung, as shown in figure 3.4. Both collagen I and IV are retained by the decellularized matrix, with collagen I noted principally around the larger airways and vasculature, and collagen IV noted throughout the parenchyma. Similar staining patterns are noted for both native and decellularized lung. The preservation of these collagen subtypes in their anatomically appropriate locations may enable the selective deposition of cell types during the development of engineered lung tissue.

![Collagen staining in native and decellularized lung](image)

**Figure 3.4:** Collagen staining in native and decellularized lung. Collagen I is stained green, collagen IV is stained red, and nuclei are counterstained blue with DAPI. Collagen I is found near large vessels while collagen IV is distributed throughout the parenchyma. Note that in native lung, red blood cells in the parenchyma appear green due to autofluorescence.

Fibronectin. Fibronectin is another key matrix component that is important for endothelial cell attachment. Immunofluorescence demonstrates, however, that fi-
bronectin is absent from the decellularized scaffolds, as shown in figure 3.5. This loss of fibronectin is also discussed in section 7.3.2, in which the lack of fibronectin was found to affect our ability to culture endothelium on the decellularized scaffolds. Fibronectin is critical for endothelial attachment and this lack of fibronectin impacts the ability to seed endothelial cells onto the decellularized scaffolds, which is discussed in detail in section 7.3.2.

Figure 3.5: Fibronectin staining in native and decellularized lung. Fibronectin is stained red, and nuclei are counterstained blue with DAPI. Very faint fibronectin staining is noted in decellularized lung. Green is autofluorescence of the lung scaffold, shown in overexposure to aid visualization of the scaffold. In native lung, remnant red blood cells autofluoresce green.
3.3.6 Scanning EM evaluation of decellularized scaffolds

We used scanning electron microscopy (SEM) to evaluate the microstructure of the decellularized lung scaffolds. Figure 3.6 shows sample images, demonstrating cellular removal yet overall maintenance of alveolar architecture. The alveoli in decellularized lungs appear slightly deflated, which is an artifact of fixation. Native lung is fixed by inflating the lung with fixative; the decellularized lung, however, cannot contain the fixative fluid within the alveolar compartment when pressurized, thus giving the lung a deflated appearance. However, there is a general similarity in alveolar architecture with preservation of the alveolar septae, although some fraying of the matrix is evident in the decellularized samples. These results, together with findings from histology studies, suggests that the overall pulmonary airway architecture and alveolar structure, including alveolar septae, are intact in the decellularized scaffolds.

3.3.7 Impact of perfusion pressure on scaffold ultrastructure

In addition to the scanning EM studies, we have used transmission EM to study the capillary-alveolar basement membrane. This is a critical feature of the decellularized scaffolds as the presence of an intact capillary network will allow the decellularized scaffold to resist macromolecular transit into the alveolar spaces and will also provide a suitable substrate for the growth of capillary endothelium in engineered lung tissues.

In figure 3.7(a) and 3.7(b) we show TEM images of native lung and lung that was decellularized without control of vascular perfusion pressures. Under such conditions, the alveolar basement membrane was at times not identifiable and no capillaries could be found. We hypothesized that we could minimize damage to the basement membrane and ultrastructure by minimizing the perfusion pressures during the decellularization process and maximally vasodilating the vasculature before
Figure 3.6: Scanning EM of native and decellularized lung. Alveolar septae are intact although some damage to the matrix is evident in the decellularized tissues. Scale bars are 100 µm in left panels and 20 µm in right panels.
beginning decellularization. Although we perfuse the decellularization fluid through the vasculature at sub-physiologic flow rates, vascular perfusion pressure can become supraphysiologic during decellularization due to massive cell lysis and buildup of cellular protein and DNA in the vasculature. We therefore carefully monitored the pulmonary arterial pressure and modified the decellularization bioreactor and perfusion rate in order to keep this pressure strictly below \( \sim 30 \) mmHg. The vasodilator sodium nitroprusside was added to the perfusate in order to minimize the initial perfusion pressures. This vasodilation was important in order to counteract a normal physiologic response to hypoxic conditions. When a region of the lung is not well ventilated, the vessels in that region constrict to reduce perfusion, thus preventing the flow of blood to a region that would not enable exchange of oxygen [23]. After explantation of the lung, there is no ventilation of any region of the lung, and so the entire vasculature of the lung constricts due to this normal physiologic behavior. Therefore, the use of a vasodilator to counteract this effect was important in our efforts to maintain low perfusion pressures during decellularization and therefore retain as much ultrastructure as possible.

In figure 3.7(c) we show TEM images of scaffolds decellularized with pressures kept below \( \sim 30 \) mmHg. Under these conditions, we see an intact, continuous alveolar basement membrane. Collagen fibers and other matrix components are retained within the alveolar septae. However, we do not notice the presence of any clear capillary structures, which should be present in abundance surrounding the alveoli.

**Retention of capillary structures in decellularized scaffolds.** The typical pressure in the pulmonary vascular system of the rodent is less than 15mmHg [114], significantly lower than the 30mmHg utilized in the above studies. Despite reducing the perfusion flow rate and using a vasodilator to lower perfusion pressures, we were unable to maintain the decellularization perfusion pressure below 30mmHg.
Figure 3.7: Transmission EM of native and decellularized lung. The alveolar basement membrane is retained when decellularization perfusion pressure is maintained below 30mmHg. C indicates capillaries, A indicates alveoli, and S indicates the alveolar septae. Scale bars are 2 µm in all panels.
However, we discovered that a slight modification in the decellularization protocol did enable perfusion during decellularization at pressures less than $\sim 20\text{mmHg}$. Of significance, this enabled the retention of at least some capillary structures. This modification consisted of lavaging the airway compartment with decellularization fluid before beginning perfusion of the decellularization fluid through the vasculature. The result was the significant lowering of the vascular perfusion pressure, especially at the beginning of the decellularization process. As shown in figure 3.8, this technique enabled the retention of capillary structures in the decellularized scaffolds.

These findings are the most recent improvements in the decellularization process, and have not been fully evaluated. We only have TEM imaging from one lung that was decellularized via this method. However, the retention of capillaries is an exciting and significant development in the creation of decellularized lung scaffolds, and we are therefore including this data despite the results being preliminary. Clearly, future studies will continue to evaluate this technique as we continually attempt to improve the decellularization process.

### 3.3.8 Permeability Assessment

In order for a lung to function in vivo, it must possess a continuous, patent and non-leaky vasculature in order to avoid massive blood loss into the alveolar and interstitial spaces. We evaluated the ability of decellularized lung scaffolds to retain $5 \mu m$ microspheres in the airway compartment, without allowing transport of these macromolecules into the vasculature.

We use $5 \mu m$ particles in order to mimic the size of red blood cells, the principle component of blood, which would need to be retained in the vasculature. However, we inject the microspheres into the airway and not into the vasculature. This is because the decellularized scaffolds are permeable to water and other small molecules, and
Figure 3.8: Transmission EM of decellularized lung demonstrating preserved capillaries. Perfusion pressure was less than 20mmHg. C indicates capillaries, while A indicates alveoli. Scale bars are 2 $\mu$m on top panels, 1 $\mu$m on bottom left panel, and 500nm on bottom right panel.
therefore the airway compartment cannot be lavaged to assess microsphere leak into the airway. We can, however, perfuse the vasculature in order to rinse out particles that have leaked out of the airway compartment. We thus evaluate the leak of 5 µm particles out of the airway and into the vasculature, and assume that there is no significant directionality to the movement of such particles across a decellularized membrane.

We determined the permeability of native lungs, lungs decellularized with uncontrolled perfusion pressures (constant perfusion flow rate), and lungs decellularized after vasodilation and with controlled perfusion pressures (less than 30mmHg). The results are shown in figure 3.9, and confirmed the TEM findings on a larger scale. We find that decellularization with high (uncontrolled) perfusion pressure leads to a 39% leak, compared to 5.7% for low-pressure decellularization and 2.1% for native lung.

![Permeability to 5µm Particles](image)

**Figure 3.9:** Retention of 5 µm microspheres by decellularized scaffolds. Microsphere assay demonstrates that low perfusion pressure (<30mmHg) during decellularization enables the retention of 95% of 5 µm particles in the airway compartment. * indicates p<0.05 compared to native.
3.3.9 Micro-CT Imaging

We used micro-CT imaging to evaluate the patency of the airway and vascular compartments of decellularized lung scaffolds. This technique can allow us to obtain 3-dimensional images of the lung scaffolds, and may facilitate the identification of the degree of patency of the airway and vascular compartments.

In figure 3.10 we show images of the vasculature, with resolution of 58 μm. At this resolution, the large vessels are shown to be intact (top panels of figure 3.10), and the native and decellularized samples are generally similar, although some leak is evident in smaller vessels, shown in the lower and middle panels. Higher resolution images (6.5 μm) of the vasculature are shown in figure 3.11, where vessels are shown as 3-dimensional projections (maximal intensity projections). In these images, vascular leak is identified as the haziness seen in some areas of the decellularized scaffold.

We also imaged the airway tree by injecting contrast agent into the trachea. The resultant images are shown in figure 3.12, where significant differences are noted between native and decellularized lung. Whereby discrete pockets of contrast (i.e. alveoli) are identified throughout the native sample, the decellularized scaffold is almost entirely filled by contrast agent. This appears to indicate significant leak across the alveolar basement membrane, which is in contrast to findings from scanning and transmission EM. This may be due to greater compressibility of the vasculature of the decellularized scaffolds, thus obscuring the identification of individual alveoli. Alternately, there could be leak of the contrast agent out of the airway, even in the absence of any damage to the extracellular matrix or alveolar basement membrane. The contrast agent consists of 20% bismuth in a 5% gelatin solution. Bismuth is the functional contrast agent, while gelatin provides a medium which can be polymerized and thus solidified by cooling the lung after injection of the gelatin/bismuth
Figure 3.10: Micro CT of the vasculature of native and decellularized lung. Overall, decellularized scaffolds appear similar to native, when imaged with a resolution of 58 µm.
Figure 3.11: High resolution micro CT of the vasculature of native and decellularized lung. Significant leak is seen as hazy regions in the decellularized tissue. Resolution of these scans is 6.5 µm.
mixture. The gelatin used has a reported molecular weight of 50,000Da (Sigma product information, item G9391). The precise molecular size of the gelatin is difficult to determine, but we can safely assume it is less than 50nm, as based on measurements of molecular radius of higher molecular weight gelatins [28]. This is a relatively small molecule, and could easily translocate the alveolar basement membrane, even if the membrane were undamaged. It is possible that there was significant movement of gelatin/bismuth across the alveolar basement membrane before the gelatin solidified.
Figure 3.12: Micro CT of the airways of native and decellularized lung. It is difficult to distinguish alveoli in the decellularized tissues. Resolution of these scans is 58 $\mu$m.
3.4 Discussion

We have developed a decellularization technique for whole lung tissues. To our knowledge, this represents the first work describing the decellularization of rodent lung. Some work was performed 20 years ago to decellularize human lung [121], but since that time no reports have been found describing the decellularization of lung tissue.

3.4.1 Removal of cellular material and DNA

The complete removal of cellular material is important for several reasons. First, if this scaffold is intended to be used for tissue engineering applications, one must be certain that all the cells from the scaffold are removed before seeding the scaffold with a new cell source. In addition to complicating the evaluation of the reseeded scaffolds, any remaining cellular material would cause immune complications if the engineered tissue is used for in vivo applications [43, 122, 8]. As a result, we have confirmed that both MHC Class I and II antigens are not present in the decellularized scaffolds. Second, in order to evaluate separately the contributions of the extracellular matrix to lung mechanics, we must be certain that all cellular components are removed. The two classes of components that can contribute to peripheral lung mechanics are cellular contributions and the extracellular matrix. This latter category can be further divided primarily into collagen, elastin, and proteoglycans [36, 59, 100, 184]. By ensuring removal of cellular components from the decellularized scaffolds, we can simplify analysis of the mechanics as we know the only possible contributions are from the extracellular matrix components. In chapter 4, we explore in greater detail the contributions of these components to the mechanical properties of the scaffold.

In the decellularized scaffolds, we demonstrate removal of almost 99% of DNA. A small amount of DNA remains in the matrix, but is present as elongated strands of DNA, as shown in figure 3.2. We note no organization of this remnant DNA in
nuclear structures, based on DAPI staining. The fact that small amounts of remnant DNA are noted via the quantitative assay is not surprising, and even commercially available biological scaffold materials contain small amounts of remnant DNA [74]. We see the removal of 98.8% of DNA compared to native lung, with a remaining DNA concentration of 1.83 ng DNA per mg of tissue (dry weight). This compares favorably to levels of 16.6 ng/mg remnant DNA seen by Ott et al. for decellularized heart tissue [152], especially considering that level is standardized to wet weight, not dry weight as in this and other studies [74]. However, the levels of remnant DNA we observe are higher than those seen for commercially available and laboratory produced ECM scaffolds used for skin grafts, where most scaffolds show less than 0.2 ng DNA per mg dry weight, although some scaffolds had as much as 1.13 ng/mg remnant DNA [74].

### 3.4.2 Extracellular matrix components

Extracellular matrix components were analyzed via immunofluorescence, demonstrating retention of collagen types I and IV, but loss of fibronectin in the decellularized scaffolds. The collagens are the most important structural component of the lung, with types I and V being the principal structural collagens and type IV being a key basement membrane component [59, 184]. Type IV collagen interacts with other components of the basement membrane, as well as with cells either via direct binding or indirectly through laminin [110]. Retention of collagen type IV may aid in attachment and proliferation of cells on the scaffolds. In addition, the demonstration that both collagen types I and IV are retained suggests that the mechanical integrity of the scaffolds may be retained. These findings are confirmed and explored in much greater detail in chapters 4 and 6.

Fibronectin is a key matrix component that is important for endothelial cell attachment [116], and that also affects cell proliferation, migration and differentiation
Immunofluorescence demonstrates, however, that fibronectin is absent from the decellularized scaffolds, as shown in figure 3.5. We hypothesize that this loss of fibronectin may be associated with two factors. First, the primary mode of decellularization is by pumping the decellularization solution through the vasculature. Thus, the vasculature is subject to more vigorous decellularization than the airways, which are primarily decellularized via the slower movement of decellularization fluid into the alveoli and up the airway tree. The second hypothesis for the loss of fibronectin is based on the underlying biochemistry. Fibronectins are large, dimeric glycoproteins, consisting of a 70kDa aminoterminal region that binds noncovalently with collagens and other ECM molecules, a central 120kDa domain that contains the cell-binding RGDS repeats, and a 65kDa carboxyterminal region that cross-links with another fibronectin molecule to form a dimer [116]. Therefore, the means of attachment of fibronectin to the rest of the ECM is through noncovalent bonds in the aminoterminal region. These noncovalent interactions could be disrupted relatively easily when subjected to the decellularization conditions, especially with a high ionic salt solution.

### 3.4.3 Assessment of Scaffold Architecture

A critical feature of the decellularized matrix is the preservation of the native 3-dimensional structure. In order to evaluate the extent to which the structure of the decellularized scaffolds was preserved, we utilized a combination of scanning and transmission EM, micro-CT, and a microsphere permeability assay. We examined the ultrastructural characteristics of decellularized lung using SEM, and demonstrated maintenance of alveolar architecture and alveolar septae despite some fraying of the extracellular matrix. Transmission EM demonstrated a completely intact alveolar basement membrane as well as collagen and elastin fibers. These EM findings are
consistent with other work in decellularizing lung matrix, where such structures are retained \cite{121}. With strict control of vascular perfusion pressure during decellularization, we demonstrate the retention of some capillaries. Micro-CT imaging demonstrates retention of the vascular network down to vessels of 100 \( \mu \text{m} \) diameter, based on conservative estimates, with a substantial number of smaller vessels also intact. However, there is also leak evident from the microvasculature.

While these imaging studies offer useful insights, they have limitations. Electron microscopy is limited to the area of the scaffold that can be examined, and microCT has a maximum resolution of 6.5 \( \mu \text{m} \), which does not allow identification of individual capillary structures. As a result, we developed a microsphere permeability assay and found that 5 \( \mu \text{m} \) microspheres would not substantially cross between the airway and vascular compartments (95\% of particles are retained, compared to 98\% for native lung). Particles of this size approximate the size of red blood cells, and the ability of the decellularized scaffolds to resist movement of these particles is likely to be critical to the ability of engineered tissues to adequately function \textit{in vivo}, as any significant blood loss into the airway compartment would not be compatible with a functional engineered lung tissue.

### 3.4.4 Progress towards objectives of a decellularized matrix

In section 3.1.1 we outlined a series of objectives for the production of a decellularized lung scaffold. Here we will review our progress compared to these objectives, and identify future directions for study.

**Cellular toxicity.** The scaffold must not be toxic to the culture of mammalian cells. We have not evaluated this objective in this chapter, but will extensively demonstrate in future chapters that the scaffold is not cytotoxic.
Removal of cellular material and DNA. All cellular material and DNA should be removed from the scaffold. We have shown that DNA is removed to 1.8ng/mg dry weight of the scaffold, compared to our objective of less than 1.0ng/mg and the level in several commercially available skin matrix scaffolds of 0.1ng/mg. Although we fall short compared to these levels, the level we observe is far less than that observed for decellularized rat heart (16.6ng/mg, and this is using wet weight; this level would be significantly higher if standardized to dry weight). Therefore, we certainly demonstrate the removal of sufficient DNA to justify further study of this matrix and its use in lung tissue engineering.

Removal of antigenic components. All antigenic molecules should be absent from the scaffold. We have shown the removal of both MHC Class I and II antigens from the decellularized scaffolds. These findings suggest that the scaffolds would be unlikely to provoke an immune response if implanted into a host. However, in order to truly assess the removal of antigenic components, one must implant the decellularized scaffolds into a host. Therefore, while we have tested the likely immunogenicity of the scaffolds using in vitro methods, this objective could only be definitively met with in vivo experiments.

Extracellular matrix components. The scaffold should retain all key extracellular matrix molecules. We have demonstrated retention of collagens I and IV, although fibronectin is almost completely absent from the decellularized scaffolds. Fibronectin is an important matrix molecule that is a substrate for both epithelial and endothelial attachment, and future studies will be used to determine if fibronectin can be retained. First, we need to determine at what point in the decellularization process fibronectin is lost, which can be accomplished with staining at various time points during decellularization. Then, alterations in the decellularization solution or tech-
nique can be evaluated, such as a lower pH or different chemical compositions. In addition, evaluation of the fibronectin content of matrices decellularized with low pressures would be insightful, as some fibronectin may be retained along with capillary or microvascular structure.

**Retention of vascular and airway structures.** The scaffold should retain an intact airway tree and vascular network. Using scanning and transmission electron microscopy in addition to micro-CT imaging, we have demonstrated that, overall, the scaffold is remarkably well preserved after the decellularization process. Scanning EM, as well as routine histology, demonstrates that the scaffold is grossly intact without large defects (i.e. alveoli and alveolar septae appear intact). Transmission EM demonstrates that the alveolar basement membrane is well preserved and that at least some capillaries are retained. Micro-CT imaging demonstrates that the vasculature is intact down to vessels of 100 μm diameter, although some leak from the microvasculature is observed on high-resolution imaging. Despite these findings, it is difficult to completely evaluate the scaffolds, as each methodology has limitations. For example, TEM can only examine 2-dimensional slices of tissue, while micro-CT is limited to a resolution of 6.5 μm, which does not enable visualization of capillary structures. Future directions of study for this objective include continued TEM evaluation to evaluate capillary preservation with low-pressure decellularization, which will be coupled with attempts to retain fibronectin, a key extracellular matrix molecule.

**Barrier function.** The matrix should provide an effective physical barrier between the airway and vascular compartments to prevent the movement of cell-sized objects between the compartments. We have shown retention of 95% of 5 μm particles by the decellularized scaffolds. Although promising, this falls short of the level of native lung, and of our objective of 99%. Continued efforts to minimize perfusion pressure
and retain capillary structure will likely improve the barrier function of the scaffolds.

### 3.5 Conclusions

We have undertaken a series of studies in order to evaluate the hypothesis that native lung tissue can be decellularized to remove cellular components and antigenic molecules, yet retain key extracellular matrix molecules and retain an intact airway-vascular barrier. We have demonstrated for the first time that whole rodent lungs can be decellularized to produce a scaffold that is devoid of cells and DNA. The decellularized lung scaffold does not retain either MHC Class I or Class II antigen, a promising finding if these scaffolds are used for *in vivo* tissue engineering applications.

We extensively characterized the makeup of the extracellular matrix, via histology, immunofluorescence, and electron microscopy. We demonstrate that collagen types I and IV are retained, which are key extracellular matrix components; however, fibronectin, a key endothelial cell attachment factor, is lost. The lack of fibronectin is an important deficiency of the scaffolds, and will be a focus of future efforts at improving the decellularization process.

Finally, we demonstrate that key ultrastructural features of the lung scaffold are preserved, including alveolar spaces, alveolar septae, the alveolar basement membrane, large vascular structures, and some capillaries. The retention of a continuous, intact alveolar basement membrane is a key finding that indicates that these scaffolds may be able to resist blood leakage across this barrier and thus be promising for *in vivo* 3-dimensional tissue engineering applications, which couples with our finding that the airway can retain 94% of 5 µm particles. In addition, we demonstrate the preservation of some capillaries when the perfusion pressure during decellularization is strictly minimized.

These studies demonstrate that the decellularized scaffolds are a promising sub-
strate for use in lung tissue engineering applications. However, we have identified several key deficiencies of the scaffolds, primarily the loss of fibronectin, the loss of many capillary structures, and vascular leak that is evident by microCT imaging and permeability studies using 5 \( \mu \)m particles. Future studies will continue to evaluate the scaffolds and aim to correct these deficiencies.
Chapter 4

Contribution of Extracellular Matrix Components to the Mechanical Integrity of Decellularized Lung Tissue

In chapter 3, we described the production of a decellularized lung scaffold that is devoid of cellular material and DNA, that is likely not immunogenic, and that retains several key features of lung architecture and ultrastructure. In this chapter, we evaluate the composition of the decellularized scaffolds in more detail with a focus on the mechanical properties of the scaffolds and will use mathematical modeling to examine the contributions of collagen and elastin to matrix mechanics. We will evaluate the hypothesis that decellularized lung scaffolds retain salient mechanical features of native lung, due principally to contributions from collagen and elastin, and these features can be evaluated using a simple mechanical model. This work also demonstrates the utility of decellularized lung tissue as a platform to study lung mechanics independent of cellular contributions.

4.1 Introduction

The principal determinants of the mechanical integrity of lung tissue are collagen and elastin fibers, while proteoglycans and cells are other potential sources of mechanical strength [59, 36]. In this chapter, we examine the contribution of these components to the mechanics of lung tissue by comparing native and decellularized lung tissues.

The decellularization of organs or tissues has been used in many applications to create material for tissue engineering applications, including heart valve [16], blood vessels [46], and small intestinal submucosa [19]. The goal of the decellularization
process is to remove cellular and nuclear material while minimizing impact on the ECM by the decellularization process. The development of a decellularized lung scaffold is valuable as a scaffold for the growth of engineered lung tissue. In addition, however, a decellularized scaffold can be used to investigate the contribution of the various extracellular matrix components to the mechanical properties of lung tissue.

The collagens are the most important structural component of the lung, as they are responsible for the overall mechanical strength of the lung [59]. Their strength is derived from triple helical domains of three polypeptide α-chains which are coiled around each other to form a right-handed superhelix. A single collagen molecule is estimated to have Young’s modulus of approximately 3-9GPa [172, 166], while the modulus of collagen fibers are on the order of 100MPa [83].

Elastin is an important ECM component in tissues that require reversible distension, and elastin allows for the intrinsic recoil property of lung tissue. Elastin is rich in hydrophobic amino acids and scarce in acidic residues, with the hydrophobic regions believed to be important in conferring its elastic properties [59]. Elastin fibers combine with microfibrils to make up the functional elastic fibers of the lung, which are very extensible and maintain a linear stress-strain relationship out to ~200% strain [110]. The Young’s modulus of single elastin fibers has been reported as 1.2MPa [2], while the modulus of elastin measured in tissues is approximately 300-400kPa [2, 83].

Proteoglycans are proteins that are found on cell surfaces, within intracellularized vesicles, and incorporated into the ECM [110]. Proteoglycans are defined by a core protein that has undergone post-translational modification with a glycosaminoglycan (GAG), a polysaccharide that can be modified to provide heterogeneity across the spectrum of GAGs. GAGs can impact a protein’s structure and function, and also help control macromolecular and cellular movement across the basal lamina [110]. The size and complexity of proteoglycans can vary greatly, and they can associate to
form even more complex structures, and may play a role in the mechanical integrity of the lung by stabilizing collagen and elastin networks [36].

Outline of the results. In this chapter, we investigate the composition of decellularized lung tissues for collagen, elastin and proteoglycan content. We will demonstrate that collagen content is retained, elastin content is retained at $\sim 40\%$ of native levels, while glycosaminoglycans are largely lost from the decellularized scaffolds. We then utilize modelling studies to investigate the roles of collagen and elastin in matrix mechanics.

4.2 Materials and Methods

Organ harvest and decellularization

Lung tissue was harvested and decellularized as described in sections 3.2.1 and 3.2.3.

4.2.1 Histological analysis

Histology was used to characterize many decellularized lung scaffolds, and to confirm the removal of cellular material. Tissues were fixed, paraffin-embedded and sectioned. Analysis was performed with standard hematoxylin and eosin staining (H&E), Masson’s trichrome for collagen, Verhoff van Gieson for elastin, and Alcian blue for proteoglycans, as well as staining for DNA using 4’,6-diamidino-2-phenylindole (DAPI).

4.2.2 Collagen assay

Collagen was quantified with a colorimetric assay to detect OH-Proline using a modified Grants method [77]. Lung samples were lyophilized and weighed, then incubated in papain (140 $\mu$g/ml) at 60°C overnight (Sigma). Papain-digested samples were incubated in 6 N HCl at 115°C for 18 h, neutralized, oxidized with chloramine-T,
reacted with p-dimethylaminobenzaldehyde. Absorbance was measured at a wavelength of 550 nm and a 1:10 w/w ratio of hydroxyproline to collagen was used to calculate the collagen content of the tissue. At least 4 samples were measured for native and decellularized samples.

4.2.3 Elastin assay

Elastin was quantified using the Fastin Elastin assay kit (Biocolor, Belfast, N. Ireland). Lung samples were first lyophilized and weighed, and then the elastin was extracted following the method described in Foronjy et al. [65]. Samples were incubated with 0.25M oxalic acid at 100°C, then centrifuged at 10,000g and the supernatant saved. The supernatant from 5 extractions was pooled, and the supernatant from the 6th extraction was also measured to ensure that no more elastin remained in the tissue. The oxalic acid was cleared using a 10,000 molecular weight cutoff filter (Millipore), then resuspended in dH$_2$O and analyzed using the Fastin Elastin kit according to the manufacturer’s instructions. At least 4 samples were measured for native and decellularized samples.

4.2.4 Sulfated glycosaminoglycan assay

Sulfated glycosaminoglycans (sGAGs), including chondroitin, dermatan, heparan and keratan sulfates, were quantified using the Blyscan GAG assay kit. Papain-digested samples (prepared as described for the collagen assay, above) were assayed according to the manufacturer’s instructions. Briefly, sulfated GAGs were labelled with 1,9-dimethyl-methylene blue dye and absorbance was measured at 650 nm.
4.2.5  Mechanical testing

Native and decellularized lung samples were analyzed using an Instron 5848 equipped with a 10N load cell. Slices of tissue of known dimensions were cyclically pre-stretched for 10 cycles to 20% strain to investigate elastic properties and then stretched until failure to evaluate ultimate tensile strength (UTS). See figure 4.1 for a schematic of the testing protocol. Using tissue dimensions, engineering stress and engineering strain were calculated from force and distance.

**Figure 4.1:** Mechanical testing protocol: a strip of lung tissue is attached to the upper plate, which is then lowered and the tissue attached to the lower plate. The tissue is cyclically stretched to 20% strain and then stretched until failure.

4.2.6  Modeling

We model the parenchymal pulmonary tissue using a simple linear model. The model is shown schematically in figure 4.2, where we model the system (a strip of lung tissue) as a linear series of elements, each of which is a spring in parallel with a string that has a defined maximum length (stop length). The string, which models collagen fibers, exerts no opposing force except when stretched to its stop length $l_i$, after which it cannot extend further. The spring models elastic fibers, and behaves like a Hookean spring with constant $k_i$.

This model is based on the description by Maksym *et al.* [125, 124]. We apply this model to our system, and evaluate both the collagen and elastin content of the
decellularized scaffolds. The model is summarized here, but the reader is referred to Maksym et al. [125] for further details.

First, stress-strain data were fit to the following exponential relationship:

\[ \sigma = G(e^{\epsilon/H} - 1) \]  \hspace{1cm} (4.1)

where \( \sigma \) is stress, \( \epsilon \) is strain, and \( G \) and \( H \) are fit parameters.

![Diagram of lung mechanics model](image)

**Figure 4.2:** System used to model lung mechanics. The tissue is represented by a linear series of elements, each of which is a spring (elastin) in parallel with a string (collagen).

We represent the distribution of stop lengths by \( N(l) \), and so the total length of the tissue is

\[ L = L_r + \int_0^\infty lN(l)dl \]  \hspace{1cm} (4.2)

where \( L_r \) is the tissue length at rest.

For a given length \( L \) we divide the elements into those that are stopped and those that have not yet stopped. The stopped elements have stop lengths less than some value \( l_0 \), and the unstopped elements have stop lengths greater than \( l_0 \). As the tissue is stretched, \( l_0 \) increases and more elements become stopped.

We then rewrite the above equation, separating the integral over \( N(l) \) to represent the stopped elements and the unstopped elements separately:

\[ L(l_0) = L_r + \int_0^{l_0} lN(l)dl + l_0 \int_{l_0}^\infty N(l)dl \]  \hspace{1cm} (4.3)

We then differentiate with respect to \( l_0 \) to yield

\[ \frac{dL}{dl_0} = \int_{l_0}^\infty N(l)dl \]  \hspace{1cm} (4.4)
and we then multiply each side by $\frac{dl_0}{dF}$ and use the chain rule to write

$$\left.\frac{dL}{dF}\right|_{l_0} = \frac{dl_0}{dF} \int_{l_0}^{\infty} N(l)dl \quad (4.5)$$

From Hooke’s law, for each spring element, we have the tension $F = kl$ and thus $k = dF/dl_0$ for the spring that has just stopped. Since we have assumed that all the springs have the same elastic constant $k$, we can replace $k$ in equation 4.5 to yield

$$\left.\frac{dL}{dF}\right|_{l_0} = \frac{1}{k} \int_{l_0}^{\infty} N(l)dl \quad (4.6)$$

We differentiate the left side of equation 4.6 with respect to $l_0$ and again use the chain rule to write

$$\frac{d}{dl_0} \left( \left.\frac{dL}{dF}\right|_{l_0} \right) = \frac{d}{dF} \left( \left.\frac{dL}{dF}\right|_{l_0} \right) \frac{dF}{dl_0} = k \frac{d}{dF} \left( \left.\frac{dL}{dF}\right|_{l_0} \right) = k \frac{d^2L}{dF^2} \left.\right|_{l_0} \quad (4.7)$$

and then differentiate the right side

$$\frac{d}{dl_0} \left( \frac{1}{k} \int_{l_0}^{\infty} N(l)dl \right) = \frac{1}{k} (N(\infty) - N(l_0)) = -\frac{1}{k} N(l_0) \quad (4.8)$$

We now equate the two sides of the differentiated equation 4.6 and reorganize to yield

$$N(l_0) = -k^2 \frac{d^2L}{dF^2} \left.\right|_{l_0} \quad (4.9)$$

However, we have made no assumptions about $l_0$, and so the above is true for all values of $l_0$, allowing us to write

$$N(l) = -k^2 \frac{d^2L}{dF^2} \quad (4.10)$$
From their definitions, we have the stress $\sigma = F/A_r$ and strain $\epsilon = \frac{L}{L_r} - 1$. Using their derivatives and a series of substitutions, we can obtain the relation

$$\frac{d^2 \epsilon}{d\sigma^2} = \frac{A_r^2}{L_r} \frac{d^2 L}{dF^2} \quad (4.11)$$

We use this relation and the second derivative of equation 4.1, $-\frac{H}{(\sigma + G)^2}$ to yield

$$N(l) = -\frac{k^2 L_r}{A_r^2} \frac{d^2 \epsilon}{d\sigma^2} = \frac{k^2 L_r}{A_r^2} \frac{H}{(\sigma + G)^2} \quad (4.12)$$

We now replace $\sigma$ with $l$ by recalling, as above, that the tension $F$ in each spring is the same, allowing us to use $\sigma = F/A_r$ and $F = kl$ to write

$$N(l) = \frac{L_r H}{(l + \frac{G A_r}{k})^2} \quad (4.13)$$

We now have a relation for $N(l)$ in terms of the fitted parameters $G$ and $H$, the tissue dimensions $L_r$ and $A_r$, and $k$. We need a way to determine $k$ from the available data, which is accomplished by evaluating equation 4.6 at $l_0 = 0$

$$\left. \frac{dL}{dF} \right|_{F=0} = \frac{1}{k} \int_0^\infty N(l) dl = \frac{X}{k} \quad (4.14)$$

Thus, $X$ is the total area under the distribution of stop lengths. However, there is no guarantee that $X$ is finite. Thus, we follow the approach in [125] and define $X$ as 10,000 given a maximum tissue strain of 2. We assume that $X$ is the same for both native and decellularized samples. We can make this assumption based on the quantitative collagen assay results in section 4.3.1, where we found indistinguishable collagen concentrations in native and decellularized lung. Then, we use the definitions of stress and strain, their derivatives $\frac{d\sigma}{dL}$ and $\frac{d\epsilon}{d\sigma}$, and the derivative of equation 4.1 to yield

$$k = \frac{A_r G X}{L_r H} \quad (4.15)$$
We now can determine \( k \) from experimental parameters, allowing us to completely determine \( N(l) \) as given in equation 4.13 for each tissue sample. Thus, for a given strip of lung tissue, knowing the stress-strain curve and the tissue dimensions, we can obtain a distribution of the stop lengths for the collagen fibers as described for this model.

We then go back to the model in figure 4.2 and assume that the collagen fibers all have identical stop lengths \( l \), and we obtain a relation for the distribution of spring constants \( k_i, M(k) \). We follow a similar derivation to obtain

\[
M(k) = \frac{kHLr/l}{(k + GA_r/l)^2}
\]  

(4.16)

Thus, for a given strip of lung tissue, knowing the stress-strain curve and the tissue dimensions, we can obtain distributions of the stop lengths for the collagen fibers and the spring constants of the elastin fibers, within the framework of the model shown in figure 4.2.

4.3 Results

4.3.1 Collagen and elastin content

As shown in figure 4.3(c), collagen content in decellularized scaffolds is indistinguishable from native lung. This preservation of collagen is important as collagen plays a key role in the mechanical strength of the lung. Collagen content was also maintained on histochemical staining via Masson’s trichrome, shown in figure 4.3. Also noted in figure 4.3(c) is a decreased collagen content in scaffolds decellularized with SDS, one of the decellularization methods that was not found to be suitable. This loss of collagen correlates with decreased mechanical integrity, and is discussed in detail in section 4.3.3.
Figure 4.3: Collagen staining and content of native and decellularized lung. Masson’s trichrome stain reveals wavy dark blue fibers in both native and decellularized lung. Quantitative assay demonstrates preservation of collagen in native and decellularized lungs, but loss of collagen after decellularization using sodium dodecylsulfate (SDS). * indicates p<0.01.
Elastin content is also preserved, although diminished, in the decellularized scaffolds, as demonstrated by both quantitative assay and histological staining (figure 4.3.1). Elastin fibers allow for the elasticity of the lung, critical to the natural recoil of the tissue that plays a key role in the relaxation and thus exhalation of the lung after inhalation. The retention of these fibers through the decellularization process is critical, as it will allow the lung scaffold to be properly ventilated during efforts at reseeding the scaffold with pulmonary cell populations. Although the scaffolds do lose 60% of the native elastin content, the remaining elastin is sufficient to allow elastic function of the lungs, as seen from mechanical testing, as described in section 4.3.3.

Overall, the retention of these key ECM components will allow the scaffold to undergo physiological levels of mechanical stress, which is important as a variety of developmental and cell differentiation processes rely on mechanical stimuli. In addition, the ECM is critical in aiding cell attachment to the matrix, and the retention of these native ECM components will facilitate cell attachment and spreading and thus the development of bioengineered lung tissues.

### 4.3.2 Proteoglycan content

Proteoglycans consist of a core protein linked to one or more glycosaminoglycan (GAG) chains. Most GAGs are sulfated, enabling their detection via quantitative assay, the results of which are shown in figure 4.5(c). We find that the GAG content of the decellularized scaffolds is significantly lower than native lung (∼6% of native lung levels). Proteoglycans are found either on the cell surface or within the extracellular matrix [63], and their removal is due in part to the removal of cell-bound GAGs. However, the GAGs found within the ECM can also be solubilized via the decellularization solutions. In figure 4.3.2 we show Alcian blue histological staining
Figure 4.4: Elastin histochemistry (Verhoff-van Geison) is shown for native and decellularized lung. Note wavy dark elastin fibers in both native and decellularized lung. Quantitative assay demonstrates preservation of some elastin in decellularized lungs compared to native. * indicates $p<0.01$. 
for proteoglycans, demonstrating that the amount of GAGs remaining in the decellularized lung scaffolds is noticeably diminished compared to native lung, confirming the quantitative assay findings.

![Native lung](image1.png) ![Decellularized lung](image2.png)

**Figure 4.5:** GAG histochemistry (Alcian blue) is shown for native and decellularized lung. Note blue GAG staining in native lung but their absence in decellularized lung. Quantitative assay demonstrates loss of sulfated GAGs in decellularized lungs compared to native lung. * indicates p<0.01.

### 4.3.3 Mechanical characterization

Mechanical testing of peripheral lung strips was used to evaluate the quasistatic mechanics of both native and decellularized lung samples. The elastic regions of the stress-strain curves indicate that both native and decellularized samples demonstrate hysteretic behavior. Hysteresis demonstrates that lung is a viscoelastic material, and
the difference between the expanding and relaxing curves represents energy that is not recovered during relaxation. In addition, samples do not creep, as shown in figure 4.6. If lung tissue were to creep, it would not deflate to its original position after an inflation; thus, the lung would never fully deflate and gas exchange would clearly be impaired. This preservation of appropriate elastic lung behavior is important for a lung scaffold; the loss of pulmonary elasticity is seen in several disease states, notably emphysema [72].

![Stress-strain curve, elastic region](image1)
![Stress-strain curve](image2)

**Figure 4.6**: Stress-strain curves of native and decellularized lung. SDS indicates a lung treated with sodium dodecylsulfate.

Ultimate tensile strength is the stress on a sample at failure, and is a measure of a material’s strength. As demonstrated in figure 4.7, the UTS of decellularized samples is indistinguishable from that of native samples. If samples are decellularized in buffers containing sodium dodecyl sulfate (SDS), however, mechanical integrity is compromised as demonstrated by the decrease in UTS. SDS can degrade collagen, causing fragmentation and swelling of tissues [26, 75] and has also been shown to increase tissue extensibility [133]. SDS is a highly ionic, amphipathic detergent, and its hydrophobic region can interact with proteins while the hydrophilic portion, especially when negatively charged, binds water and causes tissue swelling [26]. Although
other studies have not always seen a decrease in UTS with SDS treatment [133], this may be due to tissue differences. Mirsadraee et al. studied pericardial tissue, which contains much more densely packed collagen fibers than lung. In lung, due to the geometry of the tissue, collagen fibers are highly distributed, and SDS-induced swelling can much more easily lead to collagen removal, as seen in the quantitative collagen assay.

![Ultimate Tensile Stress](image)

**Figure 4.7:** Ultimate tensile strengths of native, decellularized and SDS-decellularized lung. SDS indicates a lung decellularized using sodium dodecylsulfate. * indicates p<0.01 compared to native.

Taken together with the retention of elastic behavior, the findings of retained collagen and retained matrix mechanics indicate that the decellularized scaffolds can likely withstand relevant *in vivo* physiological forces.

### 4.3.4 Model of observed mechanical behavior

In order to understand the behavior of the decellularized matrices, data were fitted to a model that has been previously developed to explain the mechanical properties of lung parenchyma [125, 124].

In figure 4.8(a) we show the distribution of stop lengths, $N(l)$, for native and decellularized lung tissue, based on stress-strain data obtained for both tissues. Several
curves are shown for each condition, each representing one sample. Both the native and decellularized lungs show similar stop length distributions, consistent with the similar stress-strain data. However, in analyzing the SDS-treated lungs, there is a trend towards shorter stop lengths, although this difference is not significant (p = 0.15 using a student’s T-test on the asymptotes). We hypothesize this is due to partial collagen degradation and removal, thus effectively shortening the remaining collagen fibers. This process would be facilitated by the SDS-induced swelling of the tissue, and is consistent with the decreased overall collagen content of the SDS-treated tissues (figure 4.3(c)).

In summary, this model demonstrates that the collagen fiber makeup of the decellularized scaffold is very similar to native lung. In particular, we have analyzed the lengths at which each collagen fiber stops extension, and found that this distribution of collagen fiber stop lengths in the decellularized lung correlates with that of native lung. In addition, the quantitative collagen assay performed in section 4.3.1 and shown in figure 4.3(c) confirmed that we have similar total collagen amounts in both native and decellularized samples. When taken together, therefore, this data demonstrates that the collagenous makeup and resultant mechanical properties of decellularized lung are similar to native lung.

We show in figure 4.8(b) the distribution of elastic spring constants, for both native and decellularized lung samples. Again, several curves are shown for each condition, each representing one sample. No significant differences are detected for any samples, including those decellularized with SDS. Therefore, similar distributions of elastin spring constants are found for all samples. This finding does not conflict with the finding that decellularized tissue only contains 40% of the elastin of native tissue, as this is the distribution of elastin spring constants, and does not analyze the total number of elastin fibers in the tissue. The findings of the model are therefore
Figure 4.8: Distribution of collagen stop lengths $N(l)$ and elastin spring constants $M(k)$ in native, decellularized, and SDS-treated lung.
consistent with the mechanical and functional testing as well as the quantitative elastin assay.

4.4 Discussion

In this work, we demonstrate the ability to decellularize rodent lung tissue using a technique that removes cellular material but that retains key components of the extracellular matrix. The development of a decellularized lung matrix is important as a scaffold for tissue engineering applications. Furthermore, by removing cellular material from the lung we are able to probe the contributions of the various extracellular matrix components to lung mechanics in the absence of cellular influence.

We have demonstrated that collagen and, to a lesser extent, elastin are retained by the decellularized lung matrices, via both quantitative assay and histochemical staining. In addition, mechanical testing demonstrates that the decellularized scaffolds are indistinguishable from native lung in terms of overall strength (ultimate tensile strength, UTS). Strain was applied under quasistatic conditions. The applied rate of strain is sufficiently slow to enable the approximation of mechanical behavior as a static system, as it would take infinitely long to make truly static measurements on a viscoelastic tissue. The applied rate of strain (1mm/min) is sufficiently slow compared to dynamic movements of lung tissue (70-115 breaths per minute in the adult rat[171]) to enable this assumption [68]. Dynamic mechanical properties include the flow-resistive behavior of airways, parenchymal viscoelasticity and resistivity [119], and are not evaluated in this work.

The principal ECM component responsible for the strength of lung tissue is collagen [59]. Mechanical testing confirms that the collagen that is retained in the decellularized scaffolds is functional, and has not been damaged by the decellularization process. Furthermore, the scaffolds retain elasticity, as shown in figure 4.6(a).
There are two key features that are retained by the scaffolds that are demonstrated in this figure. First, the scaffolds exhibit hysteresis, or path-dependence. Thus, the stress-strain curve follows a different path when being stretched as when it is relaxing. Hysteresis demonstrates that lung is a viscoelastic material, and the difference between the expanding and relaxing curves represents energy that is not recovered during relaxation. This type of behavior is analogous to (but quite distinct from) pressure-volume curves of whole lung, where hysteresis is also important to the macroscopic function of the lung, although in that case it is largely due to the effects of alveolar recruitment [60]. The second characteristic demonstrated in figure 4.6(a) is the absence of creep. In other words, the scaffolds relax to the same stress after application of strain. If the scaffolds exhibited creep, with every expansion (strain application) the tissue would not relax fully but would return to a slightly stretched state. With repeated applications of strain, such as breathing, if any creep were observed, a lung scaffold would soon completely lose its shape and be unable to relax significantly. This would preclude normal breathing, during which exhalation occurs due to the passive relaxation of the lung.

Mechanical distension is important in aiding the differentiation of type I pneumocytes [54, 81] and the final differentiation of type II pneumocytes [96]. During fetal development, the importance of fetal breathing movements (FBMs) has been documented and is important for the advanced development of pulmonary tissue [97]. In the absence of FBMs, type I pneumocytes cannot undergo late differentiation and thus cannot flatten [98]. The clinical result of a lack of FBMs is pulmonary hypoplasia, a common neonatal disorder that has been found in 15% of neonatal autopsies [209]. In order to successfully develop engineered lung tissues, the lung scaffold must be able to withstand such physiological movements and pressures, underlying the importance of the mechanical integrity of the lung scaffolds.
As shown in figure 4.7, decellularization using sodium dodecyl sulfate (SDS) significantly degrades the mechanical strength of the decellularized matrices. This correlates with significantly reduced collagen content in these scaffolds (figure 4.3(c)). As discussed in section 4.3.3, this may be related to the amphipathic structure of SDS, allowing it to bind water and increase tissue water content, thus facilitating removal of collagen during decellularization [26].

The complete removal of all cellular components from the lung scaffolds enables us to better understand the contributions of collagen and elastin to the mechanical integrity of the scaffolds. We find no noticeable effect on matrix mechanics in the absence of the various cell populations. Of note, however, in this study we are investigating peripheral lung mechanics, and tissue samples were purposely chosen to avoid the large airways and vessels near the hilum.

We have used a simple model of peripheral lung matrix to investigate our mechanical testing results. We modelled the collagen and elastin components of the lung matrix, and found our modelling results to be comparable with our mechanical testing and quantitative assay results for native and decellularized lung. Furthermore, when collagen is degraded and removed via decellularization with SDS, the effects of this are apparent in the model, in which both the number and length of collagen fibers are diminished. However, this effect is not noted for elastin, where the spring constants of elastic fibers are found at similar distributions for native, decellularized and SDS-treated lung tissue.

Collagen and elastin are generally accepted to be the principal determinants of the mechanical properties of a lung matrix. This is supported by findings in this study, whereby the mechanics of native and decellularized lung are indistinguishable when collagen and some elastin are preserved, while proteoglycans and cells are removed. However, proteoglycans can contribute to the mechanics of lung tissue in smaller
ways, as these large molecules can resist compression and shear [33]. Cavalcante et al. [36] noted small changes in the stress-strain curves of lung tissue with changes in the tonicity of the surrounding liquid, and furthermore noted that these changes were reduced after proteoglycan digestion. Therefore, proteoglycans play at least some role in the mechanics of the lung matrix. In this study, where proteoglycans are largely removed from the decellularized matrices, no significant effect of their removal was noted. However, future studies could explore this area in greater depth to investigate the possibility of smaller contributions of proteoglycans to lung matrix mechanics.

4.5 Conclusions

In this chapter, we have evaluated the collagen, elastin and proteoglycan content of decellularized lung tissue, and have investigated the mechanical properties of the decellularized matrix with particular focus on the contributions of collagen and elastin. Mechanical testing and the application of a simple model confirm that both collagen and elastin are preserved at functional levels. These findings confirm that the principal contributors to lung mechanics are from collagen and elastin, and not from cellular constituents or proteoglycans, as both are removed in the decellularized scaffolds.

Together with the work described in chapter 3, we have demonstrated the production of decellularized lung scaffolds and conducted a comprehensive evaluation of the scaffolds with regards to the makeup of the remaining extracellular mechanics, ultrastructure, and mechanical integrity. These scaffolds display characteristics that make them promising substrates for tissue engineering applications as well as a platform for the study of detailed matrix mechanics and lung biology, development and physiology.
Chapter 5

Design and Validation of a Bioreactor for the \textit{In Vitro} Culture of 3-Dimensional Lung Tissues

In this chapter, we will describe the design of a bioreactor for the \textit{in vitro} culture of whole rodent lung tissue. The bioreactor was designed to meet a series of design constraints aimed at the ability to provide sufficient nutrient supply and mechanical stimulation to the lung tissue in order to support cell survival and differentiation. We undertake a series of studies intended to evaluate the hypothesis that the bioreactor can support the \textit{in vitro} culture of whole lobes of lung tissue, demonstrated by maintenance of cell viability and differentiation state. In the process of evaluating this hypothesis, we also study the effects of perfusion and ventilation on lung survival in the bioreactor. The overall objective of the work in this chapter is to develop a bioreactor that can be used for \textit{in vitro} lung tissue culture, with the end objective being the ability to utilize this bioreactor for engineered lung tissue culture.

5.1 Introduction

A bioreactor is needed in order to culture 3-dimensional lung tissue \textit{in vitro}. The development of such a bioreactor would be beneficial not only to research on the growth of engineered lung tissue, but to the study of pulmonary biology. There are no currently available systems that allow the long term \textit{in vitro} culture of adult lung tissue.

For the work described in this thesis, our primary focus is the development of a bioreactor that is suitable to use for culturing engineered lung tissue using the
decellularized lung scaffolds described in the prior chapters. Existing attempts at pulmonary tissue engineering are limited to small tissue sizes that allow nutrient delivery via diffusion [39, 135, 136, 45, 10]. The development of a bioreactor capable of the in vitro culture of true 3-dimensional segments of lung tissue is an important step in the development of clinically useful engineered lung tissue.

In addition to tissue engineering applications, a bioreactor for in vitro lung culture would have numerous applications to assist the study of pulmonary biology, physiology, and development. For example, the interactions of lung endothelial and epithelial cells to form the alveolar-capillary barrier are not well studied, in large part because there complexity of the alveolar-capillary network and the lack if in vitro culture systems [89]. Researchers would be able to study lung behavior in a more controlled environment than the various animal models currently used, which is especially important as the mechanisms underlying many lung diseases are incompletely understood, in addition to the high cost and variability of animal models. An in vitro lung platform could also allow pharmacologic testing and investigation in human or animal tissue before proceeding to time-consuming and costly human or animal trials.

5.1.1 Prior work on in vitro culture of whole lung tissue

Culture of whole embryonic rodent lungs The in vitro culture of whole rodent lungs has been demonstrated by several groups over the past few decades [218]. However, this prior work has been done with fetal rodent lungs, which are extremely small and do not require active perfusion or ventilation. Perfusion is not required as diffusion supplies sufficient nutrient supply, and ventilation is not critical as fetal lungs do not require regular breathing. Such studies are generally performed by placing the tissue on a mesh at the air-liquid interface (ALI) in relatively simple media
(without many growth factors). Funkhouser et al. cultured fetal rat lungs on a mesh at the ALI and demonstrated lung development and surfactant production that were grossly similar to in vivo studies after 14 days of culture [67]. However, the rate of development was slower in vitro and organ growth slowed as lung size increased. This study also demonstrated that there are sufficient self-contained developmental signals to allow development to proceed in the ex vivo environment. Similar studies have also been conducted in mice [102], demonstrating embryonic organ culture for up to 27 days and confirming that, after certain embryonic stages, pulmonary embryonic development can proceed independent of exogenous in vivo signals. These studies demonstrate that the in vitro culture of embryonic rodent lungs is feasible without the need for a bioreactor.

**Culture of whole adult rodent lungs** The above studies on in vitro embryonic and neonatal lung culture demonstrate that the in vitro culture of adult lung tissue is a feasible objective. However, the lessons learned from these studies do not easily apply to the growth of engineered lung tissue. First, the size of any useful engineered lung tissue will preclude relying on diffusion for nutrient delivery. The above approaches to in vitro organ culture are only successful in fetal rodent lungs. Thus, a means of perfusion is required, adding great complexity to the experimental design. In addition, breathing movements will almost certainly be required for the successful culture of a functional engineered lung tissue. Although breathing movements were not required for the fetal organ cultures discussed above, the importance of fetal breathing movements (FBMs) has been documented and is important for the advanced development of pulmonary tissue [97]. In the absence of FBMs, type I pneumocytes cannot undergo late differentiation and thus cannot flatten [98] and type II pneumocytes may not be able to achieve their final differentiated state [96]. The clinical result of a lack of FBMs is pulmonary hypoplasia, a common neonatal
disorder that has been found in 15% of neonatal autopsies [209]. Thus, a system for the culture of engineered lung tissue must be capable of perfusion and ventilation. Some insights into the development of such a system can be drawn from prior work on the *ex vivo* culture of adult rodent lungs.

Some work has been performed culturing whole adult rodent lungs *in vitro*. However, the length of culture is typically 1-4hrs, with most focusing on very short-term studies lasting 30-60 minutes. Most of these studies utilize a commercially available system, the isolated perfused lung system from Hugo Sacks Elektronik (Hugstetten, Germany) [211, 199, 86]. There is no evidence that this system, or others that have been separately developed [156], has been utilized or validated for long-term cultures lasting longer than 24 hours.

### 5.1.2 Utility of a bioreactor for *in vitro* whole lung culture

Studies of pulmonary biology are restricted by the limited availability of *in vitro* systems for tissue and organ culture. While the *in vitro* culture of human pulmonary epithelium has been extensively demonstrated from both adult and fetal human lung tissue [115], it has proven more difficult to culture rat epithelium in the laboratory, as they tend to rapidly dedifferentiate [170]. Therefore, besides difficulty in culturing rodent lung epithelium *in vitro*, there are no available systems for long-term lung organ culture. As a result, studies of pulmonary biology have had to rely more heavily on *in vivo* work. While many important findings have been realized to date, the ability to culture whole rodent lungs in the laboratory for several days could greatly expand the range of work that can be performed. In the ensuing paragraphs we briefly outline studies that could be significantly expanded using *in vitro* bioreactor cultures.
Study of lung biology and development  Many aspects of lung biology and development could be studied using an in vitro bioreactor system. Vu et al. studied lung development and vascular formation by implanting embryonic lung fragments under the renal capsule [200]. Chetty et al. studied the effect of IGF-1 on cell proliferation in slices of neonatal (3-day-old) rat lung [40] while Muratore and colleagues evaluated the effect of endotoxin on fetal rat lung explants [139]. Studies such as these could be coupled with bioreactor experiments to explore effects over longer time periods and examine the impacts of ventilation and perfusion on lung biology and development.

Study of lung mechanical injury and mechanical ventilation  There is a large body of research investigating the effects of mechanical ventilation, which are limited by short-term experiments in animal models. The effects of mechanical stimulation on lung behavior and the lung injury response was studied by Oeckler and colleagues [146, 34]. Similarly, mechanical ventilator injury is a major problem in pulmonary medicine. Spieth et al. used a pig model to show that adding noise to mechanical ventilation pressure profiles improved oxygenation and arterial pressure [174]. Sundaresan et al. studied ventilator injury and using in silico modelling to determine optimal parameters for ventilator settings (primarily PEEP) [186].

In all of the above cases, the bioreactor system we have developed could expand the breadth and utility of the studies that can be performed. The bioreactor offers improved control over experimental conditions compared to in vivo systems, and can aid the study of pulmonary biology, pulmonary development, and ventilator injury, among other uses.

In summary, we have taken lessons from fetal lung development and adult lung physiology to design and implement a bioreactor that can maintain adult rodent lung tissue in the laboratory for up to 1 week. The bioreactor enables the perfusion of me-
dia through the vasculature, the movement of media or air in and out of the airways, and the ventilation of the lungs via negative (as well as positive) pressure. In order to more fully validate the bioreactor, we have performed experiments using freshly explanted whole rat lungs. The explanted lungs, which were not decellularized, were placed directly into the bioreactor and cultured for up to 1 week. We demonstrate that ventilating the cultured lungs with air causes damage to the pulmonary architecture and airway epithelium compared to ventilation with media, and we also examine the effects of vascular perfusion on cell survival and differentiation. Under optimized bioreactor conditions, we demonstrate the maintenance of cellular phenotype and pulmonary architecture, with minimal cellular apoptosis, for 1 week of culture. The developed bioreactor will be useful for the growth of engineered lung tissues as well as provide a platform to study lung biology, development and injury response in a controlled ex vivo environment.

5.2 Materials and Methods

5.2.1 Whole lung culture

Lungs were harvested from young adult (3 month-old) male Fischer 344 rats. All animal experimental work was approved by the Yale University Institutional Animal Care and Use Committee. Animals were anesthetized via intraperitoneal injection of sodium pentobarbital (Sigma, 40 mg/kg). After induction of anesthesia, the chest and abdomen were sprayed with ethanol and a transverse incision made just below the costal margin, entering the abdominal cavity. The diaphragm was punctured, and the ribs retracted, taking care not to touch the lungs. The inferior vena cava was severed and the lungs perfused via the right ventricle with 20-30 ml of PBS containing 50 U/ml heparin (Sigma) and 1 µg/ml sodium nitroprusside (Sigma). The trachea was then dissected and cut as high as possible. All remaining connections to the
heart and lungs were dissected free, allowing removal of the heart, lungs and trachea 
*en bloc* from the animal.

**Cannula attachment**

After removal of organs, cannulae were connected to the trachea and to the pulmonary artery trunk via the right side of the heart. The apex of the heart was cut off with a scalpel, and a right angle cannula was inserted through the right ventricle and into the pulmonary trunk. A syringe was attached to this cannula and 5-10ml of heparinized saline was injected to ensure proper cannula placement and adequate perfusion of the lungs without leakage. This cannula was then secured with suture to the heart. A separate straight, barb-end cannula was inserted into the trachea and secured with suture. The lungs were then connected to the bioreactor, and decellularized following the protocol described below.

Cannulae were attached to the pulmonary artery via the right ventricle and to the trachea, and the lung was connected to the bioreactor. The airway was lavaged with 2% amphotericin, penicillin and streptomycin in PBS, followed by two lavages with PBS, and the bioreactor was then filled with media and culture begun. Vascular perfusion and ventilation were performed as dictated by the experimental conditions.

### 5.2.2 Bioreactor components and assembly

All bioreactor components were obtained from Cole-Parmer (Vernon Hills, IL) unless otherwise noted. A silicone stopper and 500ml glass jar formed the basis of the bioreactor. PharMed tubing (Westlake, OH), sizes L/S 14 and L/S 16, was inserted through the silicone stopper to enable the necessary connections to the lung, including a perfusion loop, tracheal connection, air ventilation, and media exchange ports. Pressure was monitored using a TruWave pressure transducer (Edwards Life-
sciences, Irvine, CA) between the perfusion pump and the connection to the pulmonary artery. Perfusion was accomplished using a Masterflex L/S variable speed roller pump (Masterflex, Vernon Hills, IL). Ventilation was performed using a multi-channel programmable syringe pump (Cole Parmer), with inhalation and exhalation each performed over 30 seconds using a volume of 10ml. A diagram of the bioreactor is shown in figure 5.1.

5.2.3 Histology and immunofluorescence

After the desired culture period, lungs were fixed, paraffin-embedded and sectioned. Routine histology (H&E) was performed, as well as immunofluorescence for aquaporin-5 (type I epithelium), surfactant protein C (type II epithelium), CCSP (Clara cells), and PECAM-1 (endothelium). Sections were deparaffinized in xylene, rehydrated, and incubated with PBS with 0.2% triton-X (buffer) for 15 minutes. Antigen retrieval was performed using 0.02M citric acid in PBS for 20 min at 75-85°C, after which sections were rinsed in buffer. Blocking was performed for 1 hour at room temperature with PBS + 1% bovine serum albumin and 0.75% glycine. Primary antibodies were rinsed off with buffer and secondary antibody was applied for 1 hour at room temperature at 1:500 dilution. Secondary antibodies were obtained from Invitrogen (AlexaFluor 555 or AlexaFluor 488x). Images were acquired with a Zeiss Axiovert 200M inverted fluorescent microscope.

Cell proliferation was assessed via staining for proliferating cell nuclear antigen (PCNA) (Zymed, San Francisco, CA), and apoptotic nuclei were detected with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stain (Calbiochem, San Diego, CA). Manufacturer’s instructions were followed for both assays.
5.2.4 Microsphere ventilation assay

In order to determine if ventilation of lungs in the bioreactor is sufficient to induce movement of media to perfuse the vasculature, we developed a simple assay using 5 µm polystyrene microspheres (SPI Supplies, West Chester PA). Lungs were connected to the bioreactor, as described above, and ventilated but not perfused. The bioreactor chamber was filled with 100ml of media containing 10 million microspheres (0.1 million microspheres per ml of media). The culture was allowed to proceed, with ventilation only, for 3 hrs. The lung was then fixed, paraffin-embedded, sectioned and analyzed using routine histology (H&E).

5.3 Results

5.3.1 Bioreactor design requirements

We identified a set of design goals for the lung bioreactor, and then built and tested several designs, eventually achieving the design described herein. The bioreactor incorporates key features of the rodent in vivo environment but is also designed to be flexible, allowing the user to modify several key parameters depending upon the desired conditions.

Design goals. The design goals that we identified were as follows:

- System must be capable of perfusing media through the vasculature at a rate specified by the user and within the physiological levels of a rodent (up to 80ml/min)
- System must be capable of ventilating the lungs with air or media through the trachea. Negative pressure ventilation and the ability to constantly ventilate
the lungs is preferable, in order to be consistent with normal physiological conditions.

- Bioreactor should preferably allow for different media types to bathe the vascular and airway compartments of the lung.
- Bioreactor must allow for gas exchange into the culture medium, while simultaneously meeting the above requirements for ventilation.
- Bioreactor must have ports to allow for pressure measurements of the pulmonary artery and tracheal pressures. Pressures should ideally be within normal rodent physiological values, with a pulmonary artery pressure of less than 15-30mmHg [114].
- Bioreactor must have a means of allowing media exchange on a periodic basis.
- Bioreactor must be small and self-contained such that it can fit within the physical confines of a standard tissue culture incubator.
- All bioreactor components must be inexpensive and easily available.
- Bioreactor and all components must be able to be sterilized, preferably via autoclave.

After several iterations, a bioreactor was designed and built that meets the above criteria. A schematic of the bioreactor is shown in figure 5.1. In the ensuing sections, the detailed functions of the bioreactor are described.

### 5.3.2 Bioreactor perfusion system

Perfusion to the lung is provided via a roller pump that circulates media from the main bioreactor into the pulmonary artery. The perfusion rate can be specified by the user.
Figure 5.1: Schematic diagrams of the bioreactor used for \textit{in vitro} lung culture.
The heart of the rat is kept attached to the lung in order to facilitate the connection of a cannula to the pulmonary arterial trunk through the right ventricle of the heart. However, the pulmonary vein is not connected directly to the perfusion loop. Rather, the pulmonary veins drain through the left side of the heart directly into the main bioreactor reservoir. This was done for several reasons. First, it is technically difficult to have two connections to the vasculature of the lung and increases the likelihood of tears in the heart tissue, especially after decellularization. Second, if the perfusion loop was a closed system (with the venous return going directly into the perfusion loop and returning back to the pulmonary arteries), this would not allow mixing of media in the main bioreactor reservoir and would complicate the ability to replace the perfusion media with fresh media. Third, if the venous outflow of the lung was connected directly to the roller pump, the pump would exert a negative pressure on the venous system with every pulsation. This type of pressure would not be physiologic and would lead to stresses on the venous system that could impair proper perfusion. The venous drainage of the lung exits directly into the main bioreactor.

The perfusion rate through the lungs can be set to a user’s specifications. Physiologic flow rates in the adult rat are 40-80ml/min, although for engineered tissue culture the flow rate is typically much less than this value. In an adult rat, the entire blood volume must pass through the lungs in order to become oxygenated, whereas during engineered tissue culture we only must perfused sufficient media to support the growth of the pulmonary cells. Thus, perfusion rates during engineered culture are closer to that in a fetal rat, where the blood flow to the lung is only 8-10% of the cardiac output due to a normal physiologic shunt [91]. The pressure profile can be controlled to a limited degree using vasodilators such as sodium nitroprusside, which can be used to reduce pulmonary vascular pressure. Typically the perfusion pressure is kept below ∼30mmHg, the maximum value typically seen in the pulmonary arterial
system [114].

5.3.3 Bioreactor ventilation system

The bioreactor is capable of both positive and negative pressure ventilation. In vivo, breathing is normally accomplished via negative pressure ventilation. The diaphragm contracts and the rib cage expands to create a negative pressure within the thoracic cavity, causing air to flow into the lung to relieve this pressure imbalance. After inhalation, the breathing muscles relax and the lung passively deflates [23].

Negative pressure ventilation is the primary mode of ventilation in the bioreactor. In order to effect a negative pressure surrounding the lungs, the main chamber of the bioreactor must be completely airtight. This is accomplished by closing off all air and pressure-monitoring vents. Then, a syringe pump is used to withdraw a set volume of air from the main bioreactor, creating a negative pressure. The only pathway for this pressure to be relieved is for media (or air) to flow into the lungs via the trachea, which is connected to a separate reservoir. The syringe pump then reverses direction to push air back into the main bioreactor. This reverses the buildup of negative pressure inside the chamber, and media (or air) flows back into the tracheal reservoir. The lung deflates passively during this time.

Tracheal cannula utilizes one-way valve. As noted in figure 5.1, the connection to the trachea actually involves a Y-connector and a one-way valve open to the main bioreactor. This type of connection is necessary due to leakage of fluid out of the airway compartment, which is especially common during engineered lung culture. During inhalation, a volume of media enters the lung. However, some of this media leaks across the alveolar membrane into the interstitial space or vasculature. Therefore, not all of the media that entered the lung during inhalation can be returned to the tracheal reservoir during exhalation. Over time, this leads to pressure and volume
imbalance that lead to leaks and/or pump failures. As a result, we incorporated the design shown in figure 5.1, whereby during inhalation all the media enters the lung. However, during exhalation, media can return to the tracheal reservoir either from the lung or from the main bioreactor via the one-way valve.

The bioreactor can also utilize positive pressure ventilation, by connecting the syringe pump directly to the tracheal cannula or tracheal reservoir. However, positive pressure ventilation, such as used in mechanical ventilation of patients during surgery and in critical care situations, is known to induce pulmonary injury [146, 34, 174, 186]. As a result, we have exclusively utilized negative pressure ventilation for in vitro lung cultures.

**Tracheal inlet modification.** As discussed in section 5.3.6, we found during validation of the bioreactor design that during ventilation, the lung airway compartment was not being supplied with sufficient fresh media. We hypothesized that this was because largely the same media was being ventilated in and out of the trachea due to the volume of media contained in the tubing between the trachea and the separate tracheal reservoir, with insufficient fresh media entering the trachea. The “dead space” in the airway medium flow loop prevented fresh medium from reaching the lung tissue during breathing. As a result, we modified the bioreactor such that the media followed a different path into and out of the lung during ventilation, as outlined in figure 5.1(b). Due to this modification, most of the media entering the trachea with each ventilation was sourced directly from the tracheal reservoir (and thus ‘fresh’ compared to the media that is exiting the trachea).

**Oxygen supply during bioreactor culture.** We have measured the oxygen content of tissue culture medium in the bioreactor during lung cultures, in order to ensure that there is sufficient oxygen content. In particular, we want to ensure that there
is sufficient oxygen delivery during negative pressure ventilation, during which the main bioreactor is air-tight and the only portal for oxygen entry is via the tracheal reservoir. We have found that the oxygen tension does not drop significantly over the course of culture, and remains at 6.0-7.0mg/L, which is the same as the level in normal tissue culture media. These levels exceed normal physiological levels of 80-100mmHg (6-7mg/L corresponds to a partial pressure of 137-159mmHg).

5.3.4 Bioreactor pressure profiles

We have measured the pressure profiles in the trachea and pulmonary artery of engineered lung tissue cultured in the bioreactor, in order to ensure pressures are within expected or physiological limits. Figure 5.2 shows representative profiles. The perfusion pressure is typically kept between ∼2 and 30 mmHg. In the example given, the baseline perfusion pressure varies between 10-17 mmHg. However, the effects of the negative pressure ventilation are superimposed on this profile, thus lowering the perfusion pressure to 0-7mmHg during a negative pressure ‘breath’. This effect is seen physiologically, but at a much smaller magnitude, wherein the pressure drops in the pulmonary vasculature with inspiration. In the bioreactor the pulmonary vein drains directly into the main chamber, which also serves as the ‘thorax’, which is where negative pressure is created in order to ventilate the lung. This serves to increase transmission of negative pressures from the bioreactor to the perfused vasculature.

From the perfusion pressure profile, we can see that the maximum negative ‘thoracic’ pressure is ∼ -12 mmHg, approximately consistent with physiological values (see section 2.5). Therefore, during an inhalation, this negative pressure is exerted on the airways, driving fluid (or air) into the lungs from the tracheal reservoir. This pressure gradually decreases up the airway tree, and is -3mmHg at the tracheal inlet.
Figure 5.2: Pulmonary artery and tracheal pressures during *in vitro* lung culture. Perfusion rate is \( \sim 5 \text{ml/min} \).

Note that physiologically, the pressure at the inlet to the trachea is always essentially zero, as it is tied to atmospheric pressure. However, in the bioreactor, this pressure remains slightly negative during inhalation due to the length of tubing between the trachea and the tracheal reservoir, where the pressure reaches zero.

### 5.3.5 Media and oxygen requirements

In this section, we show a series of calculations intended to help determine the volume of media and air required for a rodent lung cultured in the bioreactor.

**Tissue culture comparison.** During *in vitro* tissue culture, it is common to feed 5 million cells with 12ml medium every 3 days. If we assume the adult rodent lung contains 100 million cells, this corresponds to a media requirement of at most 240ml every 3 days. However, this would be an overestimate as cells in tissue culture are generally actively replicating, while many cells in an intact rodent lung are quiescent and thus have lower media requirements.
Glucose consumption requirements. Kerr et al. demonstrated that the glucose consumption of a perfused rat lung is 43µmol per gram dry weight per hour [106]. The lung of an adult rat has a dry weight of ∼150-250mg [171, 99] while tissue culture medium typically contains 5.5mmol/L glucose. Therefore, the lung of an adult rat would require 28-47ml of tissue culture media per day in order to supply its glucose consumption requirements.

Oxygen requirements. Pulmonary artery endothelial cells consume 6nmol of oxygen per million cells per minute [214], while rat type II epithelial cells consume 1.25nmol per minute [52]. Assuming 100 million cells in an adult rat lung and assuming all cells in the lung consume oxygen at the higher rate, a rat lung would require at most 26mg of oxygen per day. Tissue culture media contains approximately 6mg of oxygen per liter, and the bioreactor contains approximately 300ml of media. Thus, the media can provide 1.8mg of oxygen with each exchange of fresh media (every 3 days). In addition, oxygen is contained in the air in the bioreactor; there is approximately 200ml of air in the main bioreactor. Air in the incubator contains ∼20% O₂, which at sea level and 37°C, corresponds to ∼260mg of oxygen per liter of air. Therefore the air in the bioreactor contains ∼52mg of oxygen.

The bioreactor that we have designed can provide for the media and oxygen requirements of a cultured rodent lung based on the above calculations. We routinely supply a total of 240ml of medium in the bioreactor (180ml in the main bioreactor and 60ml in the tracheal reservoir), and we exchange the air in the bioreactor daily. These conditions should be sufficient to provide more than enough nutrients and oxygen to a cultured lung.
5.3.6 Whole lung culture

In order to validate and optimize the design of the lung bioreactor, we have utilized the *in vitro* culture of whole native rodent lungs. We have cultured lungs for up to 7 days in the bioreactor, and have demonstrated that the bioreactor provides sufficient nutrient supply and mechanical stimulation to maintain cell survival and differentiation as well as lung morphology.

In addition, we have utilized the culture of native lung in the bioreactor to examine the effects of bioreactor conditions on cell survival, lung morphology, and maintenance of cellular differentiation state. We first compare the effects of air versus liquid (media) ventilation on lung morphology, then evaluate the effects of ventilation technique and nutrient delivery on cell survival. We also evaluate the effect of vascular perfusion pressure on cell survival and differentiation. Finally, we evaluate the final bioreactor design for the maintenance of cellular differentiation during 7-day cultures.

**Effects of ventilation with air versus media on overall lung morphology**

We evaluated the effects of ventilating lungs cultured in the bioreactor with either media or room air (∼20% O₂). We hypothesized that ventilation with media would offer improved cell survival as this would provide improved nutrient delivery, which may be more important in the bioreactor as there is no perfused bronchial circulation to supply the large airways. However, ventilation with air is the condition to which adult lungs are conditioned, and pulmonary epithelium is frequently cultured in the presence of an air-liquid interface, which has been shown to enable appropriate pulmonary development in fetal rat lungs [67]. Therefore, we also examined the hypothesis that ventilation with media may result in loss of epithelial differentiation state, due to the lack of an air-liquid interface.
Figure 5.3: Effect of ventilation with air versus liquid on lung architecture and airway epithelium. Air ventilation causes airway dilation and destruction of the airway epithelium after a 3 day culture.
After 3 days of culture, significant differences were noted between lungs ventilated with media versus air. As shown in figure 5.3(b), media ventilation appear similar to native lung; however, air-ventilated lungs show greatly dilated airways, with cell debris evident in the airway (figure 5.3(c)). Furthermore, the center panel of figure 5.3(c) shows that the bronchial and bronchiolar epithelium of air-ventilated lung was completely absent, a finding that was consistent across the entire lung. In addition, dilated peripheral airspaces were evident, as shown in the right panel of figure 5.3(c).

We note that the airway epithelium is also denuded if media is perfused through the vasculature (in addition to ventilation with air), while if media is perfused through the vasculature with no ventilation, the airway epithelium is still intact. This suggests that the loss of airway epithelium is not due to a lack of sufficient media, but is related to effects of air ventilation. We note that the bronchial circulation is not perfused for any cultures.

Epithelial cells are often cultured at an air-liquid interface, consistent with their physiologic locations. An air-liquid interface (ALI) is often utilized to induce epithelial differentiation [78, 212, 94], and a lack of an air-liquid interface can lead to reduced ciliogenesis [151, 216]. In addition, an air-liquid interface enables the maintained secretion of surfactant by type II epithelium when cultured *in vitro* [129]. As a result, we expected that we may observe differences in cellular differentiation state in the absence of an ALI. However, we did not observe significant changes in the expression patterns of Clara cell secretory protein (CCSP), surfactant protein C (SPC), or aquaporin (AQP) in lungs ventilated with media, as shown for cultures performed out to 7 days in figure 5.10 and discussed in section 5.3.6.

In summary, we demonstrate that ventilation with air in the bioreactor causes destruction of the airway epithelium and dilation of peripheral airspaces. These effects are observed regardless of the perfusion of media through the vasculature.
Effect of perfusion on cell survival

We examined the effect of vascular perfusion on cell survival and cellular differentiation in cultured native lungs in the bioreactor, with the aim of determining if perfusion alone could support *in vitro* lung culture, and if so what perfusion pressure was optimal.

Complicating these experiments is the fact that, after explantation of a lung, vascular permeability is rapidly increased. Isolated lung perfusion using pressures of 10mmHg can cause pulmonary edema within 10 minutes [208]. In addition, our own tests have demonstrated vascular leak within 5-10 minutes of explantation (see figure 7.3), with 3-4% of small particles (28nm radius) leaking across the alveolar-capillary membrane. Extensive pulmonary microvascular leak could result in less or even no media delivery to the distal capillaries and venous structures. Therefore, higher vascular perfusion pressures than the physiological levels of \( \sim 1-10\)mmHg may be required in order to deliver flow distally and keep distal capillaries patent [114].

We examined the effect of vascular perfusion pressures on cell survival during 3 day native lung culture in the bioreactor. As demonstrated in figure 5.4, higher perfusion pressures of up to 30mmHg resulted in fewer apoptotic cells as well as higher cell density, compared to pressures of 10 or 20mmHg. However, regardless of perfusion pressure, maintenance of cellular differentiation was poor with vascular perfusion. Substantially lower CCSP and SPC levels were observed (figure 5.5), while aquaporin expression was almost completely absent (not shown). PECAM expression was observed in the larger vessels of the vasculature, but decreased expression was observed in capillaries, as shown in figure 5.6. These experiments demonstrated that perfusion alone was not sufficient to maintain sufficient cell survival or cellular differentiation.
**Figure 5.4:** Effect of vascular perfusion and pressure on cell apoptosis and cell number during native lung culture. * indicates $p<0.01$ and # indicates $p<0.05$ compared to native.

**Effect of media flow path in the airway compartment on cell survival**

While ventilation with media permitted the maintenance of lung morphology and cell differentiation, we found significantly higher rates of apoptotic cells in ventilated cultured lungs compared to native (see figures 5.7 and 5.8). We hypothesized that this was due to insufficient fresh media delivery, and thus modified the bioreactor design in order to increase the delivery of fresh media to the airway compartment during ventilation. As shown in figure 5.1 and described in section 5.3.3, there is a single line connecting the main bioreactor to the tracheal reservoir. This length of tubing is approximately 40-45cm and contains 3-3.5ml of media. During ventilation, ∼2.5-3.0ml of media are drawn into the lung during a negative pressure inhalation, and the same volume of media returned via the tubing to the tracheal jar. Therefore, of the ∼2.5-3.0ml of media that enters the lung during each ‘breath’, this media is not fresh but simply returns into the lung from the tubing. Therefore, the actual media delivery to the lung is far less than would be delivered by ventilation with fresh media.
Figure 5.5: Comparison of CCSP and SPC expression in native lung and perfused cultured lung. CCSP and SPC expression are significantly reduced with vascular perfusion of 30mmHg. CCSP and SPC are stained in red, with nuclei counterstained blue with DAPI.
We modified the bioreactor design to add a second connection between the lung in the main bioreactor and the tracheal reservoir. Using one-way check valves, one connection was used for media delivery during inhalation and the other connection was used for media return during exhalation. This modification reduced the ‘recycled’ media from \( \sim 2.5-3.0 \, \text{ml} \) to only \( \sim 0.75 \, \text{ml} \) with each ventilation cycle, and therefore greatly increased the delivery of fresh media.

The effects of this bioreactor modification are shown in figure 5.8, where the additional breathing line was shown to improve cell survival. The percent of apoptotic cells was reduced to 3.9% for ‘loop’ ventilation from 21.5% for ventilation with a single line (‘vent only’ on figure 5.7). This compares to 0.5% for native lung.

While ‘loop’ ventilation increases the delivery of media to the lung by reducing the amount of ‘recycled’ medium, media delivery can also be increased with the addition of vascular perfusion. Perfusion together with ventilation reduces cell apoptosis to 7.9% from 21.5% for single-line ventilation alone (figure 5.7).

The ‘loop’ ventilation modification slightly increased overall cell number compared
Figure 5.7: Effect of ventilation on cell apoptosis and cell number during native lung culture. * indicates $p<0.01$ and # indicates $p<0.05$ compared to native.

Figure 5.8: Apoptotic nuclei in native and ventilated cultured lung. Ventilation with a single connection led to a much higher rate of apoptotic nuclei, as compared to native lung or ventilation with an airway ‘loop’. Apoptotic nuclei are stained brown via TUNEL, with normal nuclei counterstained green.
to single-line ventilation (figure 5.7(b)), but this was not a significant difference. For both single-line and ‘loop’ ventilation, cell number was reduced compared to native but not statistically significant.

In summary, we demonstrate that ventilation alone enables the survival of native lung tissue in the bioreactor for during 3-day cultures, provided sufficient fresh media is delivered to the lung using either the ‘loop’ ventilation modification or supplemental vascular perfusion. Loop ventilation demonstrates the best overall results, minimizing cellular apoptosis while maximizing cell number in cultured lung tissues. This set of optimized bioreactor conditions is evaluated in the next section using longer-term cultures and staining for cellular differentiation markers.

**Maintenance of cellular morphology, cellular differentiation, and alveolar structure**

In order to more fully validate the bioreactor design, we performed 7 day cultures of native lung. These cultures utilized ventilation with media with the ‘loop’ modification described above, but without any vascular perfusion. Vascular perfusion was not utilized, although future studies could explore the addition of perfusion to long-term ventilated cultures using ‘loop’ ventilation.

Lungs were evaluated via histology for cell proliferation, apoptosis, and maintenance of cellular differentiation via staining for aquaporin-5 (type I epithelium), surfactant protein C (type II epithelium), Clara cell secretory protein (Clara cells), and PECAM-1 (endothelium). Overall pulmonary architecture was preserved, including alveolar structure, as shown in figure 5.9. Lower magnification images are not distinguishable from those shown in figure 5.3 for media breathing. In addition, as shown in figure 5.10, patterns of expression of cellular markers were not substantially different from native lung.
Ventilation alone enables passive perfusion of the vasculature of the lung

Above, we demonstrate that ventilation alone can enable cell survival and maintenance of cellular phenotype of several key lung cell types, including endothelium, for up to 7 days. However, this is in the absence of active perfusion of medium through the vasculature, which was initially surprising. In order to investigate why the lack of perfusion did not seem to affect endothelial survival or differentiation, we performed an experiment using 5 µm microspheres to investigate the effect of ventilation on the movement of fluid into the vasculature. We had hypothesized that the physical movements induced by ventilation were sufficient to cause passive movement of media into and out of the vasculature, which is open to the media in the bioreactor. In this experiment, lungs were ventilated for 3 hours in the bioreactor which was filled with media containing 5 µm microspheres. If microspheres are noted in the vasculature of the lung, this would demonstrate that passive perfusion is induced by ventilation. As demonstrated in figure 5.11, microspheres were found in both large vessels as well as some capillaries. These results indicate that ventilation alone is sufficient to induce
Figure 5.10: Maintenance of pulmonary cell differentiation after 7 days of *in vitro* ventilated lung culture.
media movement in the vasculature, thus allowing maintenance of the endothelium despite the lack of perfusion.

This movement of media in the vasculature is a result of the physical motion of the lung due to ventilation. Diffusion alone would be insufficient to move such large particles into the vasculature. We can approximate the expected microsphere movement into the vasculature due to diffusion using Fick’s second law, which relates concentration \( \phi \) to time \( t \) and distance \( x \) via the diffusion constant \( D \) by

\[
\frac{\partial \phi}{\partial t} = D \frac{\partial^2 \phi}{\partial x^2}. \tag{5.1}
\]

In the simple case of one-dimensional diffusion with a boundary at \( x = 0 \) where the concentration of a substance, \( \phi(x, t) \), is constant at \( \phi(0) \), the solution to equation 5.1 is

\[
\phi(x, t) = \phi(0) \text{erfc} \left( \frac{x}{2\sqrt{Dt}} \right). \tag{5.2}
\]

The denominator in the above equation, \( 2\sqrt{Dt} \), is known as the diffusion length and is useful for understanding the scale of diffusion in a given time. In this case, \( \phi(x, t) \) is the concentration of microspheres along the main pulmonary vein of the lung, with \( x = 0 \) located at the junction between the bioreactor media (containing microspheres) and the entrance to the pulmonary venous trunk. In the case of a 5 \( \mu \)m microsphere, we can calculate the diffusion length in order to determine if diffusion alone would be sufficient to explain the movement of these particles into the lung during a 3 hour culture period. For this situation, we first make the approximation of the vasculature as a 1-dimensional system, with the axis of interest along the main pulmonary vein. The size of the pulmonary vein is significantly larger than 5 \( \mu \)m, allowing us to ignore the axial dimensions and treat it as a one-dimensional system.

In order to obtain a value for the diffusion coefficient \( D \), we use the Stokes-Einstein
relation, which states that

\[ D = \frac{k_B T}{6\pi \eta R} \tag{5.3} \]

where \( k_B \) is the Boltzmann constant, \( T \) is the temperature in Kelvin, \( \eta \) is the viscosity and \( R \) is the particle radius. At 37°C, the viscosity of tissue culture medium was measured using a viscometer and found to be 0.84 cP. Using equation 5.3, we calculate the diffusion constant for a 5 \( \mu \)m particle to be approximately \( D = 1.07 \cdot 10^{-13} \text{ m}^2/\text{s} \). Over a 3 hour period, this gives a diffusion length of 68 \( \mu \)m.

This length scale is far too small to justify diffusional movement of the microspheres into the vasculature of the lung. The distance that would need to be travelled for a microsphere to enter the vasculature of the lung is at least several millimeters, at least 100 times farther than the diffusion length. Therefore, we can be confident that the microspheres observed in figure 5.11 are due to passive perfusion resulting from the ventilatory movement of the lung. During inspiration, negative pressures outside the lung induce lung expansion. This lung expansion pulls fluid into the airway compartment, but also presumably induces fluid flow into the vasculature. Re-pressurization of the bioreactor jar causes exhalation of the lung tissue, and also presumably expulsion of fluid that flowed into the vasculature upon inspiration. In this way, bulk fluid flow may move into and out of the lung vasculature with tidal breathing movements.
Figure 5.11: Ventilation enables passive perfusion of pulmonary vasculature. Microspheres are found in vessels and capillaries due solely to ventilatory motions of the lung during \textit{in vitro} culture.
5.4 Discussion

In this chapter, we demonstrate the design and validation of a bioreactor for the \textit{in vitro} culture of whole rodent lungs. The bioreactor is capable of replicating key aspects of the \textit{in vivo} environment. In order to validate the bioreactor, as well as demonstrate its utility for performing \textit{in vitro} studies of lung behavior and physiology, we evaluated ventilation with media versus room air, as well as various perfusion and ventilation conditions. We examined outcomes with regards to cell viability, cell number, lung morphology, and maintenance of cellular differentiation state over 3-day culture periods. Finally, we performed 7-day cultures as a demonstration of the utility of the bioreactor for long-term culture of lung tissue.

5.4.1 Effects of ventilation with media versus filtered air on lung morphology

We examined the effects of ventilation with either medium or filtered room air on cultured native lung tissue. Ventilation with air was found to have clear negative effects on the airway epithelium, with almost complete loss of the airway epithelial lining. In addition, some dilation of the peripheral airspaces was evident (figure 5.3). These findings were surprising given that pulmonary epithelial cells are frequently cultured at an air-liquid interface, and the air interface has been shown to induce epithelial differentiation [78, 212, 94]. In addition, fetal rat lungs have been successfully cultured \textit{in vitro} at an air-liquid interface for up to 14 days [67]. Therefore, the air interface \textit{per se} may not be directly responsible for the loss of airway epithelium. Rather, it may be due to physical forces related to the bioreactor environment.

In the bioreactor, inhalation is performed via negative pressure ventilation - a negative pressure is created in the bioreactor by the withdrawal of air using a syringe pump, causing media or air to flow into the lung from the tracheal reservoir. This is
consistent with inhalation *in vivo*. However, exhalation *in vivo* is a passive process. In the bioreactor, exhalation is partly passive (due to elastic recoil of the lung), but there is also an increase in the pressure inside the bioreactor (resulting from the syringe pump returning the withdrawn air back into the bioreactor). This pressure increase may cause the airways to collapse upon end-exhalation. Some airway collapse at end-expiration is normal, but the amount resulting from the increased pressure upon exhalation may cause more or larger airways to collapse. With repeated collapsing and reopening, the airway epithelium may become striped or sheared off.

An air-liquid interface enables the maintained secretion of surfactant by type II epithelium when cultured *in vitro* [129]. As a result, we expected that we may observe differences in cellular differentiation state in the absence of an air interface, i.e. by ventilation of the lungs with medium versus air. However, we did not observe changes in the expression patterns of Clara cell secretory protein (CCSP), surfactant protein C (SPC), or aquaporin (AQP) in lungs ventilated with media. This was also seen in cultures performed out to 7 days, as shown in figure 5.10 and discussed in section 5.3.6. This appears somewhat contradictory to studies of *in vitro* growth of airway epithelium and type II pneumocytes, where isolated cells generally dedifferentiate in the absence of an air-liquid interface [79, 56, 215, 161, 217, 48, 151, 216]. This may be due to the native environment, which remains relatively undisturbed in these experiments but yet is highly disrupted during cell isolation procedures for *in vitro* cell culture experiments. However, perfused lung cultures do not demonstrate this maintenance of cellular differentiation. Therefore, this indicates the importance of breathing movements together with a native lung scaffold compared to coated tissue culture plastic, and suggests that breathing movements in a whole lung culture can maintain cellular differentiation similar to the effects of an air interface in isolated cell culture.
From figure 5.3(c), we note dilation of peripheral airspaces and destruction of some alveolar walls caused by air ventilation. These findings are consistent with those seen during high pressure ventilation in an *ex vivo* mouse model [199]. In our system, this may be due to over-distension resulting from differences during media versus air ventilation. During all lung cultures, a 10ml volume of air is removed from the bioreactor to effect negative pressure ventilation. This results in a tidal volume of $\sim 2.0$-$2.5$ml, which is measured during ventilation with medium. The tidal volume cannot be measured in this fashion during ventilation with air, and may be higher than $2.0$-$2.5$ml. During media ventilation, the negative pressure in the bioreactor gradually drops along the tubing connecting the trachea, in the bioreactor, to the tracheal reservoir, which is at atmospheric pressure. However, during ventilation with air, the trachea is effectively directly connected to the tracheal reservoir, without the pressure drop that is seen during media ventilation. This would result a higher pressure differential between the trachea and the alveoli during air ventilation and this would likely result in an increased ventilation volume. High tidal volume ventilation is associated with an increased occurrence of death in ventilated patients in critical care settings and with increased time requiring mechanical ventilation [11]. Therefore, future studies of native lung cultures should evaluate the effect of air ventilation using lower tidal volumes, as well as stricter control of the tracheal pressure during ventilation.

5.4.2 Effect of bioreactor design and media delivery on cell survival

In order to optimize the bioreactor design, we performed a series of iterated experiments to minimize cellular apoptosis, maintain cellular differentiation state, and minimize damage to the pulmonary architecture.
Perfusion alone was found to be insufficient to maintain cellular differentiation, as demonstrated by substantial declines in staining for the epithelial markers CCSP, SPC and AQP (figure 5.5), as well as diminished expression of the endothelial marker PECAM in capillaries (figure 5.6). Perfusion pressure did not significantly affect expression of these cell markers. However, higher perfusion pressure corresponded to decreased rates of cell apoptosis and increased cell density (figure 5.4). This is presumed to be due to increased media delivery to the tissue. While normal pulmonary vascular pressures are less than 15 mmHg in the rat [114], perfusion pressures in these studies were 10, 20 and 30 mmHg. These relatively high vascular pressures may be necessary due to increased vascular permeability upon explant of the lung, resulting in decreased effective media delivery to distal tissue. Pulmonary edema can occur in as little as 10 minutes at perfusion pressures of 10 mmHg [208], and in the absence of strict efforts to reduce vascular permeability, we would not expect for vascular permeability to be retained at native levels during the course of a 3 day lung culture. Therefore, higher perfusion pressures would be required to maintain perfusion of the distal vasculature, and would explain the reduced rates of apoptosis seen at higher perfusion pressures.

The loss of epithelial markers with perfusion, compared to ventilation, underscores the importance of physical breathing movements for the maintenance of epithelial differentiation. This has been shown in development, where the lack of fetal breathing movements impairs type II epithelial production of surfactant and the differentiation of type I pneumocytes [96, 210]. This has not been studied in adult lungs, as the lack of breathing movements would not be possible in an animal model and no system exists, before this work, that enables the long-term in vitro maintenance of adult lung tissue. In these studies, we have seen that epithelial differentiation is lost when breathing movements are not present. Further studies using the bioreactor system
could be used to examine if minimal breathing movements are sufficient to maintain surfactant production and epithelial differentiation, as well as the effects of noisy ventilation. The introduction of noise into mechanical ventilation profiles has been shown to be beneficial to lung function variables [174, 12, 13]. However, these studies have only been carried out for short time periods, usually 30 minutes, but some lasting up to a few hours; the bioreactor we describe here could be used to examine the long-term effects of such ventilatory techniques.

5.4.3 Maintenance of cellular morphology and differentiation and alveolar structure

The final bioreactor design was validated using 7-day cultures with continuous ventilation with media, using the ‘loop’ ventilation bioreactor modification. These culture conditions allowed the maintenance of cell survival, as well as maintenance of epithelial and endothelial phenotype. Surfactant expression was retained by type II epithelial cells, capillary beds were retained as demonstrated by PECAM staining, and flattened type I epithelium was present that retained expression of aquaporin-5. In addition, the airway epithelium was intact and Clara cells were noted at similar densities to native lung.

The ability to culture whole lung tissue in the laboratory for as long as 7 days enables a wide range of studies of lung physiology that previously were not possible. The bioreactor conditions can allow ventilation and/or perfusion at desired rates, and chemical or growth factors can easily be administered. Lung injury models can be applied to the bioreactor, and lung repair can be assessed under controlled conditions. Due to the absence of a blood supply and thus potential progenitor cell recruitment from the bone marrow or other sources, lung repair from tissue-resident stem cells or differentiated cells can be assessed.
In addition, lung physiology studies that were previously restricted to short time periods, lasting less than a few hours, can be carried out over several days, enabling the study of long-term cell and organ changes to applied physiological conditions. A prime example of this application is the study of lung ventilation, which is of importance due to the negative clinical implications of mechanical ventilation in critical care settings. Mechanical ventilation can be assessed over long time periods under highly controlled conditions, enabling the expansion of studies that were previously restricted to a few hours [174, 12, 13].

Despite the potential utility of this system, more detailed validation and study of lung behavior in the bioreactor is needed. First, it would be very useful to perform pulmonary function testing on lungs cultured in the bioreactor, including ventilation mechanics, perfusion-ventilation studies (V/Q testing), and lung volume measurements. In addition, measurement of pulmonary vascular leak would be useful, including the study of whether the addition of exogenous agents or modifications in perfusion or ventilation conditions affects vascular permeability. Detailed mechanical studies would also be important, such as those performed in chapter 4, to determine if lung elasticity or strength is affected by prolonged in vitro culture.

In addition, further bioreactor improvements may enable reduction of cell apoptosis rates below the current level of 3.9%, compared to native levels of 0.5%. It may be helpful to combine perfusion with the ‘loop’ ventilation utilized for bioreactor validation, which may lead to reduced cell death. Finally, more precise control of ventilation would enable more detailed study of ventilatory effects on the cultured lungs. Currently, ventilation is controlled indirectly via the volume of air removed from the bioreactor to create a negative pressure. The use of a simple computer-controlled feedback loop would enable negative ventilation under control of the pressure measured at the tracheal inlet. This would enable both negative and positive
pressure ventilation studies using pressure as a controlled variable, more consistent with studies in the literature, which control ventilation based on pressure, not volume [174, 12, 13]. Such a system would also enable more rigorous studies of the effects of ventilation with air, and may facilitate the ability to culture lungs with air ventilation without the negative effects observed herein.

5.5 Conclusions

In this chapter, we describe the design of a bioreactor for the \textit{in vitro} culture of whole rodent lung tissue. The bioreactor was designed to meet a series of design constraints aimed at the ability to provide sufficient nutrient supply and mechanical stimulation to the lung tissue in order to support cell survival and differentiation.

We demonstrated that vascular perfusion alone was not sufficient to support cell survival and cell differentiation, including surfactant production by type II epithelium. However, negative pressure ventilation with media was sufficient to support extensive cell survival (to 95.1\% of native levels) as well as maintain the differentiation of epithelium and endothelium. In comparison, ventilation with air was shown to cause virtual complete destruction of the airway epithelium as well as some breakdown of alveolar septae in the lung parenchyma.

The overall objective of the work in this chapter was to demonstrate the valid design of a bioreactor capable of culturing whole rodent lungs \textit{in vitro} for long time periods, with the objective of using this bioreactor for the future culture of engineered lung tissues. While we have achieved a design that is suitable for future use for engineered lung culture, much more study and validation of the bioreactor for native lung culture is possible. Many long-term \textit{in vitro} studies can be performed using native lung culture in the bioreactor to study lung physiology, development, injury response, and more.
Chapter 6

Epithelial Development in Engineered Lung Tissues

In the preceding chapters of this thesis, we demonstrated the production of a decellularized scaffold from native rodent lungs (chapters 3 and 4), with the goal being to demonstrate that a decellularized lung scaffold is a promising substrate for the growth of engineered lung tissue. In chapter 5, we demonstrated the design and validation of a bioreactor that can support the in vitro culture of whole lung tissue. These chapters have therefore addressed two of the three key components of an engineered tissue: a scaffold and a bioreactor. The final component is a suitable cell source for lung regeneration. In this chapter and chapter 7, we will describe our choice of cell source, evaluate the growth of these cells on the scaffolds, and examine the impact of bioreactor conditions on cell growth and differentiation in the engineered tissues.

6.1 Introduction

In this chapter, we will first demonstrate that the decellularized scaffolds are not cytotoxic, and further that they are compatible with the attachment and proliferation of a wide range of pulmonary cell types, including epithelial, endothelial, and mesenchymal cells. We then evaluate the hypothesis that the development of engineered lung epithelium is affected by key bioreactor conditions, including medium type, perfusion, ventilation, and the presence of an air-liquid interface.

Choice of cell source for engineered tissue growth. The choice of cell source for the regeneration of lung tissue on a decellularized scaffold is important. In this
work, we have chosen to obtain a heterogeneous mixture of pulmonary cells from the lungs of neonatal rats at 6-10 days after birth. This source was chosen because we can obtain a large population of cells that are still relatively plastic, because this cell source is syngeneic with the decellularized scaffolds, and because we can obtain a wide range of cell types, including all the key pulmonary cell types. Although this cell source is not applicable to the future development of human engineered lung tissue, for which a stem cell source will likely be pursued, the use of neonatal rat pulmonary cells for this work provides several advantages while allowing us to demonstrate the feasibility of this approach. If appropriate, future work would explore using stem cell-derived populations for the development of engineered lung tissue using the approach we are describing in this work.

**Engineering of distal lung tissue: prior work.** Although there has been significant progress towards the development of tissue engineered substitutes for the trachea and mainstem bronchi, there has been far less research towards the growth of distal lung tissue. In addition, there have been no reported attempts at the development of whole segments of engineered lung tissue.

There have been several reports of tissue engineered distal lung tissue, which generally combine a collagen or polymer scaffold with a pulmonary cell source [39, 135, 45, 10]. This work is described in greater detail in section 2.7. All of these approaches suffer from a lack of 3-dimensional organization, which is a critical deficiency. In order for engineered lung tissue to be useful, it must be able to connect to a vasculature and an airway. The airway must be continuous with alveoli, while the vascular connections must lead to a dense capillary network surrounding the alveoli. All work to date, while promising, has simply demonstrated the ability to induce the growth of alveoli in various 3-dimensional substrates such as gels or polymers. However, there is no organization to these alveoli; they are not connected to a larger airway or even to each
other. Furthermore, there is no vascular network surrounding the alveoli, which is an absolutely critical feature of functional lung tissue in order to allow gas exchange. Below, we outline in greater detail the approaches to lung tissue engineering to date.

**Outline of results.** In this chapter, we will first demonstrate that decellularized lung scaffolds are not cytotoxic and support the adherence and proliferation of epithelial and mesenchymal cells; in chapter 7 we demonstrate that the scaffolds can also support the culture of endothelial cells. We will then evaluate the hypothesis that the development of engineered lung tissues is affected by key bioreactor conditions, including medium type, perfusion, ventilation, and the presence of an air-liquid interface. We will demonstrate that ventilation with culture medium (i.e. “liquid ventilation”) aids in the differentiation of airway structures and epithelial cells. In addition, we will show that the static culture of small pieces of engineered tissue at an air-liquid interface has significant effects on tissue growth, and that in the bioreactor, ventilation with air affects cellular differentiation and the development of epithelial structures.

### 6.2 Materials and Methods

#### 6.2.1 Scaffold preparation

The lungs of adult Fischer 344 rats were harvested and decellularized as described in section 3.2.3. Following the decellularization procedure, the scaffolds were rinsed in 10 changes of sterile water, followed by rinsing for at least 12 hours in 10% penicillin/streptomycin in PBS. In later cultures, lungs were also rinsed in 10% FBS to aid removal of DNA remnants. The lungs were then transferred to a new, sterile bioreactor with a complete perfusion and breathing system attached. The lungs were subsequently rinsed twice in PBS and once in the media to be used for culture.
6.2.2 Neonatal cell isolation

Lungs were isolated from neonatal (~7 day-old) rats following the protocol in section 3.2.1. Lungs were then rinsed for 10 sec in 70% ethanol and rinsed twice in Dulbecco’s modified Eagle’s medium (DMEM, Gibco), and then transferred to a sterile, dry Petri dish. Lungs were minced for 5 minutes with a scalpel, and then transferred to a conical tube for elastase digestion. DNase, collagenase and elastase were obtained from Worthington Biochemical (Lakewood, NJ). Elastase digestion was performed for 20 minutes at room temperature with agitation, using 4U/ml elastase in DMEM with 100U/ml DNase. Tissue chunks were subsequently filtered through a 70 µm nylon filter and rinsed with DMEM. Undigested chunks were transferred to a clean tube and digested with collagenase for 1 hour at room temperature with agitation, in a solution of 1mg/ml collagenase in 1:1 DMEM:PBS with Ca^{2+} and Mg^{2+}. Collagenase-digested tissue was again filtered through a 70 µm filter and undigested pieces were physically crushed using a syringe plunger. The remaining tissue was rinsed with DMEM and filtered through a 70 µm filter. Cells from the collagenase and elastase digestions were combined, then washed three times in DMEM and once in the media to be used for culture.

Cell viability was assessed using trypan blue dye exclusion and cells were then seeded into the decellularized scaffolds as described in section 6.2.3.

6.2.3 Neonatal cell seeding

After pulmonary cell isolation and preparation of the decellularized scaffold, the isolated cells were suspended in the medium to be used for culture. For seeding of the airway compartment, 15ml of cell suspension per bioreactor was injected into the tracheal reservoir and cells were seeded by negative pressure ventilation to transfer the cells into the airway compartment of the lung. For seeding of the vasculature,
3ml of cell suspension per bioreactor was injected into the pulmonary artery. The cells were allowed to adhere overnight without perfusion or ventilation, after which perfusion and/or ventilation was begun depending on experimental conditions.

6.2.4 Engineered tissue culture

After seeding, the lungs were cultured statically overnight, after which perfusion or ventilation was begun. Perfusion and ventilation were varied according to experimental conditions. Culture medium was replaced twice weekly. For ventilation conditions, lungs were ventilated continuously except for a brief pause daily in order to allow manual exchange of air in the bioreactor. For the culture of pieces of engineered tissue, after overnight seeding, scaffolds were removed from the bioreactor and cut into small (1-3mm) pieces using sterile scissors. The pieces were transferred to Petri dishes for culture and, if indicated, later transferred to a Petri dish with a 0.4 µm filter insert for air-liquid interface culture.

6.2.5 Flow cytometry

After cell isolation, cells were rinsed in buffer (PBS with 2mM EDTA and 0.5% bovine serum albumin). For staining of intracellular antigens, cells were fixed with 1% formaldehyde for 15 minutes at R.T., then permeabilized with 0.2% triton-X in PBS. Primary antibodies were applied in buffer for 30 minutes at R.T. at 1:100 dilution. After 3 rinses in buffer, secondary antibodies were applied for 20 minutes at R.T. at 1:100 dilution. Antibody sources were as given in table 3.1. Cells were analyzed on Becton-Dickinson FACSCalibur machines at the Yale School of Medicine Cell Sorting Facility.
Immunohistochemistry and immunofluorescence

Tissue sections were prepared and analyzed as given in section 5.2.3.

Western blotting

Tissue samples were prepared and analyzed as described in section 3.2.5.

6.3 Results

We first performed a number of experiments intended to validate the decellularized scaffold as a suitable substrate for the growth of pulmonary cells and to validate our choice of cell population. We demonstrate that the decellularized scaffolds are not cytotoxic, indicating that components of the harsh chemical decellularization regimen have been removed. We then describe the isolation and characterization of a heterogenousous population of neonatal rat pulmonary cells, which are then utilized in subsequent experiments. Using this cell population, we performed a series of screening experiments to determine conditions that were suitable for engineered lung culture. We examine variables such as cell seeding method, medium type, length of culture, and the utility of flow cytometry sorting to isolate specific cell populations. Next, we demonstrate that a wide variety of epithelial cell types can adhere to and proliferate on the decellularized scaffolds, indicating the utility of the scaffold for the growth of whole segments of engineered lung tissue.

We then evaluate the specific effects of medium type on epithelial development and surfactant production in engineered tissues. We describe the effects of an air-liquid interface and ventilation with air on the growth and differentiation of engineered lung epithelium. Finally, we evaluate the effects of perfusion and ventilation on epithelial cell growth and differentiation.
6.3.1 Scaffold is not cytotoxic

We used a tumor-derived lung epithelial cell line for preliminary culture experiments on the decellularized lung scaffolds. These experiments were initially performed to demonstrate that the scaffold was not cytotoxic, as well as to demonstrate the first-order validity of the bioreactor system described in section 5.3 for cultures utilizing decellularized scaffolds. The MLE-12 cell line is a mouse tumor-derived epithelial cell line, and demonstrated robust cell growth during culture periods of up to 10 days on decellularized scaffolds in the bioreactor, with perfusion of media through the vasculature. Histology is demonstrated in figure 6.1. Cells appeared to form very primitive alveolar structures at 3 days, but soon proliferated extensively, and uncontrolled cell growth is shown by 7 days, an expected outcome as this is a tumor-derived cell line. These experiments were a preliminary step in the validation of the bioreactor and the decellularized lung scaffolds, and justified the subsequent experiments using freshly isolated neonatal pulmonary cells.

![Figure 6.1: H&E stain of the immortalized epithelial cell line MLE-12 cultured on decellularized scaffolds.](image)

(a) 3 days  
(b) 7 days
6.3.2 Harvest of neonatal pulmonary cells

The cell population that we utilized for subsequent cultures of engineered lung tissue was isolated from neonatal rat lungs. We chose this source for several reasons, including the ability to isolate a large number of cells which represent a heterogeneous mix of pulmonary cell types, because rodent lung epithelium is difficult to culture in vitro and because the pulmonary cells of neonatal rats are young and relatively plastic [130, 132]. The selection of this cell source is discussed in further detail in section 6.4.1.

Conditions for cell isolation were optimized based on cell number, viability, and distribution of cell types based on flow cytometry. Primary markers used were surfactant protein C (SPC; type II pneumocytes), aquaporin-5 (AQP; type I pneumocytes), Clara cell secretory protein (CCSP; Clara cells), and platelet endothelial cell adhesion molecule-1 (PECAM-1; endothelial cells). Conditions for cell isolation were chosen as a result of iterated experiments that optimized overall cell number and viability; the evaluated and final conditions are identified in table 6.1. Complete data documenting each choice of condition are not shown, though the selection of enzymes and incubation conditions was optimized based on cell yield and viability, as assessed by total cell number, trypan blue dye exclusion, and flow cytometry analysis.

Flow cytometry data of a sample lung isolation that was obtained under an ‘optimized’ isolation regimen, as outlined in table 6.1, is shown in figure 6.2. In a typical isolation, we see 5-10% of cells CCSP-positive, 40-60% of cells SPC-positive, 2-8% of cells AQP-positive, 1-2% of cells cytokeratin-14-positive, 10-30% of cells PECAM-1 positive, and 5-10% of cells α-actin-positive. Using cytospin preparations, we confirmed staining for CCSP and SPC, as shown in figure 6.3. While most of these percentages are within expected range, one would expect higher yield of type I pneu-
<table>
<thead>
<tr>
<th>Variable</th>
<th>Conditions</th>
<th>‘Optimized’ Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastase concentration</td>
<td>2, 4, 8 U/ml</td>
<td>4 U/ml</td>
</tr>
<tr>
<td>Collagenase concetration</td>
<td>0.5, 1.0, 2.0 mg/ml</td>
<td>1.0 mg/ml</td>
</tr>
<tr>
<td>Collagenase digestion time</td>
<td>1hr, 2hr</td>
<td>1hr</td>
</tr>
<tr>
<td>Mechanical crushing</td>
<td>With, without</td>
<td>With</td>
</tr>
<tr>
<td>Macrophage depletion</td>
<td>With, without</td>
<td>Without</td>
</tr>
<tr>
<td>Temperature</td>
<td>22°C, 37°C</td>
<td>22°C</td>
</tr>
<tr>
<td>Pulmonary perfusion to remove RBC</td>
<td>With, without</td>
<td>With</td>
</tr>
</tbody>
</table>

Table 6.1: Conditions that were optimized for isolation of neonatal pulmonary cells

mocytes (AQP-positive), based solely on its prevalence in native lung. However, type I pneumocytes are very fragile and many of them are unlikely to survive the cell harvesting process. Total cell yield from a litter of pups (7-12 pups) is approximately 100 million cells, with viability of 75-85%. We thus demonstrate the ability to obtain a large, viable, and heterogeneous population of pulmonary cells from neonatal rats. We utilize this cell population for experiments described in the rest of this chapter for the growth of engineered lung tissue.

### 6.3.3 Preliminary identification of bioreactor conditions for engineered lung culture

Using the cell population described in section 6.3.2, we performed a series of screening experiments intended to identify conditions that were compatible with the growth of engineered lung tissue in the bioreactor. We explored many variables and selected suitable conditions based primarily on cell density, viability and morphology via histology, as well as some evaluation of protein expression. The conditions that were evaluated are outlined in table 6.2 and briefly addressed below.
Figure 6.2: Flow cytometry staining of a panel of pulmonary markers of isolated neonatal pulmonary cells. Blue or green curves are isotype control stains and red is the antigen indicated.

Figure 6.3: Staining for CCSP and SPC on cytopsin preparations, qualitatively confirming flow cytometry analysis of neonatal cell population (figure 6.2)
<table>
<thead>
<tr>
<th>Variable</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>DMEM, BGJb, EGM-2, MCDB-131</td>
</tr>
<tr>
<td>Serum %</td>
<td>0, 10, 15 %</td>
</tr>
<tr>
<td>Perfusion</td>
<td>0 to 5 ml/min</td>
</tr>
<tr>
<td>Ventilation frequency</td>
<td>None, Daily, Continuous</td>
</tr>
<tr>
<td>Ventilation media</td>
<td>Liquid (medium), air</td>
</tr>
<tr>
<td>Cell seeding method</td>
<td>Bolus, repeat injection</td>
</tr>
<tr>
<td>Cell seeding location</td>
<td>Airway, vasculature</td>
</tr>
<tr>
<td>Cell sorting</td>
<td>Unsorted, PECAM-negative</td>
</tr>
</tbody>
</table>

Table 6.2: Bioreactor and culture conditions that were evaluated during pilot lung engineering studies.

**Medium choice.** We evaluated several medium compositions, varying both the base medium and serum concentration. We found evidence of epithelial repopulation, principally type II epithelium, using several medium conditions, including BGJb (serum-free), DMEM with 10% FBS, EGM-2 with 15% FBS, and a 3 part to 1 mix of EGM-2+15%FBS and BGJb. We did not note substantial epithelial growth using the endothelial-specific media MCDB-131+10%FBS, although only a total of two lungs were cultured using this medium. The medium types BGJb and DMEM+10%FBS appeared to provide superior conditions for overall epithelial growth, based on histology and immunofluorescence staining. As a result, these media were the focus of future experiments comparing epithelial development in engineered tissues using these medium types (section 6.3.5).

**Perfusion and ventilation.** We utilized both perfusion and ventilation during these preliminary experiments. Continuous ventilation was attempted but largely unsuccessful due to technical complications that had not yet been resolved at this early point in our studies. Therefore, these cultures were perfused at 2-5ml/min in order to provide a nutrient supply. We evaluated the effect of ventilation once-daily with a single breath, but did not detect significant differences in the resulting en-
engineered lung cultures. Despite not observing a clear benefit from the once-daily ventilation, we felt that this minimal level of ventilation would provide a more physiological culture environment. Therefore, for the majority of the experiments outlined in the rest of this section, the cultures were perfused and ventilated once daily with a single breath.

**Cell seeding location.** We evaluated the seeding location, comparing injecting cells into the airway versus the vasculature. We noted improved outcomes with seeding into the airway, primarily because the airway can better contain the injected cells, whereas in the vasculature many of the injected cells will quickly leak out of the pulmonary vein. In addition, for many preliminary experiments, the vasculature was highly damaged, as described in chapter 3, sections 3.3.5, 3.3.7 and 3.3.8.

**Cell seeding method.** We also evaluated the method of cell seeding into the airway. We compared injecting cells via negative versus positive pressure ventilation, as well as single versus repeated injections. Negative pressure injection allowed the cell suspension to better distribute through the lung parenchyma compared to positive pressure injection, and was felt to be more physiologic. In addition, a single injection was found superior to repeated injections. This was likely due to the slow adherence rate of epithelial cells, which can often take 12-18 hours to adhere onto tissue culture plastic. Therefore, repeated cell injections may have served to detach cells that were previously injected.

**Use of selected cell populations via flow cytometry sorting.** The cell population isolated from neonatal rat lungs contains both epithelial and endothelial cells, as described in section 6.3.2. Therefore, both principal cell types are seeded together into either the airway or vascular compartment, which we suspected could
negatively impact engineered tissue development. In order to separate epithelial and endothelial cell types, we used fluorescence-activated cell sorting (FACS) to select cells that expressed PECAM. We used PECAM-positive cells for injection into the vasculature, while the PECAM-negative population, which would encompass both epithelial and mesenchymal cells, was seeded into the airway. However, tissue growth after FACS was generally quite poor, presumably due to the significantly increased time and handling required for cell sorting experiments and consequent decreased cell viability.

**Summary of initial screening experiments.** These screening experiments, as described above, allowed us to identify sets of conditions that were initially compatible with engineered lung culture. Outcomes were assessed primarily via histology for cell density, viability, morphology and, in some cases, expression of cell-specific markers. The above set of experiments were not intended to be comprehensive or in-depth. However, before we were able to begin in-depth studies of engineered lung growth and development, we first had to identify conditions that enabled adhesion and survival of lung epithelium inside the decellularized matrix. While not an exhaustive survey of all possible conditions, these pilot studies encompassed approximately 75 engineered lung culture experiments spanning at least 18 months. The result of these pilot studies was a set of conditions that enables cell growth inside the scaffolds, such that we could then systematically evaluate the impact of discrete conditions on engineered lung tissues.

To summarize, the conditions that were identified as promising as an initial starting point for experimentation were: the choice of BGJb or DMEM+10%FBS culture medium for epithelial growth; neonatal cell seeding into the airway as a single bolus injection; and no use of selected cell populations via FACS; and fibronectin-coated scaffolds for endothelial cell seeding (see chapter 7).
6.3.4 Decellularized scaffolds support the growth of epithelial, endothelial and mesenchymal cells

Using the conditions identified in section 6.3.3, we performed a set of experiments intended to demonstrate that the decellularized scaffolds can support the adherence and proliferation of a wide range of pulmonary cell types, including epithelial, endothelial and mesenchymal cells. These experiments are intended to demonstrate the validity of the decellularized scaffolds, the lung bioreactor, and the isolated neonatal pulmonary cell population for the development engineered lung tissue. In the ensuing sections of this chapter, we will then evaluate the specific effects of medium type, perfusion versus ventilation, and an air interface on the development of engineered lung epithelium. In chapter 7, we will address the development of engineered lung endothelium in greater detail.

The precise conditions used in the culture of these engineered tissues are identified in table 6.3. Also described in the table are conditions that were specifically probed to evaluate the effects of those conditions on engineered tissue growth, as described in the subsequent sections of this chapter.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Media</th>
<th>Ventilation</th>
<th>Perfusion</th>
<th>Length of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Validation</td>
<td>BGJb; DMEM+10%FBS</td>
<td>Continuous; Once daily</td>
<td>2 ml/min</td>
<td>4-8 days</td>
</tr>
<tr>
<td>Perfusion</td>
<td>DMEM+10%FBS</td>
<td>None</td>
<td>2 ml/min</td>
<td>8 days</td>
</tr>
<tr>
<td>Ventilation</td>
<td>DMEM+10%FBS</td>
<td>Continuous with media</td>
<td>2 ml/min</td>
<td>8 days</td>
</tr>
<tr>
<td>Air-liquid interface</td>
<td>DMEM+10%FBS</td>
<td>Continuous with media for 4 days, then air for 4 days</td>
<td>None</td>
<td>8 days</td>
</tr>
<tr>
<td>Media screen</td>
<td>DMEM+10%FBS to BGJb transition</td>
<td>Continuous</td>
<td>None</td>
<td>8 days</td>
</tr>
</tbody>
</table>

Table 6.3: Bioreactor conditions for engineered lung culture.
Demonstration of epithelial cell repopulation

In this section we demonstrate the adherence and proliferation of epithelial cells on decellularized lung scaffolds. For these experiments, we use DMEM+10%FBS medium, perfusion of the vasculature at 2ml/min, once daily ventilation, uncoated decellularized scaffolds, and unsorted neonatal pulmonary cell populations (the ‘optimized’ conditions described in section 6.3.2). We will show that these conditions are compatible with epithelial cell adherence and proliferation. However, no endothelial cells are noted with these conditions, based on immunostaining for PECAM.

In figure 6.4 we show H&E staining of engineered lung tissue. At 4 days we see a significant number of organized, cuboidal-epithelial-lined developing epithelial structures, while at 8 days fewer such structures are noted and many cells adopt a more mature phenotype. At 4 days, many cells are proliferating, while at 8 days fewer proliferating cells are noted, as shown in figure 6.5. At both 4 and 8 days, we do not see significant numbers of apoptotic cells (figure 6.6).

![Figure 6.4: H&E stain of engineered lung at 8 days of culture. Conditions here are optimized for epithelial cell growth.](image)

We used immunofluorescence to documented the expression of key epithelial cell markers, using aquaporin-5 for type I pneumocytes, surfactant protein C for type II pneumocytes, and Clara cell secretory protein for Clara cells. Type II epithelial cells
Figure 6.5: PCNA staining of engineered lung at 4 and 8 days of culture. Proliferating nuclei stain brown for PCNA; negative nuclei are counterstained with hematoxylin.

Figure 6.6: TUNEL staining of engineered lung at 4 and 8 days of culture. Positive nuclei are brown, while negative nuclei are counterstained with methyl green.
generally predominate in the cultures, especially at later time points, as shown in figure 6.8. Clara cells are noted at high densities at 4 days, with fewer cells at 8 days (figure 6.7). We also note staining for aquaporin at 4 days, although significantly less aquaporin staining is noted at later time points (figure 6.9).

![Figure 6.7: Clara Cell secretory protein (CCSP) staining of native and engineered lung at 4 days. CCSP is stained red, while nuclei are counterstained blue with DAPI.](image)

![Figure 6.8: Surfactant protein C staining of native and engineered lung at 4 days. SPC is stained red, while nuclei are counterstained blue with DAPI.](image)

Note that aquaporin staining for type I epithelium depicts cells that are cuboidal in shape at 4 days of culture, which is contrary to their usual flat morphology, as the cells that line alveoli in functioning lungs. In addition, these cuboidal cells also frequently stain positive for either SPC or CCSP, as seen in figures 6.8 and 6.7. Therefore, it is unlikely that the cells that express aquaporin at 4 days are mature type I epithelium, as would be suggested by a flattened morphology and expression of
Aquaporin-5 staining of native and engineered lung at 4 days. AQP is stained red, while nuclei are counterstained blue with DAPI.

Figure 6.9: Aquaporin-5 staining of native and engineered lung at 4 days. AQP is stained red, while nuclei are counterstained blue with DAPI.

aquaporin without other markers. Type I pneumocytes are derived in vivo from type II epithelial cells, which are a locally resident precursor cell for alveolar epithelium. In native lungs during development, type I pneumocytes do not achieve final differentiation state in the absence of fetal breathing movements [98] and remain cuboidal in shape on histology and TEM [96]. In these engineered tissue cultures, breathing was only performed once per day, principally to exchange nutrients. As a comparison, fetal breathing movements in a late-stage fetal rodent occur at 15/min [3]. Therefore, the lack of type I pneumocyte differentiation is not surprising in these cultures that were not regularly ventilated. The cells that do express aquaporin most likely arise from a local precursor cell that has not fully differentiated to type I epithelium.

Therefore, the decellularized scaffolds can support the attachment and proliferation of pulmonary epithelium. We see robust growth of type II epithelium, as well as Clara cells and cells that are likely the precursors to fully differentiated type I epithelial cells. We observe these findings under conditions of medium perfusion through the vasculature with only occasional breathing movements.
Epithelial progenitor cell repopulation

We also note the growth of two types of pulmonary epithelial progenitor cells on the decellularized scaffolds. Cells that are dual-positive for CCSP and SPC are reported to be local progenitor cells, termed bronchoalveolar stem cells, which can differentiate into both Clara cells and type II pneumocytes and are found at the bronchoalveolar duct junction [112, 107]. Figure 6.10 shows such dual-positive cells, which are found in structures consistent with the appearance of terminal bronchioles, the expected physiological location of these cells.

![Figure 6.10](image)

**Figure 6.10**: Dual staining for SPC and CCSP in engineered lung tissue. SPC is stained green, CCSP is stained red, and nuclei are counterstained blue with DAPI. SPC-CCSP dual positive cells appear yellow.

Basal cells are a local stem cell for pulmonary airways; they reside below the columnar epithelium and serve as a regenerative cell source for epithelium of the proximal airways. Figure 6.11 demonstrates cells that are positive for cytokeratin-14, which is a basal cell marker. For comparison, native lung is shown in figure 6.11(a). In addition, dual staining for cytokeratin-14 and CCSP is shown in figure 6.12, which also demonstrates that the Clara cells are lining the airway and the basal cells lying beneath them, consistent with their normal anatomic locations. These cells
are sometimes found beneath larger airway structures, consistent with their location in native lung (figure 6.11(b)), but are also found in clusters that do not appear to be associated with large airways (figure 6.11(c)).

(a) Native lung  (b) Engineered lung  

(c) Engineered lung

**Figure 6.11**: Cytokeratin-14 staining for basal cells in native and engineered lung. Cytokeratin stains red, while nuclei are counterstained blue with DAPI.

**Mesenchymal cell repopulation**

In addition to epithelial and endothelial cell growth, mesenchymal cells can repopulate the decellularized lung scaffolds. Figure 6.13 shows immunofluorescence staining of α-actin, which stains smooth muscle and myofibroblasts. These engineered tissues were cultured under the same conditions that were shown to favor epithelial growth.
Figure 6.12: Dual staining for cytokeratin-14 and CCSP in engineered lung. Cytokeratin-14 is stained red, CCSP is stained green, and nuclei are counterstained blue with DAPI.

We also note the location of the mesenchymal cells, which are found beneath and between the developing epithelial structures, consistent with their location in native lung. Therefore, we demonstrate that the decellularized scaffolds are also suitable substrates for the growth of mesenchymal cells, and document that viable mesenchymal cells are contained within the population of neonatal pulmonary cells.

Summary of cell growth on decellularized scaffolds

In summary, we have demonstrated the culture of a wide range of epithelial cell types on the decellularized lung scaffolds, including type II epithelium, Clara cells, basal cells, bronchoalveolar stem cells, and presumptive type I epithelial precursors. We also document the growth of mesenchymal cells, which are found in supportive locations relative to epithelial structures.

These findings indicate that the decellularized scaffolds are suitable substrates for the attachment and proliferation of pulmonary epithelial and mesenchymal cells. In addition, these results demonstrate the utility of the heterogeneous neonatal pul-
Figure 6.13: α-actin staining of native and engineered lung. α-actin is stained green, while nuclei are counterstained blue with DAPI.
monary population isolated from neonatal rats. This population is able to give rise to a wide range of epithelial cell types, an advantageous feature in our efforts to repopulate entire segments of lung tissue during engineered tissue growth.

However, there is much more to the development of engineered lung tissue than simply the growth of a variety epithelial cells on a decellularized scaffold. First, the overall cell density is less than native; typically less than 25-50% of the scaffold contains cells. Additionally, functional lung tissue requires that the growing epithelium be organized in complex fashions. The tissue must retain a 3-dimensional structure that can conduct air through patent airways towards alveoli. The cellular repopulation must be organized, with selected cell populations regenerating appropriate levels of the airway tree. At this time, we cannot make any firm conclusions regarding these aspects of ‘higher-order’ lung tissue growth. We have demonstrated the formation of lined epithelial structures consistent with small airways and alveoli, with cell populations that are appropriate for such structures. However, whether these structures are organized into a 3-dimensional airway tree or are simply randomly growing in the scaffold is not known.

In the ensuing sections we will evaluate the effects of specific medium composition, ventilation versus perfusion, and the presence of an air-liquid interface on the development and differentiation of epithelial cells and structures. In chapter 7 we will discuss in greater detail the growth of endothelial cells on the scaffolds, while in chapter 8 we will evaluate co-cultures of endothelial and epithelial cells and evaluate the engineered tissues using several functional assays.
Figure 6.14: Effect of media composition on epithelial development. Note that in BGJb media, epithelial structures are driven towards apical expression of SPC granules with loss of CCSP expression. In DMEM media, cells retain expression of both SPC and CCSP, with SPC expression diffusely cytoplasmic.
6.3.5 Effect of media composition on epithelial differentiation

Medium type can have significant effects on cell growth and differentiation, and thus on the development of engineered lung tissues. In order to investigate some of these differences in more detail, we compared the growth of engineered lung cultures using a serum-free media (BGJb) versus a serum-containing media (DMEM+10%FBS) on epithelial differentiation. In these experiments, cells were first seeded onto the scaffolds in DMEM+10%FBS and allowed to culture in this medium for 2 days, after which a gradual 4-day transition to BGJb (serum-free) media occurred, with the final 2 days of culture in pure BGJb media.

The transition to serum-free medium caused substantial effects on the expression of surfactant. On immunofluorescence, we note that serum-free medium leads to a more apical expression of surfactant (SPC) as compared to DMEM+10%FBS (figure 6.14(c) and 6.14(d)). This corresponds to significantly increased expression of surfactant on Western blot with the serum-free medium (BGJb) (figure 6.15; compare lanes labelled ‘DMEM’ and ‘BGJb’). In addition, the form of surfactant is much more consistent with native lung (with most surfactant noted as the 21kDa pro-SPC form in BGJb medium).

Figure 6.15: Surfactant expression in engineered epithelial tissues. ‘Lad’ is a protein ladder; the indicated bands are 20 and 25kDa; ‘Nat’ is native lung tissue; ‘Vent’ is engineered lung tissue ventilated with DMEM medium; ‘Perf’ is engineered lung tissue perfused with DMEM medium; ‘DMEM’ is statically cultured engineered lung in DMEM medium; ‘BGJb’ is statically cultured engineered lung in BGJb medium; ‘ALI’ are engineered lung ventilated with air; and ‘Decell’ is decellularized scaffold.
In addition, the transition to serum-free medium leads to a decrease in CCSP expression, noted via immunofluorescence in figure 6.14(f) and 6.14(e). Note that in both medium types, diffuse CCSP expression is noted in the lumens of the developing epithelial structures; this is due to lack of perfusion or ventilation, as these cultures were performed in small tissue slices.

These findings may indicate that the serum-free medium (BGJb) is causing a maturation of the engineered epithelium towards distal pulmonary epithelium, leading to a loss of expression of CCSP and induction of surfactant expression. Clara cells are a known progenitor for alveolar epithelium, and we therefore may be observing the maturation of these Clara cell-lined epithelial structures towards alveolar epithelium. Consistent with this hypothesis, we observe a high density of small Clara cell-lined epithelial structures in early lung cultures (i.e. 4 days; see figure 6.7(b). By 8 days, we see fewer of these structures and an increased density of more mature, type II epithelium (see figure 6.7(c) and 6.8(c)). Therefore, there may be some differentiation of these CCSP+ structures towards alveolar epithelium even in DMEM+10%FBS medium, although it appears to be accelerated when tissues are transitioned to the serum-free BGJb medium.

These findings clearly indicate the need for studies using isolated, characterized cell populations of particular cell types, such as Clara cells, type II pneumocytes, bronchoalveolar stem cells, etc. Such studies would lend interesting insight into the progenitor potential of each cell type, and would allow the evaluation of particular interactions of each cell type with the decellularized lung scaffolds.

6.3.6 Effect of air-liquid interface on static culture

An air-liquid interface is often utilized to induce the differentiation of in vitro cultured epithelium, including airway epithelium [217] as well as type II epithelium [56, 215,
Therefore, we compared the growth of engineered tissues in the presence and absence of an air interface.

For these experiments, we cultured neonatal pulmonary cells on small pieces of decellularized scaffold. Small pieces of scaffold were used for these experiments as the ability to create an air interface in the bioreactor is difficult. As discussed in section 5.3.6, ventilation of native lungs with air in the bioreactor was found to have significant negative impacts on the airway epithelium and overall lung morphology. In addition, by performing these experiments on small pieces of decellularized scaffold, we can better control the experimental conditions, eliminating variability from different scaffolds, isolated cell populations, and bioreactors.

Cells were seeded onto a decellularized scaffold in identical manner as for typical engineered tissue growth. However, after allowing 24 hours for cell adherence, the tissue was cut into small pieces and cultured in the liquid phase of culture medium. After 4 days, some samples were transferred to an air-liquid interface (ALI) for an additional 4 days of culture while the remaining samples were kept within the liquid layer. We found substantial effects of the air interface under these conditions, as shown in figure 6.16. In the absence of an ALI, developing epithelial cells are clearly detached from the substrate, although these cells are not apoptotic and still stain positive for the proliferative marker PCNA.

These results were surprising, as the vast majority of engineered tissues that are cultured in the bioreactor are not exposed to an air interface. This may have less to do with the introduction of an air interface than the induction of media flow through the tissue. Due to surface tension, it is unlikely that an air-liquid interface existed in most of the tissue, despite the tissue piece being place at the air interface. However, due to capillary action and evaporation of liquid from the top surface of the tissue, increased media movement may have occurred that may explain these findings.
Figure 6.16: Effect of an air-liquid interface on cell proliferation and attachment. Histological staining showing that cell attachment is diminished in the absence of an air interface, although cells do not stain for the proliferation marker PCNA or appear apoptotic via TUNEL stain. PCNA stains proliferating nuclei brown, with negative nuclei counterstained blue with hematoxylin. TUNEL stains apoptotic nuclei brown, with negative nuclei counterstained green with methyl green.
6.3.7 Effect of air-liquid interface on lung development in the bioreactor

An air-liquid interface substantially improves the attachment of epithelial cells to the scaffolds when cultured as static pieces. As a result, we attempted to replicate the air-liquid interface in the bioreactor. In order to create an air-liquid interface, we cultured the engineered lungs first for 4 days under ventilation with media, to allow cell attachment and proliferation as usual. For the final 4 days of culture, the ventilation was switched from media to filtered room air.

Unfortunately, as described in section 5.3.6, ventilation with air causes severe damage to the airway epithelium as well as destructive changes to some alveolar walls (figure 5.3). Therefore, we reduced by approximately 50% the tidal volume used for air ventilation, from the previous value of ∼2.0-2.5ml for liquid or air breathing used in chapter 5, in an effort to reduce the damage caused to native lungs by air ventilation.

In contrast to the findings in section 6.3.6, we did not note changes in cell attachment or morphology due to the presence of air ventilation in the bioreactor.

However, we found that ventilation with air in engineered lungs cultured in the bioreactor led to induced expression of aquaporin, a differentiation marker for type I epithelium. This was noted both in cells in the parenchyma, which typically stain solely for surfactant protein C (indicate of type II epithelium) as well as occasional staining of cuboidal cells in developing epithelial structures. Although dual staining studies to demonstrate co-localization of AQP and SPC are not possible with these antibodies, the observed staining patterns indicate that it is highly likely that most of the aquaporin-expressing cells also express SPC. As can be seen from figure 6.17(b), virtually all of the cells in the parenchyma express SPC, while a subset of the parenchymal cells express AQP (figure 6.17(a)).
These findings are highly suggestive that the air-liquid interface is inducing differentiation of type II epithelium to type I epithelium. Type II epithelium is a known local progenitor cell for type I epithelium, and so this differentiation is not surprising [4, 62]. Furthermore, we note reduced expression of surfactant protein C in air-ventilated engineered lungs, as shown in figure 6.15 (compare lanes ‘ALI’ to lungs that were ventilated with medium (‘Vent’) and perfused (‘Perf’)). This is also consistent with the differentiation of type II to type I epithelium.

![Image](a) Aquaporin

![Image](b) SPC

![Image](c) CCSP

Figure 6.17: Effect of ventilation with air on epithelial development in engineered lung tissue. AQP expression is noted in parenchymal cells (top left) that are also positive for SPC (panel b), as well as occasional strong expression in cuboidal epithelial cells (top right). CCSP expression of cuboidal epithelium is also noted (panel c).

In addition, we noted the growth of a very limited number of ciliated epithelial
cells, as shown in figure 6.18. This is the first evidence we have seen of ciliated epithelial growth in engineered lung tissues, and is likely a result of the introduction of an air interface. When airway epithelium is cultured in vitro, the transition of the cells from liquid to the air interface induces cilia expression [217, 48]. Furthermore, the lack of an air-liquid interface can lead to reduced ciliogenesis [151, 216]. Therefore, while these results are not surprising given the behavior of epithelial cells during in vitro culture, it is an interesting finding and is also evidence that the air interface has important impacts on our efforts to regenerate lung tissue in vitro.

Figure 6.18: Ciliated epithelium in native and engineered lung. Ciliated cells are highlighted in red for engineered lung.

6.3.8 Effect of perfusion and ventilation on the development of epithelial structures in engineered lung tissues

Perfusion and ventilation can have significant impacts on the culture of lung tissue in the bioreactor, as we demonstrated in section 5.3.6 for native lung cultures. Ventilation also has significant impacts on the lung epithelial development, including the differentiation of types I and II pneumocytes [96, 98, 97]. As a result, we compared the effects of perfusion and ventilation on engineered lung development during 8-day cultures in the bioreactor.
For these experiments, the conditions were the same as utilized during the validation experiments of section 6.3.4, with culture medium of DMEM+10%FBS, uncoated scaffolds, and an unsorted neonatal pulmonary cell population. However, cultures were either perfused through the vasculature at 2ml/min or ventilated continuously with medium at 1 breath/min (identical conditions to those utilized in 5.3.6).

![Figure 6.19: Effect of perfusion and ventilation on engineered lung culture.](image)

Both ventilation and perfusion enable the growth of a substantial number of epithelial cells, as noted on routine histology in figure 6.19. However, there are many more developing epithelial structures lined with cuboidal epithelium in perfused
Figure 6.20: Effect of perfusion and ventilation on cell proliferation and apoptosis in engineered lung culture.
cultures. Many of the cells that form these epithelial structures stain positive for CCSP, as shown in figure 6.21. We still note staining for CCSP in ventilated cultures, but the cells attain a more flattened morphology. This may indicate that ventilation is inducing the CCSP-expressing cells to differentiate towards alveolar epithelium (either type I or II pneumocytes). Further detailed study will be required to examine these findings in greater detail.

We note that in the absence of ventilation, the lumens of developing epithelial structures are filled with an eosinophilic, and thus likely proteinaceous, material, visible on H&E staining in figure 6.19. This material also stains positive for CCSP, as seen in figure 6.21. This buildup of CCSP indicates that the Clara cells lining these epithelial structures are producing CCSP. In addition, the removal of this material with ventilation indicates that the airway tree is still intact and can conduct fluid, and furthermore suggests that these developing epithelial structures are a part of the airway tree and do not proliferate randomly within the matrix.

Perfusion versus ventilation did not have significant effects on the expression of SPC, as shown in figure 6.22. The majority of cells are positive for SPC under both ventilation and perfusion conditions. In addition, aquaporin expression was not noted under either condition (data not shown).
Figure 6.21: Effect of perfusion and ventilation on CCSP expression in engineered lung tissue. CCSP is stained red, while nuclei are counterstained blue with DAPI.
Figure 6.22: Effect of perfusion and ventilation on SPC expression in engineered lung tissue. SPC is stained in red, and nuclei are counterstained blue with DAPI.
6.4 Discussion

In this chapter, we first described extensive screening experiments that were used to validate the utility of the decellularized scaffolds, the bioreactor, and the neonatal pulmonary cell population for the growth of engineered lung tissue. We then used these validated components to probe specific aspects of the growth and differentiation of engineered lung epithelium.

6.4.1 System validation for engineered tissue culture

In order to validate the system we have developed for engineered tissue culture, we first demonstrate that the scaffolds are not cytotoxic and can support cell adherence and proliferation. We then describe our choice of a pulmonary cell population that was utilized in most experiments on the growth of engineered tissue. This cell population was obtained from the lungs of neonatal rats, and contained a heterogeneous mix of pulmonary cells. Finally, we performed an extensive series of screening experiments in order to identify conditions that were compatible with engineered lung growth.

Scaffold cytotoxicity and compatibility with cell culture

Using a tumor-derived epithelial cell line, we demonstrated that the scaffolds were not cytotoxic and did not retain components of the harsh decellularization treatments that would impact cell culture. We also here demonstrated that the scaffolds could be sterilized and suitably prepared for tissue culture.
Selection and isolation of a pulmonary cell population for seeding of engineered tissues

We chose to use pulmonary cells freshly isolated from neonatal rat pups (7 days old) as a cell population to use for the growth of engineered lung tissue. This choice was made based on several considerations. First, rodent epithelium is difficult to culture in vitro. While in some cases the culture of rodent lung epithelium on tissue culture plastic has been shown, it is generally considered difficult, frequently leads to cell de-differentiation, and does not allow for extensive expansion of cell numbers. In order to perform meaningful experiments using whole decellularized lung scaffolds, each scaffold must be seeded with millions of cells, and it would be difficult to obtain enough cells from the in vitro culture of rodent lung epithelium.

Second, cell isolations from neonatal lungs yield a wide range of cell types, making it suitable to use as a starting population for the growth of entire segments of lung tissue. Such a heterogeneous cell population may be considered disadvantageous for studies probing the growth of particular cell types or investigating specific interactions between cell types or between cells and the extracellular matrix. However, here we are attempting to engineer whole segments of lung tissue, and we have no a priori justifications for focusing our efforts on any one of the many cell types found in the lung.

Third, we can obtain a large population of neonatal pulmonary cells that are young and relatively plastic. The lungs of neonatal rats are not simply small lungs, but are actively undergoing development. Alveolar development in post-natal rats continues out to at least day 13 [130], with a rapid period of alveolar development between days 3 and 8 and subsequent arterial development between days 8 and 11 [132].

Finally, the cells of neonatal rats are syngeneic with the decellularized adult rat
lung scaffolds. Taken together, these reasons provide compelling justification for the use of neonatal rat pulmonary cells as a cell population for the growth of engineered lung tissue.

However, the use of neonatal pulmonary cells as a cell population does not apply to the eventual, long-term goal of this work, which is to develop functional engineered lung tissue for human patients. Rather, we use neonatal cells in order to demonstrate the feasibility of growing engineered lung tissue in the laboratory as well as to assist in the development of tools, techniques and platforms that will be useful for future efforts at lung tissue engineering. Future studies can harness the capabilities of stem cells, for example, in order to pursue the growth of lung tissue using a model more directly applicable to the eventual goal of developing tissues for use in humans.

**Screening of conditions for engineered lung culture**

There are many variables that can affect the growth of engineered lung tissue, and countless combinations of conditions. Furthermore, how one determines what constitutes the “best” conditions for engineered lung growth is not clear. The diversity of possible conditions and outcomes makes it extremely unwieldy to attempt to fully evaluate all relevant variables. In the results described in section 6.3.3, we attempted to screen the variables which we felt were key to engineered lung growth. We did not intend or attempt to comprehensively evaluate many of these variables. Rather, we used simple histological evaluation to assess cell density, viability and morphology. In some cases, we supplemented this with evaluations of protein expression using immunofluorescence or Western blotting.

As a result this extensive set of experiments, we identified the following conclusions: the medium types BGJb and DMEM+10%FBS enabled substantial cell adherence and proliferation; flow cytometry sorting to isolate specific cell popula-
tions was generally unsuccessful; seeding of cells into the airway compartment was superior to seeding into the vasculature; a single bolus airway seeding was superior to multiple seedings; and scaffold coating with fibronectin and endothelial-specific media was required for endothelial cell attachment and proliferation.

Preliminary demonstration of epithelial, endothelial and mesenchymal cell growth on decellularized scaffolds

Using the set of conditions identified in the screening experiments, we demonstrated that the decellularized scaffolds could support the attachment and proliferation of epithelial, endothelial and mesenchymal cells. We documented the culture of type II epithelium, Clara cells, and presumptive type I epithelial precursors, which stained positive for aquaporin-5, a type I marker, but that did not display the typical flattened morphology of type I pneumocytes. We also demonstrated the growth of two epithelial progenitor cell types: basal cells and bronchoalveolar stem cells.

In addition, mesenchymal cells were shown to be able to proliferate on the scaffolds. Furthermore, and importantly, these cells appeared to grow in physiologically appropriate locations, as they were found beneath and between developing epithelial structures (figure 6.13). The culture of endothelial cells has proven more difficult on the decellularized scaffolds. First, fibronectin coating of the scaffolds was required, as fibronectin was lost during the decellularization regimen and rat lung EC require fibronectin for attachment. In addition, endothelial cells did not significantly adhere or proliferate in media other than EC-specific media (such as MCDB131+10%FBS with additional growth factors). However, when endothelial cells were cultured on fibronectin-coated scaffolds in EC medium, we demonstrated robust attachment and proliferation.

In summary, we demonstrated that the decellularized scaffolds can be sterilized
and prepared for subsequent cell culture; that we can isolate a heterogeneous pop-
ulation of pulmonary cells from neonatal rats; and that the decellularized scaffolds
can support the attachment, survival and proliferation of a wide range of cell types,
including multiple types of epithelium as well as mesenchymal cells and endothe-
lial cells. These experiments provide substantial validation of this system for use in
pulmonary tissue engineering.

6.4.2 Effect of an air-liquid interface on static-cultured and
ventilated engineered lung tissues

We compared the effects of an air-liquid interface on the development of engineered
epithelium in both static culture (using small slices of scaffold) and in ventilated
cultures in the bioreactor.

Air interface in static-cultured engineered lung tissues

When cultured in vitro, rat type II epithelium require an air-liquid interface to prevent
dedifferentiation and loss of morphological characteristics [79, 56, 215, 161]. This
appears consistent with results we observed in static cultures, as shown in figure 6.16,
where the air interface significantly improved epithelial growth and attachment. In
addition, the demonstration that surfactant is expressed by these cells is promising,
as surfactant expression is rapidly lost when cells are grown on tissue culture plastic
[129].

However, engineered tissues that are cultured in the bioreactor are not cultured in
the presence of an air interface (which in the bioreactor is obtained by ventilating the
lungs with air). Yet, the overall results appear similar, in terms of cell morphology,
differentiation state and marker expression (compare static culture in figures 6.16
and 6.14 to liquid-ventilated bioreactor cultures in figures 6.4, 6.8, and 6.7).
Therefore, the air interface does not *per se* appear to be critical for epithelial culture on the decellularized scaffolds. There may be two explanations for this observation. First, in the bioreactor, even in the absence of an air interface, we are frequently providing mechanical stimulation, typically in the form of ventilation; this has been shown to stimulate the growth and differentiation of both types I and II epithelium, as well as facilitate overall fetal lung development [96, 98, 54, 210]. Second, in cultures on static slices of lung scaffold, the air interface may induce increased delivery of oxygen and nutrients from capillary action that may be more important than the possible induction of an air interface.

In static engineered tissue cultures, an air interface was induced by culturing the pieces of scaffold on top of a mesh that was placed at the air-liquid interface. However, it is not clear that this actually created an air interface within the piece of tissue. Rather, the air interface may have simply increased the delivery of media, and thus oxygen and nutrients, through the scaffold. The lung pieces used for static culture were 2-3mm in maximal dimension and were generally 1-2mm thick. However, this may still be too thick to allow sufficient diffusion of oxygen and nutrients into the tissue. Oxygen can become limiting in as little as 100-200 µm depth into a tissue, depending on cell density and oxygen requirements [42]. In cartilaginous constructs, oxygen tension drops off significantly by \(~600\,\mu m\) and further by \(~1000\,\mu m\) [126], while in normal cartilage tissue growth is hindered at \(~1200\,\mu m\) depth [105]. In neural tissue, diffusion of growth factors is significantly reduced at 500 \(\mu m\) depth [111]. Therefore, we may be observing effects of reduced diffusion, and thus insufficient oxygen or nutrient delivery, in the pieces of engineered tissue.

However, the effect of the air-liquid interface may indirectly improve epithelial growth by assisting nutrient delivery through an induction of media movement through the tissue, resulting from capillary action to replenish liquid lost to evap-
oration. This is consistent with data showing that 2mm slices of adult mouse lung can be kept in culture for up to 9 weeks if cultured at an air-liquid interface [173], despite such slices also exceeding the thickness expected to allow diffusion of oxygen and nutrients through the tissue.

**Air interface in ventilated engineered lung tissues**

In engineered tissues that were ventilated with air, we found that the air interface led to induced expression of aquaporin, a marker of type I epithelium. Aquaporin expression was commonly found in the parenchyma, staining cells that also express surfactant protein C (figures 6.17(a) and 6.17(b)). Therefore, although co-localization studies were not performed, it is highly likely that the air interface is inducing differentiation of type II epithelium towards type I epithelium. Type II pneumocytes are known local progenitors for type I cells [4, 62], and so the potential for this differentiation is not surprising. However, *in vitro* cultures of isolated type II cells appear to display behavior opposite that observed in the engineered tissues. During *in vitro* type II cell culture, an air-liquid interface is required to maintain a type II differentiation state, and prevent the cells from attaining certain features of type I cells, including a flattened morphology and reactivity with antibodies raised against type I cells [57, 56, 161, 215]. This contrasts with the maintenance of type II cell phenotype in engineered tissues cultured in liquid and the apparent differentiation of some of these cells towards a type I phenotype when ventilated with air.

This finding is similar in some ways to behavior observed in chapter 5 using native lung culture in the bioreactor, where we noted maintenance of epithelial phenotype (Clara cells and types I and II pneumocytes) during liquid-ventilated culture, as opposed to de-differentiation that is observed with *in vitro* culture of epithelial cells. Together, these results suggest that the extracellular matrix plays critical roles in
maintaining and inducing epithelial differentiation. Future studies can be used to explore the precise interactions between particular extracellular matrix components and various populations of epithelial cells, in order to identify particular factors that drive epithelial differentiation.

**Observation of ciliated epithelium.** Ventilation with air also led to the development of a very limited number of ciliated epithelial cells (figure 6.18). This is the first evidence we have seen of ciliated epithelial growth in engineered lung tissues, and is likely a result of the introduction of an air interface. When airway epithelium is cultured *in vitro*, the transition of the cells from liquid to the air interface induces cilia expression [217, 48]. Furthermore, the lack of an air-liquid interface can lead to reduced ciliogenesis [151, 216]. Therefore, while these results are not surprising given the behavior of epithelial cells during *in vitro* culture, it is an interesting finding and is also evidence that the air interface has important effects on our efforts to regenerate lung tissue *in vitro*.

### 6.4.3 Ventilation enables the differentiation of developing epithelium in engineered lung tissues

We compared the effects of ventilation versus perfusion on engineered lung development. We found that in the absence of ventilation – with perfusion alone or during static culture – the developing epithelial structures were filled with proteinaceous material that stained positive for Clara cell secretory protein (figures 6.14 and 6.21). This demonstrates the effectiveness of ventilation in moving fluid into and out of the airways and suggests that the airway tree is intact and effectively transmits fluid through the lung. Furthermore, this indicates that the epithelial structures are in communication with the airway tree and are not simply randomly proliferating in the scaffold. In addition, this finding also indicates that CCSP is being actively
secreted, an important finding as CCSP is the most abundant secreted protein in normal pulmonary airways. However, we cannot rule out that perfusion stimulates increased CCSP production, rather than ventilation increasing the clearance of CCSP out of the lung. Quantification of CCSP production can be estimated using Western blotting, but was not utilized in these studies. Although the functions of CCSP are not known, it is thought to regulate the local inflammatory response [180] and is decreased in diseases such as asthma, COPD and lung cancer [21, 201]. Therefore, this additional demonstration of CCSP production by engineered tissues is encouraging.

Ventilation was also found to impact the morphology of cells expressing CCSP. With perfusion alone, the developing epithelial structures remained lined by columnar cells that express CCSP (figure 6.21). However, in the presence of ventilatory movements these cells attain a less cuboidal morphology. This may be due to differentiation of Clara cells to type II epithelium, as these cells are also positive for SPC, as shown in figure 6.22. This may be due to induction of cell differentiation due to physical distension induced by ventilatory movements.

Ventilatory movements have been shown to induce type II cells to attain characteristics of type I epithelium. This has been shown in studies of fetal sheep lungs [103, 7], in vitro fetal rat lung explants [80], and in vitro culture of isolated rat type II cells [81, 82]. In these studies, we do not note changes in type II cell phenotype or expression of the type I marker aquaporin-5 due to ventilatory movements. However, these engineered tissue cultures were also perfused, which may have supplied sufficient mechanical stimulation such that we did not detect differences in surfactant expression. Surfactant expression was the key method of analysis for the above-referenced studies examining breathing movements on type II differentiation, and in-depth comparisons were performed on surfactant types A, B and C. We only utilized immunofluorescence and Western blotting for pro-surfactant C. Ventilation
may induce changes in type II cells that we have not been able to detect in these studies, but future experiments can be used to clarify these matters.

6.5 Conclusions

In this chapter, we first described extensive screening experiments that were used to validate the utility of the decellularized scaffolds, the bioreactor, and the neonatal pulmonary cell population for the growth of engineered lung tissue. We then used these validated components to probe specific aspects of the growth and differentiation of engineered lung epithelium.

In order to validate the system we have developed for engineered tissue culture, we first demonstrated that the scaffolds are not cytotoxic and can support cell adherence and proliferation. We then described our choice of a pulmonary cell population that was utilized in most experiments on the growth of engineered tissue. This cell population was obtained from the lungs of neonatal rats, and contained a heterogeneous mix of pulmonary cells. Finally, we performed an extensive series of screening experiments in order to identify conditions that were compatible with engineered lung growth.

Using these validated components, we then probed the effects of an air-liquid interface on the growth and differentiation of engineered lung epithelium in both static cultures and ventilated tissues. We demonstrated that an air interface was necessary for epithelial growth in static cultures, while in ventilated tissues, an air interface induced the differentiation of type I pneumocytes, presumably derived from type II cells that are found in abundance in engineered tissues and that are known local progenitors for type I epithelium [4, 62].

We evaluated the effects of medium ventilation versus perfusion on the development of engineered lung epithelium. We found that ventilation affects epithelial devel-
opment by inducing the differentiation of Clara cells towards alveolar epithelium. We do not find evidence for the differentiation of type I epithelium in medium-ventilated tissues, in contrast to the ability of air ventilation to induce this transition.
Chapter 7

Endothelial Development in Engineered Lung Tissues

In the prior chapters of this work, we have described the ability to produce decellularized lung scaffolds (chapters 3 and 4) and the design and validation of a bioreactor for the in vitro culture of lung tissue (chapter 5). We then turned our focus to the creation of engineered lung tissue, and demonstrated in chapter 6 the ability of a wide range of pulmonary epithelial and mesenchymal cell types to adhere to and proliferate on the decellularized scaffolds. In chapter 6 we also discussed how key bioreactor conditions impact the development of the engineered lung epithelium.

In this chapter, we will first demonstrate the ability of the decellularized scaffolds to support the growth of engineered lung endothelium, and will identify conditions that are compatible with engineered lung endothelial growth. We then evaluate the effects of several specific factors on the development of engineered endothelium, with a focus on the ability of these factors to impact the formation of a functional endothelial barrier between the vascular and airway compartments. This is one of the key functions performed by the endothelium in vivo and will be required in order for an engineered lung tissue to achieve the design objectives we described in section 1.4.

7.1 Introduction

Functional engineered lung tissue must possess a patent, perfused vasculature that facilitates gas exchange with the airspaces. The endothelium plays key roles in these functions. The endothelium forms a confluent lining of the vasculature, and actively
inhibits thrombosis [154]. In addition, the pulmonary capillary endothelial network brings a large volume of blood into extremely close proximity with the alveolar spaces in order to allow gas exchange, which occurs by diffusion across the alveolar epithelium, extracellular basement membrane, and capillary endothelium into the blood [23, 120].

In order to enable gas exchange across the capillary-alveolar membrane, the endothelium must form a functional barrier to prevent fluid and blood movement into the airways. Buildup of fluid in the alveoli significantly increases the distance required for gas to diffuse from the alveoli into the capillaries, and this has significant negative impacts on oxygen transport [131]. The principle source of fluid movement across an endothelial layer occurs at intercellular junctions, thus in a paracellular manner, as opposed to transcellular movement [123]. Thus, endothelial cell junctions must be tightly formed in order for a lung to resist fluid leak and thus prevent pulmonary edema, a source of significant clinical morbidity [150, 127].

The endothelial barrier is affected by many factors, including a variety of noxious agents that can induce leakage and pulmonary edema [150]. However, a variety of factors also decrease endothelial permeability. We are interested in methods that reduce the permeability of the endothelium in engineered lung tissues, in order to reduce fluid leak across the endothelium and thus help enable gas exchange, one of the key goals of an engineered lung tissue, as discussed above and in section 1.4. In this chapter, we will investigate the ability of two compounds, sphingosine-1-phosphate and angiopoietin-1, to reduce the permeability of pulmonary endothelium and thus affect the endothelial barrier in engineered lung tissue.

Sphingosine-1-phosphate (S1P) is a sphingolipid that mediates many cellular processes via G-protein coupled receptors, of which there are at least five known types. Endothelial cells express various combinations of S1P receptors, and S1P can induce
a variety of effects via these various receptors [164]. S1P can affect cell survival, migration, morphogenesis, proliferation, and differentiation [202]. Both in vivo and in vitro studies have demonstrated that S1P affects endothelial permeability and thus the integrity of the endothelial barrier [70, 164, 155, 188, 113].

Angiopoietin-1 (Ang-1) is one of the angiopoietins, a class of angiogenic growth factors [127]. Ang-1 is expressed primarily by mesenchymal cells and induces EC migration and the formation of capillary structures [189]. In addition, Ang-1 reduces vascular permeability and inflammation and increases vascular integrity [127]. Furthermore, the protective effects of Ang-1 on vascular leakage occur even in adult animals, as shown in a mouse model by Thurston et al. [190].

Outline of results. In this chapter, we will first identify conditions that are compatible with the growth of engineered lung endothelial tissue. As part of these studies, we demonstrate the importance of fibronectin for endothelial attachment and proliferation on decellularized lung scaffolds. We will then examine the effect of S1P and Ang-1 on endothelial monolayer permeability, as well as the effect of Ang-1 on cell junction formation in engineered tissue cultures. Finally, we will examine the effect of bioreactor conditions on the growth of engineered lung endothelium, with a focus on the development of a functional endothelial barrier using a permeability assay and transmission EM.

7.2 Materials and Methods

Scaffold preparation

Decellularized scaffolds were prepared as described in section 6.2.1.
Neonatal cell isolation and seeding

Neonatal rat pulmonary cells were isolated as described in section 6.2.2. Cells were seeded into the scaffolds as described in section 6.2.3.

7.2.1 Endothelial cell culture

Rat lung microvascular endothelial cells were obtained from VEC Technologies (Rensselaer, NY) and grown on fibronectin-coated (~ 1 μg/cm², Gibco) tissue culture vessels in MCDB-131 complete media including 10% FBS and supplemental growth factors (VEC Technologies). Angiopoietin-1 was obtained from R&D Systems (Minneapolis, MN) and sphingosine-1-phosphate was obtained from Cayman Chemical (Ann Arbor, MI).

7.2.2 Optimized conditions for engineered endothelial culture

Scaffolds were coated with 1mg of fibronectin (Gibco) perfused through the vasculature in 60ml of PBS at 37°C, then rinsed with PBS and media. Each scaffold was seeded twice at days 0 and 2 or 3 of culture with 8-10 million rat lung microvascular EC at each time point (two T150 culture flask was used per lung for each of two seedings). Cells were trypsinized from tissue culture plates using 0.25% trypsin (Gibco), filtered through a 40 μm filter to remove cell clumps, and injected into the pulmonary artery as a single bolus injection in ~3ml of media. After allowing cell adherence for 1 hour, perfusion was begun through the vasculature at ~1.5ml/min. After 1-2 hours, the perfusion rate was increased to 3ml/min for the remainder of the culture period of 7-10 days. Medium was changed every 3-4 days.
7.2.3 *In vitro* testing of endothelial attachment and barrier formation using ECIS

Cell culture chambers with electrode arrays were purchased from Applied BioPhysics (Troy, NY), and coated with either fibronectin (∼1 µg/ml, Gibco), gelatin (1%, Sigma), or collagen (10 µg/ml, BD Biosciences). Electrical cell-substrate impedance readings were made on an ECIS Model 1600R (Electric cell-substrate impedance sensing) unit from Applied BioPhysics (Troy, NY). Cell attachment was evaluated based on changes in impedance in the first 24 hours of culture, while monolayer permeability assessments were made over 24 hours after cultures reached confluence. Substrate coatings, media, and growth factors were varied as dictated by experimental conditions.

**Immunofluorescence**

Tissue samples were prepared and stained as described in section 3.2.6.

**Transmission electron microscopy (TEM)**

Samples were prepared and analyzed as described in section 3.2.8.

7.2.4 Microparticle retention

We developed a simple yet useful assay to evaluate the permeability of whole rat lungs to smaller particles, which have sizes on the order of large macromolecules. In this assay, we quantify the leakage of a FITC-labelled dextran solution across the airway-vascular barrier. FITC-labelled dextran with a molecular weight of 2,000,000Da was obtained from Sigma (St Louis, MO). Assay validation was performed by measuring the permeability of native lung and native lung that was treated with 0.025% trypsin for 2 min. Lungs were perfused with heparinized PBS and connected to the usual
bioreactor cannulae. A baseline lavage sample was obtained, and then the trypsin-treated lung was perfused with 10ml of 0.025% trypsin in PBS and allowed to dwell for 2 min at RT, then rinsed with 10ml of PBS. The FITC-labelled dextran solution (1 mg/ml) was injected into the pulmonary artery, and then flushed with 20ml of PBS. Two lavage samples were then immediately taken in succession from the trachea. Fluorescence was measured using a fluorescent plate reader and data were fit to a standard curve.

When performed on decellularized or engineered lungs, the assay was performed through the airway, as with the microsphere assay (see section 3.2.9). Thus, the FITC-dextrans were injected into the airway, and the vasculature was flushed with PBS.

7.3 Results

In this chapter, we first describe the importance of fibronectin for the attachment and proliferation of rat microvascular endothelial cells on decellularized lung scaffolds. We compare the effects of perfusion and ventilation on the development of engineered lung endothelium, and demonstrate that perfusion enables improves cell growth and proliferation versus ventilation. We then evaluate the efficacy of two compounds, sphingosine-1-phosphate and angiopoietin-1, in reducing the permeability of lung microvascular endothelium in static tissue culture, and then examine the effect of angiopoietin-1 on endothelial junction formation in engineered tissue culture. We then evaluate the barrier function of engineered endothelium using transmission EM and a permeability assay.
7.3.1 Preliminary identification of bioreactor conditions for engineered lung endothelial culture

As discussed in section 6.3.2, we are able to isolate a population of neonatal rat pulmonary cells. This cell population is of high viability (75-85%) and contains a significant number of endothelial cells (10-30%; see section 6.3.2 and figure 6.2). In our initial efforts at the growth of engineered lung tissues, we intended to be able to culture both endothelial and epithelial populations using this starting cell source.

We therefore performed a series of screening experiments intended to identify conditions suitable for the culture of engineered endothelium. These experiments were performed in parallel with those described in section 6.3.3, where our focus was on the identification of conditions for engineered epithelial growth. We also performed several studies specifically focused on the identification of conditions for the growth of engineered endothelium, as outlined below and summarized in table 7.1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>EGM-2, MCDB-131</td>
</tr>
<tr>
<td>Serum %</td>
<td>2, 10, 15 %</td>
</tr>
<tr>
<td>Cell seeding method</td>
<td>Bolus, repeat injection</td>
</tr>
<tr>
<td>Cell source</td>
<td>Neonatal pulmonary cells; rat lung microvascular EC</td>
</tr>
<tr>
<td>Cell sorting (for neonatal cells)</td>
<td>PECAM+; unsorted</td>
</tr>
<tr>
<td>Scaffold coating</td>
<td>Fibronectin, gelatin</td>
</tr>
</tbody>
</table>

Table 7.1: Bioreactor and culture conditions that were evaluated during pilot lung engineering studies to identify conditions suitable for engineered endothelial growth.

Screening experiments performed in parallel with epithelial screening experiments. In section 6.3.3 we document a wide range of screening experiments that were primarily intended to identify conditions suitable for engineered epithelial growth. In addition to evaluating these conditions for epithelial cell attachment and survival, we used immunofluorescence to identify endothelial cell growth. We will not
review all of the conditions that were evaluated with these screening studies; please refer to section 6.3.3 for details. However, none of the conditions evaluated in section 6.3.3 resulted in the growth of endothelial cells in the decellularized lung scaffolds.

**Use of selected cell populations via flow cytometry sorting.** The majority of the screening experiments were performed using the heterogeneous neonatal pulmonary cell source described in section 6.3.2. However, this cell population contains both epithelial and endothelial cells, and we suspected the seeding of both principal cell types together could negatively impact endothelial adherence or proliferation. In order to separate epithelial and endothelial cell types, we used fluorescence-activated cell sorting (FACS) to select endothelial cells based on PECAM expression. However, this selected endothelial population also failed to demonstrate growth on the scaffolds. We presume this was due to significantly increased time and handling required for cell sorting experiments and consequent decreased cell viability.

**Scaffold coating.** We evaluated the utility of adding exogenous substrate materials to coat the decellularized scaffolds before cell seeding. This was motivated by the findings described in section 3.3.5, where fibronectin was found to be absent from the decellularized scaffolds. Therefore, we evaluated the utility of fibronectin and gelatin coating of the decellularized scaffolds before seeding with either the heterogeneous neonatal pulmonary cell population or PECAM+ cells selected via FACS. However, neither gelatin nor fibronectin had positive impacts on outcomes.

**Cell source.** Due to the failure to demonstrate endothelial growth on the scaffolds using the isolated neonatal pulmonary cells under any of the above screened conditions, we used a commercially purchased source of rat lung microvascular EC. When these cells were seeded into the scaffolds that were fibronectin-coated and cultured
in the presence of EC-specific medium, we found substantial growth of endothelial cells, as shown in figure 7.1.

**Figure 7.1**: H&E stain of a fibronectin-coated decellularized scaffold seeded with rat lung microvascular endothelial cells

**Summary of endothelial screening experiments.** These screening experiments, as described above, allowed us to identify a set of conditions that was compatible with engineered endothelial culture. Outcomes were assessed primarily via histology for cell viability and expression of PECAM on immunofluorescence. These experiments were not intended to be comprehensive or in-depth. The result of these pilot studies was a set of conditions that enables endothelial cell growth inside the scaffolds, such that we could then systematically evaluate the impact of discrete conditions on engineered lung endothelium.

To summarize, the conditions that were identified as suitable for the culture of engineered endothelium were: the use of a purified, *in vitro* expanded population of rat lung microvascular EC; fibronectin-coated scaffolds; and the use of EC-specific medium (MCDB-131 with 10%FBS and supplemental growth factors).
7.3.2 Rat lung microvascular endothelial cells require fibronectin for attachment and proliferation on decellularized lung matrix

During initial experiments described in section 6.3.3 and section 7.3.1, we identified a set of conditions that was suitable for the initial growth of engineered lung endothelium. We found that the decellularized scaffolds had to be coated with additional fibronectin in order to enable endothelial attachment and proliferation.

As described in section 3.3.5, we found that the decellularized scaffolds did not retain fibronectin after the decellularization process. Given the known importance of fibronectin for the attachment of a variety of endothelial cell types [18], as well as the results of the screening experiments described in section 7.3.1, we performed in vitro studies to evaluate the ability of rat lung microvascular endothelial cells to attach to tissue culture plastic that was coated with a variety of substrates, including fibronectin.

Utilizing electric cell-impedance substrate sensing (ECIS), we quantified cell attachment to surfaces that were coated with fibronectin, laminin, and collagen. ECIS is an established technique that is primarily used to evaluate the permeability of cell monolayers, but can also be used to study cell attachment and spreading [203]. The cell attachment data is shown in figure 7.2 and demonstrates that rat microvascular EC preferentially adhere to fibronectin-coated surfaces, but do not adhere to uncoated or collagen- or laminin-coated substrates. Therefore, the lack of fibronectin on the decellularized scaffolds is consistent with the failure to see endothelial growth in that environment in the absence of fibronectin replacement.
7.3.3 Validation of FITC-dextran permeability assay

We developed a simple yet useful assay to evaluate the permeability of whole rat lungs to small particles, which have sizes on the order of large macromolecules. In this assay, we quantify the leakage of a FITC-labelled dextran solution across the airway-vascular barrier. This assay can be used repeatedly over the course of a culture, involves materials that are cell culture-friendly, and provides a measure of the permeability of the entire lung. In addition, if the assay is performed immediately before fixation, the FITC-dextran could be identified on histologic sections using anti-FITC antibodies.

The FITC-labelled dextran has a molecular weight of 2,000,000 Da. For a monodisperse dextran, the Stokes-Einstein radius (nm) is related to molecular weight by

\[ r_s = 0.0488(MW)^{0.437} \]  

[197, 148]. For a 2MDa dextran, this yields a radius of 27.7nm.

We validated this assay by evaluating the permeability of native lung and na-
tive lung that was made ‘leaky’ by brief perfusion of the vasculature with dilute trypsin. The FITC-labelled dextran solution was injected into the pulmonary artery, and then flushed with saline. Two lavage samples were then immediately taken in succession from the trachea. As shown in figure 7.3, the permeability of lung is increased by trypsin treatment, as expected due to disruption of endothelial attachment to the basement membrane. However, even native lung provides a measurable leak via this assay. This degradation of vascular permeability is the result of delays between animal sacrifice and the injection of the dextran, as well as the handling of the lung tissue. However, a decellularized lung is currently too leaky to be evaluated by this method; the lung is so leaky that one cannot get any return after a lavage.

![FITC-Dextran Permeability Assay](image)

**Figure 7.3:** Permeability of native and trypsin-treated lungs to 2 megadalton FITC-labelled dextrans. Native lung exhibits a small amount of leakage, which is increased upon treatment of the vasculature with trypsin. Lavage 1 and 2 are sequential lavage samples of the airway after perfusion of the vasculature with FITC-dextrans.

When performed on decellularized or engineered lungs, the assay was performed through the airway, as with the microsphere assay (see section 3.2.9). Thus, the FITC-dextrans were injected into the airway, and the vasculature was flushed with saline. Dextrans that translocated into the vascular compartment were measured as leak. The assays were performed in this fashion because in decellularized or en-
engineered lungs, the tissue is highly permeable to fluid and a return sample cannot be obtained after an airway lavage. As such, the dextran was injected into the airway as a single bolus lavage, and the vasculature was flushed to measure leak of FITC-dextrans across the airway-vascular barrier.

Our objective for this assay was to develop a reliable method to evaluate the permeability of the whole lung without destroying the tissue, thus allowing repeated measurements on a single sample. Many methods of evaluating permeability require fixation of the tissue and histologic examination, thereby preventing repeated measurements on a single lung culture. The assay we have described can be used repeatedly over the course of a culture, involves materials that are cell culture-friendly, and provides a measure of the permeability of the entire lung.

### 7.3.4 Effects of perfusion versus ventilation on engineered lung endothelium

In the above sections, we identified conditions for engineered lung endothelial culture (section 7.3.1), confirmed the importance of fibronectin for lung microvascular endothelial attachment (section 7.3.2) and described the design and validation of a permeability assay for whole lung tissues to 55nm dextran particles (section 7.3.3). With this work completed, we are now able to begin evaluating the effects of specific conditions on the development of engineered endothelial tissues, with a focus on the formation of a functional endothelial barrier.

We compared the effects of culturing engineered lung endothelium with perfusion versus ventilation. While perfusion would be suspected to provide a more physiological environment for endothelial cell growth, we showed in chapter 6 that ventilation improves epithelial cell growth and differentiation in engineered lung tissues. Therefore we evaluated both ventilation and perfusion with regards to endothelial cell
survival and proliferation and the formation of cell-cell junctions using transmission EM.

We found that perfusion substantially improved the growth of engineered lung endothelium, as shown on histology in figure 7.4. In addition, more apoptotic cells were noted with ventilation, as shown in figure 7.5 and consistent with their poor appearance on H&E histology.

![Image](a) Engineered endothelium, with vascular perfusion at 3ml/min

![Image](b) Engineered endothelium, with ventilation at 1 breath/min

**Figure 7.4:** H&E staining of perfused versus ventilated engineered lung endothelium.

In addition, we analyzed perfused and ventilated cultures for the presence of cell junctions using transmission EM and VE-cadherin staining. Tight junctions between endothelial cells are an important means of barrier function, as they tightly

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(a) Engineered endothelium, with vascular perfusion at 3ml/min

(b) Engineered endothelium, with ventilation at 1 breath/min

**Figure 7.5:** TUNEL staining of perfused versus ventilated engineered lung endothelium. EC cultured with ventilation are substantially more apoptotic than perfused lung. Apoptotic nuclei stain brown via TUNEL while negative nuclei are counterstained with methyl green.
link adjoining cells together and thus inhibit the movement of fluid between these paracellular spaces [123]. If these cell junctions are weak or absent, fluid leak can occur out of the vasculature and cause pulmonary edema [150, 127]. This can severely impair gas exchange, one of the key objectives in the development of engineered lung tissue (see section 1.4).

Using TEM, we found cell-cell junctions in the perfused engineered tissues, as shown in figure 7.6. Not all cells demonstrated tight junction formation. We did not attempt to quantify the number or percentage of tight junctions as TEM is limited by a very small field of analysis. Given possible variations of cell growth and proliferation over the lung, we did not have sufficient samples over the entire lung tissue to be able to obtain meaningful, statistically significant numbers.

![Figure 7.6](image)

**Figure 7.6:** Demonstration of tight junction formation between endothelial cells in engineered lung tissue. Endothelial cells are marked with asterisks, separated by an extended cell-cell junction. Scale bar is 500nm.

In ventilated tissues, no cell-cell junctions were found, consistent with the poor appearance of these cultures on histological examination. Indeed, we found few cells
with normal morphology on TEM in ventilated cultures.

We also assessed cell junction formation using immunofluorescence for VE-cadherin. We found robust staining for VE-cadherin in perfused engineered lung endothelium, as shown in figure 7.7. Ventilated tissues were not stained for VE-cadherin due to their poor appearance on histology and TEM.

![Figure 7.7: Expression of VE-cadherin in native and engineered lung. VE-cadherin is stained red, with nuclei counterstained blue with DAPI.](image)

While evaluating the engineered endothelium for the presence of tight junctions using transmission EM, we noted that the endothelial cells in perfused cultures were attached to the decellularized scaffold, but were found in the alveoli. Images are shown in figure 7.8, where it can clearly be seen that the EC are located outside of the alveolar septae and in the alveolar spaces. This was a very surprising finding; the EC are seeded into the vascular compartment, and we have shown that cell-sized (5 µm) particles do not significantly translocate the airway-vascular barrier (figure 3.9).

Examination of ventilated lung tissues also demonstrated that EC were located in the alveolar spaces, as shown in figure 7.9. This indicates that the cells are not migrating across the alveolar basement membrane due to forces generated by the vascular...
Figure 7.8: Endothelial cell deposition in perfused engineered tissues. With a perfused culture, endothelium is noted on the alveolar basement membrane, and not within capillary structures in the alveolar septae. Alveoli are indicated with ‘A’, and alveolar septae with ‘S’. Scale bars are 2 µm.
perfusion pressure. The vascular perfusion pressure during these cultures was low, less than 10mmHg, at flow rates of 1.5-3ml/min. Rather, these findings suggest that the EC are either forced across the basement membrane during the initial bolus seeding process, when the cells are injected into the vasculature and when vascular pressure is not monitored; or, the EC may preferentially migrate across the alveolar basement membrane due to some unidentified factor, such as a preferred substrate material that is more conducive to EC growth and proliferation.

We recall that the majority of capillary structures are lost in the decellularized scaffolds (section 3.3.7). Therefore, there is no normal microvascular endothelial substrate in these tissues. The primary available substrate in the microvasculature is the inside of the alveolar septae, which is then coated with fibronectin. It is possible that this surface is not conducive to endothelial attachment, leading to migration of the EC into the airways and thus attachment to the alveolar basement membrane.

### 7.3.5 Effects of sphingosine-1-phosphate and angiopoietin-1 on endothelial monolayer permeability

One of the key functions of the pulmonary endothelium is the prevention of fluid movement out of the vasculature into the interstitium or alveolar spaces. In order to prevent this movement of fluid, endothelial cells must form functional cell-cell junctions in order to reduce the permeability of the endothelial layer. We are therefore interested in identifying conditions that reduce endothelial permeability, and here utilize *in vitro* measurements to quantify the effects of sphingosine-1-phosphate and angiopoietin-1 on endothelial monolayer permeability.

**Sphingosine-1-phosphate reduces endothelial permeability in the absence of serum.** S1P is a sphingolipid that has been shown to promote endothelial barrier integrity in both *in vitro* and *in vivo* experiments [70, 113, 164, 155, 188]. We studied
Figure 7.9: Endothelial cell deposition in ventilated engineered tissues. With a ventilated culture, endothelial cells appear severely apoptotic and are also found within alveoli, not within capillary structures. Alveoli are indicated with ‘A’, capillaries with ‘C’, alveolar septae with ‘S’, and endothelial cells with asteriks. Scale bars are 5 µm.
the effect of added S1P on endothelial monolayer resistance using ECIS, and found that S1P had demonstrable effects on monolayer resistance, but that these effects were abrogated in the presence of serum (figure 7.10). These findings are consistent with others in the literature, in that the effects of S1P are frequently evaluated in serum-free medium [70, 188]. Serum is an important component of endothelial culture medium, and the removal of serum for extended culture is not generally compatible with in vitro endothelial proliferation [76]. Although there has been some recent work to develop serum-free culture media, serum is still an important part of the typical endothelial culture conditions, and as such we did not evaluate the effects of S1P in engineered tissue cultures.

Figure 7.10: Effect of sphingosine-1-phosphate on endothelial permeability. Monolayer resistance was measured using ECIS, demonstrating an increase in resistance with S1P treatment only in the absence of serum.

Angiopoietin-1 reduces endothelial permeability Angiopoietin-1 (Ang-1) is an angiogenic growth factors that reduces vascular permeability and inflammation
and increases vascular integrity [127, 189, 190]. We studied the effect of angiopoietin-1 on endothelial resistance using ECIS and found that Ang-1 effectively increases monolayer resistance, as shown in figure 7.11. In addition, these effects persist even in the presence of serum. Due to the ability of Ang-1 to reduce EC permeability in the presence of serum, we utilized Ang-1 for subsequent experiments on engineered lung endothelium.

Figure 7.11: Effect of angiopoietin-1 on endothelial permeability in the presence and absence of serum. Monolayer resistance was measured using ECIS, demonstrating an increase in resistance with Ang-1 treatment that persisted in the presence of serum.

7.3.6 Effect of angiopoietin-1 on cell junction formation in engineered tissue

Based on the ECIS findings of section 7.3.5, we evaluated the effect of Ang-1 on cell junction formation in engineered lung cultures. High-dose Ang-1 was administered (500ng/ml) as this induced the maximal resistance change on ECIS studies. In order to assess resistance in engineered tissues, we used expression of VE-cadherin, a
cell junction molecule that is a key component of adherens junctions that join together neighboring endothelial cells. As such, VE-cadherin is a key contributor to the formation of a functional endothelial barrier [196].

For these studies, engineered endothelial tissue was first cultured for 7-10 days as described in section 7.2.2, with vascular perfusion of medium at 3ml/min. The engineered tissue was then cut into thin slices and cultured in medium either with or without Ang-1. After 24 hours of additional culture, VE-cadherin expression was assessed via Western blotting. As shown in figure 7.12, we did not detect significant changes in VE-cadherin expression for engineered tissues that were treated with Ang-1.

Figure 7.12: Effect of angiopoietin-1 on VE-cadherin expression in engineered tissues. ‘Nat’ is native lung, ‘EC’ is cultured lung microvascular EC, ‘Eng’ is engineered lung endothelium, and ‘+Ang’ is engineered lung endothelium in the presence of angiopoietin-1.

7.3.7 Assessment of barrier function of engineered lung endothelium

We are interested in the ability of engineered lung endothelium to form a functional barrier between the vascular bed and the airspaces. This is important in order to reduce fluid leak into the alveoli and thus enable gas exchange, and it is a key component of the objectives we described for engineered lung tissue (section 1.4). In order
to evaluate the barrier function provided by the lung endothelium, we used a permeability assay to measure the translocation of small (55nm) FITC-dextran particles from the airspaces into the vasculature. This assay was developed and validated as described in section 7.3.3.

For this assay, we inject FITC-dextran into the airway compartment, and measure the amount that leaks across the alveolar-vascular barrier by flushing the vasculature with saline. For decellularized scaffolds, there is essentially no barrier function to such small particles, with virtually all (98.4%) of the dextran translocating the alveoli into the vasculature and recovered with vascular rinsing. This compares to native lung, which when treated similarly shows a leak of 12.9% (figure 7.13).

In engineered lung endothelial tissues that were perfused, we demonstrate the retention of up to 30% of dextrans in the airway compartment, after culture periods of 7-10 days. Ventilated endothelial tissues demonstrated a permeability of 87%. Even this degree of barrier function is surprising given the poor appearance of the ventilated tissues on histology and transmission EM.

Due to the failure to observe effects of Ang-1 on endothelial junction formation in cultured lung tissue, we did not evaluate the ability of Ang-1 to affect permeability using this assay. This does not indicate that Ang-1 does not affect the barrier function of engineered endothelium; rather, the method we utilized to assess barrier function (via Western blotting for VE-cadherin) likely does not allow us to discern the effects of Ang-1 on engineered endothelium. Future studies can use this permeability assay to quantify barrier function and determine the effects of growth factors, media composition, or bioreactor conditions on barrier function of cultured engineered lung tissue.

These findings for perfused engineered lung endothelium, especially when coupled with the findings of robust VE-cadherin expression via immunofluorescence (figure
Figure 7.13: Permeability of engineered lungs seeded with endothelial cells alone to 2 megadalton FITC-labelled dextran. * indicates p<0.05 compared to decellularized scaffolds.

7.7) and cell-cell junction formation via TEM, indicate that we are observing the beginnings of the formation of a functional endothelial barrier in engineered endothelial tissues.
7.4 Discussion

In order to enable gas exchange, the vasculature of an engineered lung, and in particular the endothelial lining, must present a sufficient barrier to fluid and protein movement in order to prevent pulmonary edema, or the filling of airspaces with fluid. In this chapter, we compared the effects of perfusion and ventilation on the development of engineered lung endothelium with a focus on the development of an endothelial lining of the vasculature. We evaluated the effects of perfusion and ventilation on endothelial cell survival, endothelial cell junction formation via transmission EM and VE-cadherin immunostaining. We then described the effects of two exogenous agents (angiopoietin-1 and sphingosine-1-phosphate) on the permeability of rat lung microvascular EC using *in vitro* resistance measurements and assessed the ability of angiopoietin-1 to impact VE-cadherin expression in engineered lung endothelial cultures. Finally, we assessed the barrier function of engineered lung endothelium using a permeability assay to measure the translocation of small 55nm particles across the alveolar basement membrane.

7.4.1 Pulmonary endothelium requires fibronectin for attachment to decellularized scaffolds

Fibronectin is a dimeric cell-adhesive glycoprotein that aids cell attachment to the extracellular matrix via a repeated peptide sequence, the Arg-Gly-Asp (RGD) sequence [163]. Fibronectin is important for the adherence of a variety of cell types to the extracellular matrix or to tissue culture plastic [193, 18]. In many cases, sufficient fibronectin is provided by the serum contained in tissue culture media, as fibronectin is found in the serum of all vertebrates [194, 84]. However, we found that rat lung microvascular EC require a fibronectin-coated substrate in order to attach to both tissue culture plastic and to decellularized lung scaffolds (figure 7.2); the level of fibronectin
contained in the serum was insufficient to enable significant cell attachment.

As described in section 3.3.5, the decellularized scaffolds were found to lose fibronectin during decellularization. As a result, during all attempts at reseeding the scaffolds with endothelial cells, we first coat the scaffolds with fibronectin by perfusion through the vasculature. This was found to significantly increase endothelial attachment to and proliferation on the decellularized matrix. The loss of fibronectin is likely related to the removal of capillary structures during decellularization (section 3.3.7). Fibronectin is a component of the endothelial basement membrane [175] and is thus likely lost during decellularization along with the loss of the majority of capillary structures.

7.4.2 Deposition of endothelial cells in engineered lung tissue

As shown in figure 7.8, endothelial cells in engineered tissues were found in the alveolar spaces. This was surprising as the endothelial cells were seeded into the vasculature, and we have shown that very few cell-sized particles (5 µm) can translocate the alveolar-vascular barrier (figure 3.9). We can identify three potential causes for the movement of endothelial cells across this barrier. First, shear stress due to perfusion during culture may induce endothelial movement across the barrier. Second, increased vascular pressure during the initial cell seeding process may drive cells across the membrane, where they remain during culture. During the seeding step, vascular pressure are not monitored and may be higher than 10mmHg, the typical perfusion pressure during tissue culture. Third, the alveolar basement membrane may be a more hospitable substrate for cell adherence and proliferation than the microvascular environment of a decellularized scaffold.

Shear stress experienced by EC during culture of perfused engineered endothelium. We can estimate the forces experienced by endothelial cells when
cultured with perfusion in engineered tissues. With vascular perfusion, one may expect a shear stress would be applied to cells in the vasculature, which may account for the improved growth of endothelial tissues with perfusion compared to ventilation. These shear forces are extremely small, however. To estimate these forces, we first assume that there are 1 million alveoli in the adult rat lung, and that each capillary network (fed by a single terminal arteriole) spans 4 alveoli in a 2x2 arrangement. Thus there are 250,000 capillary networks in the rat lung. Assuming a dense capillary network with capillary diameters of 5 µm, intercapillary spacing of 5 µm, and an alveolar diameter of 200 µm, each capillary net would have approximately 40 capillary segments in any given cross-section. In engineered tissue culture, vascular perfusion is 3ml/min. For 250,000 capillary networks, each containing 40 capillary segments in parallel, the flow rate through each capillary segment is approximately 8 nl/min.

Using the Hagen-Poiseuille equation, \( \tau = \frac{4\mu Q}{\pi R^4} \), where \( \mu \) is the fluid viscosity (0.7cP for tissue culture media), \( Q \) is the flow rate and \( R \) is the capillary radius, we obtain a shear stress of \( \tau = 0.03 \text{ dyn/cm}^2 \). For a physiological blood flow rate of 40-80ml/min and using the viscosity of blood, the same calculation yields a shear stress of 1.1-2.3 dyn/cm\(^2\), consistent with the expected physiological values.

Engineered endothelial tissue would therefore experience a maximal shear stress of 0.03 dyn/cm\(^2\). However, the actual shear stress would be far less than this value due to the highly leaky nature of the decellularized scaffold, which will reduce the effective capillary blood flow as fluid leaks out of the vasculature into the airways and interstitium. During perfusion, there is a net movement of fluid across the vasculature into the airspaces, as suggested by permeability measurements showing that 98.4% of 55nm particles translocate the airway-vascular barrier (figure 7.13). This net fluid movement will reduce the flow of medium to downstream vessels, further reducing the shear stress experienced by the endothelium.
Additionally, if engineered tissues are ventilated during culture and not perfused, thus removing shear stress as a potential driving force, cells are still found in the alveoli (figure 7.9). We therefore suspect that either the cells are driven across the alveolar basement membrane during the seeding process, when they are injected into the vasculature, or the alveolar basement membrane is a more hospitable substrate for cell adherence and proliferation.

The alveolar basement membrane as an endothelial substrate. The alveolar basement membrane is principally composed of collagen type IV [59]. Although fibronectin is a common endothelial cell growth substrate, some evidence indicates endothelium can also proliferate on collagen IV substrates. Brown et al. showed growth of human microvascular EC on decellularized scaffolds which were shown to contain collagen IV; the scaffolds studied included urinary bladder matrix, urinary bladder submucosa, small intestinal submucosa, and liver stroma [31]. However, other components are also present in these substrates, including laminin and collagen type VI, and so we cannot conclude that collagen IV was an attractive substrate by itself for endothelium. Studies of EC attachment to artificial vascular grafts demonstrate minimal attachment of EC to collagen IV-coated grafts [104]. Therefore, it seems unlikely that native alveolar basement membrane is a preferential substrate for EC attachment as compared to the vasculature.

However, in these studies, the scaffolds were coated with fibronectin. While the fibronectin was perfused through the vasculature, it will also translocate into the alveoli and thus coat the alveolar basement membrane (fibronectin has a molecular weight of $\sim 440$ kDa, and so is smaller than the $2$ MDa dextrans which we know easily leak across this membrane; figure 7.13. Thus, it is possible that the fibronectin-coated alveolar basement membrane is a more attractive substrate for cell attachment and proliferation than the fibronectin-coated vasculature, especially if a significant
number of capillaries are lost during decellularization, which would therefore remove
the normal endothelial basement membrane.

**High vascular pressures during initial cell seeding.** The vascular pressure
during the initial seeding of endothelial cells is not controlled. These pressures were
not measured, and it is therefore not known whether the pressure may have exceeded
the typical maximal pulmonary vascular pressure of 30mmHg [114] (see section 2.5).
Future experiments can be used to determine if control of vascular pressure during
this step impacts the deposition of endothelial cells in engineered tissues.

**Summary of endothelial deposition in engineered tissues.** Future studies
will continue to evaluate this interesting finding, in order to identify conditions that
enable endothelial growth in the vasculature and prevent endothelial movement into
the alveoli. Specifically, we first will minimize the applied pressure during cell seeding,
in order to ensure that cells are not forced across the alveolar basement membrane at
this step. In addition, improved decellularization regimens, such as continued reduc-
tions in perfusion pressure, that maximize capillary and native fibronectin retention
will likely facilitate endothelial growth in the vasculature.

### 7.4.3 Effect of ventilation on cell survival in engineered end-

**dothelial tissues**

As described in section 7.3.4, when engineered endothelial tissues were ventilated as
opposed to perfused, cell morphology, survival and barrier formation were diminished
(figures 7.4, 7.5 and 7.9). The severe impact of ventilation on endothelial survival is
surprising. Recall the native lung cultures that were used in the validation studies
of the lung bioreactor (see chapter 5, section 5.3.6). Here, we demonstrated that
overall cell survival of ventilated lung approaches that of native lung (figure 5.7),
while endothelial differentiation state can be maintained in culture for 7 days with only ventilation (figure 5.10). Therefore, it is surprising that ventilation has such negative effects on endothelial survival and adherence in engineered tissue cultures.

The movement of fluid in ventilated lung tissues that are largely devoid of cells may play a role in the effects of ventilation on engineered endothelial culture. In native lung, ventilation induces the movement of fluid (or air) into the airway compartment, where it enters the alveoli and remains until exhalation. Decellularized lungs, however, are highly permeable to fluid and macromolecules (98.4% of 55nm dextran particles translocate the alveolar basement membrane; figure 7.13). Therefore, with each inhalation, approximately 2.5-3.0ml of fluid enters the lung and is transmitted into the alveolar network. The vast majority of this fluid then filters across the alveolar basement membrane, into the vasculature and the interstitial space. This movement of fluid is most likely turbulent in nature, due to the rapid divisions of the airway tree as well as the presence of the alveolar basement membrane as a barrier.

Ventilated engineered endothelium shows somewhat increased staining for apoptosis (figure 7.5). Laminar shear stress is known to inhibit endothelial apoptosis [50], while turbulent flow patterns can lead to increased endothelial turnover and apoptosis [49, 192]. This may account for the poor growth of endothelial tissues with ventilation.

7.4.4 Effect of sphingosine-1-phosphate on endothelial permeability

Sphingosine-1-phosphate reduces the permeability of an endothelial monolayer in culture. We demonstrated this for rat lung microvascular EC, while other investigators have performed similar studies using human and bovine pulmonary artery and mi-
crovascular endothelial cells [70]. However, we also noted that the effects of S1P on monolayer resistance are lost in the presence of serum. This is consistent with other studies, where serum is removed or kept at very low levels (less than 0.5%) during the application of S1P and measurement of resistance [70, 188]. Due to the importance of serum for endothelial cell culture, we therefore did not utilize S1P for further experiments on engineered lung tissue. However, S1P may be useful during future co-culture experiments using both endothelial and epithelial cells in engineered tissues, as epithelium can be cultured in low-serum or serum-free medium [217, 161] and we have seen evidence of increased epithelial differentiation in the absence of serum in engineered tissues (figure 6.14).

7.4.5 Effect of angiopoietin-1 on endothelial permeability and cell junction formation

We found that angiopoietin-1 can reduce the permeability of lung microvascular endothelium in monolayer culture, as shown in figure 7.11. This is consistent with the known properties of Ang-1, and at similar doses, Ang-1 has been shown to increase monolayer resistance in human kidney glomerular endothelium [167]. Furthermore, we note that the effects of Ang-1 persist even in the presence of serum. As a result, Ang-1 was identified as a potential agent to help increase the formation of a functional endothelial barrier in engineered lung tissue.

In order to assess barrier formation in engineered tissues, where resistance measurements are not possible, we used Western blotting to analyze VE-cadherin expression. VE-cadherin is a key component of adherens junctions that connect adjacent endothelial cells, thereby reducing paracellular fluid movement and contributing to the formation of a functional endothelial barrier [196]. We measured VE-cadherin expression in statically cultured engineered tissues in the presence and absence of
Ang-1 as a means of evaluating the barrier formation of the engineered endothelium. As shown in figure 7.12, we did not detect a change in VE-cadherin expression due to Ang-1. In addition, even in the absence of Ang-1 we note strong staining for VE-cadherin via immunofluorescence (figure 7.7). Robust VE-cadherin expression, even without added Ang-1, is indicative of promising endothelial barrier formation in the engineered lung tissue.

7.4.6 Evaluation of endothelial barrier function in engineered lung tissue

In order to further evaluate the formation of an endothelial barrier in engineered tissues, we utilized transmission EM, VE-cadherin immunostaining, and a permeability assay we developed using FITC-labelled dextrans. We demonstrate robust expression of VE-cadherin via immunostaining in perfused endothelial cultures. Also in perfused cultures, we document the presence of tight junctions between endothelial cells using TEM, as shown in figure 7.6. We did not quantify the number of tight junctions or evaluate the effect of Ang-1 or other factors on the density of tight junctions. However, the finding that tight junctions are forming in the engineered tissues is promising for the development of an endothelial barrier.

In order to evaluate the permeability of engineered tissues, we developed an assay using FITC-labelled dextrans (2MDa, 55nm diameter), as described in section 7.3.3. Briefly, in this assay we inject dextrans into the airway and quantify the amount of leakage across the alveolar basement membrane by flushing the vasculature. In figure 7.13, we demonstrate a reduction in leak of up to 30% after 7-10 days of culture of perfused engineered lung endothelium. Although this is still distant from our goal of a 75% barrier for engineered lung tissue (see section 7.4.7), this is a promising step towards this objective.
7.4.7 Objectives for engineered endothelial tissue and identification of key next steps

In section 1.4, we state that functional engineered lung tissue should have a patent, perfused vasculature and should be able to exchange gas between the airway and vascular compartments. Here, we outline more specific objectives for the endothelial compartment and assess our progress towards the development of engineered lung endothelium.

Objectives for engineered lung endothelium:

**Percent endothelial coverage.** The endothelium of an engineered lung tissue must provide a non-thrombogenic lining of the vascular compartment. Capillary structures that are not lined with endothelium will thrombose and will therefore not be perfused. In order to estimate a goal for endothelial coverage of engineered endothelium, we look at pulmonary dead space. The dead space fraction of a lung provides an estimate of the fraction of the inspired air that does not participate in gas exchange with the vasculature. In healthy humans, a pulmonary dead space fraction of 0.3 is considered normal; this represents air that occupies the conducting airways and therefore does not participate in gas exchange in the alveoli. Dead space fractions above 0.58 correlate with marked increases in morbidity in patients with ARDS [145]. A dead space fraction of 0.58 implies that approximately 60% (0.42/0.7) of inspired air contributes to alveolar dead space, or non-perfused alveoli. Therefore, we set as a minimal goal that an alveolar dead space of no more than 50% is acceptable. However, in an engineered lung, alveolar dead space can be caused by either a non-perfused capillary network surrounding an alveolus or by insufficient epithelial repopulation of the alveolus. Therefore, we set as objectives that 70% of the capillary network be perfused and 70% of the alveoli be repopulated with epithelium. If these levels are
achieved, the combined tissue would meet the goal of 50% alveolar dead space. Thus, 70% of the capillary network should be covered with a confluent endothelium. This can be assessed to approximate levels using histological sections and transmission EM to confirm the proper location of endothelial cells.

**Barrier function to 5 µm particles.** The endothelium, together with the matrix, must form an intact barrier to large, cell-sized particles. We assessed the permeability of native and decellularized lung to leakage of 5 µm microspheres, and found that native lung retained ~98% of microspheres while decellularized scaffolds retained ~94% (figure 3.9). We propose that engineered endothelium should be able to match the performance of native lung regarding the leakage of 5 µm particles. The leak of blood-sized particles into the lung could lead to uncontrolled blood loss and thus failure of an engineered tissue. Therefore, we set as an objective that engineered endothelium prevent the leak of 98% of 5 µm microspheres.

**Barrier function to 55nm particles.** In addition, the endothelium must contribute to the formation of a functional barrier to fluid leak out of the vasculature. Using the permeability assay that we developed, native lung has a leak of ~13% (figure 7.13, section 7.3.3). Since significant leakage out of the vasculature would lead to fluid loss and pulmonary edema, and thus severely compromise gas exchange, we set as an objective that an engineered endothelium prevent the leakage of 75% of 55nm dextran particles. It is difficult to precisely justify a goal for this assay, as we do not know how the measurements of permeability made using this *in vitro* assay correlate with clinical pulmonary function.
Key next steps in the development of engineered lung endothelium:

**Endothelial deposition.** As shown in figure 7.8 and discussed in sections 7.3.4 and 7.4.2, endothelial cells in engineered tissues are not found in the vasculature, but are rather adherent to the alveolar basement membrane. This is clearly a critical issue. The focus of continued work on this problem will be the decellularization process, and continued attempts to minimize vascular perfusion pressure during decellularization and retain capillary structures. Although we demonstrate some retention of capillaries, significant more improvement is needed. Strict control of perfusion pressure as well as time-course experiments to identify when capillaries are lost during decellularization will be key experimental objectives.

**Fibronectin retention.** As well as the loss of capillary structures, fibronectin is removed during decellularization. This may also have important impacts on endothelial deposition, but the retention of native fibronectin would likely improve the adherence and proliferation of endothelial cells in the decellularized scaffolds. Therefore, we will strive to retain fibronectin, principally by retaining capillary microstructure. If fibronectin is found to be lost despite capillary retention, modifications in the decellularization solution may be required, such as reductions in the alkalinity of the solution.

**Improved cell coverage.** A secondary objective will be to improve the endothelial coverage of engineered endothelial tissues. This can likely be accomplished with increased cell seeding densities, improved cell seeding techniques, and longer culture times. Improvements in this area will also have positive impacts on the development of a functional endothelial barrier.
**Endothelial barrier formation.** The formation of a functional endothelial barrier will be continually assessed in engineered endothelial cultures using the 55nm dextran assay (section 7.3.3). It is possible that addressing the above points may demonstrate substantial increases in endothelial barrier formation. If additional barrier formation is needed, future experiments could employ the addition of angiopoietin-1 to engineered culture medium.

### 7.5 Conclusions

In this chapter, we focus on the endothelium of engineered lung tissue. We first describe the attachment of endothelium to the decellularized lung scaffolds, and the dependence of this attachment on fibronectin. We then evaluate the ability of two compounds, sphingosine-1-phosphate and angiopoietin-1, to affect the permeability of cultured endothelial monolayers. We find that both S1P and Ang-1 reduce the permeability of an EC monolayer, but that only Ang-1 has this effect in the presence of serum.

Using engineered tissue cultures, we evaluate the effect of Ang-1 on cell junction formation, and do not detect a change in VE-cadherin expression due to Ang-1. However, we do note robust VE-cadherin expression as well as tight junction formation via TEM even in the absence of Ang-1. In addition, using a permeability assay to evaluate the ability of labelled 55nm dextran particles to translocate the alveolar-vascular barrier, we show that engineered tissue can provide up to a 30% barrier to the passage of these particles.

The findings described in this chapter demonstrate the ability to culture endothelium on a decellularized lung scaffold, the ability to couple in vitro permeability studies to engineered tissue culture, and, most importantly, the potential of engineered endothelium to form a preliminary barrier between the airway and vascular
compartments. These findings are initial steps towards two of the key objectives for an engineered lung tissue (see section 1.4): the presence of a patent, perfused vasculature and the ability to exchange gas between the airway and the vascular network.

Clearly, much more work is required to accomplish these long-term goals. The permeability of engineered endothelium must be reduced to far below the current levels of 70%, which can likely be accomplished with improved cell seeding techniques, higher densities of seeded cells, and the optimization of culture and bioreactor conditions. In addition, retention of endothelial cells within the vasculature will likely assist in reducing permeability. This also may be aided by the retention of fibronectin and capillary structures in the decellularized scaffolds, thus improving the available substrate structure in the microvasculature. Although we coat the scaffolds with exogenous fibronectin, this certainly does not replicate the native distribution of fibronectin. Advances in the decellularization technique in order to retain native fibronectin may greatly improve cell attachment and proliferation on the scaffolds.
Chapter 8

Functional Evaluation of Engineered Lung Tissue, Conclusions, and Directions for Future Study

8.1 Introduction

In the first chapter (section 1.4), we identified four long-term objectives for the creation of viable engineered lung tissue:

- **Vasculature and airway.** There must be a patent, perfused vasculature and a patent airway tree that can be ventilated.

- **Gas exchange.** The lung must be capable of exchanging sufficient gas between the airway and vascular compartments to meet the physiological needs of the animal. In particular, the partial pressure of oxygen in the pulmonary vein should be at least 50 mmHg.

- **Mechanics.** The tissue must be strong enough to withstand all needed movements, in particular breathing motions and vascular perfusion, as well as manipulation during surgical implantation. Therefore, the tissue should mimic the mechanical properties of normal lung in terms of strength and elasticity.

- **Immunogenicity.** The tissue should not provoke an immune response when implanted into a host.

In the previous chapters of this dissertation, we described the decellularization of adult rodent lung to create scaffolds for tissue engineering (chapters 3 and 4), and
then described the culture of engineered epithelial and endothelial tissue using the decellularized scaffolds (chapters 6 and 7).

In the development of decellularized lung scaffolds, we evaluated several scaffold characteristics that are relevant to the attainment of these long-term objectives. We evaluated the patency of the airway and vascular networks using imaging and electron microscopy studies. We found that the vasculature is intact down to vessels of at least 100 µm yet the majority of capillaries are lost during decellularization, while the alveolar ultrastructure and basement membrane appears intact on EM studies. We also demonstrated that the scaffolds do not retain either MHC class I or II antigen, suggesting that the scaffolds would not generate an immune response. Finally, we evaluated scaffold mechanics and demonstrated overall retention of mechanical properties, although a significant amount of elastin is lost during decellularization.

Studies of engineered epithelium demonstrated the beginnings of key epithelial functionality that will be required for gas exchange. These key epithelial components are the presence of type II pneumocytes that produce surfactant, and the lining of the alveolar surface with flat, differentiated type I pneumocytes. Under selected conditions, we demonstrate surfactant production and evidence for type I pneumocyte differentiation in engineered epithelial tissues.

Studies of engineered endothelium, meanwhile, indicate that barrier function by an engineered endothelial tissue is possible. We found that engineered perfused endothelial tissues can prevent the movement of up to 30% of 55nm particles between the airway and vascular compartments (figure 7.13). Endothelial barrier function to prevent the movement of fluid out of the vasculature will be critical for effective gas exchange; buildup of fluid in the airspaces or interstitium will significantly increase the diffusion distance and effectively eliminate functional gas exchange between the alveoli and capillary network.
Therefore, we see preliminary evidence that engineered epithelium and endothelium can contribute to attaining the functional goals outlined above. However, a functional engineered lung tissue requires both an epithelium and an endothelium, together and functioning in the same tissue. In this chapter, therefore, we will discuss aspects of the functionality of engineered epithelium and endothelium and address the co-culture of these two cell populations in engineered lung tissues.

The co-culture of rodent lung endothelium and epithelium has not been studied significantly in in vitro systems, although human cells are more conducive to co-culture [87, 88]. This may be due to difficulty culturing rodent lung epithelium in vitro and the difficulty in maintaining epithelial phenotype during culture. A literature review identified only one study of rodent epithelial and endothelial co-culture, which utilized rat type II pneumocytes together with bovine microvascular EC grown on opposite sides of a polycarbonate filter [79].

The co-culture of lung endothelium and epithelium in an engineered tissue is important not only for their separate functionality. The cell populations must also interact and must occupy the same ‘space’. An engineered lung must not only have alveoli with type I and II pneumocytes, but these alveoli must be surrounded by a perfused capillary network. Type II pneumocytes are required to produce surfactant or the alveolus will not inflate and deflate; flattened type I pneumocytes are required to enable gas exchange across their thin membranes; and endothelium is required to line the capillary network and prevent thrombosis of the vasculature, thus enabling the movement of blood to within close proximity of the alveoli.

Therefore, the co-culture of lung endothelium and epithelium is important. A key issue in the continued development of engineered lung tissues is the identification of conditions, principally a consensus medium composition and a strategy for seeding the airway and vascular compartments, that enables the culture of both epithelial
and endothelial cells together in an engineered tissue.

8.2 Materials and Methods

Decellularized scaffolds were decellularized and prepared following the protocols described in sections 3.2.3 and 6.2.1. Neonatal cells were isolated as described in section 6.2.2 while endothelial cells were cultured as described in section 7.2.1. Endothelial cell growth supplement (ECGS) was obtained from Sigma and used at 50 µg/ml. Engineered endothelial tissue was cultured as described in section 7.2.2. Engineered epithelial tissue was cultured as described in section 6.2.4.

8.2.1 Endothelial and epithelial cell co-culture on lung scaffolds

Scaffolds were prepared as for isolated endothelial or epithelial engineered tissue culture, although all scaffolds were coated with fibronectin as described in section 7.2.2. Neonatal cells were isolated and seeded into the airway compartment at either day 0 or day 4-5 of culture, as described in section 6.2.3. Endothelial cells were cultured as described in section 7.2.1 and seeded as described in section 7.2.2 at either day 4-5 or day 0, as appropriate based on timing of neonatal cell seeding. During endothelial-only culture, tissue was perfused through the vasculature at 1.5-3ml/min. During epithelial-only culture, tissue was ventilated with medium at 1breath/min. During co-culture periods, tissue was both perfused and ventilated at the above rates.

8.2.2 acLDL uptake

Acetylated low-density lipoprotein (acLDL) was obtained from Invitrogen (Eugene, OR). Cultured endothelial cells were rinsed in PBS and incubated with 1 µg/ml acLDL in PBS with supplemental Ca$^{2+}$ and Mg$^{2+}$ at 37°C for 1 hour. Cells were
then fixed using 4% formaldehyde in PBS. Total cells and percent demonstrating acLDL uptake were counted high-power microscopic field using a Zeiss Axiovert 200M inverted fluorescent microscope.

**Mechanical testing**

Tissue segments were analyzed as described in section 4.2.5.

### 8.3 Results

In this chapter, we address the co-culture of endothelium and epithelium on decellularized scaffolds, and identify key issues that must be addressed for the continued development of functional engineered lung tissue.

#### 8.3.1 Functional assessments of engineered lung tissue

**Retained mechanics in engineered lung tissues.** We measured the mechanical properties of engineered lung tissues that were seeded with neonatal pulmonary cells and cultured as described in section 6.3.4 for 7 days. We show in figure 8.1 that the overall mechanical strength of the engineered tissues is similar to native and decellularized lung. Although these engineered tissues were not seeded with endothelial cells, this finding is promising that the mechanics of the lung matrix are not degraded during culture periods, indicating that there is not substantial remodelling, degradation or deposition of the matrix by the seeded cell population.

In previous chapters, we demonstrated that, when cultured separately, engineered lung endothelium and epithelium show some signs of the functionality that will be required in engineered lung tissue. We found that engineered perfused endothelial tissues can prevent the movement of up to 30% of 55nm particles between the airway and vascular compartments (figure 7.13). Additionally, engineered epithelial tissues
Figure 8.1: Ultimate tensile strength of engineered tissues. Native and decellularized lung strengths are also shown.

demonstrate surfactant production and evidence of type I pneumocyte differentiation. However, the next challenge is to incorporate these attributes into the same engineered tissue via co-culture of engineered endothelium and epithelium.

8.3.2 Preliminary identification of media suitable for endothelial and epithelial growth

A critical hurdle for the co-culture of engineered epithelium and endothelium is the identification of a consensus culture medium that is compatible with the survival and function of both cell types. The primary media utilized in the growth of engineered epithelium are DMEM with 10%FBS and BGJb, a serum-free, chemically defined medium; the primary endothelial medium is MCDB-131 with 10%FBS and supplemental growth factors (the precise composition is proprietary).

Epithelial culture in endothelial-specific media. We attempted to culture engineered lung epithelium in the base medium of the primary endothelial growth medium (MCDB-131+10%FBS; we did not include the supplemental endothelial growth factors). However, we did not note any evidence of cell attachment after
short-term culture periods of 4 days (data not shown). Although we cannot rule out that epithelial cell growth is possible in this medium, such as with the addition of epithelial growth factors, the modification of seeding technique, or the adjustment of bioreactor culture conditions, we did not evaluate this in greater depth. It is very difficult to use engineered tissue culture as a platform for the screening of medium types or other culture conditions. Therefore, we have focused our efforts at co-culture on modifying the conditions of endothelial culture.

**Endothelial cell culture in epithelial media.** We performed screening experiments to evaluate the growth of endothelial cells during *in vitro* culture in the medium that was utilized for most engineered lung epithelial cultures (DMEM+10%FBS). We compared endothelial growth and acLDL uptake in MCDB complete medium (which contains 10% FBS and supplemental growth factors), MCDB + 10%FBS, and DMEM + 10%FBS. Morphology is demonstrated in figure 8.2 along with images of acLDL uptake. After 4 days of culture, removal of supplemental growth factors leads to a reduction in cell number by 43%, while cell number is reduced by 65% in DMEM medium, compared to complete MCDB medium (figure 8.3). It is also evident that DMEM medium negatively impacts cell morphology. acLDL uptake was maintained by all cells regardless of culture medium.

DMEM medium causes alterations in cell morphology, although uptake of acLDL is retained (figure 8.2). These effects on cell morphology and cell number are also observed in MCDB medium without supplemental growth factors, although to lesser extents. These *in vitro* studies indicate that endothelial cell cultures can be maintained in DMEM media, although proliferation is severely reduced. DMEM-based media may therefore be useful for engineered tissue co-culture, although seeding and proliferation of the endothelial cell population may be best done first and using medium that facilitates endothelial proliferation.
In order to improve endothelial growth and proliferation in DMEM-based media, the use of selected endothelial growth factors may be beneficial. In figure 8.2 we also demonstrate that endothelial morphology is improved by addition of endothelial cell growth supplement (ECGS) to DMEM media. This also leads to increased endothelial proliferation compared to DMEM media alone, resulting in a 65% increase in cell number compared to DMEM medium without ECGS, although this is still reduced by 44% compared to MCDB-complete medium (figure 8.3).

These preliminary screening experiments demonstrate the ability to modulate endothelial growth using the selected addition of endothelial growth factors. Continued work is needed to identify optimal endothelial growth in an epithelial-favorable medium, such as DMEM or BGJb. Additionally, any growth factors utilized for EC growth must be tested to assess their effects on engineered epithelial tissue development.

**Engineered endothelial growth in epithelial media.** The poor endothelial cell growth observed *in vitro* using DMEM-based medium and the removal of endothelial growth factors from MCDB-based medium was also observed in engineered tissue culture. In figure 8.4 we compare histology of engineered endothelium cultured in EC-specific medium (MCDB-131 complete) compared to MCDB-131 medium with 10%FBS but without supplemental growth factors and to DMEM medium with 10%FBS. Cell morphology is clearly affected in these media, and an increased density of apoptotic cells is noted. These findings are consistent with the *in vitro* screening studies described above, and indicate that we can use *in vitro* screening of endothelial media conditions to assist in the identification of a consensus medium composition that is compatible with the growth of engineered lung endothelium. Future studies are needed to evaluate the effect of supplemental ECGS on engineered endothelial growth, as the *in vitro* screening studies described above suggest this may be a
Figure 8.2: Endothelial growth and phenotype in MCDB and DMEM media formulations. The ability of endothelial cells to proliferate and maintain an endothelial phenotype was assessed. Right panels show acLDL uptake in red.
Figure 8.3: Endothelial proliferation in MCDB and DMEM media formulations. Cells per high powered microscopic field were counted after 4 days of culture in the indicated media. * indicates $p<0.01$ compared to MCDB-complete and # indicates $p<0.01$ compared to DMEM+10%FBS.

8.3.3 Identification of conditions for endothelial and epithelial co-culture in engineered lung tissues

We performed a series of engineered lung cultures that were seeded with both endothelial and epithelial cells. We compared the effects of seeding the epithelial population before and after seeding of the endothelium. In all cultures, we utilized DMEM+10%FBS medium during epithelial cell growth. Thus, when the epithelium was seeded first, we utilized DMEM medium for the entire culture period, including after the seeding of endothelial cells. When the endothelium was seeded first, we evaluated the effects of performing a gradual versus a sudden transition from endothelial medium (MCDB) to epithelial medium (DMEM). Cultures were performed for 8-10 days, with one cell population seeded at day 0 and the other population seeded at day 4-5. Unfortunately, no conditions demonstrated substantial cell viability at the end of the culture period. Histology is not shown.
Figure 8.4: Medium impacts the growth of engineered endothelial tissue. Engineered perfused endothelium was cultured in the indicated medium type. H&E histology is shown in the left panels, while right panels show apoptotic nuclei in brown (via TUNEL) while normal nuclei are counterstained with methyl green.
In tissues where the endothelium was seeded first, we demonstrated in section 7.3.4 that the endothelial cells adhere to the alveolar basement membrane. Therefore, the usual epithelial substrate, the alveolar basement membrane, would have been obscured by the growing endothelium. It is likely that epithelial cells could not attach sufficiently to the matrix under these conditions. Furthermore, the switch of medium to DMEM with epithelial seeding would have simultaneously begun to degrade the culturing endothelium. The combination of non-adherent, and thus apoptotic, epithelial cells with a struggling endothelium in suboptimal medium could readily lead to the apoptosis of virtually all cells.

In the engineered co-culture tissues that were seeded with epithelium first, the culturing epithelium would first occupy the alveolar basement membrane. This is also the preferred substrate for the endothelial cells which are subsequently seeded (section 7.3.4). Additionally, the fibronectin coating of the scaffold was applied at the beginning of the epithelial culture, 4-5 days before endothelial seeding, and may have been removed or degraded over the course of 4-5 days of epithelial growth. Therefore, the seeded endothelial cells may not have had a suitable substrate for adherence. The sudden presence of millions of apoptotic endothelial cells could have then resulted in the concomitant death of the growing epithelium.
8.4 Discussion

8.4.1 Separate endothelial and epithelial tissues demonstrate functionality

We have demonstrated that engineered lung endothelial and epithelial tissues can display certain key aspects of functionality that will be required in a viable engineered lung tissue. Although levels of function fall short of what is needed, these findings are promising signs that, with continued improvements, we can culture whole segments of functional engineered lung tissue.

Engineered endothelium demonstrates preliminary barrier function

We showed that engineered lung endothelium displays some barrier function, reducing the leak of 55nm dextran particles by up to 30% compared to decellularized scaffolds. Although 30% is far less than our target barrier function of 75% (compared to native levels of 87%), it is very promising that we see the development of any level of barrier function. Using improvements in seeding method, in order to deliver a higher density of cells to a wider range of the vasculature, and using longer culture periods, continued improvements in barrier function will be possible.

Decellularization improvements will facilitate growth of functional engineered endothelium. Continued efforts to improve the growth of engineered endothelium will couple with improvements in decellularization, whereby we aim to retain more of the capillary network. Retention of capillary structures will improve the ability of seeded endothelial cells to remain in the vasculature, and may prevent their translocation to the airway compartment. Furthermore, a capillary network will provide a native framework for the growth of engineered endothelium, thus facilitating the formation of a microvasculature surrounding the alveolar network. The retention
of capillaries also may prevent the loss of native fibronectin. This would facilitate endothelial adherence to the microvasculature and would thereby likely reduce the translocation of endothelial cells to the airway compartment.

**Engineered epithelium demonstrates surfactant production and lined airway structures**

Engineered epithelial tissues demonstrate the production of surfactant, especially when tissues are cultured in a serum-free epithelial differentiation medium (BGJb). The production of surfactant by lung epithelium is a critical requirement for the growth of functional engineered lung tissue. Surfactant is primarily secreted by type II epithelium, and serves to reduce surface tension at the air-liquid interface in alveoli, thus preventing alveolar collapse at end-expiration as well as reducing the pressures required to expand the alveoli during inspiration [51]. We showed that engineered tissues produce surfactant, and also that surfactant production can be modulated using culture conditions, principally via choice of culture medium.

We have demonstrated the growth of numerous lined epithelial structures in engineered tissues. An additional important finding discussed in section 6.3.8 is that these epithelial structures remain filled with cellular secretions (that stains positive for CCSP) in cultures that are perfused. However, in ventilated cultures the epithelial structures are free of exudate. This suggests that ventilation can effectively transmit fluid into the airway of the engineered lungs. More importantly, however, this also indicates that the developing epithelial structures are a part of the airway tree, and are not simply growing randomly within the matrix. This speaks to the advantages of a decellularized matrix, and demonstrates the importance of the overall 3-dimensional architecture of the decellularized scaffolds.
8.4.2 Co-culture of engineered endothelium and epithelium is challenging

We have evaluated several conditions in order to culture engineered tissues with both endothelium and epithelium. However, this has proven to be a challenge. Rat lung EC grow poorly in the medium used for engineered epithelial growth, when cultured both on tissue culture plastic and on decellularized scaffolds. In addition, we do not observe any lung epithelial growth in media that is conducive to endothelial growth.

The co-culture of epithelium and endothelium is generally difficult, and few in vitro systems have demonstrated the culture of these cell types together, especially in rodent models. One study of epithelial and endothelial co-culture demonstrated the growth of rat type II pneumocytes together with bovine microvascular EC in DMEM medium with 10% fetal calf serum [79].

Future work in this area will focus on the identification of a consensus medium that is compatible with the growth of both engineered lung endothelium and epithelium. We feel that the optimal approach will be to identify conditions that enable endothelial cell maintenance in the media used for engineered epithelial growth (BGJb and DMEM+10%FBS). Rat lung EC can easily be cultured in vitro; rodent lung epithelium is far more difficult to grow on tissue culture vessels. Therefore, we can screen endothelial medium conditions in a rapid and efficient manner, as demonstrated in section 8.3.2. Attempting to modify the epithelial culture medium to mirror the favored endothelial medium (MCDB+10%FBS and growth factor supplements) would be far more difficult due to the need to utilize engineered tissues for these studies, a time-consuming and tedious process. Modifying the endothelial medium is also preferable because endothelial cultures can be maintained in vitro in quiescent states using low-serum media formulations [70, 188].
8.4.3 Approaches for continued efforts at co-culture of engineered endothelium and epithelium

Based on the findings in this dissertation, the co-culture of engineered endothelium and epithelium is most likely to be successful if performed as follows. The endothelium should be seeded first and allowed to adhere, proliferate and mature. The scaffolds require fibronectin coating for EC attachment, and this coating should ideally be applied immediately before endothelial cell seeding. If epithelial cells were already seeded onto the scaffolds, fibronectin coating would be difficult and could negatively impact the viability of cultured epithelium. Additionally, the neonatal epithelial cell population was shown to not adhere or proliferate in the endothelial medium (MCDB-131+10%FBS) and thus if the epithelium were seeded first one would need to maintain the cultures in the epithelial medium (DMEM+10%FBS) throughout the culture period. This would severely impair the ability of seeded endothelial cells to proliferate as needed to create a functional endothelial lining, as we showed that EC growth and morphology is impaired in the DMEM medium (figures 8.2 and 8.3). Therefore, the best approach would likely be to first seed the endothelium onto fibronectin-coated scaffolds and EC-specific medium for culture. During this period, the tissues would be perfused but not ventilated.

During the culture of the endothelium, the permeability assay described in section 7.3.3 can be utilized to assess the growth and development of the endothelial network. Importantly, this assay can be performed repeatedly on the same engineered lung tissue. A preliminary set of experiments would be utilized to optimize the culture time and endothelial seeding method. However, the ability to make repeat permeability measurements on each lung will allow us to tailor the growth of each engineered tissue so that the endothelium is fully developed before the seeding of epithelial cells.

After the maturation of the engineered endothelium, the culture medium can be
switched to epithelial-favorable medium. This consensus medium will need to be determined as discussed above. Freshly isolated neonatal rat pulmonary cells can then be seeded into the airway compartment, and the epithelium can be allowed to proliferate. During this period we anticipate using both perfusion and ventilation, due to the effects identified in isolated cultures described in chapters 6 and 7. Based on the growth of isolated epithelial cultures, we expect that a co-culture period of at least 4-8 days will be required, in addition to the endothelial culture period.

Use of both perfusion and ventilation in engineered tissue co-cultures. We discussed at length the effects of perfusion and ventilation on the development of engineered epithelium and endothelium. Briefly, we found that epithelial growth was significantly improved with ventilation, while endothelial growth required perfusion. Therefore, co-culture experiments will likely require both the perfusion and ventilation of engineered tissues. Initial experiments will need to be performed to evaluate if the combination of ventilation and perfusion has negative impacts on the development of either the epithelium or the endothelium, when cultured separately. Although we have no a priori suspicions that perfusion and ventilation will together impact engineered tissue culture, these screening experiments will be necessary and worthwhile.

8.5 Overall Conclusions of this Dissertation

In this dissertation, we demonstrate progress towards the growth of engineered lung tissue. Our efforts encompass the three key components of an engineered tissue: a scaffold, a cell source, and a bioreactor. We first described the production of a scaffold by decellularizing native rodent lung tissue, and performed a series of studies to evaluate this scaffold as we attempted to demonstrate that it is a suitable scaffold for
the growth of engineered lung tissue. We then detailed the design and validation of a bioreactor that can be used to culture lung tissue in the laboratory under physiological conditions. We cultured native rodent lungs in the bioreactor to demonstrate retention of cell viability, cellular differentiation state, and lung morphology. We then utilized a heterogeneous population of pulmonary cells harvested from neonatal rats to demonstrate that the decellularized scaffolds can support the adherence and proliferation of a wide range of pulmonary cell types, including epithelium, epithelial progenitor cells, mesenchymal cells, and endothelium. We demonstrated that bioreactor conditions have significant impacts on the development of both engineered epithelium and endothelium. Finally, we demonstrated preliminary evidence of the functionality of these isolated engineered epithelial and endothelial tissues.

8.5.1 Evaluation of Decellularized Lung Scaffolds

In chapters 3 and 4, we described the decellularization of rodent lung tissue and a host of studies intended to evaluate these scaffolds, with the overall primary objective being to evaluate their suitability as scaffolds for lung tissue engineering.

**Removal of DNA and Immunogenic Markers** We demonstrated that the decellularized scaffolds contain 1.2% of the DNA of native lung, and we do not detect any immunogenic markers (MHC Class I and II antigen). This level of decellularization is close to levels found in commercially available decellularized skin scaffolds [74], and the lack of MHC Class I and II antigen is suggestive that an immune response would not be provoked by these scaffolds, although *in vivo* studies will be needed to ascertain the true immunogenic nature of the scaffolds.

**Retention of Extracellular Matrix Components** We demonstrated retention of collagen in the decellularized scaffolds using histochemical staining, immunofluo-
rescence for collagen types I and IV, and quantitative assay, which together demonstrate similar collagen content and distribution to native lung. Elastin was shown to be preserved based on histological staining, although only 40% of native levels are preserved on quantitative assay. In addition, fibronectin was found to be absent from the scaffolds, and glycosaminoglycans were also not found. Future studies will focus on the retention of these components of native lung during the decellularization process, as elastin is an important matrix molecule for the mechanical properties of lung, while fibronectin is critical for cell attachment and glycosaminoglycans affect macromolecular and cellular movement in the scaffold.

**Mechanical Properties of Decellularized Scaffolds** We demonstrate mechanical properties of the decellularized scaffolds that match native lung. Measurements of ultimate tensile strength and elastic behavior do not identify any differences between native and decellularized lung. The retention of mechanical properties occurs despite only 40% of native elastin levels in the decellularized tissue. This may be due to the retention of sufficient elastin in order to demonstrate normal elastic properties, but more likely indicates the need for more in-depth study of the elastic properties of the decellularized lung scaffolds. Additionally, more careful control of the perfusion pressure during decellularization will likely aid in the retention of elastin, as less microvascular disruption will reduce the removal of elastin from alveolar septae.

**Microstructural and Ultrastructural Evaluation of Decellularized Lung Scaffolds** In order to evaluate the integrity of the vasculature and airway tree, we utilized a variety of imaging modalities – scanning EM, transmission EM, and micro CT. SEM demonstrated intact alveoli and alveolar septae, while TEM demonstrated an intact alveolar basement membrane and the retention of some capillaries, if the decellularization process is performed with low, controlled perfusion pressures.
Micro CT demonstrates retention of the vasculature down to vessels of approximately 100 µm diameter, although some leak is evident from smaller vessels.

The primary focus of decellularization improvements will focus on the retention of the capillary network. Current ‘best-case’ decellularization regimens demonstrate some capillary retention, but a substantially reduced density compared to native lung. Careful time-course experiments will allow the identification of when capillaries are removed during the decellularization process. Once this is established, modifications can be evaluated, likely focusing on continued reductions in vascular perfusion pressure, such as using further reduced flow rates.

8.5.2 Design and Validation of a Bioreactor for the *in vitro* Culture of Lung Tissues

We described the design of a bioreactor that can support the culture of whole lung tissue in the laboratory. The bioreactor is capable of perfusion through the vasculature as well as ventilation with liquid or air via either negative or positive pressure. Using cultures of whole native lungs, we demonstrated that ventilation with air results in damage to the airway epithelium and the pulmonary parenchyma; this may be a result of control of ventilation via volume as opposed to pressure, resulting in over-distension of the lung and repeated opening and collapsing of the airways. We also found that ventilation is required to maintain the differentiation of lung epithelium, and that particular features of bioreactor design have significant impacts on cell viability. Finally, we demonstrated that cell viability can be maintained at near-native levels, cellular differentiation state is retained, and lung morphology is similar to native lung.

This bioreactor will be useful for both the culture of engineered lung tissue as well as the study of native lung biology, development and physiology. Many stud-
ies are possible, utilizing native lung cultures in the bioreactor. Lung physiology can be probed under strictly controlled conditions of ventilation and perfusion. Mechanical ventilation can be studied over long time periods. Lung biology and injury repair can be studied without immune system effects or stem cell recruitment from extra-pulmonary sources. Drug screening can be performed under stricter controls than most animal models. This is just to name are few; there are indeed countless opportunities for future study in this area.

8.5.3 Demonstration of Cell Growth on Decellularized Lung Scaffolds

We demonstrated that the decellularized scaffolds are suitable substrates for the adherence and culture of a range of pulmonary cell types, including epithelium (types I and II, and Clara cells), epithelial progenitor cells (basal cells and bronchoalveolar stem cells), mesenchymal cells, and endothelium. This is an important finding as a scaffold for the development of engineered lung tissue will need to support the growth of a wide range of cell types.

8.5.4 Evaluation of the epithelium of engineered lung tissue

We utilized cultures of neonatal pulmonary cells on the decellularized scaffolds in order to study the effects of medium type, bioreactor conditions, and an air interface on the growth and differentiation of engineered lung epithelium.

Medium type impacts epithelial differentiation. We have shown that medium type affects the differentiation of epithelial structures in culture on the decellularized scaffolds. Culture in a serum-free defined medium, BGJb, induces surfactant expression to levels approaching native lung, which may be due to differentiation of Clara cells to type II epithelium. Further study of medium composition, including the
use of key growth factors, will be useful to both study and guide the differentiation of engineered lung epithelium. In particular, we will focus on the differentiation of cultured epithelium towards mature alveolar epithelium (type I and II pneumocytes).

**An air interface enables differentiation of type I epithelium.** In the bioreactor, engineered tissues cultured with an air interface, provided by ventilation with air, demonstrated increased expression of aquaporin-5, indicative of type I epithelium, and reduced surfactant expression. This indicates the differentiation of type II to type I epithelium, as type II cells are a known local precursor cell for type I pneumocytes. These findings therefore demonstrate that breathing movements alone are not sufficient to induce a type II to type I differentiation, but in the presence of an air interface, this differentiation is observed. Together with findings using native lung culture in the bioreactor, these studies indicate the importance of the decellularized scaffold in driving epithelial regeneration and differentiation.

**Ventilation enables epithelial differentiation and adherence** We compared the effects of ventilation and perfusion on epithelial adherence and differentiation in engineered lung tissues, and found that both ventilation and perfusion enabled the growth of Clara cells and type II epithelium. However, cells expressing the Clara cell marker CCSP adopted a more flattened morphology with ventilation, whereas they retained a cuboidal morphology with perfusion. A significant number of these cuboidal cells also express surfactant protein C (SPC), indicating they may be the local progenitor cell type termed bronchoalveolar stem cells. Therefore, ventilation appears to promote the differentiation of these progenitor cells towards a more mature epithelial phenotype.

Epithelial differentiation towards alveolar phenotypes is beneficial to our efforts at engineering lung tissue as our focus is on the distal, alveolar epithelium, the site of gas
exchange. However, we also wish to retain Clara cells in the airways of engineered tissues, and so must therefore ensure that we do not induce the differentiation of the airway epithelium towards an alveolar epithelial phenotype. Stricter control of ventilation conditions may facilitate these efforts; we have seen that overdistension of native lungs with air has strong negative impacts on native lung culture in the bioreactor (chapter 5). Current tidal volumes during ventilation are at the upper limit of normal (2.0-2.5ml in engineered tissues compared to 0.8-2.0ml in native rat). Better control of ventilation, possibly including the use of pressure-controlled rather than volume-controlled ventilation, will likely facilitate these efforts and enable reductions in tidal volume.

8.5.5 Evaluation of the endothelium of engineered lung tissue

We developed engineered lung endothelium using culture of rat lung microvascular endothelial cells on the decellularized scaffolds. We first demonstrated the importance of fibronectin for the attachment of endothelium to the decellularized lung scaffolds. We then evaluated the ability of two compounds, sphingosine-1-phosphate and angiopoietin-1, to affect the permeability of cultured endothelial monolayers. We found that both S1P and Ang-1 reduce the permeability of an EC monolayer, but that only Ang-1 has this effect in the presence of serum. Additionally, we demonstrate that vascular perfusion facilitates engineered endothelial growth while ventilation is damaging.

We assessed the formation of an endothelial barrier in engineered tissues using transmission EM, assessments of VE-cadherin expression, and a permeability assay to quantify the permeability of 55nm dextran particles between the airway and vascular compartments. We did not observe an impact of Ang-1 on cell junction formation in engineered tissues, but in the absence of Ang-1 demonstrated robust VE-cadherin
staining as well as tight junction formation. Finally, we demonstrated that perfused engineered tissues can provide up to a 30% barrier to the passage of 55nm particles out of the airway compartment.

Future efforts on endothelial development will include the identification of improved cell seeding techniques, higher densities of seeded cells, and the optimization of culture and bioreactor conditions; the focus of these improvements will be continued reductions in permeability of the vascular compartment. In addition, retention of endothelial cells within the vasculature will likely assist in reducing permeability. Advances in the decellularization technique in order to retain native fibronectin and capillary structure may greatly improve these efforts.

8.5.6 Co-culture of engineered epithelium and endothelium

Current efforts at the combined culture of engineered epithelial and endothelial tissue were largely unsuccessful. This area will be an important area for future work, as clearly the combined growth of endothelium and epithelium will be required for a functional engineered lung tissue. Key points for co-culture are the identification of a consensus culture medium composition and a strategy for seeding the airway and vascular compartments.

8.6 Key Future Efforts for the Development of Functional Engineered Lung Tissue

Throughout this dissertation, we have identified areas of importance for future study. The following issues are of key importance for the continued development of functional engineered lung tissues.
Retain microvasculature during decellularization. Reduction of vascular perfusion pressures to 20mmHg during decellularization enables the retention of some capillaries in the decellularized scaffolds. Control of perfusion pressure to lower levels, such as 10-15mmHg, will likely enable the retention of a significant fraction of the capillary network. This will be a primary focus in ongoing efforts, as the retention of the capillary network will also likely enable the retention of fibronectin, an important substrate for endothelial attachment and proliferation. Capillary and fibronectin retention will provide a native substrate for engineered endothelial growth, in regions surrounding the alveolar spaces, thereby enabling the growth of an engineered tissue that possesses sufficient perfusion of the capillary network surrounding alveoli.

Improve the functionality of isolated endothelial and epithelial lung tissues. Although we have seen preliminary evidence of function in separate epithelial and endothelial lung tissues, these levels of functionality are not sufficient to meet the objectives of an engineered lung tissue. Using improved seeding techniques, prolonged culture times, and optimization of bioreactor conditions, the continued improvement of the functionality of separate epithelial and endothelial tissues must be demonstrated.

Identify a consensus medium and seeding strategy for the co-culture of engineered endothelium and epithelium While the isolated culture of engineered epithelium and endothelium has demonstrated promising evidence of functionality, this functionality must be combined in order to create a functional engineered lung tissue. The identification of a consensus medium that can support the growth of both endothelial and epithelial cells will be a key effort. Additionally, a strategy will need to be identified to determine the optimal method for the seeding, proliferation, and maturation of the epithelium and endothelium.
In this dissertation, we have demonstrated the feasibility of engineering lung tissue in the laboratory. We demonstrated the production of a decellularized scaffold that can support engineered tissue culture, and have developed a variety of key techniques and platforms that enable the growth of engineered lung tissue. We demonstrated that engineered epithelial and endothelial tissue can be produced, and furthermore that these tissues demonstrate preliminary evidence of functionality. In total, this work provides a foundation for the continued development of whole, functional engineered lung tissue.
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Biography

Thomas H Petersen, born February 6, 1981 in Danbury, CT
Princeton University, BSE Electrical Engineering, magna cum laude, June 2002
Duke University, PhD Candidate, Biomedical Engineering, Degree expected December 2009
Duke University, MD Candidate, Degree expected December 2010

PUBLICATIONS AND CONFERENCE PRESENTATIONS


HONORS AND DISTINCTIONS

Whitaker Foundation Graduate Fellowship

Phi Betta Kappa, member, inducted 2002

Tau Beta Pi, National Scholarship Recipient, 2001

Barry M. Goldwater Scholar, 2000-2002