DIFFERENTIATION OF NORMAL AND CYSTIC FIBROSIS HUMAN LUNG EPITHELIAL CELLS IN A DECELLULARIZED AND RECONSTITUTED MOUSE LUNG

APPROVED BY SUPERVISORY COMMITTEE

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I would like to dedicate this work to my wonderful family, my endlessly patient wife, my graduate committee, and my mentors Woody and Jerry.

Additionally, I would like to thank the many coworkers, collaborators, and friends I've had the pleasure to get to know at UT Southwestern. This work would have been impossible without them.

DIFFERENTIATION OF NORMAL AND CYSTIC FIBROSIS HUMAN LUNG EPITHELIAL CELLS IN A DECELLULARIZED AND RECONSTITUTED MOUSE LUNG

by

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Thesis

Presented to the Faculty of the Graduate School of Biomedical Sciences The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2017

DIFFERENTIATION OF NORMAL AND CYSTIC FIBROSIS HUMAN LUNG EPITHELIAL CELLS IN A DECELLULARIZED AND RECONSTITUTED MOUSE LUNG

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The University of Texas Southwestern Medical Center at Dallas, 2017

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Engineered lung tissue may eventually address the chronic shortage of transplantable lung tissue and permit modeling of lung disease in a controlled *ex vivo* environment. However, there are presently no sources of primary lung stem cells which can be expanded at sufficient scale to permit engineering multiple lungs from a single donor. I have developed a method for conditionally reprogramming primary human bronchial epithelial cells in culture to extend their functional lifespan, and have used these cells to reconstitute lung epithelium in a decellularized lung matrix. For conditional reprogramming, I cultured primary human bronchial epithelial cells derived from patients with or without cystic fibrosis with a small molecule Rho-associated coiled kinase inhibitor and co-cultured it with irradiated J2 3T3 fibroblasts. I determined the ability of the human bronchial epithelial cells to differentiate after 40 population doublings by culture at an air liquid interface for 35 days as confirmed by transepithelial electrical resistance measurement, histology, Ussing chamber analysis, and immunofluorescence staining of differentiation factors. I also found that this conditional reprogramming method permits cloning of human bronchial

epithelial cells, and that these cells can support genetic modification by CRISPR. Next, I developed a method for decellularizing and reconstituting murine lungs in a bioreactor with vascular perfusion and simulated breathing. Lungs reconstituted with the conditionally reprogrammed human bronchial epithelial cells formed both upper and lower airway structures after only 12 days of culture. I confirmed the formation of a bronchial pseudostratified epithelium and alveolar formation in the reconstituted lungs by histology, western blotting, and immunofluorescence staining. To develop an eventual universal donor paradigm for engineered tissue, I developed an *in vivo* luciferase rejection screen in mice using luciferase expressing life-extended primary skin fibroblasts transplanted intradermally. The methods developed for long-term culture of primary lung epithelial cells permits rapid scale-up of patient derived human bronchial epithelial cells and clonal selection without the need for genetic manipulation; facilitating the study of lung diseases and optimization of organ reconstitution in tissue engineered models.

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PRIOR PUBLICATIONS

LaRanger, R., Peters-Hall, J. Coquelin, M., Alabi, B., Chen, C.T., Wright, W.E., Shay, J.W. Reconstituting Mouse Lungs with Conditionally Reprogrammed Human Bronchial Epithelial Cells. Tissue Engineering Part A (Submitted).

Robin, J.D., Ludlow A.T, **LaRanger, R**., Wright, W.E., Shay, J.W. Comparison of DNA Quantification Methods for Next Generation Sequencing. Nature Scientific Reports 6 Article number: 24067 (2016). Doi: 10.1038

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LIST OF DEFINITIONS

ALI – Air liquid interface

CF – Cystic fibrosis

CFTR – Cystic Fibrosis Transmembrane Conductance Regulator

CR – Conditionally reprogrammed

CRISPR – Clustered regularly interspaced short palindromic repeats

ECM – Extracellular Matrix

ESC – Embryonic Stem Cell

iPSC – Induced pluripotent stem cell

HBEC – Human bronchial epithelial cell

ROCK – Rho associated coiled kinase

H&E – hematoxylin and eosin

TEER – Trans epithelial resistance

CHAPTER ONE
Introduction

Chronic obstructive pulmonary diseases have no cure except transplantation, and are the third leading cause of death in the United States (Association, 2013). The only cure for chronic obstructive lung disease is transplantation, and there is a chronic shortage of transplantable lung tissue in the United States (Valapour et al., 2015). Further, many pulmonary fibrotic diseases such as cystic fibrosis (CF) are difficult to study *ex vivo* because primary lung stem cells rapidly lose their ability to differentiate after *in vitro* culture and study of these disease depends on the creation of 3-D differentiated epithelium (Gentzsch et al., 2016). Engineered lung tissue may permit the study of complex fibrotic diseases of the lung in a 3-D setting and the generation of transplants to meet this clinical need. A major challenge in the tissue engineering field is identification of an source of cells for lung tissue engineering applications which is easy to isolate, genetically stable, and which can differentiate into the various epithelia of the lung (Gilpin & Ott, 2015). I have developed a bioreactor system for extended *ex vivo* culture of reconstituted mouse lungs and demonstrated that conditionally reprogrammed (CR) human bronchial epithelial cells (HBECs) can differentiate into both upper and lower airway cells after expansion *in vitro*. These results have produced a population of cells for lung tissue engineering applications which can be grown rapidly from a biopsy and recapitulate the entire airway epithelium. Further, I have demonstrated the capacity of decellularized mouse matrices for optimizing lung tissue engineering. To recapitulate the native lung environment *ex vivo,* I have developed a method for decellularizing the mouse lung matrix that preserves structural cues such as rhythmic breathing, extracellular matrix (ECM) stiffness, and ECM composition. To abrogate

the need for constantly deriving fresh primary human cells to study lung reconstitution and to create disease models, a Rho-associated coiled kinase (ROCK) inhibition-based conditional reprogramming culture method was used to preserve the ability of these human bronchial epithelial cells (HBECs) to clonally expand and differentiate long-term *in vitro* without genetic manipulation. To create tissue engineered models of CF, clonal selection and CRISPR mediated genetic manipulation of primary HBECs was utilized to simulate the delta-F508 mutation in otherwise normal cells for the purposes of modeling the disease in engineered tissue. Developing these methods has enabled creation of reconstituted lungs with isogenically derived normal and CF HBECs. Finally, pursuant to the effort of creating a universal donor paradigm for engineered tissue, a method for measuring allogeneic rejection with luciferase imaging of intradermal luciferase-expressing transplants in a mouse model was developed.

In this introduction, the relevance of 3-D culture versus 2-D culture will be discussed. Then, the complexities of the lung and why it is difficult to study human lung diseases in simplified models will be reviewed. Next, the field of tissue engineering transplantable material, beginning with cell sheets and continuing to complex tissue decellularization/recellularization will be introduced. An overview of the current strategies for complex tissue reconstitution will be discussed, including the cells which are presently being used in these applications. This will be followed by a brief overview of CF, the efforts employed to model this disease, and how tissue engineered constructs may provide novel insights into the mechanisms of CF. The introduction will conclude on the advantages of the conditional reprogramming methods developed and the need for cell populations other than embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) for tissue engineering.

Importance of Dimensionality Mechanics, and Architecture Interactions

2-D monolayer culture was first developed by Harrison and co-workers in 1907 (Harrison & Jackson, 1907). Cell culture has been instrumental for performing most aspects of modern science to the point where as a technique it is largely ubiquitous. Embryonic stem cells and induced pluripotent stem cells, for instance, can be grown on tissue-culture treated plastic and their ability to differentiate is very well preserved over the course of culture in these conditions (Amit et al., 2000; Okita, Ichisaka, & Yamanaka, 2007; Yu et al., 2007). Alternatively, it has been shown that some adult human stem cells lose their ability to differentiate during culture on tissue culture treated plastic (Montarras et al., 2005; X. Z. Yan et al., 2014). Some primary cancer cells also demonstrate a significantly altered gene expression profile after culture on tissue culture treated plastic relative to the parent tumor (Tanner & Gottesman, 2015). It has been hypothesized that differences in cell behavior between 2-D and 3-D culture are due to the failure to recapitulate aspects of the native niche of the cell.

New tissue culture methods which recapitulate structural cues present in the *in vivo* environment have been developed to maintain the character of adult stem cells *in vitro.* Cells sense cues from their environment such as local tensional gradients, ultrastructural influences on their shape, and the extracellular matrix protein composition of their surroundings through integrin signaling as well as their cell-cell interactions and polarity sensing (Chiquet, Gelman, Lutz, & Maier, 2009; Pozzi & Zent, 2003; Shih, Tseng, Lai, Lin, & Lee, 2011; Soucy & Romer, 2009). Efforts to simulate these cues *in vitro* have focused on recapitulating the stiffness, 3-D architecture, and rhythmic mechanical stimulation which cells experience *in vivo*. (Mishra et al.,

2014; Sasai, 2013; Shamir & Ewald, 2014; Su et al., 2013; Tanner & Gottesman, 2015). Optimizing these elements of tissue culture has resulted in preservation of stemness and an *in vivo*-like expression profile in many cell types.

The stiffness of tissue culture substrates influences behavior of cells on their surface through integrin signaling (Shih et al., 2011). Rigid structures such as glass and plastic are orders of magnitude more stiff than materials found *in vivo*, which can lead to altered integrin-driven behavior driven by altered mechanosensing (Lauridsen & Gonzalez, 2017). Mechanosensing influences migration (Lo, Wang, Dembo, & Wang, 2000), focal adhesion formation (Tee, Fu, Chen, & Janmey, 2011), cell proliferation (Balestrini, Chaudhry, Sarrazy, Koehler, & Hinz, 2012), growth factor or surfactant production (Balestrini et al., 2012), and stem cell differentiation (Engler, Sen, Sweeney, & Discher, 2006). This research has recently led some investigators to attempt to improve the functional life span of adult stem cells by culturing them on substrates with a physiological-like stiffness. One of the first applications of this biomechanics research to cell transplantation was performed by the Blau laboratory, which was interested in preserving the regenerative capacity of satellite muscle stem cells. They observed that while muscle satellite cells which are directly transplanted from one mouse to another could take part in muscle regeneration, satellite cells which have been expanded on tissue culture plastic could not. By culturing cells on a soft hydrogel whose stiffness mimicked the stiffness found in muscle tissue (12kPa) as opposed to tissue culture treated plastic stiffness (100,000kPa) (Skardal, Mack, Atala, & Soker, 2013), the Blau laboratory produced cultured satellite cells which could take part in the regeneration process after transplantation as if they were freshly isolated satellite cells even after expansion *in vitro* (P. M. Gilbert et al., 2010). This research

provided *in vivo* evidence of the impact that culture in *in vivo*-like conditions can have on the ability of stem cells to differentiate, which had been previously theorized (Guilak et al., 2009). Cancer cell behavior and drug resistance profiles have also been shown to be driven by stiffness sensing in a Rho-A driven fashion (Lin et al., 2015; Tse et al., 2012). Optimizing the mechanical aspects of the culture environment for primary adult stem cells can result in more *in vivo*-like behavior and preservation of stemness.

While 2-D culture on hydrogels which recapitulate the *in vivo* mechanical environment can improve tissue culture outcomes, cells in this culture system are largely limited to forming either sheets or clumps of cells on a 2-D surface. The role that 3-D shape sensing has in cell behavior has been studied in the developing embryo. It has been found that cell shape and localized forces can impact the differentiation and behavior of cells (Heisenberg & Bellaiche, 2013). As cells divide, they apply a mechanical pull on surrounding cells at that axis of division, which can in turn impact the polarity of their axis of division. In a sphere shape these interplaying forces play a critical role in the development of zebrafish embryos (Xiong et al., 2014). These developmental studies may explain in part the preservation of stemness which has been observed in spherical organoid culture.

Many labs have explored the concept of 3D organoid culture to preserve the stemness of adult stem cells during expansion *in vitro*. In brief; organoid culture involves culture of cells in a 3-D sphere pattern suspended in media or in ECM or hydrogels. One of the first examples of culturing normal and tumor derived epithelial mammary cells in organoid spheres in Matrigel was performed by the Bissell laboratory (O. W. Petersen, Ronnov-Jessen, Howlett, & Bissell,

1992). This system was developed to address the difficulty in differentiating primary normal breast epithelial cells from breast cancer derived cells in 2-D culture, which was previously determined by life span of the cells in culture (Stampfer & Bartley, 1985). Using this system, the Bissell laboratory could distinguish cancerous lines from normal lines by the size of their organoids after 12 days in culture and their pattern of deposition of collagen in the Matrigel. This organoid culture system has since prompted other laboratories to use variations of this method to permit expansion and differentiation of epithelial cells derived from other tissues. For example, the Clevers lab in 2009 found that colonic epithelial cells could be expanded in floating organoid culture to preserve their capacity for differentiation after extended culture *in vitro* (T. Sato et al., 2009). They used this method to demonstrate the ability of stem cells expanded in organoid culture to renew colon epithelium in an *in vivo* transplantation model, which was not possible with standard 2-D culture (T. Sato et al., 2011). The Clevers lab then attempted to develop this organoid culture system as a tool for studying CF. Their hypothesis was that the CFTR channel, when active, would transport chloride ions into the organoid and that the resulting change in osmotic pressure would cause it to swell. This swelling would be easy to observe, and could be used as a way to measure CFTR function. Cells derived from CF patients did not demonstrate CFTR mediated swelling unless the cells had CFTR corrected either by a drug or CRISPR-mediated gene therapy (Dekkers et al., 2016; Schwank et al., 2013). Organoid culture has become a very useful tool for expanding primary adult stem cell populations while preserving their stemness and in recapitulating some of the *in vivo* like behavior of cells *in vitro*.

Organoid culture systems only simulate a spherical mechanical niche and structural niche. Our lab has previously shown that HBECs immortalized through ectopic expression of

hTERT and Cdk4 (Ramirez et al., 2004) can show dramatically different expression profiles depending on the mechanical niche they are exposed to. For example, when cultured embedded in Matrigel in organoid culture they form spheroids which secrete surfactant, but the spheroids never produce cilia or goblet cells of the upper airway (Delgado et al., 2011). These cells do form upper away structures, such as ciliated and goblet cells, but only after culture at an air-liquid interface on a collagen gel (Ramirez et al., 2004). To study the lung *ex vivo*, one would ideally have a culture system exposing a single population of cells to multiple mechanical niches to induce their differentiation into all the sub-populations of cells in the lung.

The Lung Consists of a Series of Distinct Niches

The lung is an environment which presents multiple layers of information to cells. It provides a series of microenvironments with differing mechanical and ultrastructural properties and differing extracellular matrix (ECM) protein compositions (Suki, Ito, Stamenovic, Lutchen, & Ingenito, 2005; Suki, Stamenovic, & Hubmayr, 2011). The lung also provides differing levels of rhythmic stretching and sheer force to cells depending on their location in the tissue (Suki, 2014). These differing forces in turn impact the behavior of the cells in various structural niches of the lung (Balestrini & Niklason, 2015). The lung ECM consists of several proteins such as collagen IV, fibronectin, laminin, and elastin (Price, England, Matson, Blazar, & Panoskaltsis-Mortari, 2010). Interaction with these ECM proteins influences cell signaling pathways (Bottaro, Liebmann-Vinson, & Heidaran, 2002), formation of focal adhesions and stress fibers (Clark, 1993), cell shape (Alford & Rannels, 2001), and ultimately informs the behavior of cells in their particular niche (Brown & Badylak, 2014). Cells sense the presence of these ECM proteins through activity of cell surface proteins such as integrins (Frith, Mills, Hudson, & Cooper-White,

2012; Martin-Bermudo, 2000). Cells in these environments respond to changes in stiffness or ECM proteins by modifying their own environment, such as by excreting remodeling factors or ECM (Suki et al., 2005; Suki et al., 2011). As the lung has several unique structural niches with differing levels of ECM proteins, it is important to simulate as many of these as possible when attempting to phenocopy *in vivo* cell behavior in this complex tissue.

Beyond ECM protein content, the lung provides mechanical cues and shear stress to cells depending on their location in the lung. For instance, alveolar cells are subjected to rhythmic shear forces from blood flow and stretching/contracting as the lung fills with air (Suki et al., 2011). These forces are transmitted to cells at sites of focal adhesion composed of integrin receptors with their extracellular domain bound to ECM components and their cytoplasmic domain bound to a cluster of proteins which are in turn physically linked to cytoskeletal actin (Colombelli et al., 2009; Ji, Lim, & Danuser, 2008). This direct connection to the mechanics of the ECM, for example, directs the production of ECM proteins and remodeling enzymes produced by interstitial cells (Chiquet et al., 2009). The physical and ECM forces in the lung, such as rhythmic shear stress, pre-stress, and ECM gradients creating local mechanical properties are key regulators of cellular processes in the lung (Suki, 2014). Because of this high level of complexity, efforts to mimic these conditions to induce differentiation of lung stem cells have focused on mimicking particular niches of the lung such as the bronchi or alveoli, as opposed to the entire organ.

Some tissue culture methods have been developed to mimic the upper and lower airway niches individually and produce useful models of the behavior of cells in those lung

compartments. Exposure to shear and stretch stresses, for example, has been shown to be critical for the display of differentiated alveolar type II cell characteristics in a lung on a chip model. In this system, rhythmic vacuum assisted stretching of the epithelial cells to promoted secretion of surfactant in epithelial cells on a chip (Huh et al., 2010). This system of recapitulating the lower airway mechanical environment has led to novel insights regarding pulmonary edema *in vitro* which had been impossible using standard 2-D culture (Huh et al., 2012). The upper airway of the lung can be simulated by exposing HBECs to an air liquid interface (ALI) cell culture system that permits formation of a pseudostratified epithelium by exposing a layer of cells cultured on a semipermeable membrane to air while the cells are being fed by media from below the layer (de Jong et al., 1994). This air liquid interface culture of HBECs produces a pseudostratified epithelial layer which can also simulate diseases such as CF by producing a current in response to activation of their chloride channels (Peters-Hall et al., 2015; Robert et al., 2008). However, this culture system does not introduce any of the other informational elements such as ECM protein gradients, physiological stiffness, or rhythmic shear stressors, which have been shown to influence cell behavior. To gain a better understanding of cell behavior, particularly in complex tissue such as the lungs, one needs a culture system which can recapitulate these characteristics in combination.

Decellularized Matrices for Recapitulating *in Vivo* Mechanical and ECM Characteristics

To create a tissue culture system which preserves the ultrastructural, ECM, and physical force cues of the original organ, one can remove the cells in a tissue by perfusing with detergent and DNase that largely preserves the structure. This technique was first used to maintain the gross mechanics of connective tissue while removing allogeneic cells to use in clinical

transplants (Badylak, 2004; Badylak et al., 1995). Over time, it was found that decellularized matrices influenced the behavior of cells which were seeded on them. It was later determined that decellularized matrices could be used to reconstitute complex tissues *ex vivo* because of the preserved architecture, ECM orientations, and other factors (T. W. Gilbert, Sellaro, & Badylak, 2006).

Culturing of cell-sheets was a precursor for reconstituting tissue from decellularized matrices. This paradigm was first established by the culture of epidermal cell sheets. By inducing them to excrete ECM by culture with ascorbic acid, an epidermal layer could be made which provided barrier functions to patients with severe burns who did not have sufficient tissue to expand for an autologous transplant (Gallico, O'Connor, Compton, Kehinde, & Green, 1984). This success was significant, and inspired work by other labs to create corneal transplants from layered cell sheets (Germain, Carrier, Auger, Salesse, & Guerin, 2000). There was a landmark paper in the field of corneal tissue engineering in 1999 whereby 3 layers of corneal cell lines seeded on a transparent hydrogel created a transparent cornea transplant *in vitro* (Griffith et al., 1999). The corneal transplants were, however, largely unsuccessful in clinical applications. Skin transplants made from cell sheets also had problems in translation to the clinic. Skins treated with cultured epidermal skin sheets were fragile and blistered after transplantation (Compton et al., 1989). To address this, some labs cultured sheets of epidermal cells onto hydrogel dermis equivalents containing embedded fibroblasts to make a sturdier graft and recapitulate the mechanical qualities of full-thickness skin (Daly, 1982; Lafrance, Yahia, Germain, & Auger, 1998; Lanir, 1979; P. Tong & Fung, 1976). After transplantation of these full thickness skin constructs, histological analysis of the transplant sites suggested a discontinuous lamina densa

and decreased anchoring fibrils up to 148 days after the surgery (Woodley et al., 1988). At this time, it was generally understood that the basement membrane played an important role in wound repair, but these studies and others demonstrated its importance in transplant stability (Chetty, Boissy, Warden, & Nordlund, 1992).

Decellularization was commonly used for creating mechanical allogeneic transplants which did not require immunosuppression (Kreuz, Hyatt, Turner, & Bassett, 1951; Mellonig, 1984, 1991). The first decellularized ECM scaffold material to achieve acceptance by orthopedic surgeons was a decellularized porcine small intestine mucosa (SIS-ECM) graft which was used to replace tendons (Dejardin, Arnoczky, & Clarke, 1999; Dejardin, Arnoczky, Ewers, Haut, & Clarke, 2001). Building on this, groups attempted to replace the hydrogel-based artificial dermis used in early full thickness skin constructs with decellularized ECM. The Wainwright laboratory developed the first topical clinical application of decellularized skin as a wound dressing for burns in 1995 (Wainwright, 1995). It was found that cells repopulated these matrices quickly and reconstituted the basic structural niches effectively. It was hypothesized that decellularized matrices may provide cues to cells which would direct and support their differentiation (Badylak, 2004). Further work in this field demonstrated that decellularized skin matrices promoted wound healing and the rapid creation of a new epidermal layer (Armour, Fish, Woodhouse, & Semple, 2006; Kimuli, Eardley, & Southgate, 2004). It was proposed that reconstituted tissues made from autologous cells seeded onto a cadaver-derived or xenogenic matrix would have two key advantages. First, because components of the ECM are conserved across species and the cells which present foreign antigens have been removed, the decellularized matrix is not in and of itself immunogenic, which had been borne out in transplantation studies (Bannasch, Stark,

Knam, Horch, & Fohn, 2008; Bernard, Chu, et al., 1983; Bernard, Myers, et al., 1983; Constantinou & Jimenez, 1991). Further, it was found that decellularized ECM maintains mechanical cues and low levels of embedded growth factors which induce rapid repopulation of the tissue with host cells (Reing et al., 2010). It was later found that these wound dressings were also capable of supporting cell growth and differentiation of cells *ex vivo*, permitting the construction of cellularized tissue-engineered grafts (Armour et al., 2006; Bannasch et al., 2008; Prasertsung, Kanokpanont, Bunaprasert, Thanakit, & Damrongsakkul, 2008). This led several groups to use explore the use of decellularized matrices as scaffolds for engineering complex tissue.

Reconstitution of Tissue using Decellularized Matrices as a Scaffold

Decellularized matrices provide structural and ECM cues to cells cultured on them, but they also afford another layer of information for reconstituting tissue. Several groups have designed bioreactors which, when attached to decellularized matrices, recapitulate the rhythmic shear and stretching forces that are important in determining cell fate (Bonvillain et al., 2012; Bonvillain et al., 2013; H. C. Ott et al., 2010; T. H. Petersen, Calle, Colehour, & Niklason, 2011; T. H. Petersen et al., 2010b; Price et al., 2010; Song et al., 2011).

In 2010, the Niklason lab found that decellularized lung matrices preserve the various structural niches and support embryonic stem cell (ESC) growth into tissue. They further found that this tissue could be transplanted after a short period of incubation in a bioreactor and could participate in gas exchange (T. H. Petersen et al., 2010b). Importantly, the decellularization process preserved the extracellular matrix proteins and the ultrastructural niches, which in turn

informed the differentiation of endothelial cells into some vasculature and the ESC cells formed some components of lung epithelium. While these lungs were transplantable and functioned after transplant in a rat lung lobe model, they only functioned for a few days before becoming fibrotic. In later optimizations of the protocol, a transplanted lung lobe could survive for up to two weeks before becoming fibrotic (Song et al., 2011). Clearly, there are several issues to overcome in improving the creation of tissue-engineered lungs for transplantation.

Cystic Fibrosis

Beyond the development of tissue for transplantation, engineered lung tissue permits the study of complex diseases which are not well recapitulated in animal models. CF is one such disease, as there is no mouse model of spontaneous cystic fibrosis lung disease (Z. Yan et al., 2015). CF is a recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes an anion channel regulated by ATP hydrolysis and phosphorylation (Riordan et al., 1989; Rommens et al., 1989). CF is one of the most common recessive genetic disorders in Caucasians (Stoltz, Meyerholz, & Welsh, 2015). It is caused by mutations in the CFTR gene, which was first cloned and characterized in 1989 (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989) . CFTR is expressed in ciliated cells lining the surface epithelium and submucosal gland ducts and in the serous cells of the submucosal glands (Engelhardt, Zepeda, Cohn, Yankaskas, & Wilson, 1994). This disease causes chronic bacterial infections, lung inflammation, and the creation of thick mucus plugs in the lung. The mechanism by which this mutation causes lung disease is not fully known. While it was discovered early that bacterial infections were caused by abnormal alveolar fluid secretion,

the reasons for why loss of chloride channel function leads to formation of this fluid are still unclear (Smith, Travis, Greenberg, & Welsh, 1996).

Primary patient derived cells quickly lose their ability to differentiate and express CFTR *in vitro* after 2 or 3 passages, limiting their utility for drug screening or other applications as they must constantly be re-derived from patient samples (Suprynowicz et al., 2012). Additionally, there are no mouse models of CF which model the disease in humans. CFTR knock-out mice do not spontaneously develop lung disease and instead have extreme intestinal mucus blockage as they have a compensatory chloride channel in the lung (Clarke et al., 1994; Grubb, Paradiso, & Boucher, 1994). While there are pig and ferret models of this disease (Z. Yan et al., 2015), they are expensive to maintain and very rapidly develop bacterial infections which make study of the causative events of cystic fibrosis *in vivo* difficult. *In vitro* drug discovery efforts for this disease use air-liquid interface (ALI) primary cells or cell lines that are cultured on a semipermeable membrane to evaluate the ability of compounds to restore CFTR function by measuring the ability of the resulting pseudostratified epithelium to generate current in response to forskolin-triggered increases in c-AMP. Patients heterozygous for CF mutations do not generally display symptoms (Abeliovich et al., 1992). This is in large part because as long as only a fraction of epithelial cells have functional CFTR channels, they can create a sufficient chloride gradient to produce osmotic pressure. This in turn implies that if a patient with defective CFTR has only a subset of cells with functioning CFTR because of cell or gene therapy, they would attain a normal phenotype. This makes CF a very attractive target for gene and cell therapy (Arthur, 2015; Gui, Qian, Rocco, Grecu, & Niklason, 2015; Hawkes, 2015). There is some controversy over the exact percentage of cells which would need to be corrected to cure a CF patient; while a study performed by Johnson et. al reported expression of CFTR in 6-10% of airway epithelia is

sufficient to restore the function of chloride epithelium (Johnson et al., 1992), other studies have indicated that correction takes modification of 25% the lung epithelium (Armstrong, Cunningham, Davies, & Alton, 2014; Davies, Nunez-Alonso, McLachlan, Hyde, & Gill, 2014; Griesenbach, Pytel, & Alton, 2015; Kumar et al., 2015; Schwank et al., 2013). Further, because the lung epithelial cells to target are exposed to the airway, application of gene-editing agents could be applied directly to the lung epithelium via aerosol or other topical strategies (Oakland, Sinn, & McCray, 2012). There have been some efforts to perform cell therapy to correct CFTR, which have had limited success (Gui et al., 2015; Loi, Beckett, Goncz, Suratt, & Weiss, 2006; Oakland et al., 2012). While experiments have shown considerable promise, they are often hampered by having to rely on large animal models. Developing a tissue engineered construct to test these correction strategies could significantly speed their development by permitting optimization before moving to expensive pig studies. To do this, though, the field needs a source of primary cells which can be expanded into sufficient numbers while retaining differentiation function to use in tissue engineering experiments.

Sources of Cells for Tissue Engineering Applications

Tissue engineering at the scale of a human lung requires billions of cells. The ideal source of cells for regenerative medicine or tissue engineering applications would be non-immunogenic, amenable to expansion in tissue culture, and capable of differentiating into multiple cell lineages (Gilpin, Charest, Ren, Tapias, et al., 2016). Induced pluripotent stem cells (iPSCs) were first generated by the Yamanaka group in 2007 (Okita et al., 2007). These cells are very valuable for tissue engineering experiments because of their pluripotency and capacity for multiple population doublings. In particular, they have made it possible to create tissue which

was previously very difficult to generate from adult stem cells such as differentiated cardiac cells (Eng et al., 2016) and neurons (D'Aiuto et al., 2014). Because this technique can take human skin fibroblasts and turn them into stem cells which are immortal and pluripotent, some groups have investigated seeding these cells into decellularized matrices, and have successfully gotten them to differentiate into lung epithelium while cultured on a lung matrix (Ghaedi et al., 2013; Huang et al., 2014). The ability of iPSCs to generate large numbers of cells which would otherwise be difficult to produce without using embryonic stem cells has facilitated significant advances in tissue engineering and our understanding of developmental biology. There are some concerns regarding using iPSCs for clinical applications because of reports of genetic instability and off target differentiation (Peterson & Loring, 2014). There is research being performed to overcome these challenges, such as the development of integration free iPSC reprogramming methods (Sharma, Wu, & Wu, 2013). However, in tissues which have abundant populations of stem cells and rapid turnover, such as the lung (Evans, Van Winkle, Fanucchi, & Plopper, 2001), an attractive option would be to culture those adult stem cells without reprogramming them, preserving their capacity to differentiate, and using them immediately after expansion for tissue engineering applications.

Conditionally Reprogrammed HBECs as a Tissue Engineering Reagent

Conditional reprogramming of epithelial cells with Rho-associated coiled kinase (ROCK) inhibition and co-culture with irradiated 3T3 cells was first developed by the Schlegel laboratory (Palechor-Ceron et al., 2013). While this technique can extend the lifespan of epithelial cells, there has been some controversy as to whether cells that are life-extended with this technique retain the ability to differentiate after a long period of cell culture. Further, how these life-

extended cells are able to survive in culture for so many passages without overexpression of hTERT to overcome telomere shortening-related senescence is unknown. The Shay/Wright lab's optimization of this technique has recently allowed the preservation of human bronchial epithelial cells to differentiate after extended time in cell culture (paper in preparation). This now provides an effectively unlimited number of otherwise "normal" cells which can be used to study lung biology and disease in an unaltered genetic background.

Applications for Tissue Engineered Lungs

The applications of lung tissue engineering can be put into two categories. The ultimate long- term goal of tissue engineering lungs is to address the critical shortage of transplantable lung tissue; as there is presently no way to treat chronic obstructive lung diseases except by lung transplantation (H. C. Ott et al., 2010). Further, because of the complexity of the lung matrix, diseases of the lung are very difficult to simulate *ex vivo*. Preliminary work in simulating disease in decellularized lungs have produced valuable insights into cancer, where perfusable tumor modules have been shown to produce transcriptional profiles that resemble tumors *in vitro* and produce circulating tumor cells which develop into metastatic tumors and demonstrate drug resistance not seen in 2-D culture of the same cell populations (Mishra et al., 2014; Vishnoi, Mishra, Thrall, Kurie, & Kim, 2014). Further, culture of cells on decellularized lungs of human patients with fibrotic diseases have demonstrated that the lung matrix of patients with fibrotic diseases induce apoptosis in otherwise normal cells *in vitro* through an uncharacterized mechanism (Booth et al., 2012). The work outlined in this thesis represents initial steps towards these two eventual goals of the field of lung tissue engineering.

CHAPTER TWO
Reconstituting Mouse Lungs with Conditionally Reprogrammed Human Bronchial Epithelial Cells

Abstract

Engineering reconstituted lungs using primary human lung stem-like cells would be a desirable approach for creating transplantable tissue, but primary human bronchial basal cells rapidly lose the ability to differentiate during *in vitro* expansion. Further, while basal cells collected from minimally invasive bronchial brushings could be a valuable reagent for tissue engineering, they are only known to differentiate into upper airway cells. As part of this dissertation work, I developed methods for conditionally reprogramming (CR) primary human bronchial epithelial cells (HBECs) to extend their functional life span and permit their differentiation into both upper and lower airway lung epithelium. I also developed a bioreactor to support vascular perfusion and rhythmic breathing of decellularized mouse lungs reconstituted with CR HBECs isolated from patients with and without cystic fibrosis (CF). In reconstituted lung culture, these cells differentiate into upper airway bronchial epithelium and lower airway alveolar structures after 12 days. Conditionally reprogrammed cells only differentiate into an upper airway epithelium after 35 days at the air liquid interface, suggesting that culture in a lung matrix leads to more rapid differentiation of HBECs than air liquid interface culture. These data can be interpreted to indicate that we have developed a novel source of cells for lung tissue engineering that can be isolated from an upper airway biopsy and used to recapitulate the entire airway epithelium.

Introduction

Engineering lungs by seeding native decellularized scaffolds with stem cells is a potential method for addressing the severe shortage of available transplants for organ failure and for studying disease *ex vivo* (Gilpin, Charest, Ren, & Ott, 2016). Decellularized matrices can provide cells with extracellular matrix proteins (Shojaie et al., 2015), mechanical cues (Huh et al., 2010), and ultrastructural architecture (Xiong et al., 2014); all of which influences their differentiation. The lung consists of an array of niches which are very difficult to recapitulate *in vitro* (Irvin & Bates, 2003). Others have reported engineering lungs using decellularized lung scaffolds from rats or larger mammals seeded with embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) (Ghaedi et al., 2013; Song et al., 2011). However, iPSCs generated by some methods have been shown to display genetic instability after large numbers of cell divisions and have been shown to form teratomas after transplantation (Peterson & Loring, 2014; S. M. Wu & Hochedlinger, 2011). As methods for generating iPSCs improve, these challenges may be overcome; but when engineering highly proliferative tissues such as the lung some have proposed that adult stem cells which are expanded in the correct conditions may be a preferred source of cells for tissue engineering (Butler et al., 2016; Gilpin, Charest, Ren, Tapias, et al., 2016).

HBECs are a heterogeneous population of cells containing multipotent basal cells that can be isolated from patients with minimally invasive endobronchial biopsy (Elliott et al., 2012) or bronchial or nasal brushings (Lopez-Souza, Avila, & Widdicombe, 2003). HBEC basal stem-like cells are multipotent and capable of forming airway epithelium, but using currently

established methods these cells rapidly lose the ability to differentiate *in vitro* and have not been demonstrated to differentiate into lower airway cells in *in vitro* or *ex vivo* conditions tested to date (Fulcher, Gabriel, Burns, Yankaskas, & Randell, 2005). If these challenges could be overcome, multipotent HBECs could be used to rapidly engineer transplantable lung tissue derived from a patient's own cells, abrogating the need for lifelong immunosuppression using donor lung transplants.

Primary conditionally reprogrammed (CR) HBECs cultured in the presence of an irradiated fibroblast feeder layer and ROCK (Rho-associated coiled-coil containing protein kinase) inhibitor have a significantly extended lifespan and retain the ability to differentiate in response to culture at an air-liquid interface (ALI) (Suprynowicz et al., 2012). Recently, the Randall laboratory published research showing that culturing HBECs using a technique similar to our reprogramming protocol can extend the functional lifespan of HBECs derived from multiple patients with and without CF while preserving their ability to form an epithelial layer for up to 10 passages; but their work showed that the cells lose much of their capacity to produce cilia after 5 passages and lose the capacity completely by 10 passages (Gentzsch et al., 2016). These cells also demonstrated statistically significant drops in CFTR activity over at 5 and 10 passages in their CR conditions, though CF cells cultured in these conditions at ALI did show responsiveness to treatment by VX-809 by Ussing chamber analysis. Further, it has been shown that cells cultured with this method can be used for CRISPR in one study looking at impact of CRISPR mediated knock-out of Muc18 in HBECs (Chu et al., 2015). However, while CR HBECs have recently been used for reconstituting a tracheal matrix, they have not been shown to

be capable of differentiating into lower airway cells or reconstituting lung tissue (Butler et al., 2016).

The only primary bronchial basal cells known to differentiate into both upper and lower airway cells in a reconstituted lung are distal KRT5+ TP63+ bronchial basal cells; but these cells only survive a few passages and are isolated from cells of the distal airway (Gilpin, Charest, Ren, Tapias, et al., 2016). As primary HBECs are often used to screen for cystic fibrosis (CF) drugs *ex vivo* (Gentzsch et al., 2016) lungs seeded with CR HBECs isolated from a CF patient could represent a novel model for studying this disease *ex vivo*. If CR HBECs differentiate into upper and lower airway cells in response to culture in a lung, it would mean that a patient's airway epithelium could be reconstructed from their own cells isolated using a minimally invasive procedure.

In this study, I have created an *ex vivo* reconstituted lung system using CR HBECs seeded into a decellularized mouse lung using a bioreactor with simulated breathing and vascular perfusion. I document methods for decellularizing the murine lung while accessing the vascular and tracheal compartments. Using the bioreactor system, normal human CR HBECs and CR HBECs isolated from a patient with cystic fibrosis (CF CR HBECs) were seeded into decellularized murine lung matrices and maintained for up to two weeks. These multipotent lung-derived cells rapidly reconstitute the upper and lower airway niches and differentiate into a variety of cell types, including type I and II pneumocytes. HBECs have not been previously demonstrated to differentiate into lower airway pneumocytes. Tissue engineering of lungs using a primary adult stem-like cell population with an extended life span would permit iterative

generation of tissue engineered constructs with the same population of non-genetically manipulated multipotent cells. This would immediately facilitate generation of *ex vivo* lungs for the study of disease and ultimately transplantation.

Results

Mouse Lung Decellularization Preserves the Extracellular Matrix

A bioreactor system for decellularization of murine lungs and extended culture of introduced human lung epithelial cells with vascular perfusion and simulated breathing was developed (Fig. 1A). The native murine lung (Fig. 1B, left) was loaded into the bioreactor through cannulation of the trachea and right ventricle with blunted needles. Simulated breathing was performed through a syringe pump withdrawing air from the pressurized main bioreactor chamber, lowering the pressure and drawing liquid or air from the tracheal chamber into the lung via a breathing loop originally developed by TH Peterson *et al* (T. H. Petersen et al., 2010a) while vascular perfusion was performed by circulation of culture medium into the cannulated right ventricle by a peristaltic pump (Fig. 1A).

After decellularizing the lung in this bioreactor system for 3 hours by vascular perfusion with a CHAPs solution followed by a 1hour DNAse wash, the resulting tissue takes on a “ghost-like” or translucent appearance as seen by others (H. C. Ott et al., 2008) (Fig. 1B, right). A comparison of hematoxylin and eosin (H&E) staining of native (Fig. 1C, left) versus decellularized mouse lungs (Fig. 1C, middle) demonstrates that while the cells have been removed from the lung matrix, the extracellular matrix (ECM) structure remains largely

preserved with no murine cellular regrowth even after the decellularized lung has been cultured for 12 days in the bioreactor system (Fig. 1C, right).

Decellularization of the murine lung with this protocol leaves less than 50ng of DNA/mg of lung tissue after 12 days in culture conditions in a bioreactor, conforming with generally accepted tolerances for decellularization of tissue for tissue engineering applications (Crapo, Gilbert, & Badylak, 2011) (Fig. 1D). In optimizing this protocol, I found that decellularization for 2 hours was not sufficient to fully decellularize the lung matrix while decellularization for 5 hours with the CHAPs solution had no additional effect on the amount of residual DNA present in the matrix (Supp. 1). Decellularization is known to remove a significant percentage of lung ECM proteins which are essential for the mechanical function of the lung and epithelial cell attachment and spreading (Brown & Badylak, 2014; Faulk et al., 2014; Reilly & Engler, 2010). Immunofluorescence staining of ECM proteins in the decellularized lung shows retention of elastin but some loss of fibronectin (Bonenfant et al., 2013) (Fig. 1E). To account for this loss of fibronectin, I supplement the lung with fibronectin 1 hour before reconstituting the lung. These results indicate that the decellularization protocol produces a mouse lung matrix with preserved architectural and most ECM components with no intact murine cells remaining viable to repopulate the lung over 12 days.

Lung Mechanical Integrity is Largely Preserved with Reduced Stiffness After Decellularization

Testing preservation of vascular access to the lung and biomechanical properties of the matrix after decellularization was necessary for determining the integrity of the mouse lung matrix for tissue engineering applications. In this bioreactor system, a blunted 20 1/2-gauge

needle was secured into the right ventricle to gain access to the mouse lung vasculature (Fig. 2A). The lung was then perfused with Evan's blue dye through the right ventricle to evaluate access to the vascular compartment after decellularization. The Evan's blue dye perfused through the right ventricle permeated the lung's vascular compartment without visible leakage, demonstrating that the decellularized vascular compartment could hold imposed hydraulic pressure over time (Fig. 2B). I next evaluated lung elasticity (Young's modulus) and tensile stiffness after decellularization by measuring the load/stress of strips of the lung matrix to uniaxial deformation/strain as previously described (O'Neill et al., 2013). While the lung remained intact and held tensile stress (up to 16 kPa) after decellularization, the Young's modulus and tensile stiffness were reduced by 48% and 54%, respectively (Fig. 2C); this could be due in part to lack of a cellular component. In summary, the described mouse lung decellularization protocol and bioreactor system accesses both the tracheal and vascular compartments while preserving their mechanical integrity.

Conditionally Reprogrammed HBECs Rapidly Reconstitute Mouse Lungs

Investigations of lung diseases such as CF are commonly performed with primary human bronchial epithelial cells (HBECs) cultured at the air liquid interface (ALI) by culture on a microporous membrane (Transwell^{TM}) (R. Wu, Zhao, & Chang, 1997). So far there are no studies comparing the dynamics of murine lungs reconstituted with HBECs to HBECs cultured at the ALI. In addition, I sought to determine if CR HBECs would respond to the decellularized murine lung niche by producing both upper and lower airway cells. Thus, I utilized two human cell strains isolated from primary tracheal/bronchial tissue; one derived from a patient free of

lung disease, and the other from a patient with CF caused by a homozygous Δ-F508 mutation in the CFTR gene. I first determined that both populations can divide past 50 population doublings *in vitro* using the CR conditions, but senesce much earlier in standard lung epithelial cell culture conditions (Supp. 2).

Reconstitution of decellularized mouse lungs was next performed with 10 million PD 30 CR HBECs. In order to account for the loss of fibronectin in our decellularized mouse lungs, the lungs were coated with fibronectin for 1 hour before seeding them with CR HBECs, which improved their initial attachment and differentiation in the lung. Over the course of culture in the bioreactor the lungs were fed with BEGM containing no ROCK inhibitor or IR J2 3T3 feeder layer. After 3 days in culture the lungs were switched to ALI BEGM media, which is supplemented with additional $CaCl_2$ and retinoic acid. After 4 days of culture in the bioreactor, lungs seeded with CF or wild type (WT) CR HBECs showed only partial differentiation into lung epithelium. However, after 12 days of culture in a bioreactor, H&E staining of recellularized mouse lungs seeded with WT and CF CR HBECs morphologically showed the formation of alveolar type cells in the lower airway niche and ciliated pseudostratified epithelium in the upper airway compartment that contained basal and goblet appearing cells (Fig. 3A). In optimizing the seeding protocol, I found that removing conditional reprogramming conditions from the CR HBECs 7 days before seeding them into lungs produced an exaggerated columnar upper airway epithelium with no basal cells and no lower airway epithelium; suggesting that the reprogrammed state of CR HBECs is essential to their capacity to differentiate into lower airway cells (Supp. 3). Seeding the murine lung with either 1 or 4 million CR HBECs and culturing them for 12 days produced reconstituted lungs which did not display

well differentiated epithelium, underscoring the necessity of the proper ratio of CR HBECs to lung matrix surface area for proper differentiation (Supp. 4). From a morphological standpoint, this indicates that CR HBECs respond to signals in the reconstituted murine lung system by forming both upper and lower airway niche-appropriate structures.

Immunofluorescence staining was then used to evaluate the epithelial cell lineages that CR HBECs differentiate into after culture in a reconstituted lung for 12 days. Recellularized murine lung upper airways were observed to contain cytokeratin 14 (CK14) positive basal cells, α tubulin positive cilia buds, MUC 5B positive goblet cells, and Clara cell secretory protein (CCSP) positive club cells (Fig. 3B). CR HBECs in the lower airways stained for aquaporin 5 (AQP5) and pro-surfactant protein C (Pro-SPC), indicating their differentiation into alveolar type I and II cells respectively (Fig. 3B). To further compare CR HBECs cultured in 2D, ALI, and in reconstituted lungs, Western blotting for AQP5, CK14, Pro-SPC, and CCSP was performed. CK14, an epithelial basal stem cell marker, is high in 2D culture and ALI culture. This is to be expected as CK14 positive basal cells are highly represented in pseudostratified epithelia. AQP-5 and Pro-SPC, markers of alveolar type I and II cells respectively, are more abundant in reconstituted lungs compared to ALI or 2D cultures which do not have peripheral lung differentiated markers. CCSP, a marker of terminal bronchioles, is found in 12-day reconstituted lung cultures but not in 35 day ALI cultures (Fig. 4C). These results can be interpreted to indicate that CR HBECs are multipotent and have the capacity to differentiate into both upper and lower airway cells when presented with the appropriate niches.

Multipotency and Clonogenicity of Conditionally Reprogrammed HBECs

If CF CR HBECs are able to support clonal selection *in vitro* while maintaining their capacity for differentiation, these cells may permit modeling of gene therapy or genetic manipulation in tissue engineered systems. At ALI, CR HBECs fail to undergo terminal differentiation into a pseudostratified epithelium in 12 days; indicating that these cells remain in an undifferentiated state (Fig. 4A). After culture at ALI for 35 days, CF CR HBECs form a pseudostratified epithelium displaying both ciliated cells and mucus producing goblet cells as detected by immunofluorescence staining for acetylated alpha tubulin and Muc 5B respectively (Fig. 4B). I next isolated individual cultured CF CR HBEC clones and cultured them at an air-liquid interface for 35 days. These clones produced a comparable pseudostratified epithelium displaying both ciliated and goblet cells (Fig. 4C) demonstrating that single CR HBECs are multipotent. To our knowledge this is the first successful differentiation of CR HBEC clones at the ALI. These results indicate that CF CR HBECs can tolerate clonal selection while maintaining their ability to differentiate. This makes them a valuable cellular resource for studying genetic correction approaches of diseases in tissue-engineered models. To further demonstrate that these cells are capable of simulating cystic fibrosis, we determined their ability to respond to differentiation at ALI by producing CFTR mRNA. To this end we performed ddPCR on these cultures after 3, 4, and 5 weeks of culture; finding that these cells were capable of producing CFTR protein between 3 and 5 weeks in differentiation conditions (Fig. 4D). Importantly; while these CF HBECs do form CFTR protein, this CFTR protein is non-functional because of the delta-F508 mutation. Being a class II trafficking error in the CFTR protein, this protein is never transported to the cell membrane where it could be used to perform chloride transport. These results indicate that CR CF HBECs can tolerate clonal selection while

maintaining their ability to differentiate, produce CFTR in response to differentiation conditions, and differentiate in response to culture in the lung which may make them a valuable tool for studying genetic correction of diseases such as cystic fibrosis in tissue-engineered models.

Discussion

In this investigation, I have used a novel conditional reprogramming protocol and mouse lung bioreactor system to recapitulate the upper and lower airway epithelium in a decellularized lung within only 12 days. Overcoming the small size and fragility of the mouse heart-lung block by cannulating the right ventricle to access the pulmonary vasculature, the present studies demonstrate the feasibility of using lung matrices derived from the large array of murine disease models of lung disease. Conditionally reprogrammed HBECs address an unmet need in tissue engineering for both disease modeling and transplantation; a source of primary patient derived tissue that can be rapidly expanded from a sample into sufficient numbers of cells to create engineered tissue without losing the ability to differentiate (Mendez, Ghaedi, Steinbacher, & Niklason, 2014). Further, to the best of my understanding, this is the first study to demonstrate the ability of HBECs to differentiate into lower airway type I and II cells; a trait previously only associated with lung stem cells isolated from the distal bronchial-alveolar junction (Kajstura et al., 2011). That our tissue culture method preserves the ability of these cells to different into pneumocytes (type I and type II alveolar) cells has inherit value; this is normally a difficult trait to maintain in lung stem cells (Huang et al., 2014). The isolation and culture of alveolar type I and II cells on plastic *in vitro* has been found to be very difficult; in culture these cells quickly senesce or undergo phenotypic drift and cannot be subcultured and expanded (Gonzalez et al., 2005). My HBEC population, which contains basal cells, follows the ability of basal stem cells to

differentiate towards distal lung phenotypes when properly induced (Vaughan et al., 2015). Generation of type II cells from our primary HBECs can in turn permit studies of how the resulting AEII cells, which have been found to be the stem cells of the lower airway (Barkauskas et al., 2013; Whitsett, Wert, & Weaver, 2010), respond to mutations which cause diseases of the lower airway caused by CRISPR in an otherwise controlled *ex vivo* environment. This population of life extended stem cells capable of differentiating into lower airway cells may also allow modeling of surfactant diseases such as congenital surfactant deficiently syndromes which are presently extremely difficult to model without freshly isolated embryonic stem cells (Whitsett et al., 2010).

The scalability of the methods described in this report may permit more rapid optimization of conditions for reconstituting lungs *ex vivo*. In designing our decellularization method, I focused primarily on using CHAPs as the decellularization solution. Other laboratories have demonstrated that CHAPs effectively decellularizes lungs while preserving the ECM ultrastructure and protein components (Crapo et al., 2011; Faulk et al., 2014; Huang et al., 2014; Keane, Swinehart, & Badylak, 2015; Reing et al., 2010; Wagner et al., 2014). While the Ott laboratory has used a decellularization technique which combines use of SDS decellularization with a Triton-X100 wash (Gilpin, Charest, Ren, Tapias, et al., 2016), they have only used this in rats and larger mammals. In further studies, it may be worth comparing the mouse lung matrices produced using an adoption of the Ott decellularization protocol in our bioreactor system to the CHAPs decellularization method I have been using. During our optimization of the decellularization process, I found that CHAPs based decellularization dramatically reduces the amount of fibronectin in the matrix, which has been observed by other laboratories (Wallis et al.,

2012). Fibronectin is a particularly important component of the ECM. Fibronectin plays a crucial role in mediating the communication between ECM and cells; binding to several integrin and non-integrin ECM receptors on cells and to both collagen and proteoglycans (Roman, 1997). It is rich in the RGD subunit, a tripeptide that is important in cell adhesion via the a5b1 integrin (Helfrich, Nesbitt, Dorey, & Horton, 1992). Importantly, fibronectin, along with laminin, plays a critical role in the binding of cells to decellularized matrices where it is depleted (Lecht et al., 2014). Taking this into account; I hypothesized that supplementing the lungs with fibronectin would improve the attachment of CR HBECs to our lung cultures. Further optimization of this supplementation with laminin, and other ECM components may further improve upon our recellularization results.

The broad array of genetically engineered mouse models and tools available in mice afford much more flexibility and utility compared to rat lungs, which have comparatively fewer genetic tools and disease models. Previous studies have demonstrated that matrices derived from decellularized lungs isolated from patients with chronic obstructive pulmonary fibrosis or idiopathic pulmonary fibrosis (IPF) have altered mechanical properties and that normal lung cells do not survive on these diseased matrices (Booth et al., 2012; Wagner et al., 2014). While other murine lung bioreactor systems have been developed (Godin et al., 2016), they do not support vascular perfusion of media or cells in the bioreactor, which may prevent recapitulation of a vascular endothelium, simulation of vascular delivery of compounds to the tissue engineered construct, or the collection of circulating tumor cells seen in 4D rat lung perfusable tumor models (Mishra et al., 2014; Vishnoi et al., 2014). Utilizing mouse models of lung diseases such as IPF may allow investigators to examine the role of fibrotic or damaged matrices on cell

behavior in the context of simulated breathing and vascular perfusion. These studies will be particularly important for developing gene and cell therapy applications in a controlled *ex vivo* environment. This experimental system provides a format for elucidating the causes of lung disease, and separating lung epithelial cell autonomous effects from those of the ECM.

The improved CR HBEC culture protocol was based on the initial work done to reprogram epithelial cells through small molecule inhibition of Rho-Associated Coiled Kinase (ROCK) and co-culture with irradiated J2 strain of 3T3's (Palechor-Ceron et al., 2013). Inhibition of ROCK prevents the phosphorylation of myosin light chain and the formation of stress fibers, which can contribute to cell survival in culture (G. Chen, Hou, Gulbranson, & Thomson, 2010; Jaffe & Hall, 2005). Stress fibers can form when cells are cultured on substrates with high stiffness; causing apoptosis which can be inhibited by small molecule ROCK inhibition (Y. Zhou et al., 2013). The mechanism by which co-culture with irradiated J2 3T3 cells and ROCK inhibition interact to produce the CR effect has not yet been fully characterized, however, it may be that CR HBECs are capable of clonal selection because of ROCK's role in anoikis; by abrogating this cell death pathway I may be permitting the cells to divide without the cell-cell contact normally required of epithelial cells (Samuel & Olson, 2010).

Importantly, this work may also be significant for cell therapy for chronic lung diseases. The bulk of research and clinical trials for cell therapy for chronic lung diseases has focused on immunomodulation by mesenchymal stem cells applied through the vasculature, and almost no work has been done with cell therapy using epithelial cells or by delivering cells directly to the airway epithelium (Weiss, 2014). However, a very recent study has shown that bone-marrow

derived stem cells delivered to the lung epithelium in mice can restore CFTR function (Duchesneau et al., 2017). As one of the major barriers cited by this study to lung stem cell therapy is poor engraftment and survival rates, it may be that CF HBECs could be taken by from a patient by bronchial brushing, expanded and gene corrected using the CR protocol and CRISPR, and then re-introduced via the airway.

One of the confounding factors facing the study of CF *in vivo* is that mouse models of the disease made by knock-out of CFTR do not spontaneously develop lung disease, as they have an alternate chloride channel that is not present in human lung epithelial cells (Clarke et al., 1994; Kent et al., 1996). This CR HBEC culture method may permit rapid simulation of genetic correction of CFTR in patients; which has only been done thus far using cell lines in organoid culture systems (Schwank et al., 2013). Tissue engineered lungs made from HBECs isolated from patients with cystic fibrosis should allow for simulating the behavior of CF HBECs in a lung context without chronic inflammation or infection, which are common confounding factors in the study of CF in animal models (Z. Yan et al., 2015).

The only cure for most chronic lung diseases, including chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, cystic fibrosis, and primary pulmonary hypertension, is transplantation, but there is a significant shortage of such donor lungs (Kreider & Kotloff, 2009; Nunley, Bauldoff, Holloman, & Pope-Harman, 2009; Valapour et al., 2015; Yusen et al., 2016). Even when there is access to donor lungs, because they are an allogeneic transplant, the patient must be on immunosuppression drugs for the rest of their lives. These regimes significantly harm the long-term outcomes of patients . Unfortunately, the supply of these allogeneic lung

transplants is not sufficient to meet demand; the number of patients added to the waiting list for lung transplants regularly exceeds the number of transplants performed annually (Valapour et al., 2015). Further, these transplanted allogeneic lungs have relatively low long-term survival rates, with 53% at 5 years and 31% at 10 years (M. Z. Tong, Johnston, & Pettersson, 2015). This has led the transplantation community to call for transplantation paradigms which reduce or eliminate immunosuppression post-surgery for tissue transplants (Orlando, 2010; Orlando et al., 2010). Reconstituted lungs show promise in transplantation, but using the present methods and cell sources the lungs fail a few weeks after transplantation (Gilpin, Charest, Ren, & Ott, 2016). A scalable lung culture system allows for the optimization of these methods in the context of tissue, by increasing the number of experiments that can be done with a single primary cell lineage. This may in turn permit optimization of lung tissue engineering and the generation of reconstituted lungs for transplantation.

Methods

Culture of Conditionally Reprogrammed HBECs

Primary normal HBECs were a generous gift of the UNC (University of North Carolina Marisco Lung Institute, The CF Center Tissue Procurement and Cell Culture Core). Primary CF HBECs were harvested and cultured from CF lung explant tissue under the UT Southwestern IRB approved protocol No. CR00013395/STU052011020. These cells were cultured in 50/50 Bronchial Epithelial Growth Medium (BEGM) (Lonza) plus DMEM High glucose media (ThermoFisher) supplemented with the full BEGM bullet kit +5% FBS +10uM Rock Inhibitor (RI) Y-27632 (Enzo Lifesciences); in a 10-cm tissue culture dish. Approximately 500K of these cells were seeded in co-culture with 500K of freshly irradiated (30Gy) J2 3T3's. These cells

were cultured at physiological oxygen concentrations as described previously (Wright & Shay, 2006). For ALI culture and culture of reconstituted lungs after 3 days of culture with RI, I used modified ALI BEGM medium prepared as described previously (Y. Chen, Nickola, DiFronzo, Colberg-Poley, & Rose, 2006).

Murine Lung Decellularization

To develop the lung decellularization protocol, I injected 12 mice with 100mg/kg:10mg/kg of a ketamine/xylazine solution and a 250U/kg heparin solution (H3393, Sigma) intraperitoneally prior to surgery. After surgically removing the heart-lung block, the trachea was inflated with 5 mL's of a 50U/mL PBS heparin solution in 1mL intervals, with the lung being allowed to deflate between each interval. The right atrium of the heart was then pierced with a 20 ½ gauge needle and 3mL of a 50U/mL heparin solution was slowly perfused through the pulmonary circulation system. The needle was then replaced with a 20 ½ gauge blunt ended needle and cannulated in place; and then 2mL of the heparin solution was perfused through the lung. The trachea was then cannulated using a 20 ½ gauge blunt needle.

The lung was loaded into a bioreactor containing 200mL of a CHAPs solution (8mM CHAPs, 1M NaCl, 25mM EDTA in PBS) and was placed in 37 degrees C incubator. 1mL of the CHAPs solution was infused into the tracheal compartment every half hour, and CHAPs was perfused through the lung at 1mL/min. After 2, 3, or 5 hours of CHAPs decellularization, four lungs were placed into a new jar containing 200mL of a DNAse solution (30ug/mL Benzoase (Sigma), 1.3mM MgSO4, 2mM CaCl2 in ddH_2O). 1mL of fresh DNAse solution was infused into the tracheal compartment every half hour, and DNAse was perfused through the lung at

1mL/min for 1 hour. The lung was then transferred into 200mL of a PBS+5X antibiotic: antimycotic solution (PBSAA) (Gemini Bio-Products) 1mL of the PBSAA solution was infused into the tracheal compartment, and PBSAA was perfused through the lung at 1mL/min overnight at 37 degrees C. The lungs were then transferred to storage at 4 degrees C until they were ready for use. One hour before use, the lung was inflated with 1mL of a 1mg/mL solution of fibronectin in PBS while suspended in PBS in the bioreactor. The lung was incubated in the bioreactor in a 37 degrees C incubator for one hour before initiating the recellularization protocol.

Murine Lung Pico Green DNA Quantitation

DNA Quantitation of native and decellularized lungs was performed using a Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Eugene, OR) following the manufacturer's protocol. Briefly, after papain digestion, lung tissue isolates were mixed with the PicoGreen reagent. They were then measured via spectrophotometry against a standard curve. The final measurements were normalized to wet tissue weight. The difference between native and decellularized lung DNA content was determined by a two-tailed Student's T test.

Murine Lung Recellularization and Culture

We transferred a decellularized murine lung into a bioreactor with 100mL of BEGM. The lung was infused with 1mL of BEGM in the tracheal compartment, and was vascularly perfused with BEGM at 1mL/min for at least one hour. The lung was then transferred into a jar containing 150mL of BEGM. It was then inflated with an infusion of 1mL of BEGM, and then 10 million PD30 CR HBECs suspended in 2mL of BEGM were slowly infused into the murine lung

immediately after the lung was inflated with 1mL of BEGM. The bioreactor containing the lung was then transferred to a 37 degrees C incubator for 16 hours of overnight static culture to allow the cells to attach. The next morning, the lung began vascular perfusion at 1mL/min. Four hours later I initiated simulated liquid breathing by attaching the tracheal chamber with 50mL of BEGM. Negative pressure induced breathing was performed by withdrawing 7mL of air at 1mL/min from the main tube and then re-infusing that same air over 7 minutes using an automated syringe pump (New Era Pump Systems Inc). For the next 2 days, the lungs would be gassed every day by infusing the primary chamber with 120mL of air. After 3 days of culture, the lung was transferred to new jars containing 150mL of BEGM ALI media for the main chamber and 50mL of BEGM ALI media for the tracheal chamber. The rate of liquid breathing and vascular perfusion were kept constant, and the bioreactors were manually gassed every 24 hours. Media was changed every three days until a total of 12 days of culture had passed. The lung was then taken from the bioreactor; the two right lobes were removed and flash-frozen in liquid nitrogen for protein isolation. The remaining lobe was fixed in 10% neutral buffered formalin.

ALI Culture

ALI culture was performed on human placental collagen IV coated 24 mm Transwell^{TM} permeable (0.4um pore) polyester membrane supports (Costar, Ref 3450) as described previously (Y. Chen et al., 2006). Briefly, 400K CR HBECs were seeded onto transwells with 1mL of BEGM media above the membrane and 2mL of BEGM media below the membrane. The CR HBECs became confluent after 2-3 days; after which point the BEGM media was replaced with 2mL of BEGM ALI media. This media was changed every 2-3 days.

Hematoxylin and Eosin (H&E) and Immunofluorescence (IF) Staining

We performed H&E and IF staining on slides as described previously (Kaisani et al., 2014). In brief; the right lobe of the lung was inflated with 10% neutral buffered formalin (NBF) and then stored overnight in 10% NBF with gentle rocking. The lung lobes were then paraffin embedded, and slides were made with 5 micron thick slices of the tissue. The slices were rehydrated, and underwent H&E or IF staining. Prior to IF staining, antigen unmasking was performed by boiling for 10 minutes in a 10mM sodium citrate buffer. Slides were blocked and incubated overnight with the primary antibody at 4 degrees C. After washing with PBS, slides were incubated with secondary fluorescent antibody for 1 hour at room temperature, followed by a second PBS wash and counterstaining with DAPI. For IF staining I used the following antibodies; Fibronectin: ab2413 (Abcam) (1:1000), Elastin: ab21610 (Abcam) (1:1000), CCSP: AB40B73 (Abcam) (1:1000), AQP5: EPR 3747 (Abcam) (1:1000), CK14: PA5-29608 (Thermo Fisher Scientific) (1:500), Pro-SPC: AB40879 (Abcam) (1:500), Muc5B (sc-20119) (Santa Cruz Biotechnology) (1:500), and acetylated alpha tubulin (T6793) (Sigma-Aldrich) (1:500).

Protein Isolation and Western Blotting

Protein was isolated using lysis buffer (1% Triton-X100, 1mM EDTA, 12mM NaCl in TRIS buffer at 7.4 pH with freshly added protease inhibitor (Roche Product No. 05892791001)). Flash-frozen lung lobes were pulverized with a mortar and pestle. The pulverized lung lobe was suspended in 100uL of lysis buffer and was drawn through a $20^{1/2}$-gauge needle 5 times. The suspension was centrifuged at 21,000 RCF and 4 degrees C for 15 minutes. The supernatant was transferred to a new tube and stored at -80 degrees C. Protein was isolated from ALI cultures by

first washing the membrane with sterile PBS 2 times. 100uL of lysis buffer was added to the well and the nylon membrane was scrapped with the back of a sterile pipette tip. The suspension was collected and the nylon membrane was washed with another 50uL of lysis buffer. The protein suspension was stored at -80 degrees C.

For Western Blotting, I loaded 25ug of the protein lysate for 2D culture and ALI culture and 35ug of protein for native, decellularized, and reconstituted lungs onto a Mini-PROTEAN TGX precast gel (Bio-Rad Cat. 456-1086) and ran the gel at 100V for 30 minutes. After transferring the protein to a PFA membrane using a Trans-Blot Turbo Transfer Pack (Bio-Rad Cat. 170-4157), I used blocking buffer (5% dry milk in PBST) for 1 hour and performed primary and secondary immunostaining as described previously (Delgado et al., 2011). For Western Blotting, I used the following primary antibodies; CCSP: AB40873 (Abcam) (1:1000), AQP5: EPR3747 (Abcam) (1:1000), CK14: PA5-29608 (Thermo Fisher Scientific) (1:500), Pro-SPC: AB40879 (Abcam) (1:500), and Beta Actin (ab8227) (Abcam) 1:1000.

Biomechanical Testing

We assayed the tensile strength and Young's modulus of native and decellularized lungs using an Instron 5848 with a 10-newton load cell. I took a strip of the mouse lung of approximately 9-12 mm in length and 3-6 mm in width and placed them into the load cell after sandwiching each end of the lung strip in sand paper. The sample dimensions were recorded using an area micrometer (with a resolution of 1um) by lightly compressing the sample into a rectangular cross-section. The sample was preloaded to reach the toe region and preconditioned with 6 cycles of 10% cyclic engineering strain (grip to grip) at 1 second per cycle, and then were

loaded until failure. For each lung slice strain was measured as $\varepsilon = dl / l_o$ where ε = strain, dl = change in length, and l_o =Initial length. Stress was measured as $\sigma = F_n / A$ where σ = stress, F_n = applied force and A = Area. The Young's Modulus was measured as the linear regression of stress divided by strain during deformation. The tensile stiffness was measured as a linear regression of $k = F / \delta$ during deformation where k = tensile stiffness, F = applied force, and δ = displacement. The statistical significance of differences between native and decellularized matrix bulk-Young's modulus and stiffness was determined by a two-tailed Student's T test.

Figure 1

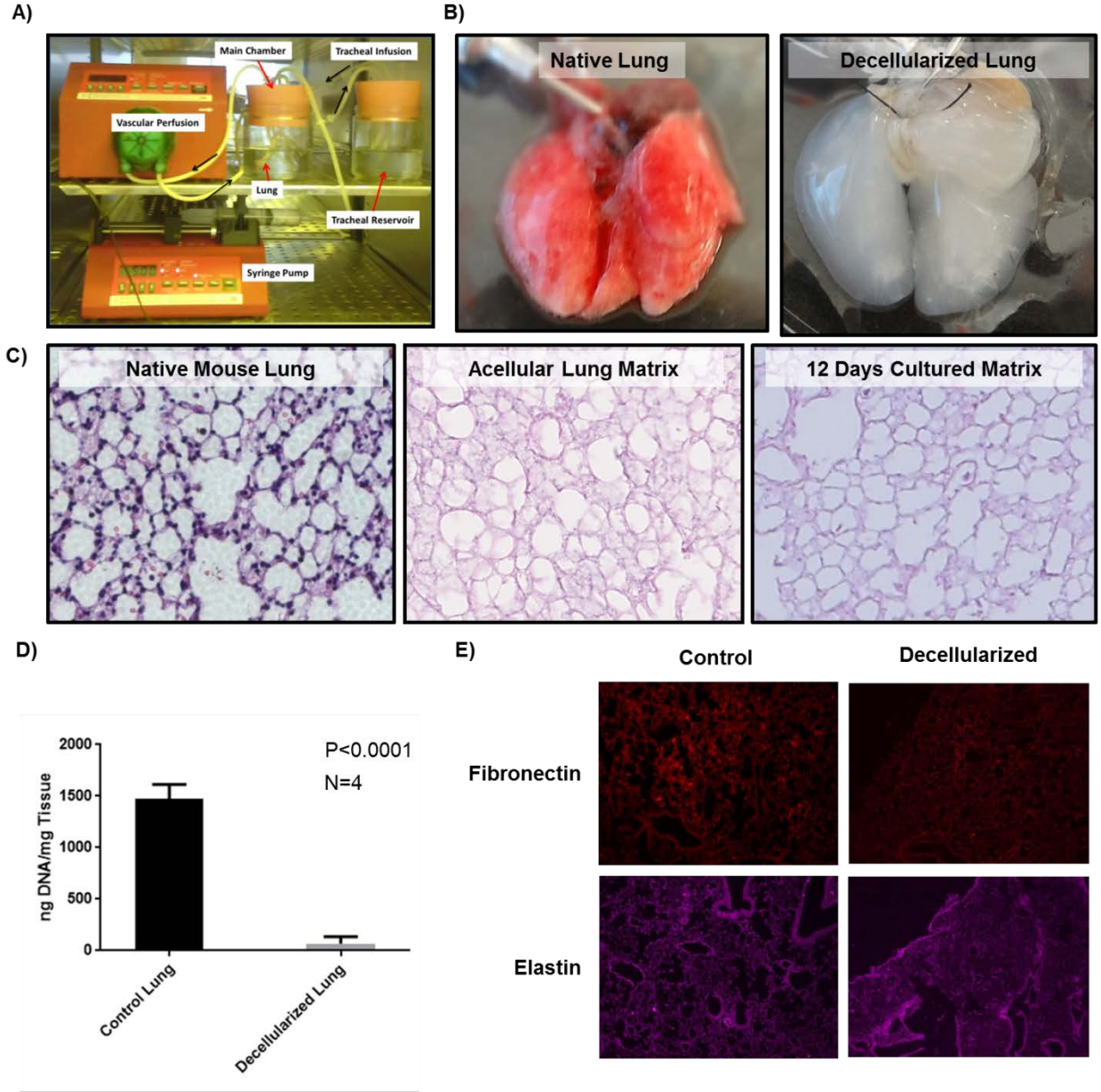


Figure 1: Mouse Lung Decellularization A) The bioreactor system for decellularization of murine lungs and culture of recellularized lungs with vascular perfusion. The Vascular perfusion is effected by a peristaltic pump, while liquid breathing is performed by a programmable syringe pump withdrawing air from the main culture chamber, creating negative pressure which induces the lung to expand. **B)** Comparison of lung and the post-decellularization "ghost lung". **C)** H+E staining comparing a native murine lung to a decellularized matrix and a decellularized matrix cultured for 12 days **D)** Pico-Green DNA quantitation of DNA after decellularization using either 2, 3, or 5 hours of CHAPs perfusion in the decellularization protocol. **E)** IF staining comparing the presence of ECM proteins before and after decellularization in a murine lung.

Figure 2

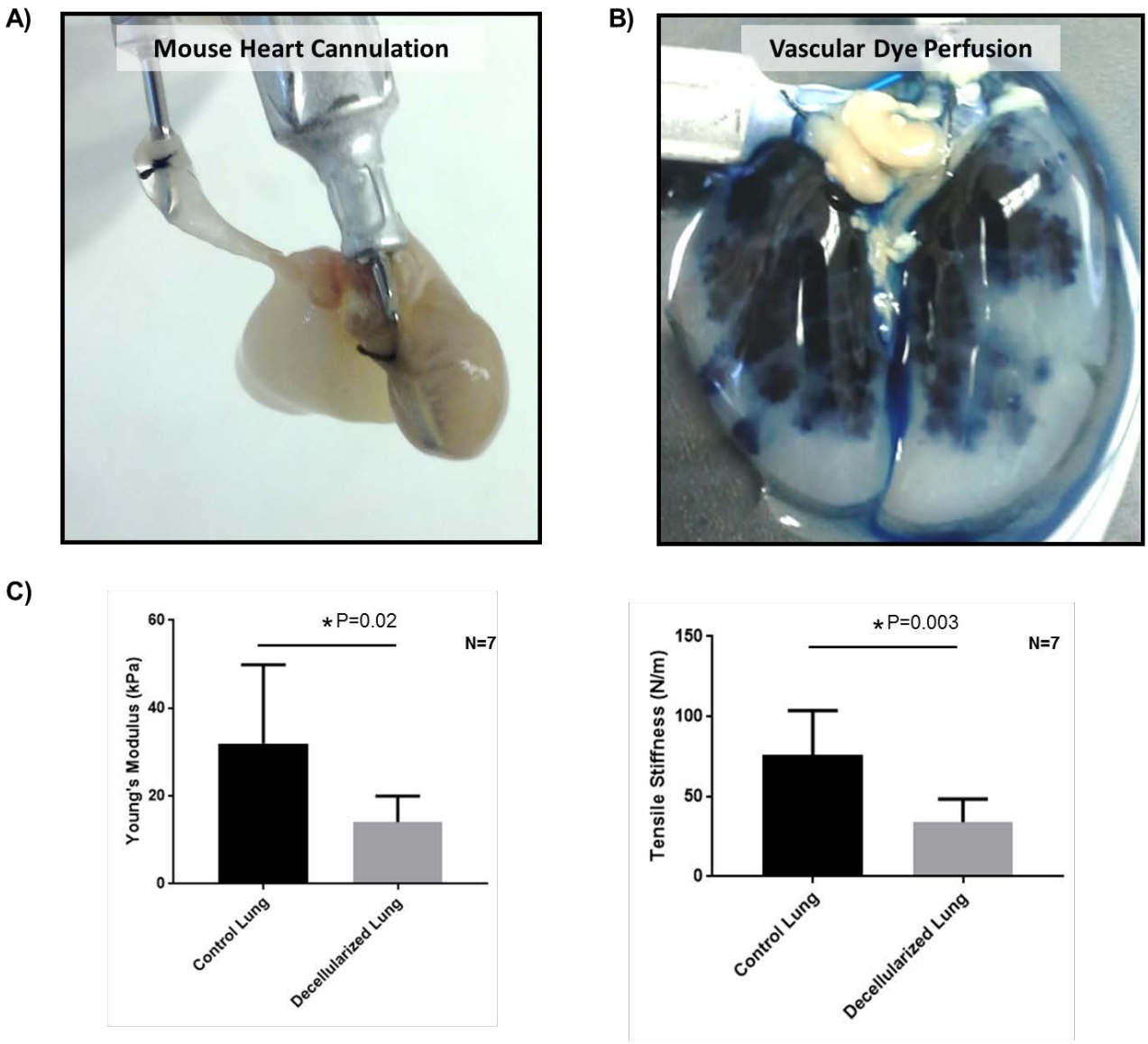


Figure 2: Preservation of Vascular Access and Lung Mechanics After Decellularization **A)** Access to the vascular system of the heart-lung bloc is attained by securing a blunt-ended needle to the right ventricle. **B)** A decellularized murine lung infused with a 15% Evan's blue dye solution in PBS. **C)** Quantitation of the Young's modulus, a measure of elasticity and tensile stiffness of the lung after decellularization as analyzed by analysis of stress/strain.

Figure 3

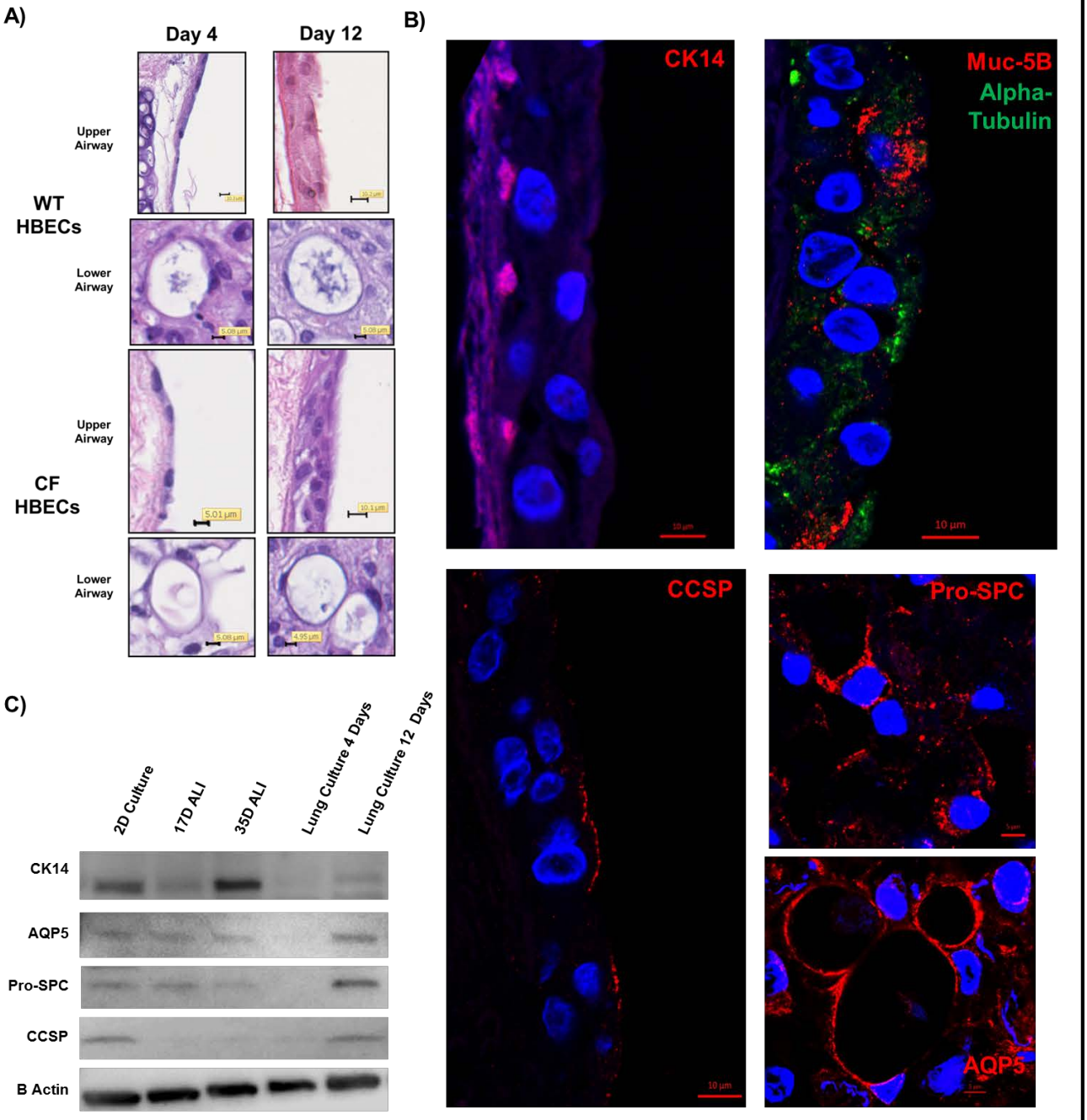


Figure 3: Differentiation of CR HBECs in Reconstituted Lung Culture A) H+E staining of reconstituted lungs seeded with WT HBECs (top four images) and CF HBECs (bottom four images) for 4 days or 12 days. **B)** IF Staining of basal cells (CK14), mucin-producing goblet cells (Muc-5B), ciliated cells (Alpha Tubulin), and Clara cells (CCSP) in the upper airways and with alveoli type I cells (AQP5), and alveoli type II cells (Pro-SPC) in the lower airways in a WT CR HBEC reconstituted lung cultured for 12 days in a bioreactor. **C)** Western blot comparing AQP5, CK14, Pro-SPC, and CCSP protein in WT CR HBECs cultured in 2-D, at ALI for 12 days and 35 days, and in reconstituted lung culture for 4 days and 12 days.

Figure 4

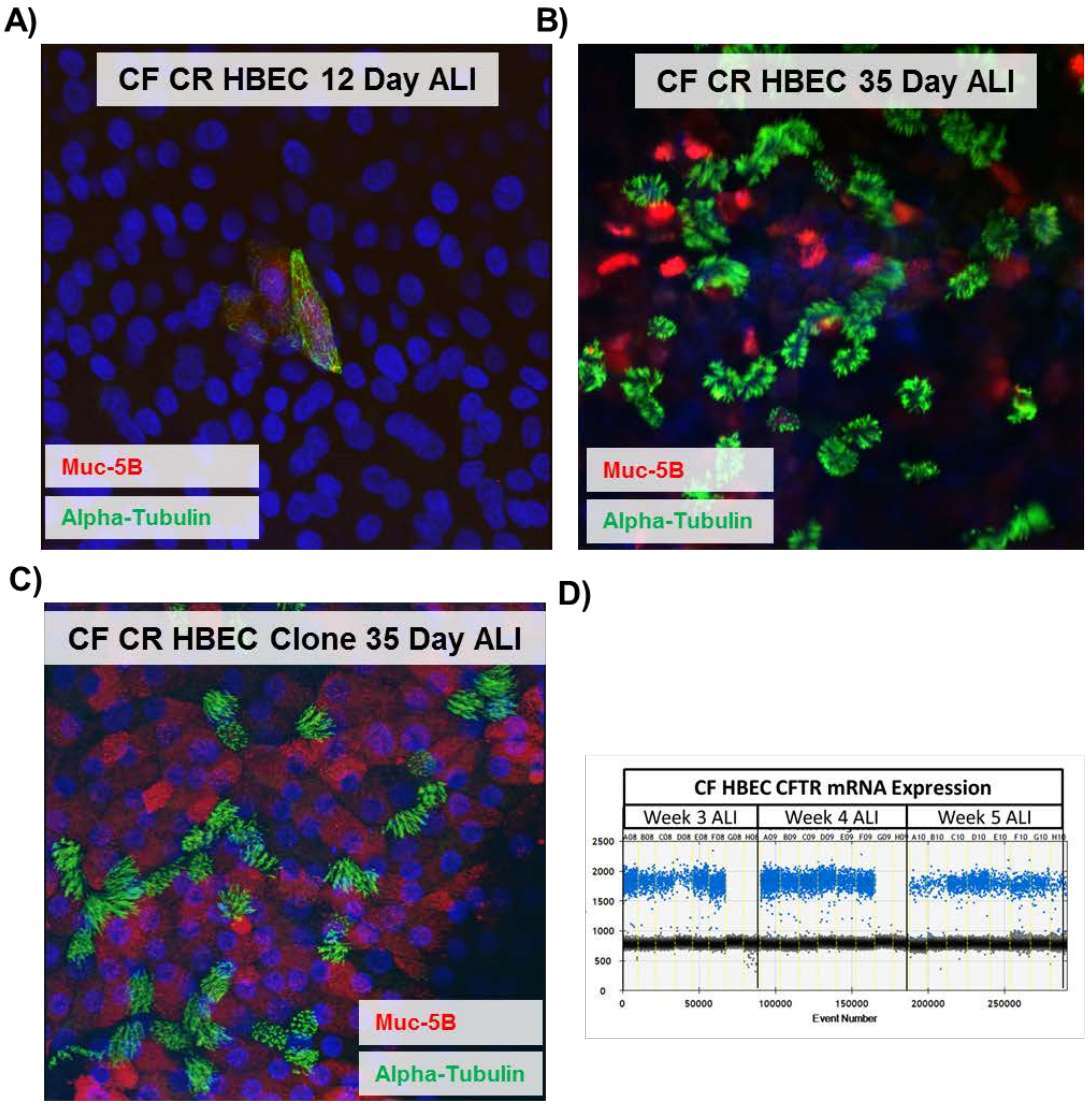
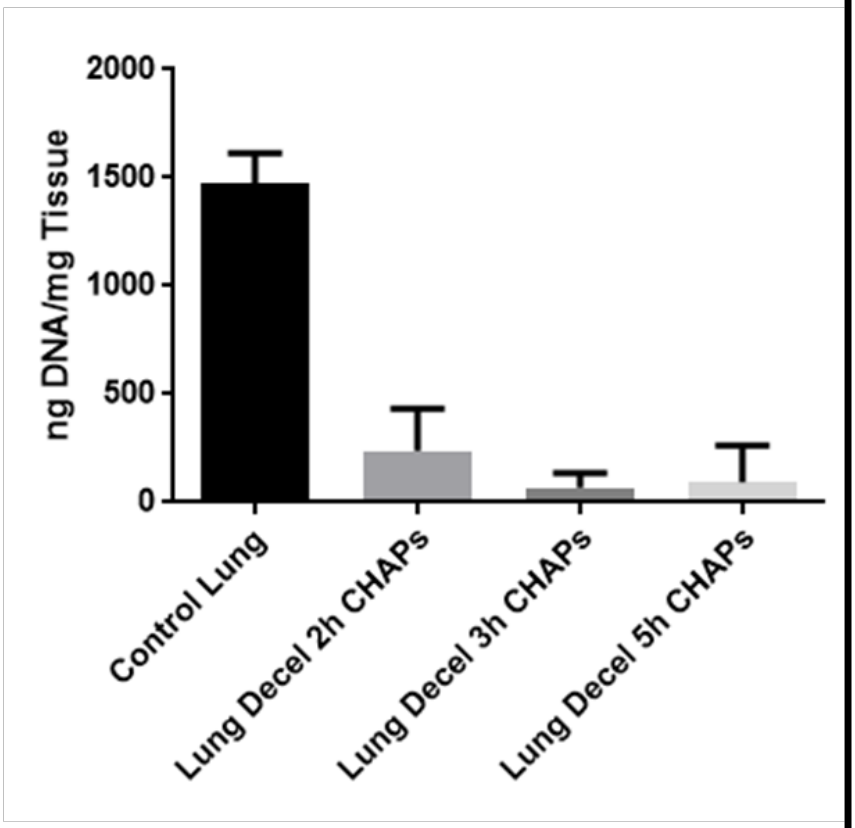


Figure 4: Differentiation and Clonogenicity of CR HBECs at ALI A) IF staining of CR HBECs cultured at ALI in a transwell for 12 days showing a lack of differentiation. **B)** IF staining for ciliated cells (alpha-tubulin) and goblet cells (Mub5-B) CR HBECs cultured at ALI in a transwell for 35 days. **C)** IF staining of a CR HBEC clone cultured at ALI for 35 days. **D)** ddPCR analysis of ALI cultures of CR CFHBECs at 3, 4, and 5 weeks ALI culture.

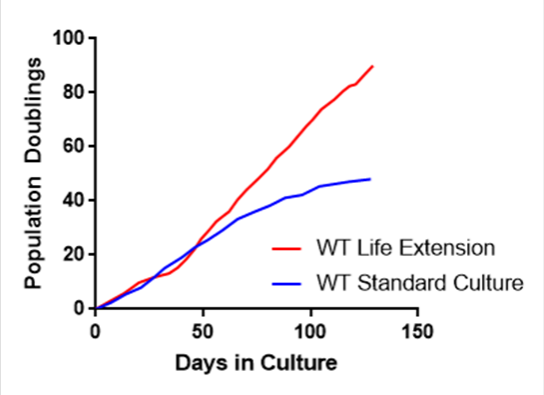
Supplemental Figure 1



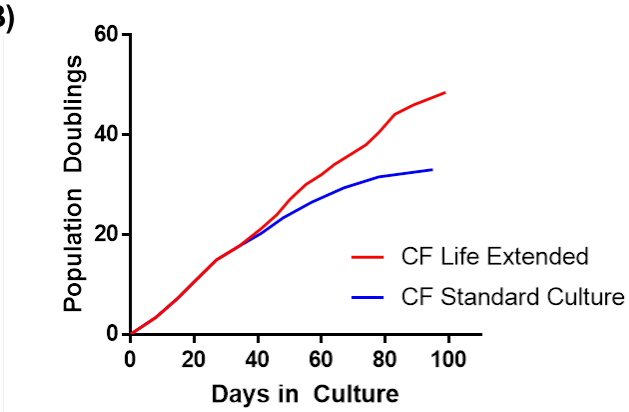
Supp. Figure 1: Optimization of Lung Decellularization: Pico-Green quantitation of Lungs decellularized with 2, 3, or 5 hours of perfusion with the CHAPs solution.

Supplemental Figure 2

A)

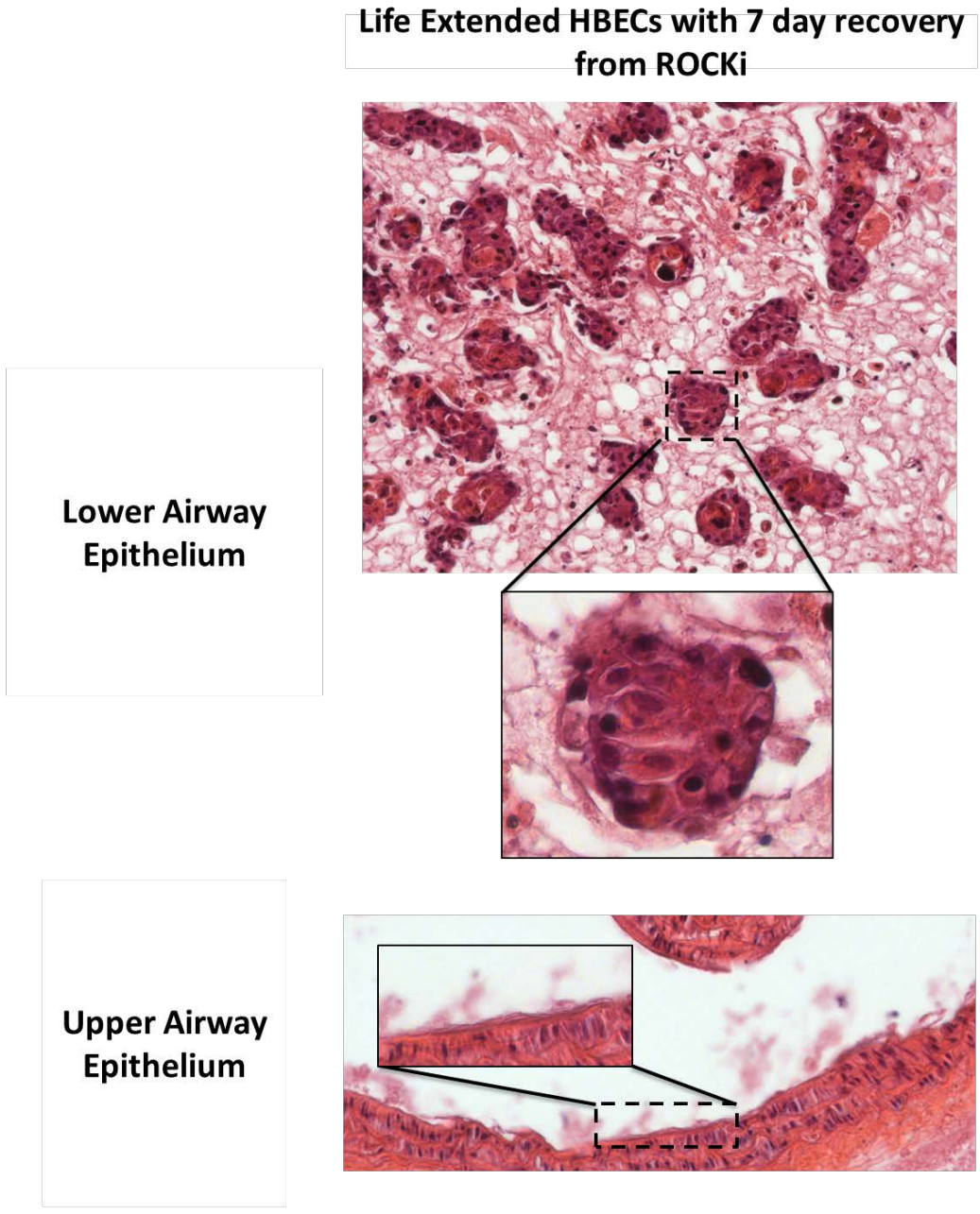


B)



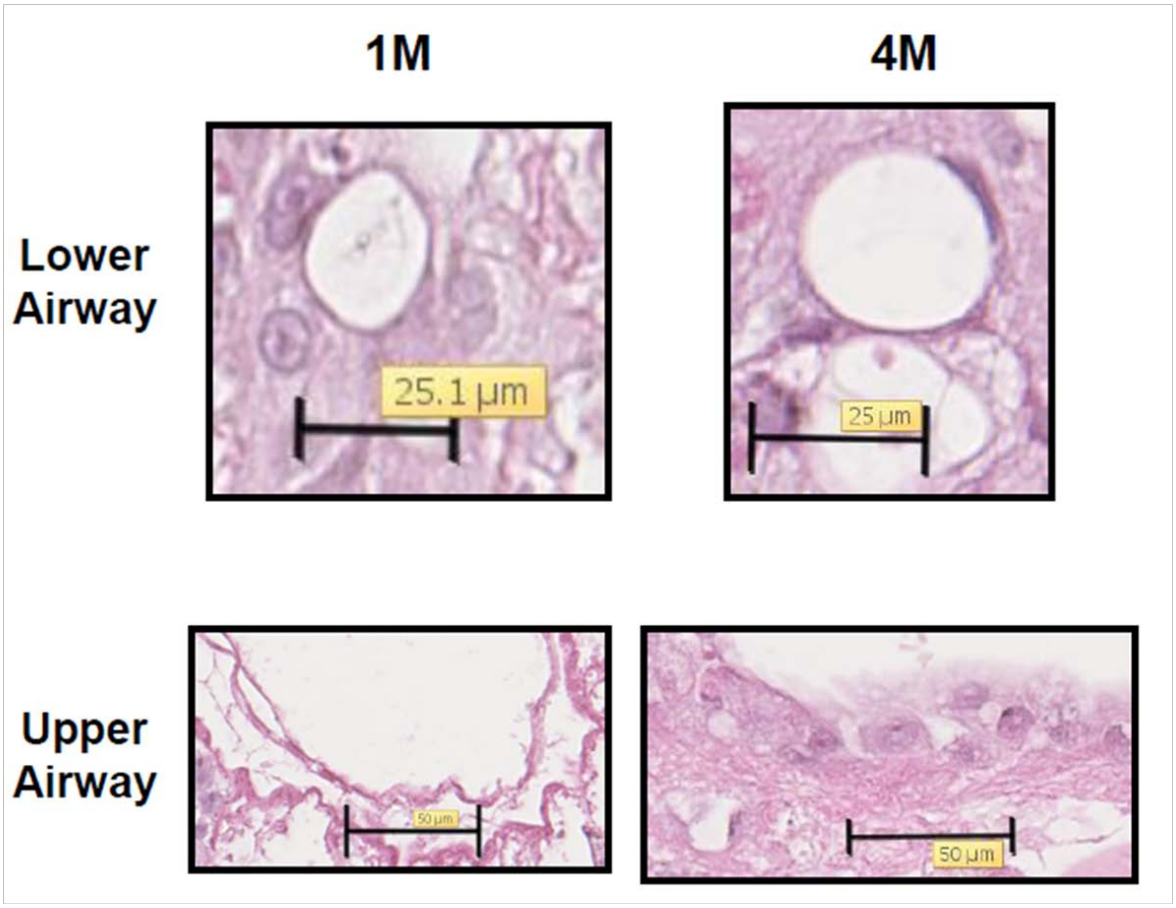
Supp. Figure 2: Conditional Reprogramming Extends the Life Span of HBECs Growth curves for CR versus standard culture HBECs from patients with and without CF.

Supplemental Figure 3



Supp. Figure 3: ROCK Inhibition Recovery Inhibits CR HBEC Differentiation in a Lung Matrix: Representative upper and lower airway epithelium of lungs seeded with CR HBECs which recovered from CR conditions for 7 days before seeding into lung matrix and 12 days of culture.

Supplemental Figure 4



Supp. Figure 4: Low CR HBEC Seeding Density Prevents Lung Reconstitution: Representative H+E staining of upper and lower airway epithelium of lungs seeded with 1 or 4 million CR HBECs before culture for 12 days.

CHAPTER THREE
Modeling Cystic Fibrosis and Gene Therapy

Abstract

Cystic Fibrosis (CF) is a complex genetic disorder that is caused by any of almost 2000 known mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. The main tool used to study all of these forms of CF at present is primary patient derived human bronchial epithelial cells (HBECs) cultured at the air liquid interface (ALI). Several cell lines and iPSC derived cell populations have been generated to produce less labor-intensive alternatives to facilitate drug screening. Unfortunately, these cells have failed to recapitulate all the characteristics of primary HBECs after their expansion *in vitro*, preventing their use as a tool to study treatment of CF. I have developed a method for culture of HBECs that are conditionally reprogrammed (CR) which allows for the passaging of primary HBECs without any genetic manipulation and for sufficient numbers of population doublings to dramatically increase the number of experiments which can be done with a single biopsy. In this work, I describe optimization of CF CR HBEC culture and demonstrate that differentiated pseudostratified epithelium made with these cells has preserved CFTR function. Further, I show that CF CR HBECs can be cloned and report initial results with CRISPR mediated creation of CF lines from native patients. This research opens the possibility of personalized medicine approaches to patients with CF to develop the best treatment strategy on a patient-by-patient basis and permits basic science investigations of primary patient-derived cells which can be genetically modified to correct the underlying disease phenotype.

Introduction

Cystic Fibrosis (CF) is an autosomal recessive disorder of the CFTR gene (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989). Failure to have normal CFTR chloride channel activity results in the buildup of viscous mucus and chronic bacterial infections in the lung, which is believed to be the primary cause of death for patients with this disease (Cantin, Hartl, Konstan, & Chmiel, 2015). As of this time, the exact mechanism by which defects in the CFTR gene lead to this disease phenotype are unknown. There are six classes of mutations which cause CF. Class I mutations result in nonsense mutations and degradation of the CFTR mRNA. Class II mutations cause misfolding and destruction of the CFTR protein in the endoplasmic reticulum. Class III CFTR mutants reach the plasma membrane, but have impaired chloride gating. Class IV CFTR mutants have decreased chloride ion conductance through the chloride channel. Class V mutations have splicing defects which result in decreased levels of CFTR protein in some patients. Class VI mutant CFTR fails to properly anchor to the plasma membrane (Bell, De Boeck, & Amaral, 2015). Each of these classes of errors in CFTR may require its own pharmacologic strategy for correction. For instance, the drugs lumacaftor and ivacaftor have been developed to treat class II CFTR mutations by correcting the folding defect in CFTR through direct binding to the protein. However, these drugs are unlikely to be effective in treating other misfolding, nonsense, or gating defect mutations (Elborn et al., 2016; Konstan et al., 2016).

One of the challenges in studying CF is that the only way to induce expression of CFTR is to differentiate the cells at an air liquid interface, which requires culture for between 20 and 35 days depending on the cell donor and type (Fulcher et al., 2009; Gruenert, Willems, Cassiman, &

Frizzell, 2004; Van Goor et al., 2011). When cultured in 2-D on tissue culture treated plastic, cells with defects in the CFTR gene behave effectively identically to cells without defects in the CFTR gene. The primary method for measuring CFTR transport of chloride ions across an epithelial layer is Ussing chamber analysis. This technique was first developed in 1950 by H.H. Ussing to study sodium ion transport across frog skin (Ussing & Zerahn, 1951). Briefly, the frog skin was stretched over a chamber which had Ringer's solution on either side. As there was no net imbalance of sodium ions, any movement of sodium ions would be caused by active transport of the otherwise short-circuited skin. The resulting change in current could then be measured by electrodes placed in the Ringer's solution on either side of the membrane. Importantly, this technique was also found to be capable of detecting changes in active ion transport caused by agents such as adrenaline added to the Ringer's solution in the Ussing chamber. While this device was originally conceived for skin, it also works very well for cultured epithelia that have fully differentiated. Because cultured airway epithelium is polarized and has tight junctions, it can prevent the passive diffusion of ions across it and any active transport will have directionality and therefore will produce a current. These polarized pseudostratified epithelium cultures made with primary HBECs on a semipermeable membrane can be placed in an Ussing chamber for measurement of CFTR chloride channel activity (Li, Sheppard, & Hug, 2004).

To develop other therapies and ultimately pursue personalized medicine for patients with this disease, we need to move beyond primary cell cultures to achieve sufficient scale to conduct genome correcting and small molecule screening efforts. The Harris lab was one of the first groups to successfully immortalize HBECs using the SV40 T antigen (Reddel et al., 1988). Other laboratories have generated cell lines through ectopic expression of BMI1 and hTERT (Fulcher

et al., 2009) or hTERT and Cdk4 (Delgado et al., 2011). However, these cells are somewhat impaired in their ability to fully differentiate into a pseudostratified ciliated epithelium, likely due to their continual production of factors which are pro-survival but which also induce a stemness phenotype (Dimri et al., 2002; Lange & Calegari, 2010; Z. Liu et al., 2013).

There have been two primary schools of thought regarding the creation of cell lines for the study of cystic fibrosis and other lung diseases. Many laboratories have attempted to create cell lines which could recapitulate native HBEC behavior through expression of a combination of oncogenes, virus-derived immortalization elements, and pre-oncogenes. However, these cells have not been able to produce a fully differentiated ciliated pseudostratified epithelium (Gruenert et al., 2004). Recently, some labs have attempted to use induced pluripotential stem cells (iPSCs) as a source of cells for the study of CF, with some limited successes but without fully recapitulating the fully differentiated ciliated pseudostratified epithelium obtained with primary HBEC cultures (Firth et al., 2015). Both of these strategies have run into some difficulty because the study of CF necessitates terminal differentiation into a pseudostratified epithelium. Other labs followed up on this work and demonstrated that these cell lines could produce a CF phenotype as measured by impaired CFTR activity at ALI after differentiation *in vitro* (Jefferson et al., 1990). However, it was found that these lines demonstrated karyotypic abnormalities which would impair the stability of the line over long-term culture.

The Randall lab and the Shay/Wright laboratory developed methods for generating HBEC lines without ectopic oncogene expression. Both lines could differentiate at ALI and were karyotypically stable. The Randall laboratory used lentiviral transfection with hTERT and BMI-1

and demonstrated that the cells could differentiate at ALI; forming a current in response to cAMP signaling after 14 passages *in vitro* (Fulcher et al., 2009). However, these cells did not produce as robust a signal as freshly isolated primary cells and did not form a ciliated pseudostratified epithelium at ALI. The Shay/Wright laboratory demonstrated that HBECs immortalized with ectopic expression of hTERT and CDK-4 could differentiate in response to ALI culture as shown by generation of ciliated and mucus producing goblet cells (Ramirez et al., 2004). However, these cells were not tested for CFTR activity after differentiation at ALI. Work done in the Shay/Wright laboratory since has shown that these cells do not produce robust CFTR expression after culture at ALI. These lines were a dramatic improvement over lines created with overexpression of viral oncoproteins because of their karyotypic stability, but still relied on viral transfection of immortalization genes and failed to fully recapitulate primary cell differentiation *in vitro*.

iPSCs have been explored as an alternative method to create HBECs from patient fibroblasts (Huang et al., 2014). The first iPSC generation protocol which produced a CFTR-expressing epithelial layer in response to culture at ALI was generated by the Rossant laboratory (Wong et al., 2012). Importantly, this method did not produce cultures capable of generating current by Ussing chamber analysis. While this technique did produce some off-target differentiation, some of the cells were able to differentiate into HBEC populations. This technique was further improved upon by the Davis laboratory, which successfully produced iPSCs from skin fibroblasts derived from patients with the delta-F508 form of cystic fibrosis. They used a zinc-finger nuclease mediated homologous recombination strategy to correct the delta-F508 mutation, and were able to demonstrate functional rescue of CFTR protein function in

cells derived from CF patients with Ussing chamber analysis (Crane et al., 2015). Even given these advances, there are still significant barriers to using iPSCs for drug discovery for CF. The Davis iPSC protocol takes 23 days to reprogram skin fibroblasts into HBECs, and they noted high variability between cell populations generated with this method. Further, iPSCs develop genetic variability and abnormal karyotypes during extended culture, which can be problematic for cells that need to be scaled up from small initial biopsies or scrape samples (Martins-Taylor et al., 2011; Peterson & Loring, 2014; Taapken et al., 2011).

As described in Chapter 1 of this thesis; CR HBECs represent a potentially useful tool for studying CF because of its relative scalability and lack of genetic manipulation. To verify this, I have developed culture methods for CF CR HBECs which preserve their ability to differentiate while maintaining CFTR function. Further, I have shown that CF CR HBECs can tolerate single cell cloning and expansion, and that CR HBECs can support the culture of clones made with CRISPR mediated gene editing. This research demonstrates the utility of CR HBECs in the study of CF.

Results

Conditional Reprogramming of CF and WT CR HBECs Extends Their Life Span in Culture

Work done in the Shay/Wright laboratory has demonstrated that WT CR HBECs are best cultured in co-culture conditions with lethally irradiated fibroblast. To verify that these cells can be used for CRISPR-mediated genetic modification I had to determine the best conditions for culture of CF-derived HBECs. The three main conditions I compared were standard culture, culture with filtered media conditioned by irradiated J2-3T3's, and cells co-cultured with

irradiated J2-3T3's. In serially passaging these cells, I found that while CF HBECs grown in standard conditions senesce at about PD 35. CF HBECs cultured in conditioned media at more physiologic oxygen (5%) and co-cultured at atmospheric (21%) oxygen both senesced at approximately PD 55. In contrast, co-cultured HBEC at 5% oxygen did not senesce until PD 60, a modest increase. Interestingly; the HBECs derived from a CF patient co-cultured in 5% oxygen senesced more quickly relative to those isolated from a WT patient; WT HBECs in culture senesced at PD 50, and the WT CR HBECs have now been cultured well past 150 population doublings. The lack of more long term growth of CF HBECs may be due to the high levels of chronic inflammation experienced by cells in a CF lung (Stoltz et al., 2015) and thus the cells when isolated had experienced many more round of cell turnover than their WT counterparts. An alternative explanation is that this simply could be due to differences between the two donors. In summary; co-culture at low O2 was the best condition for culturing HBECs derived from CF patients (Fig. 5).

CR HBECs Demonstrate a Pseudostratified Epithelium and Generate an Active

In chapter 1 I demonstrated that CR HBECs cultured at ALI form a pseudostratified epithelium by immunofluorescence staining for cilia and goblet cells. However, to demonstrate that these cells can be used for studying CF I next needed to demonstrate that these cells are capable of CFTR mediated polarized chloride channel activity in an Ussing chamber assay. I first established a baseline with freshly isolated HBECs differentiated at ALI (Fig. 6A), showing that they generate current in response to forskolin induced increases in intracellular cAMP, which is then inhibited by addition of the small molecule CFTR-inhibitor 172. This indicated that the current was generated by CFTR activity specifically. A representative trace from PD 40 CR

HBECs demonstrated similar sensitivity to Forskolin and an elimination of the current by addition of CFTR inhibitor; indicating that CFTR activity is preserved after extended culture in CR conditions (Fig. 6B). Comparing all my freshly isolated control Ussing Chamber analyses of HBECs and PD 40 CR HBEC ALI cultures, I found no significant difference between forskolin-stimulated current. Further, the differences between forskolin activated current and CFTR inhibited current were significantly different in both conditions (Fig. 6C). This indicated that I could use CR HBECs to model CF, but I had to determine if CF CR HBECs could reproduce the CF phenotype of insensitivity to forskolin-mediated current generation.

To use CF CR HBECs as a tool for drug discovery in CF, I must be able to demonstrate that the cells produce no current in response to forskolin natively, but that drug-mediated correction of the CFTR defect does result in the creation of a chloride transport mediated current. As the CF CR HBEC population used in these studies has the delta-F508 mutation, it can be corrected by treatment with the drug VX-809 (Van Goor et al., 2011). A representative trace from Ussing chamber analysis of an ALI culture of CF CR HBECs demonstrates that while treatment with amelioride does inhibit sodium channels in the epithelium, reducing the generated current, and UTP does trigger non-CFTR mediated intracellular calcium-induced chloride channel activity, there is no significant activity stimulated by forskolin treatment, indicating that CFTR is defective in these cells (Fig. 7A). When the ALI cultures were treated with 10uM VX-809 for 48 hours before Ussing chamber analysis, a representative trace shows that CFTR activity has been rescued by the drug (Fig. 7B). Analysis of multiple Using chamber analyses of treated and untreated CF CR HBEC ALI cultures demonstrates a significant difference between the forskolin current responses of the two conditions (Fig. 7C). Further, it shows that there is a

difference between the forskolin treated current and the CFTR-inhibited current in CF CR HBEC ALI cultures. These results demonstrate the potential utility of CF CR HBECs for the study of CF.

Cloning and CRISPR Gene Editing of CR HBECs

Having demonstrated that CF CR HBECs can simulate the CF phenotype and can be used to detect successful drug correction of CF, I next aimed to determine if these cells could be used to effect CFTR gene editing. As mentioned previously, there are a number of approaches being pursued for the generation of CF treatment by gene therapy *in vitro* and *in vivo* (Arthur, 2015). However, those studies which use primary cells involve heterogeneous populations of genetically altered cells because HBECs are not amenable to cloning and retaining their capacity for differentiation. Being able to clone primary HBECs would allow for the creation of pure populations with particular genetic modifications to CFTR; permitting unadulterated analysis of the effect that repair has on cells derived from CF patients. As I developed two methods for culturing CR HBECs, conditioned media and co-culture, I fist compared the utility of these two methods for clonal culture. Representative pictures of clones cultured for 5 days shows that the co-cultured clones formed denser colonies, while clones cultured in conditioned media tended to spread out (Fig. 8A). In comparing these techniques quantitatively, I found that from 600 HBECs plated at clonal density, only 2 clones from the conditioned media could be expanded up to 10 cm dish culture, while 6 clones from the co-culture condition could be expanded to 10cm dish culture (Fig. 8B). In analyzing the growth curves of three clones from the co-culture condition which were plated at clonal density at PD20; I found that transfer to 10cm culture from single

cell clones took 20 population doublings, and that all three clones could survive both subsequent cloning and transfer to ALI culture, the results of which were discussed in chapter 1 (Fig. 8C).

Following this work, I attempted CRISPR mediated CFTR gene knock-out by inducing double-stranded break mediated non homologous end joining repair at the 508 locus in CR HBECs in collaboration with other students in the Shay/Wright laboratory. After transfection with the guide RNA and screening of the resulting clones, we found 2 clones that were mutated in both alleles and 1 which was a heterozygote for mutations in CFTR (Fig. 8D). Sanger sequencing of these clones showed that both of the homologous mutants had deletions at the 508 locus, and that the heterozygote only had a deletion in one allele at that locus (Fig. 8E). These results demonstrate the potential utility of CR HBECs by showing their ability to withstand the rigors of cloning and to permit the generation of clonal populations of HBECs generated by CRISPR mediated genetic manipulation.

Discussion

The CR method developed in this study extends the lifespan of cells while retaining the ability to differentiate and increases the utility of those cells by permitting cloning and genetic manipulation. My work demonstrates that not only is CFTR function maintained in CR HBECs, but that CF CR HBECs can be used to model CFTR rescue. This flexibility permits study of CF at a more granular level, potentially facilitating the more rapid development of cures for the many different varieties of CF.

There are difficulties facing both genetic therapy and cell therapy for CF. The airway epithelium should be an ideal target for gene therapy. The cells one needs to target in order to affect restored CFTR function in the airway epithelium are directly exposed to the air and therefore can be targeted by aerosolized gene-editing factors such as virus or lipid droplets (Arthur, 2015; Griesenbach et al., 2015). Additionally, there has been research demonstrating the potential utility of cell therapy for treating cystic fibrosis; applying epithelial cells with functional CFTR to large animal models of CF (Duchesneau et al., 2017; Gui et al., 2015). However, lung epithelial cells are extraordinarily resistant to viral infection (Arthur, 2015). There have been some lentiviruses developed to overcome this resistance and effect genetic therapy for CF (Griesenbach et al., 2012; Griesenbach et al., 2015; Mitomo et al., 2010). Further, particular variants of the flu virus have been developed which are adapted to infect airway epithelium (Armstrong et al., 2014). However, these vectors are far from optimized, and may not be ideal for gene therapy, as lentivirus-mediated gene therapy can significant have off-target effects (Moiani et al., 2012). This, coupled with that there are no good mouse models of CF-initiated lung disease, means that it is extremely difficult to study the effects that various genetic therapy treatment strategies will have on an airway epithelium. In addition, the correction in mature differentiated cells instead of stem like basal cells would mean the defect is only transiently corrected; the effect would be lost over time as the epithelial cells turned over unless basal cells could be infected by these viruses as well.

There have already been some studies demonstrating CRISPR mediated correction of CFTR defects in CF patients to model genetic therapy for this disease. For instance, the Niklason laboratory demonstrated that primary HBECs exposed to a lentivirus containing the full-length

mRNA of CFTR can produce CFTR in bronchial epithelial cells in a cell therapy model (Gui et al., 2015). The Clever's lab has also shown that CRISPR can be used to correct the delta-F508 mutation in organoids derived from the intestinal epithelium of CF patients (Schwank et al., 2013). However, these studies have not been able to produce current in genetically modified HBECs cultured at ALI in an Ussing chamber. While two laboratories have successfully used CRISPR (Firth et al., 2015) or zinc finger nucleases (Crane et al., 2015) to correct CF mutations in HBECs derived from iPSCs and have shown some restored CFTR function by Ussing chamber analysis, as far as could be determined there has been no comparison of these manipulated cells to fresh native cells.

Ultimately, CR HBECs may have two advantages over iPSCs in the study of CF; 1) CR HBECs are genetically stable and permit the rapid generation of large numbers of cells (Palechor-Ceron et al., 2013), 2) while iPSCs have genetic drift and the generation of HBECs from iPSCs can take more than 20 days, while CF CR HBECs can be used immediately (Firth et al., 2015; Liang & Zhang, 2013). Many mutations can cause CF or influence this chronic lung disease, making it an excellent candidate for developing a personalized medicine approach to treatment of patients with this disease. With our normal CR HBECs and CRISPR mediated homologous recombination gene editing it becomes possible to introduce numerous CF mutations that do not have therapies at the present time and to use these cells as a drug screening cell based system. In summary, considering that potential personalized medicine applications of primary cells must be as cost-effective as possible, CR HBECs represent a valuable tool for studying CF.

Methods

Conditioned Media Culture:

Conditioned media culture was prepared by irradiating J2 3T3's with 30 Gy of X-rays. A confluent 15cm plate of these cells was incubated with BEGM media for 48 hours. After this time the media was filtered and either immediately transferred to CR HBECs or frozen at -80 degrees C for storage. 10uM of RI was added to the conditioned media immediately before application to the cells. The media was changed every 2-3 days.

CR HBEC cloning:

HBECs to be cloned were cultured as described previously; to clone them 600 cells were seeded onto a 15cm dish. For the conditioned media cloning experiments, the cells were added to 25mL conditioned media with RI. For the co-culture, 1 million irradiated J2-3T3 cells were plated onto a 15 cm dish with 25mL BEGM + RI. The cultures were observed for 2 weeks for colony formation, and individual colonies were ring-mediated trypsinized and then transferred to 24 well plates. The CR HBECs were then passaged as described previously.

Ussing Chamber Analysis:

ALI cultures were maintained for 4-5 weeks and tested for resistance of at least 700 Ohms in Trans epithelial resistance (TEER) analysis, cultures were treated with 10uM DMSO or VX809 48 hours prior to analysis. Ussing Chamber analysis was performed as described previously (Fulcher et al., 2009).

Figure 5

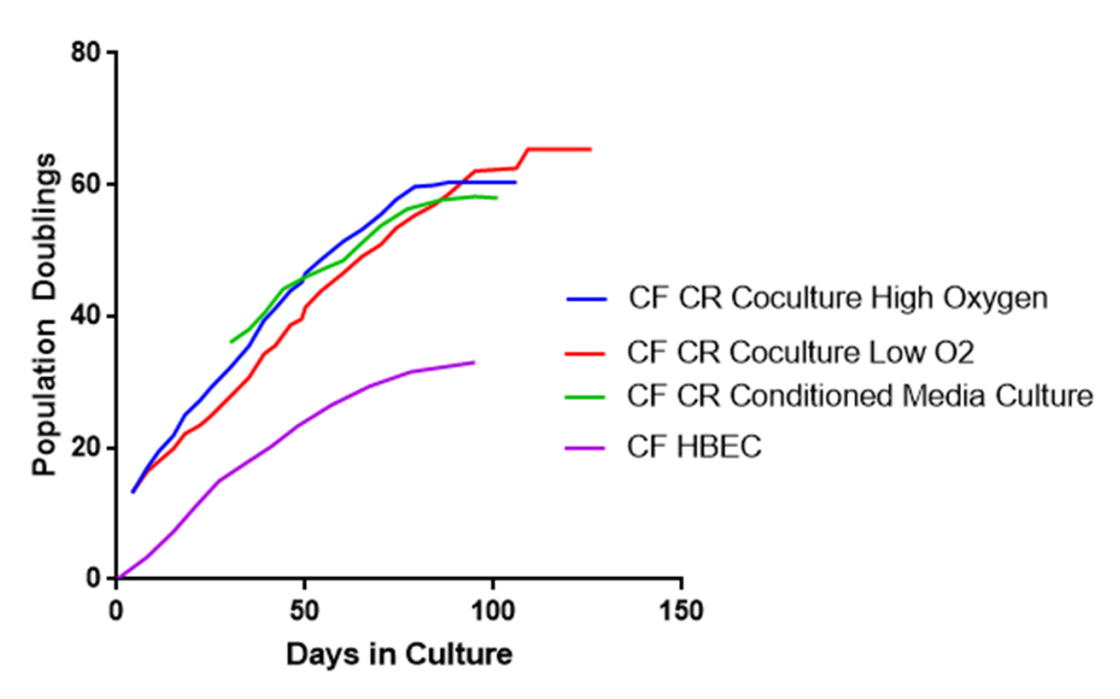


Figure 5: Determining Tissue Culture Conditions for Reprogramming CF HBECs: HBECs derived from a patient with the delta-F508 homozygous mutation causing Cystic Fibrosis were serially passaged under the following conditions; 1) Atmospheric oxygen with 10uM RI and coculture with irradiated J2 3T3s, 2) Physiologic (5%) oxygen with 10uM RI and coculture with irradiated J2 3T3s, 3) Atmospheric oxygen with 10uM RI and media conditioned by irradiated J2 3T3s for 48 hours, 4) Culture in media with no ROCK inhibition. Coculture with low oxygen permitted the longest period of tissue culture growth without excessive senescence until after 60 days of culture.

Figure 6

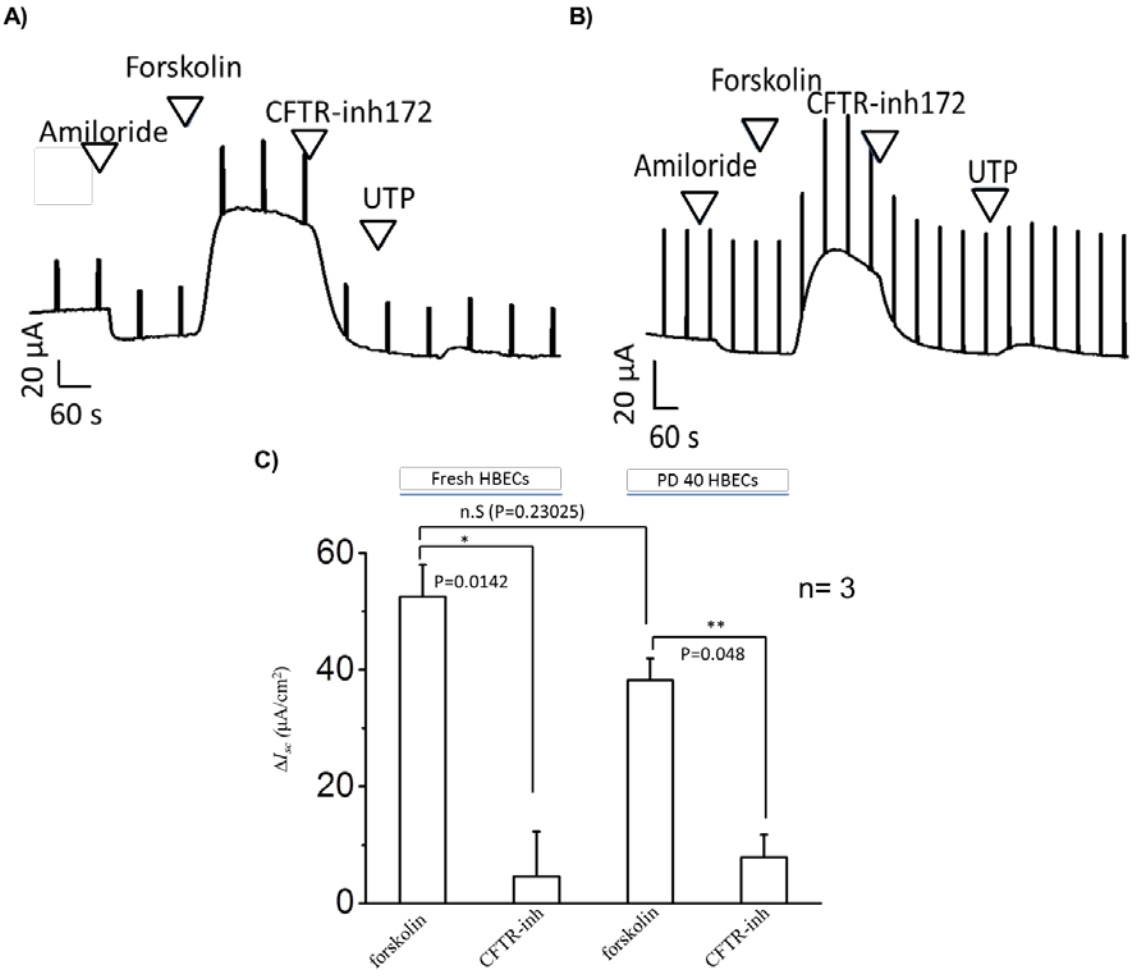


Figure 6: Measuring CFTR Activity in CR HBEC Cultures with Ussing Chamber Analysis: A) Representative trace of freshly isolated HBECs showing response to treatment with amiloride, forskolin, CFTR inhibitor 172 and UTP. **B)** Representative trace of PD 40 CR HBECs showing current generation in response to treatment with forskolin which is inhibited by inhibition of CFTR. **C)** Average current generation from 3 wells analyzed in the Ussing Chamber for the control condition (freshly isolated HBECs) and the experimental condition (PD 40 CR HBECs) indicates that there is no significant difference between forskolin induced current between the two conditions, and that both conditions respond to CFTR inhibition.

Figure 7

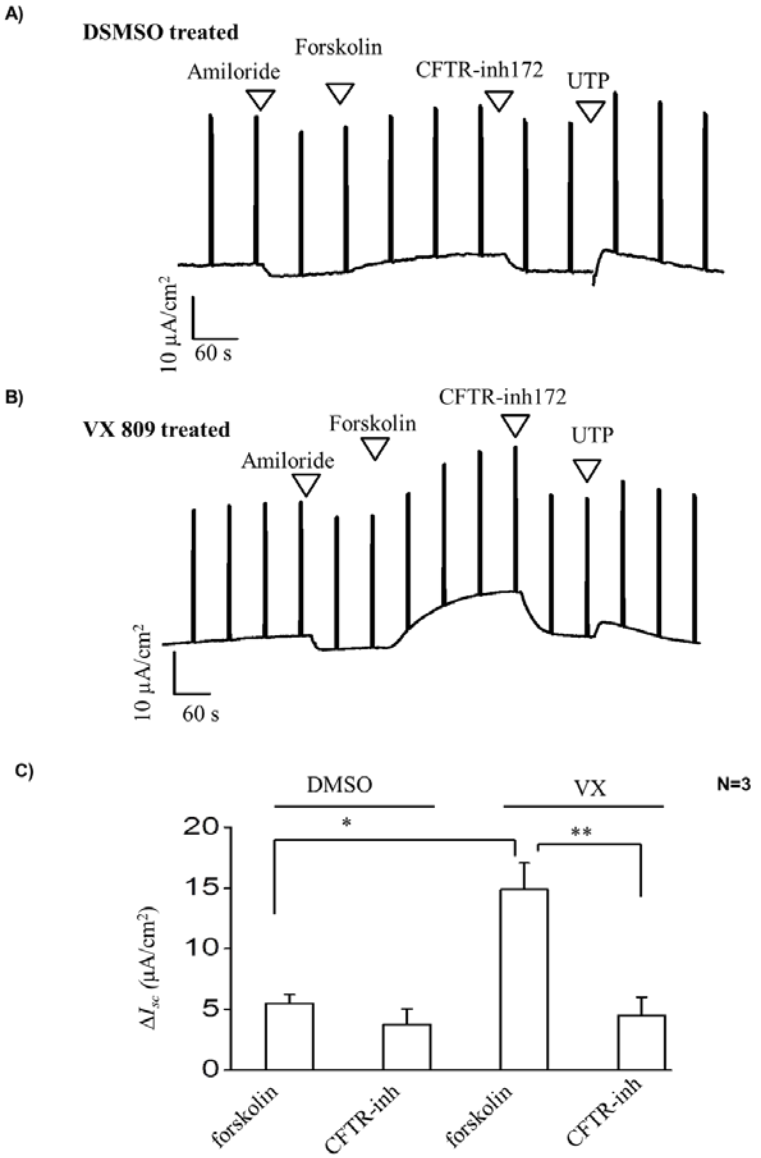
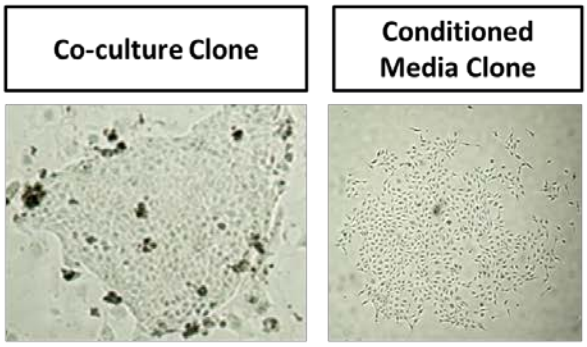


Figure 7: CF CR HBECs Have Preserved CFTR Function and are Sensitive to Corrector Drugs A) Representative Ussing Chamber Trace showing DMSO treated CF CR HBECs at ALI being insensitive to forskolin. **B)** Representative Ussing Chamber Trace of CF CR HBECs at ALI after 48 hours of treatment with 10uM VX-809 having restored CFTR activity as shown by restored current generation in response to forskolin treatment which is abrogated by CFTR inhibition. **C)** Average of three Ussing Traces showing that no current was generated in DMSO treated CF CR HBECs at ALI and that VX-809 treatment restored CFTR function in these cultures.

Figure 8

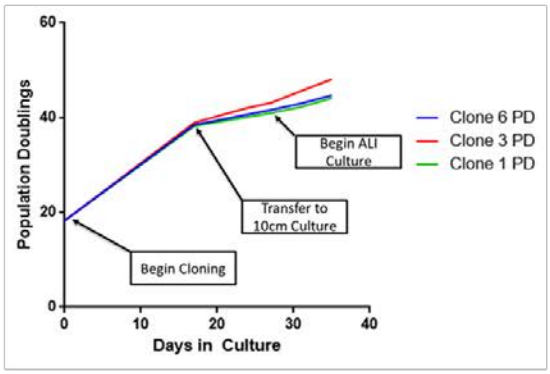
A)



B)

Condition	Number of seeded cells	Number of Clones Surviving to 10cm Dishes
Conditioned Media	600	2
Coculture	600	6

C)



D)

E)

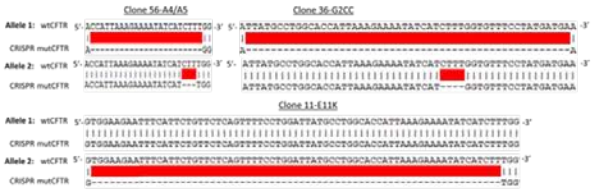


Figure 8: Clonal Selection of CR HBECs and CRISPR Mediated Generation of CFTR Mutants A) Representative pictures of CR HBEC clones cultured in either co-culture or conditioned media cloning conditions. **B)** Table demonstrating the differing capacities for CR HBECs plated at clonal density to tolerate scaling up culture after being plated at 600 cells per 15cm dish. **C)** Growth curve indicating the ability of three CR HBEC clones to tolerate clonal selection and subsequent expansion into populations sufficient for ALI culture. **D)** CFTR PCR gel of TOPO-TA cloned alleles from 3 clones generated after CRISPR mediated cutting of the CFTR gene showing in each case at least 1 cut allele. **E)** Sanger sequencing of the CR HBEC clones generated after CRISPR mediated cutting of CFTR at the 508 locus showing generation of two clones with cuts in both alleles and one heterozygote.

CHAPTER FOUR

Development of a Quantitative *In Vivo* Immunogenicity Screen

Introduction

Organ transplantation has led to dramatic clinical successes; allowing for the treatment of organ failure which would otherwise be fatal. However, patients who receive allogeneic transplants must be put on immunosuppressive drug regimens for the rest of their lives (Ruiz et al., 2013). Because of the negative health impact chronic immunosuppression can cause, some groups have explored strategies for abrogating this need by the creation of genetically modified Universal Donor grafts (Ruiz et al., 2013; Q. Zhou et al., 2013). This would be particularly valuable for the field of skin transplantation, as there is a great demand for allogeneic skin transplants and skin transplants are comparably tolerable for clinical genetic engineering applications (Andreadis, 2007). One of the challenges in creation of a Universal Donor paradigm is that *in vitro* assays of immunogenicity do not necessarily match *in vivo* rejection responses because of the complexity of rejection (Benichou et al., 2011). Assays that could evaluate the *in vivo* rejection modulation in a more high-throughput, quantitative fashion would speed research in the field of immunomodulation of transplants.

Skin transplantation is vital for patients with severe burn wounds; so much so that it has been a major area of clinical study for more than 60 years (Nam et al., 2014). While autologous

full thickness skin grafts are the gold standard for the treatment patients with burn wounds (Foster, 2014; Snell, Loh, Mahambrey, & Shokrollahi, 2013; Stanton & Billmire, 2002), if a patient has extensive burns over more than 50% of their body, they will not have enough skin to reasonably create skin grafts. Every year, there are 1000s of patients in the US who get burns over more than 60% of their body surface. A number of artificial skin constructs have been developed to meet this need. As mentioned previously, epidermal sheets were first developed as a way to quickly reestablish barrier function in patients with burns in 1984 (Gallico et al., 1984). Following this work, and the discovery that a skin transplant containing both a dermis and an epidermis is considerably more stable, investigators have attempted to develop allogenic full-thickness skin grafts for transplantation. A number of these constructs have already been clinically approved (Shevchenko, James, & James, 2010). Unfortunately, the method by which skin transplant rejection is measured is by qualitative scoring of skin transplants (Bahar, Nabai, & Ghahary, 2012). This practice is, in part, traditional. The first studies of allogeneic skin transplant rejection, which were performed by the Medawar laboratory, were done with skin grafts in humans and involved qualitative scoring of transplant survival based on transplant color and attachment (Billingham, Brent, & Medawar, 1953). In animal studies, the lack of quantitative comparison between graft survival and the requirement of generating one skin transplant per mouse process makes it difficult to screen expression of potential immunomodulatory factors. Using more quantitative assays to determine the survival of allografts would increase the quality of the information given from the assay and allow for direct comparison of different experiments.

Bioluminescence assays provide a quantitative assay of transplant survival, and are based on the activity of luminescent luciferase proteins. This class of proteins was first discovered by EN Harvey in 1914 as the underlying mechanism of firefly luminescence (Harvey, 1914, 1917). The characteristics of this luminescent reaction were further elucidated in work which determined the luminescent response of luciferase to changes in available oxygen, pressure, etc. (McElroy & Ballentine, 1944). The first time bioluminescence was used *in vivo* was in monitoring the survival of salmonella which had been transfected with luciferase expression during treatment in a mouse as a way to evaluate clearance of the bacteria (Contag et al., 1995). Modern tracking of mammalian cells expressing luciferase in tissue arguably began in 2001 in the Gambhir laboratory. They used a novel cooled charged coupled device camera to track adenovirus infection induced *in vivo* luciferase expression in mouse skeletal muscle. The camera itself was provided an enormous increase in the ability to detect and quantitate photons produced by luciferase activity. This research was particularly important, as it demonstrated that *in vivo* detection of infected muscle cells correlated to *in vitro* quantitation of MOI (multiplicity of infection) in a reproducible fashion (J. C. Wu, Sundaresan, Iyer, & Gambhir, 2001). Subsequent advancements have found that bioluminescence is an excellent non-invasive technique for measuring the survival of transplanted tissue *in vivo* (Close, Xu, Sayler, & Ripp, 2011; Lepperhof et al., 2014) and to quantitate the rejection of transplanted tissue (C. H. Chen et al., 2010; X. Chen, Zhang, Larson, Baker, & Kaufman, 2006; Ojogho, Li, Zuppan, & Escher, 2007). However, these techniques have heretofore relied either on transgenic mice expressing luciferase, or on genetic modification of established cell lines. These advances began to approach the question of accuracy of quantitation of rejection, but they did not approach scalability needed for pursuit of engineering a Universal Donor transplant.

A more ideal reagent for rapidly screening tolerogenic factors for the creation of a Universal Donor transplant would be cultured primary cells that could be quickly transplanted after transfection. Mammals have different strategies for aging; a comparative genetics study which was recently performed indicates that mice do not engage in replicative aging similarly to humans (Gomes et al., 2011). Their telomeres are very long and do not shorten significantly as they age. While it has been shown that mouse telomerase knockouts do eventually display early signs of aging which can be corrected by telomerase expression, these mice do not show this phenotype until after several generations of being telomerase negative (Jaskelioff et al., 2011). Human primary cells have a limited lifespan in culture. This phenomena is known as the Hayflick limit, which was originally described by Dr. Leonard Hayflick in 1961 and further elucidated in 1965 (Hayflick, 1965; Hayflick & Moorhead, 1961). Work following this demonstrated that the mitotic clock for human cells is the telomere; TTAGGG repeats at the end of chromosomes which shorten with cell division because of the end replication problem originally described by James Watson (Harley, Futcher, & Greider, 1990). Soon thereafter, it was found that the enzyme telomerase elongates telomeres (Morin, 1989) and that ectopic expression of this protein could extend the lifespan of primary human cells in culture (Steinert, Shay, & Wright, 2000). Mice have endogenous active telomerase, which permits the spontaneous immortalization of primary murine cells *in vitro* (Prowse & Greider, 1995). Notably, primary murine cells do have a limited lifespan *in vitro* which is largely mediated by their sensitivity to oxygen since most investigators use atmospheric instead of physiological levels of oxygen in cell culture experiments (Parrinello et al., 2003). Given this, I hypothesized that primary mouse fibroblasts cultured at low oxygen could be cultured as a population for enough doublings to

permit multiple rounds of genetic engineering; permitting them to be used for screening tolerogenic factors.

We have spontaneously immortalized fibroblasts from Balb/C and 129S2 mice using these more physiological conditions, and have infected the resulting cell lines with luciferase expression. Following this, the cell lines were infected with tolerogenic factors and injected intradermally into the backs of either syngeneic or allogeneic mice. The survival of these grafts was measured by bioluminescence imaging over 14 days in living mice. The results demonstrated clear quantitation of rejection dynamics over that period. The assay developed in this work may be useful for screening the efficacy of multiple tolerogenic factors using normal primary mouse fibroblasts.

Results

Establishment of the Immunogenicity Assay

To generate cell lines for performing the immunogenicity assays, I isolated fibroblasts from skin samples taken from Balb/C and 129S2 mice. These were serially passaged for over 90 population doublings with no signs of crisis; indicating that these cells would be amenable to multiple rounds of transfection followed by scale-up (Fig 9A). As 129S2 and Balb/C have different MHC haplotypes, cells derived from one line will be allogeneic when transplanted intradermally into the other strain, but those same transplants should be autologous when transplanted into another mouse of the same strain. I aimed to test that multiple fibroblast

transplants injected intradermally into the dorsal skin of the mouse would permit a higher N per mouse; correcting for variability which is common in transplant survival experiments. The assay design (Fig. 9B) permits 4 conditions; 1) allogeneic Balb/C transplant, 2) syngeneic Balb/C transplant, 3) allogeneic 129S2 transplant, 4) syngeneic 129S2 transplant. By testing the protection afforded by tolerogenic factors expressed in these four conditions, I was able to rapidly subtract background changes in survival by comparing survival of the syngeneic and the allogeneic transplants. Intradermal transplants are discrete and do not move after 10 days in culture. In addition, the syngeneic transplants persist beyond 10 days, while the allogeneic transplants begins to die at 7 days and are absent after 10 days (Fig 9C). Quantitation of syngeneic and allogeneic 129S2 and Balb/C transplant survival found that both syngeneic cell transplants survived well past 30 days after some-post transplant loss of viability, while allogeneic transplants using the same cells were undetectable after 10 days and never re-emerged (Fig. 9D). To confirm that the immune response was driving the difference in cell death between syngeneic and allogeneic transplants, 129S2 and Balb/C mice which had been previously sensitized to Balb/C cells were intradermally injected with fibroblast transplants. The allogeneic transplants in these sensitized mice died more quickly, being undetectable after 6 days post-transplant, while the survival of the syngeneic transplant was unaffected (Fig. 9E). These results indicated that this assay could quantitate rejection of multiple intradermal primary fibroblast transplants using a bioluminescent assay.

Preliminary Studies Show TGF-B Expression Confers Survival Advantage to Allogeneic Grafts

The ability of potential tolerogenic factors to confer resistance to rejection was evaluated in this model system. The four pilot factors tested in this study were PD-L1, TGF-B1, FasL, and IL 10. Each of these factors is purported to contribute to a tolerogenic response by a relatively

well understood mechanism. PD-L1 expression blocks binding of PD-1 to cells, preventing complement and T cell mediated cell death (Aramaki et al., 2004; Freeman et al., 2000; Keir, Butte, Freeman, & Sharpe, 2008; Keir et al., 2006; Wen et al., 2008). TGF-B1 expression triggers FoxP3 mediated conversion of T cells into an induced regulatory T cell phenotype which induces a tolerogenic response (Regateiro, Howie, Cobbold, & Waldmann, 2011). FasL is proposed to be a factor which causes localized T cell apoptosis; creating immunoprivileged sites in the eye and in some tumors (Green & Ferguson, 2001; Guller & LaChapelle, 1999; Kim, Emi, Tanabe, Uchida, & Toge, 2004). However, FasL's role in creating immunoprivileged sites is controversial. It has been suggested that FasL is involved in mediation of inflammation as opposed to immune privilege (Maher, Toomey, Condron, & Bouchier-Hayes, 2002; Restifo, 2000). IL-10 has also been implicated in immune privilege; primarily through the induction of a regulatory T-cell phenotype (Ashour & Niederkorn, 2006; Boussiotis et al., 2000; Hong, Laks, Cui, Chong, & Sen, 2002; van de Veen et al., 2013) which has been implicated in amelioration of the rejection response in other model systems. Balb/C-luc fibroblasts were engineered to express TGF-B1, FasL, PD-L1, or IL-10. After selection, these cells were injected into Balb/C mice for syngeneic transplants and 129S2 mice for allogeneic transplants, and the survival of these grafts was measured over the course of 15 days. In this study; TGF-B expressing allogeneic transplants survived for 15 days, while the other allogeneic transplants were not detectable after 10 days (Fig. 10). Importantly, the survival of syngeneic transplants in all cases was not significantly changed by expression of candidate tolerogenic factors. This indicates that any change in survival caused by TGF-B expression was due to an amelioration of the rejection response as opposed to a conferred universal post-transplant survival advantage. However, while rejection did seem to be delayed, it was not abolished in the TGF-B transplant. These results demonstrate

the ability of this assay to compare the capacity of the expression of multiple tolerogenic factors to induce a tolerogenic effect.

Discussion

This research demonstrated the utility of primary murine skin derived fibroblasts cultured in low oxygen for rapidly measuring immunogenicity *in vivo* using multiple transplants on a single mouse. TGF-B impacts the survival of transplanted fibroblasts, but does not completely abrogate rejection. Being able to detect this shift in transplant survival demonstrates the utility of this assay to detect the ability of potential tolerogenic factors to overcome the immune response.

Primary murine fibroblasts were capable of surviving multiple rounds of selection. While these cells did not form tumors after transplantation in mice (data not shown), I have not verified conclusively that these fibroblasts have not experienced at least some genetic alterations. In the future, by karyotyping these cells at a series of different passages, I would be able to determine if culture at low oxygen alone is sufficient to immortalize primary mouse skin derived fibroblasts, or if there were additional events which were required for extending the replicative life span of these cells. The mechanisms of allogeneic skin transplant rejection are very complex; in human skin transplants dendritic cells have been found to be one of the major drivers of the acute immune response (Benichou et al., 2011). As the cell populations I derived from mouse skin are heterogeneous, I may be able by FACS sorting determine if our cultures contain a sub-population of dendritic cells.

Ultimately, it is unlikely that expression of a single factor will be able to create a Universal Donor transplant. Some work has supported this hypothesis, indicating that immune-escape is often mediated by expression of more than one gene working in cooperation; such as in the case of TGF-B and IL-10 working cooperatively to induce regulatory T cell induction of tolerance (D'Orazio & Niederkorn, 1998) or CTLA-4 and PD-L1 expression working cooperatively in melanoma to avoid detection by T cells (P. A. Ott, Hodi, & Robert, 2013). This experimental system can permit the creation of cells expressing combinations of genes, or it can use to create mixed transplants with cells carrying different combinations of factors. In either case, the combination of a simple primary cell culture system, a bioluminescent assay, and a mouse model which supports multiple transplants should permit rapid screening of multiple factors for the creation of Universal Donor cells. These could in turn be used for the creation of engineered tissue constructs which could be applied to patients as needed without having to make specific constructs for each patient.

Methods

Isolation and Culture of Primary Murine Fibroblasts

2-4 month old Balb/C and 129S2 Mice were euthanized by CO2 inhalation. After follow-up cervical dislocation, the backs were shaved and washed with betadine. The dorsal skin was then removed in a sterile tissue culture hood. The skins were then incubated in 10mL 0.25% Trypsin at room temperature in a 10cm dish for 30 min. The skin was then minced with sterile surgical scissors before being transferred to a new 10cm dish with 10mL DL Liberase. The minced skins were incubated in DL Liberase for 3 hours at room temperature. The fragments

were broken up by pipetting and then spun down at .2RCF. The supernatant was removed and the extracted cells were plated onto a new 10cm dish with 10mL Media-X +10% cosmic calf serum (CCS) in 2% oxygen. After 24 hours, the media was changed to remove all non-adherent material and un-digested skin fragments. Cells were maintained in 2% oxygen and were passaged at 70%-90% confluency with 500K cells seeded onto a 10cm dish, with media being changed every 2-3 days.

Bioluminescent Immunogenicity Assay

Cells which were to be used for transplantation were lifted as described previously and were stored on ice in suspension at 250K cells per 50uL serum free Media X. Mice were anesthetized by isoflurane inhalation. Their backs were shaved, washed with betadine and then with 70% ethanol. Intradermal injections of 250K fibroblasts suspended in 50uL of serum free Media X were made using a Hamilton syringe. The mice were then transferred to normal housing and were observed for at least 30 minutes to verify their health.

For measuring immunogenicity, mice were anesthetized by isoflurane inhalation. The mice were then re-shaved. They were then injected with luciferin intraperitoneally for a final dose of 126 mg/kg of body weight. After 12 minutes the mice were imaged using a CCD lumascope for 4 pictures at 1 minute of exposure and one picture at an 8 minute exposure. Mice were then transferred to their housing and allowed to recover for at least 30 minutes of observation.

Viral Transfection

Balb/C and 129S2 cells used for transfection were cultured as described previously. For making the luciferase-expressing line, Balb/C and 129S2 cells at 50% confluency were incubated with UB-Luc in lentiviral supernatant overnight. The next morning they were split to 3 10cm dishes each. 24 hours later the cells were selected with 1mg/mL G418 selection for 5 days. The surviving population was then cultured at 2% O2 with media X +10% CCS. The plasmids used for viral transfection of the tolerogenic factors were pWZLBlast for PD-L1 and IL-10 and Precision-Lenti ORF for TGF-B and FasL. Cells infected with any of these four plasmids were selected with 1.5 ug/mL blastomycin.

Figure 9

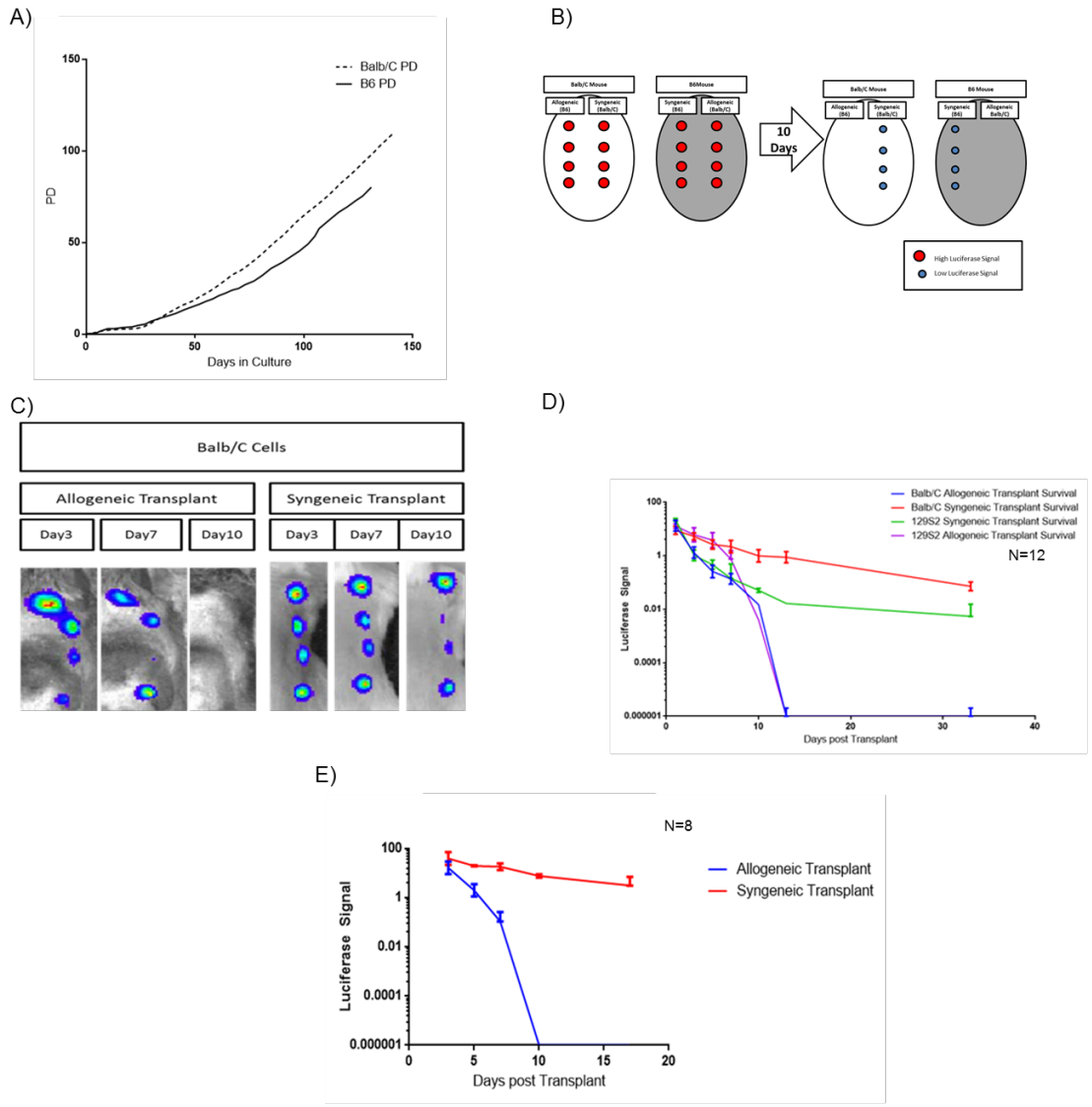


Figure 9: Primary Mouse Fibroblast Low Oxygen Culture and Transplant Rejection: **A)** Growth curves of primary mouse fibroblasts cultured at physiological oxygen (5%) for over 80 population doublings. **B)** Schematic for the *in vivo* immunogenicity assay. **C)** Representative images of allogeneic and syngeneic transplant survival over 10 days. **D)** Aggregated survival curves of syngeneic and allogeneic luciferase positive transplants measured by luminescence. **E)** Transplant survival in sensitized mice.

Figure 10

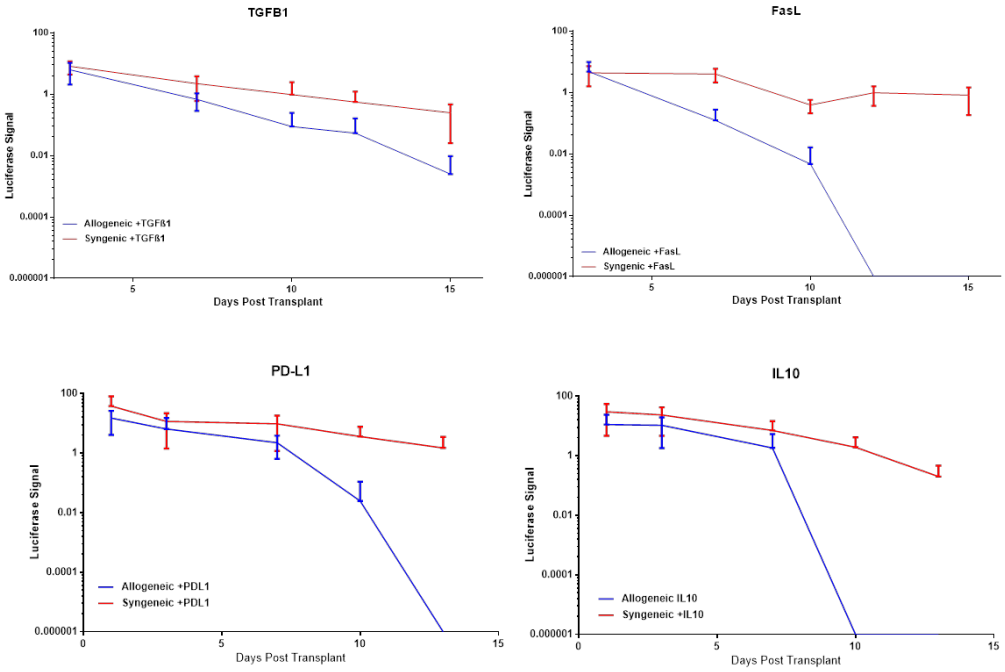


Figure 10: Survival of Transplants Expressing Candidate Tolerogenic Factors: Survival curves of allogeneic and syngeneic transplants expressing TGF-B1, PD-L1, FasL, and IL-10 along with luciferase.

CHAPTER FIVE
Overall Discussion and Next Steps

The work I have performed in this dissertation has focused on developing an *ex vivo* tissue-engineered lung system using CR HBECs as a reagent for modeling human lung diseases including cancer. I have shown that these cells can be isolated from patients with a genetic disorder, CF, and that these cells retain their CF phenotype after extended culture. Further, I have demonstrated that these cells are capable of clonal selection while preserving their capacity to differentiate, and that the conditional reprogramming method permits HBECs to not only differentiate into upper airway but also into lower airway cells. This is a result that has not been reported previously. I have also developed a novel method for rapidly assaying immunogenicity using a bioluminescence assay which will be critical for developing Universal Donor transplantable engineered tissue. This work will be continued along the four following lines; 1) supplementing my lung tissue engineering research with additional experiments, 2) tissue engineered models of lung cancer mechanobiology and drug resistance, 3) tissue engineering optimization, including adipocyte derived stem cells for engineering multiple niches of complex tissue, 4) CRISPR mediated correction of CF in CR CF HBECs. These future aims will build on the work I have already performed and have already been the basis of some ongoing collaborative research.

Lung Tissue Engineering with Conditionally Reprogrammed Stem Cells

To supplement the lung tissue engineering research described in chapter 2 of this thesis, I will perform some additional experiments. While it has been hypothesized that conditionally

reprogrammed stem cells are in a more stem-like state than other epithelial cells (X. Liu et al., 2017) it is unclear if the ability of HBECs to differentiate into both upper and lower airway cells in my reconstituted lung system is a function of conditional reprogramming, or if it is a feature of the HBEC population reacting to culture in a decellularized lung. By seeding decellularized lungs with freshly isolated HBECs which have not been reprogrammed and culturing them for 12 days in a bioreactor with simulated breathing, I will be able to determine of conditional reprogramming is required for HBECs to differentiate into both upper and lower airway cells, or if this is simply resulting from culture in a decellularized lung.

Additionally, I will be working on determining the role that reconstitution with conditionally reprogrammed HBECs has on the mechanics of decellularized lung tissue. My research has shown that decellularization of mouse lungs results in a significant loss of elasticity and tensile strength. This is to be expected, as there is a loss of cells and some ECM during the decellularization process. If conditionally reprogrammed lungs are able to reconstitute lung tissue after 12 days in culture, we should be able to detect some restoration of both the elasticity and tensile stiffness in recellularized lungs.

Tissue Engineered Models of Lung Cancer Mechanobiology and Drug Resistance

Work in our own lab has shown that normal HBECs display a markedly different transcriptional profile when cultured in different mechanical conditions (Delgado et al., 2011). Cancer cell behavior is also profoundly influenced by their microenvironment; their immunotolerance and drug resistance is predicated on the generation of a permissive

microenvironment including stroma and local ECM remodeling. In addition, cell migratory behavior is dependent on collagen fiber alignment (Swartz et al., 2012; Villasante & Vunjak-Novakovic, 2015). By way of example, recent work performed by the Min laboratory has demonstrated that lung A549 tumor lines cultured in a decellularized rat lung form perfusable nodules which are reminiscent of those seen *in vivo* (Mishra, Sakamoto, et al., 2012; Mishra, Thrall, et al., 2012). Further work done in this system demonstrated that lung cancer lines cultured in decellularized rat lungs produce tumors whose transcriptional profile matches those of tumors *in vivo* (Mishra et al., 2014) and that these tumors produce circulating tumor-like cells (Vishnoi et al., 2014).

KRAS is one of the important mutations that occur in lung cancer; KRAS is the gene that is most often mutated in small cell lung cancer (Sheridan & Downward, 2015). Mutant KRAS is linked with metabolism, but has also been implicated in mechanosensing (Kerr, Gaude, Turrell, Frezza, & Martins, 2016; Low et al., 2014). Our *ex vivo* reconstituted lung system permits study of the impact that the physical environment can have on KRAS signaling, and by extension, metabolism and drug resistance. This will permit directly comparing 2D culture, culture on a lung matrix, and culture on a lung matrix with liquid breathing to determine the reactions of KRAS-mutant cancer cells to rhythmic shear stress and stretching.

Our initial results in this investigation involved taking experimentally progressed cell lines (M. Sato et al., 2013) with the immortal normal cell line HBEC3KT as a control. In all of these experiments, the lungs were seeded with 10 million immortalized HBECs and were then cultured for 2 days in a bioreactor with or without simulated breathing. After this time, the cells

were fed with media containing U13 labeled glucose and were cultured for one hour. After this the lungs were washed and then harvested, with 2 lobes being flash frozen and the last being formalin fixed. One of the flash-frozen and pulverized lobes was analyzed for labeled metabolites by mass spectrometry. The control HBEC3KTs, cultured in a lung for 2 days, showed similar morphology when cultured in a decellularized lung for two days, but they displayed a differing metabolism; breathing lungs produced significantly more lactate than the control lungs during the 1 hour labeling interval (Fig. 11A). Interestingly; tumorigenic HBEC3KT progressed line with mutant KRAS cells formed colonies of cells in both the static culture and breathing lungs; further, they produced high levels of labeled lactate regardless of breathing (Fig. 11B). Progressed HBEC3KT cells infected with KRAS shRNA lost this phenotype; they still formed tumor clumps, but they regained sensitivity to breathing-induced lactate metabolism (Fig. 11C). As the shRNA for KRAS also contained GFP, I could visualize the presence of seeded cells in the lung after two days by visual inspection of the now-green lung (Fig. 11D). When tumor cells seeded into a lung were cultured for 10 days, they formed clumps of cells reminiscent of advanced tumors (Fig. 11E) though this remains to be confirmed for staining with Muc5B and other determinants of advanced cancer progression. These preliminary results indicate that this *ex vivo* lung culture system is robust and can be used to model tumor biology *ex vivo*. This will permit the parsing out of the importance of ECM sensing and mechanical perturbation for tumor biology in a controlled system.

Future experiments will focus on finishing the mechanosensing collaboration, introducing stroma, and reconstituting blood vessels for eventual recapitulation of metastasis. The mechanosensing experiment results shown above will be compared to 2-D culture metabolomics

results with or without physiologic surface tension to parse out the impact that interaction with the ECM has independent of mechanosensing.

As already mentioned, the normal epithelium and stromal cells surrounding a tumor can have a profound impact on the behavior of those cells; in particular; the presence of a captive stroma can result in cells having improved drug resistance, vascularization, and immune-resistance (Joyce & Fearon, 2015; Swartz et al., 2012; Tanner & Gottesman, 2015). By seeding a decellularized lung with 99% normal CR HBECs labeled with GFP and renilla luciferase and 1% experimentally progressed tumor cells labeled with RFP and firefly luciferase, it becomes possible to visualize the interaction of normal lung epithelial and potentially mixed with cancer associated fibroblasts (stromal cells) with the forming tumor. Further; the inclusion of two types of luciferase in normal and tumor cells, respectively, would allow for quantitative measurement of the total tumor burden in the *ex vivo* cultured lung over time without having to destroy a lung for each time point. This experimental paradigm would have a few distinct advantages beyond those discussed previously. Being able to quantitatively measure tumor burden over time for an entire lung would permit identification of important inflection points such as when a tumor begins logarithmic growth and when the stromal cells are altered by the presence of the tumor cells; as measured in exponential growth of firefly luciferase signal and drop off of renilla luciferase signal respectively. As renilla and luciferase emit different wavelengths of light, they can be quantitated simultaneously. These *ex vivo* engineered lungs with and without stroma could then perfused with media containing candidate therapeutic compounds. Their efficacy at killing cancer cells while preserving normal cells could be measured as a ratio of renilla and firefly luciferase signal over time, without having to generate a new recellularized lung per time

point. This could allow quantitation of the importance of the *ex vivo* environment in modeling tumor behavior and resistance to therapeutics, and could ultimately improve drug development for lung cancer.

Tissue Engineering Optimization

While I have been able to generate both upper and lower airway cells in a decellularized lung matrix, there are still some outstanding questions facing the tissue engineering field as a whole. In particular, there are still open questions regarding the best cell type for tissue engineering applications. Addressing this in future experiments will be required for making engineered tissue suitable for transplants.

Our lab has previously generated HBECs immortalized with ectopic expression of hTERT and CDK4. These cells are effectively immortal, and have been shown previously to differentiate in response to culture on top of Matrigel, in Matrigel, and at an air-liquid interface (Delgado et al., 2011; Kaisani et al., 2014; Roig et al., 2010). The main difference between these cells and CR HBECs is that HBEC3KT cells constantly express hTERT and cdk4; while conditional reprogramming conditions are reversible by removed ROCK inhibitors. Our preliminary research indicated that HBEC3KT cells could differentiate in response to culture at ALI, but that they did not express CFTR, indicating that they did not differentiate fully (data not shown). As culture on ECM can improve differentiation (Tsuchiya et al., 2014), I seeded decellularized mouse lungs with 10 million HBEC3KT cells and cultured them for 6 days to determine if ECM cues provided by the matrix could induce these cells to differentiate. H+E staining of these lungs indicated that these cells were able to attach to the matrix, particularly the

upper airway channels, but they did not form a defined epithelium in a manner similar to what was seen with the CR HBECs after 9 days (Fig 12). However, this experiment was done with KSFM media without calcium supplementation, while CR HBECs are cultured in BEGM differentiation media for 9 days in the reconstituted lung system. By culturing these cells in a lung with BEGM ALI media I may be able to improve on their ability to form an epithelium when cultured in a lung. Additionally, I am investigating the use of adipose derived stem cells for lung tissue engineering. Adipose derived stem cells have been being investigated for regenerative medicine applications for more than 20 years, in part because of their multipotency and in part because of the ease in which the primary cells can be isolated in large numbers from lipoaspirates (Zhu, Heydarkhan-Hagvall, Hedrick, Benhaim, & Zuk, 2013). As the lung consists of such a large number of stromal populations (Kotton & Morrisey, 2014; Nichols et al., 2014; Weiss, 2014) it would be extremely advantageous if a primary cell population could be used to populate multiple niches in the lung matrix. Immediate next generation experiments will be to culture human adipose derived stem cells in the lung matrix seeded either through the vasculature or the epithelium. Further, it would be ideal if I could reconstitute the vasculature of the lung using endothelial cells (Ren et al., 2015). Our work in reconstituting the vasculature is preliminary, but I will be seeding the vasculature with endothelial cells in lungs which also have been seeded with epithelial cells. Maturing a lung with multiple populations of cells may promote an additive or synergistic effect and help each of the populations recapitulate their appropriate niches.

Simulation of CFTR Correction in CF CR HBECs

Gene therapy for CF is hampered by the complexity of the disease and that there are very few models which can be used to study the potential efficacy of a given treatment, as discussed previously. The conditional reprogramming tissue culture method described in this dissertation may permit the study of gene or cell therapy for CFTR in *ex vivo* engineered models; which would permit more rapid development for cures for this disease. Importantly this would permit the development of normal and isogenically derived CF mutant HBEC to be tested in reconstitute lung matrices to separate the cell autonomous role of CFTR mutation in CF etiology form the chronic inflammation and matrix damages that occurs in vivo. In chapter 2 of this dissertation, the ability of these cells to tolerate clonal culture while preserving their ability to differentiate was demonstrated. In future experiments, I am planning to use reagents which have already been developed to test the ability of these cells to simulate CF therapy.

Using a colon organoid culture model, the Clevers lab has successfully modeled CFTR defects in CF patients. Following up on this work, they developed a suite of CRISPR reagents which use homologous recombination to correct the defect in CFTR and add G418 resistance into a nearby intron (Schwank et al., 2013). They have kindly given us their CRISPR reagents; I am transfecting CF CR HBECs with the same delta-F508 mutation with these reagents and are culturing clones. Clones which survive selection will be used for culture at ALI to determine if the genetic correction will be detectable in Ussing chamber analysis. These cells will also be cultured in decellularized lungs to determine their ability to reconstitute tissue *ex vivo*. These cells would be a very powerful basic science tool. It has been observed that HBECs isolated from CF patients have abnormal protein secretions; in particular they produce abnormally high levels

of matrix metalloproteinases (Peters-Hall et al., 2015). However, it is unclear if these secretions are caused by the chronic inflammation present in CF patient lungs, or if there is something intrinsic about the aberrant chloride channel activity in CF which causes these changes in protein expression. CF cells which have a corrected CFTR gene could be differentiated in a lung and ALI, their secretions could be collected and then subjected to mass spectrometry to quantify the protein secretions. This would give us additional insight into the mechanisms by which aberrant CFTR causes CF. These cells may also be a very valuable tool for translational research. In particular, there is still come controversy over the percentage of cells which must undergo gene correction in a patient with CF to make a therapeutic impact. By culturing mixtures of corrected and uncorrected CF CR HBECs from the same patient at ALI or in *ex vivo* reconstituted lungs, I can simulate various percentages of corrected cells achieved by virus-mediated gene therapy. By quantitating the changes in disease phenotype in these differentiated systems, I can rapidly establish a lower-bound of the percent of cells requiring gene correction in patients to produce a therapeutic impact.

Figure 11

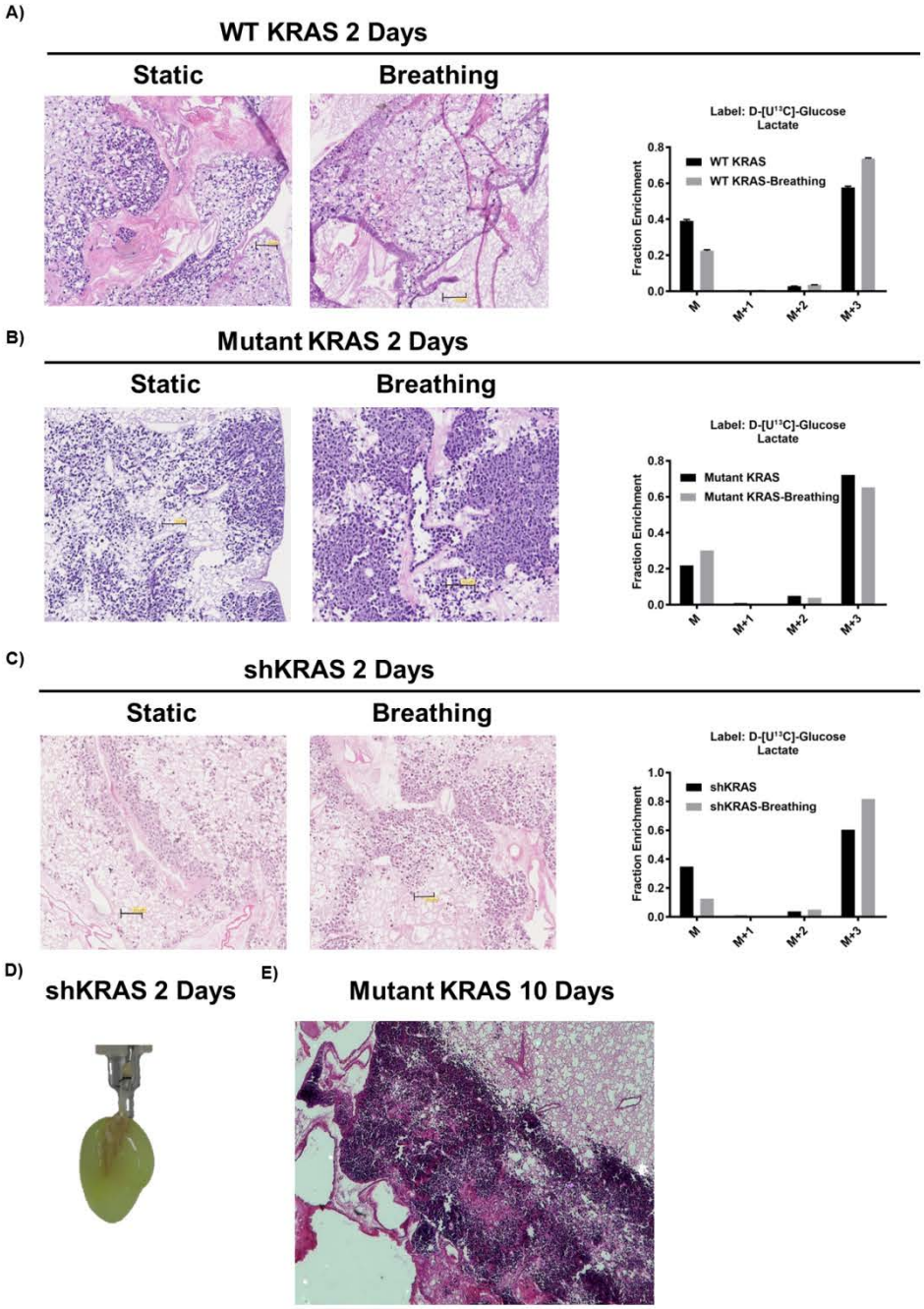


Figure11: Mutant KRAS Impacts Cancer Cell Mechanosensing and Metabolism: A) H+E staining and quantitation of mass spectrometry of HBEC 3KT cells seeded into a decellularized lung and cultured for two days with and without breathing. **B)** H+E staining and quantitation of mass spectrometry of experimentally progressed HBEC 3KT cells with mutant KRAS seeded into a decellularized lung and cultured for two days with and without breathing. **C)** H+E staining and quantitation of mass spectrometry of experimentally progressed HBEC 3KT cells with mutant KRAS and a shRNA for KRAS seeded into a decellularized lung and cultured for two days with and without breathing. D) Visualization of GFP positive experimentally progressed HBEC 3KT cells with mutant KRAS and shRNA to KRAS after 2 days of culture in a lung. **E)** H+E staining of a decellularized lung reconstituted with experimentally progressed HBEC3KT cells with mutant KRAS with simulated breathing for 10 days.

Figure 12

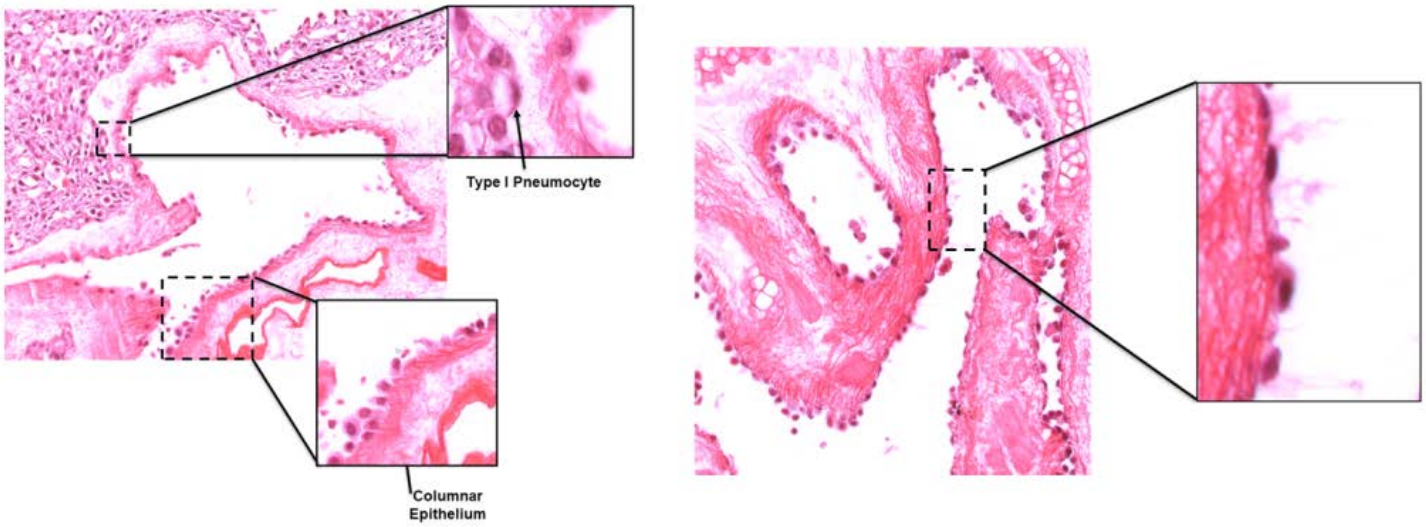


Figure 12: Seeding HBEC3KT Cells in a Lung: H+E staining of decellularized lungs reconstituted with 10 million HBEC3KT cells for 6 days.

Conclusions

Tissue engineering is a field with relatively few established best practices and tools. One of the reasons why there is so little standardization is that experiments in this field tend to be time-intensive and expensive, making it difficult to optimize approaches. In this research, I have developed tools for tissue engineering research with a focus on scalability. *Ex vivo* decellularized lung cultures using a simple bioreactor system can be adopted by labs relatively easily and cheaply using existing mouse models of disease. CR HBECs permit tissue engineering with expanded primary cells without genetic manipulations and without having to constantly derive new primary cell populations or iPSCs. These and the other methods developed in this work have already permitted basic science discovery; showing for the first time that HBECs can be induced to differentiate into both upper and lower airway cells. Further development of this system may ultimately lead to the engineering of transplantable Universal Donor tissue. In the pursuit of this end-goal, these systems will facilitate study of disease and biology in a more *in vivo* like context.

Acknowledgements:

This work was supported by the National Cancer Institute (Lung SPORE P50CA70907) and Cancer Center Support Grant. This work was also funded by the HHMI Med Into Grad Scholars Program, The TL1 Scholars Program (TL1TR001104), and an National Cancer Institute T32 training grant (2T32CA124334-08). This work was performed in laboratories constructed with support from National Institute of Health grant C06 RR30414.

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