DECELLULARIZED LUNG MATRIX AS A SCAFFOLD FOR MOUSE LUNG STEM CELLS

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

ZIBA JABERANSARI

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Dr. ANGELA PANOSKALTSIS MORTARI

MAY 2012

Acknowledgements

I sincerely thank Dr. Angela-Panoskaltsis-Mortari for giving me the opportunity of to be a part of her laboratory and for her continuous support, patience and motivations. I would like to acknowledge Andy Price and other members of the Dr.Mortari's lab for their patience and assistance with my projects. I also would like to thank Nisha Shah of the sorting center at the Masonic Cancer Center for her advice and assistance with FACS and sort analysis. I would also like to express my gratitude to Dr. Susan Keirstead for her advice and support during my Master's study. Finally, I would like to thank my father and family for their continuous support.

Dedication

This thesis is dedicated to my father who has always supported me and my beloved mother and sister.

Abstract

Decellularized tissue allows scientists to be able to examine cell proliferation and differentiation in the condition of a natural scaffold. The lung is one of several organs which were decellularized. This research proposes seeding decellularized lung with stem/progenitor cells. Endogenous lung stem cells were isolated by enzymatic digestion (Collagenase) of lung tissue and sorted based on expression of three markers: epithelial cell adhesion molecule EpCAM, (beta 4 integrin) CD104, and CD24. A cellular whole mouse lung matrix was prepared and complete decellularization shown by histology and PCR for genomic DNA. We expected the cells to survive and differentiate in this natural scaffold. However, the number of these cells was too low and cell survival was poor with no expression of any epithelial cell marker. The evidence in the literature shows that lung stem cells do exist but more work is required to investigate these cells cultured on a decellularized matrix.

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Introduction

Significance

According to the American Lung Association data base, more than 400,000 Americans die each year from lung disease (American Lung Association, 2012). Lung disease is the third cause of death and in every six deaths, one is due to lung disease (World Health Organization, 2011). Moreover, chronic airway diseases are included in the top ten global causes of death. According to the World Health Organization (WHO) deaths, due to respiratory disease, will increase from 18.7% in 1990 to 57% by 2020.

Therefore, the necessity for an efficient treatment for respiratory disease becomes more and more crucial. In addition, the only effective treatment for end-stage lung diseases is allogeneic lung transplantation and it has hurdles and complications. Some complications of lung transplantation therapies are: lack of enough donors for lung organ, incompatibility of HLA between donor and recipient cells, and chronic rejection and infections. Therefore, using stem cells and cell therapy seems to be a good alternative for patients who suffer from respiratory diseases. In this regard, repairing lung tissue may be feasible by applying stem cells for diseases such as asthma, chronic obstructive pulmonary disease (COPD) (James & Wenzel, 2007; Tzortzaki & Siafakas, 2009), cystic fibrosis (CF), and α-Anti trypsin deficiency (Kreider & Kotloff, 2009; Roomans, 2010). In addition, gene transfer therapies are good treatment strategies for lung disease. However, this technique is far from clinical feasibility (Lee & Southern, 2007; Martin, 2008) and these therapies have ethical issues due to possibility of death in human subjects (services, 2012).

One of the ethical and scientific issues is to find a proper source of stem cells for cell therapy. Scientists have recommended a variety of cell populations from endogenous to exogenous stem cell sources (Roomans, 2010).Exogenous stem cells derive from organs

other than lung. Exogenous stem cells include several sources: stem cells that are derived from other tissues such as bone marrow stem cells (Kotton et al., 2001), embryonic stem cells, side population from bone marrow called Hoechst-effluxing cells (MacPherson et al., 2005; Wong et al., 2009), epithelial stem cells that are present in bone marrow (Gomperts et al., 2006), Mesenchymal stem cells, and iPS are other exogenous stem cells (Roomans, 2010; Takahashi & Yamanaka, 2006). On the other hand, there are endogenous lung stem cells located in the lung. Some researchers believe that lung stem cells and niches exist. In fact, Anversa et al's recent study provided evidence for the existence of candidate stem cells in the human lung that express c-kit marker (2011).

Most studies on lung stem cells are done *in-vitro* and by culturing in two-dimensional or synthetic scaffolds. Little knowledge is available regarding the differentiation of endogenous stem cells and their potential to differentiate into a specific lung cell lineage in a condition similar to natural lung matrix. The current study focuses on investigation of endogenous lung stem cells cultured in a decellularized matrix. We hypothesized that epithelial progenitor cells in lung epithelium (EpCAM+) (McQualter, Yuen, Williams, & Bertoncello, 2010) can engraft in an acellular lung matrix (Price, England, Matson, Blazar, & Panoskaltsis-Mortari, 2010) and differentiate into alveolar epithelial cells. Our data showed the existence of this population in the lung. However, engrafting endogenous lung stem cells requires various elements in terms of proper lung epithelium regeneration, and angiogenesis.

Exogenous Stem Cells

Adult bone marrow stem cells can regenerate lung tissue in a mouse model with naphthalene damaged lung airways, and express Clara Cell Secretory Protein (CCSP) which is usually a marker of Clara cells. The CCSP positive bone marrow cells also expressed CD45, CD90, CD73, and CD105, which are mesenchymal markers (Giangreco et al., 2009). Some people speculated that type II alveolar cells and type I alveolar cells can be derived from CCSP positive cells. Alveolar type II cells express pro-surfactant protein (proSpc) and alveolar type I cells express aquaporin 5 (AQP5) markers. However,

using transgenic mice and a lineage specific reporter, Kotton et al.'s research revealed that hematopoietic stem cells, prepared from bone marrow cannot regenerate an injured lung, however, can generate pulmonary alveolar epithelium (Kotton, Fabian, & Mulligan, 2005) . It is now agreed that bone marrow cells have low engraftment in lungs (Weiss et al., 2011).

Another cell population which has been suggested as a good option is embryonic stem cell (ES). The most significant hurdle for the use of embryonic stem cells is the ethical issue of damaging human embryos to prepare ES cells. In addition, there is always a chance of teratoma formation (Cao et al., 2007). Another source of pluripotent stem cells is the amniotic fluid stem cells and these cells can be easily delivered to the lung through alveolar capillaries (Warburton et al., 2008). However, little research has been carried out on the efficiency of exogenous stem cell engraftment in the lung parenchyma. In other words, regarding seeding stem cells via capillaries, the most challenging problem is the "homing" problem (Roomans, 2010). In other words, when researchers inject stem cells through capillaries there is a chance that they will incorporate in another location instead of the desired location, which would be the epithelium of the lung.

Another candidate for lung regeneration is bone-marrow side population (SP) cells. According to Olmsted et al.'s study, these cells do not stain with Hoechst 33342 dye. This population expressed CD45, Sca-1, was negative for lin markers and could rescue the hematopoietic system of lethally irradiated mice (Olmsted-Davis et al., 2003). Macpherson et al. examined the engraftment of sex mismatched bone marrow, derived SP cells, and the results demonstrated the ability of the male donor cells could engraft in a female recipient's damaged respiratory epithelium (MacPherson et al., 2005).

Mesenchymal stem cells (MSCs) have also been suggested as an exogenous stem cell in lung cell therapy. These cells are multipotent, having plasticity, and are another cell population that has been used for repairing lung tissue. These cells are derived from various tissues such as bone marrow, adipose, and cord blood. They express: CD73, CD90, and CD105 (Le Blanc & Ringdén, 2006). Another important characteristic of these cells is the ability to differentiate into three tissues: cartilage, bone, and fat. Two populations of MSC have been reported for regeneration of lung tissue. The first

population expresses cytokeratin as well as CD45 but the other one expresses (MacPherson, Keir, Edwards, Webb, & Dorin, 2006) epithelial receptor cytokeratin 5 (CK5), and CXCR4, which is chemokine receptor (Gomperts et al., 2006; Ringdén et al., 2006). In addition, scientists have recently found evidence regarding their suppressive effect on an inflammatory reaction in the body. Therefore, scientists can use MSCs for treatment of severe graft versus host diseases after transplantation of bone marrow (Ringdén et al., 2006). Moreover, they can produce various growth factors and cytokines. Although, MSCs seem to be a very good option for regeneration of the lung since they have a good capability of differentiation, the engraftment results in various studies have not been satisfactory. In fact, in different injury models various engraftment results were observed. However, MSC can induce differentiation of other stem cells such as HSC (Sueblinvong & Weiss, 2010). Also, MSCs can increase engraftment of HSC (Maitra et al., 2004). The last option for lung stem cell therapy is iPS cells. These cells have embryonic stem cell properties. Regarding their pluripotent characteristic, these cells can differentiate to three germ layers including lung epithelium (Boulting et al., 2011).

These advances provided an opportunity for autologous iPS therapies. Therefore, iPS cells less likely trigger immune reactions in recipients of cell therapy. In addition, it can address the ethical issues of ES cell therapies (Pietronave & Prat, 2012). However, the most important negative aspect of these cells is the formation of teratoma.

Endogenous Lung Stem Cells and Niches

According to Warburton et al. (2008), after an acute oxygen injury in alveolar epithelial cells (AEC), more telomerase is expressed in these cells. In addition, the alveolar surface is quickly repaired after the injury. This evidence raises the possibility of resident stem cells and their potential to renew lung epithelium (Warburton et al., 2008). Indeed, lung stem cell is a controversial discussion among other stem cell researchers. There are various ideas about the existence of a stem cell niche as well as airways or

epithelial stem / progenitor cells in the lung. In terms of the lung organ anatomy for each part of the lung organ, various stem cell niches have been suggested. In fact, the earliest lung stem cell niches, the neuroepithelial bodies (NEBs) were recognized in the human embryo after the third month and were found to be responsible for segmentation of lung and its development (Khoor et al., 1996). This niche can generate Clara cells which are assumed to be lung stem cells. From NEB, the first Clara Cell Secretary Protein (CCSP) was produced (Shan et al., 2004.Stripp and Reynolds, 2006). Moreover, previous research recommended three locations for niches: cells in the tracheal submucosal gland ducts, in the bronchial and bronchiole neuroepithelial bodies (NEBs) (Susan D. Reynolds & Malkinson, 2010), and bronchoalveolar duct junction (BADJ) in the terminal bronchioles (Borthwick, Shahbazian, Krantz, Dorin, & Randell, 2001; Engelhardt, Schlossberg, Yankaskas, & Dudus, 1995; Giangreco, Reynolds, & Stripp, 2002; Hong, Reynolds, Watkins, Fuchs, & Stripp, 2004; Kim et al., 2005). The BADJ contains bronchio alveolar stem cells (BASC) and also gives rise to Clara cells as well as ATII. According to Kim et al (2005) BASC have stem cell characteristics and repair lung epithelium after an *in-vivo* injury.

On the other hand, Hong et al. suggested conducting airways in proximal and distal area airways contain niches (2001). In contrast, Borthwick et al., proposed the gas-exchange alveolar epithelium as a stem cell site (Liu, Driskell, & Engelhardt, 2006). Generally, this evidence recommends different cell types such as Clara cells, Basal cells, cells in the submucosal gland, and cells in conducting air-way as lung stem cells. These cells have self-renewal and multipotency characteristics (Borges et al., 1997; Engelhardt, 2001; Engelhardt et al., 1995). Also, it has been suggested that alveolar type II (ATII) cells can be alveolar epithelium progenitors (Mason & Williams, 1977). Moreover, in airways there is a population of cells that are the same as SP cells and have similar biochemical and phenotype properties as SP cells in bone marrow. These cells efflux the Hoechst-33342 dye, and are small and granular (S.D. Reynolds et al., 2007). According to their work, these cells repair airways at different locations after injury. Furthermore, two new cell populations have been recommended as lung stem cells: first, cells with

epithelial cell adhesion molecule EpCAM , CD104, CD24 marker (McQualter et al., 2010) and secondly, C-Kit positive cells (Kajstura et al., 2011).

Structure of Lung and Lung Stem Cells

Lungs develop from foregut endoderm (Rawlins et al., 2008) and encompass a wide variety of cell types. The lung structure is composed of three epithelial parts: (1) airways, which are made from cartilage (i.e. trachea and bronchi); (2) distal bronchioles; and (3) alveoli, which exchange gas. Of the whole lung, 85 percent is of alveolar and 15 percent non-alveolar tissue. Figure 1. illustrates lung structure and different cells that form lung epithelium. As is shown, the

non-alveolar tissue is conducting airways and half of this is a non-cellular matrix. This non-alveolar area contains pseudo-stratified epithelial cells, goblet cells, basal cells, neuroendocrine cells, ciliated and non-ciliated (Clara cells) (Figure 2) (Rawlins et al., 2008). On the other hand, alveolar epithelium contains alveolar type I and II cells. Alveolar or pneumocytes type II are squamous cells that pump ions, and produce surfactant proteins such as surfactant protein–C (SP-C) or (sftpc) which are crucial for the inhibition of lung collapse and the ability of the lung to inflate at physiologically normal pressure (Petersen et al., 2010). Moreover, previous studies revealed these cells are progenitors for ATI (Young, Kremers, Apple, Crapo, & Brumley, 1981). In addition, ATII play a role in immunological functions. ATII cells are 60 % of all alveolar cells, but they only cover 5% of alveolar epithelium. In contrast, 40% of the alveolar cells are ATI and cover 95% of the alveolar surface (K. Stone, R. Mercer, P. Gehr, B. Stockstill, & J. Crapo, 1992). As Figure1 demonstrates these cells are flat and responsible for gas exchange. Alveolar walls are single layer epithelium without any ciliated cells.

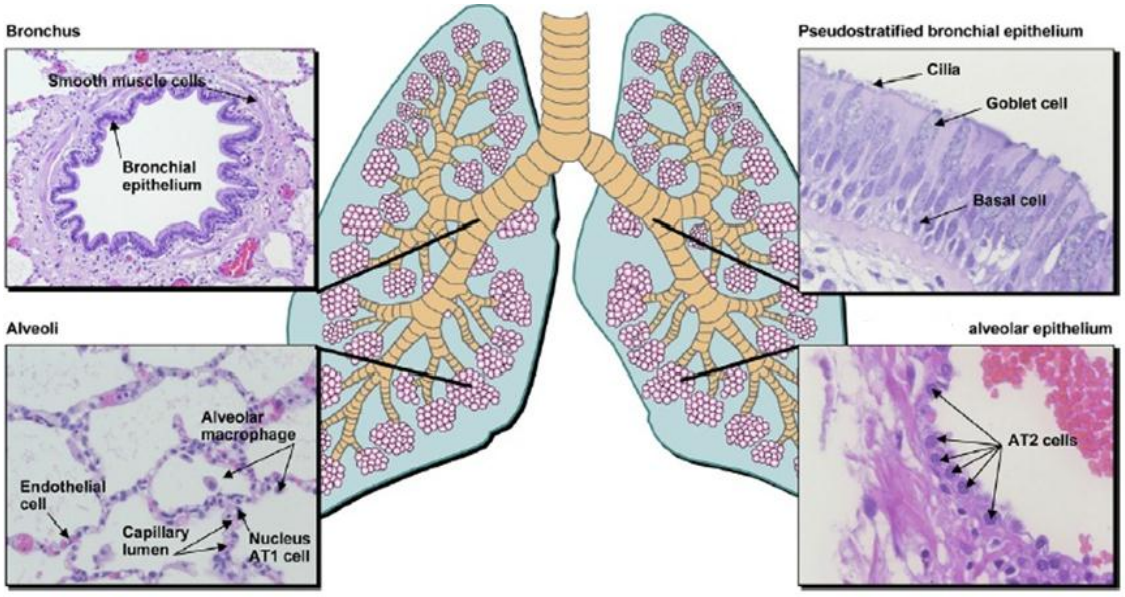


Figure 1. The structure of the lung with trachea and bronchioles end in alveolar epithelium. The smaller squares show H&E sections from lung tissue of mature cells that comprise lung tissue.

Among lung cells, Clara cells and basal cells were suggested as lung / progenitor stem cells. The current idea regarding the differentiation and hierarchy of lung stem cells is that Basal cells give rise to many cells including Clara cells. Then Clara cells differentiate to ATII and from ATII, ATI cells will be derived.

Figure 2 from (Rawlins et al., 2008) illustrates the distribution of various specialized cells and niches in the lung system.

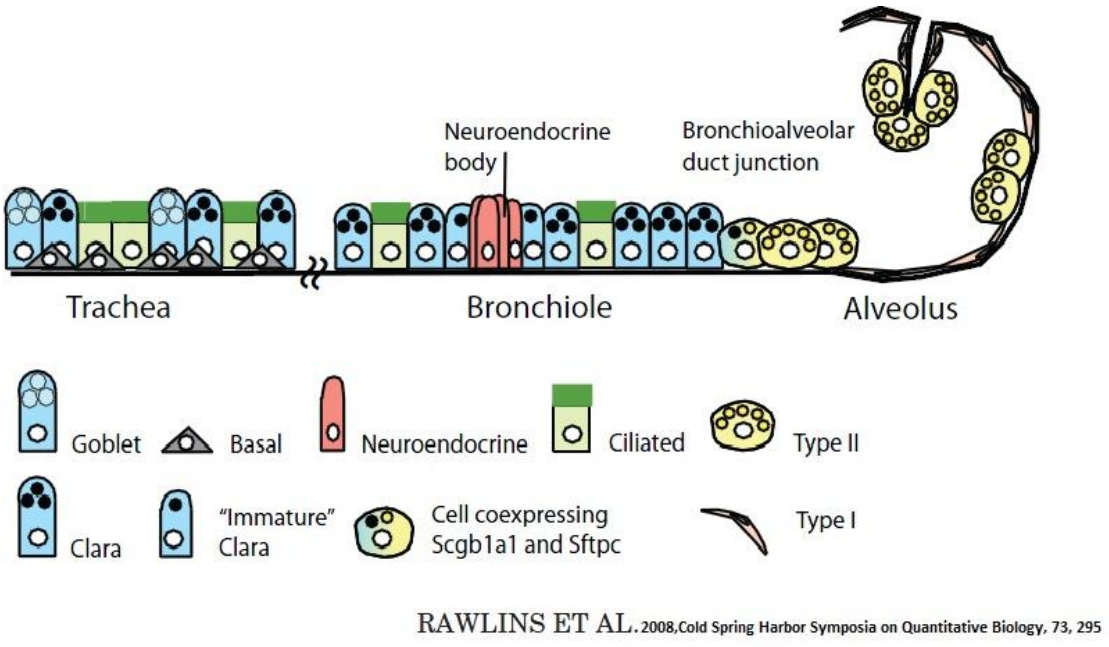


Figure 2. Epithelial Cells in Mouse Lungs.

As is shown in Figure2 basal cells are located in the trachea. These basal cells express cytokeratin Kr5 and p63.

The basal cells are undifferentiated. These cells in humans exist in all airways (Rock et al., 2009). The junction of bronchioles and alveoli the bronchiole alveolar duct junction

(BADJ) contains cells that express both secretoglobin or Clara Cell Secretary Protein (CCSP also known as Scbg1a1 and CC10), and ATII cell's protein SP-C. However, in human, the size of BADJ is larger (Rawlins et al., 2008). These cells are bronchioalveolar stem cells (BASCs) and can give rise to both airway and alveolar cells. In addition, the neuroendocrine bodies (NEB) exist as a cluster in the bronchiole. Rawlins et al. suggested Clara cells are progenitors of ciliated cells and ATII.

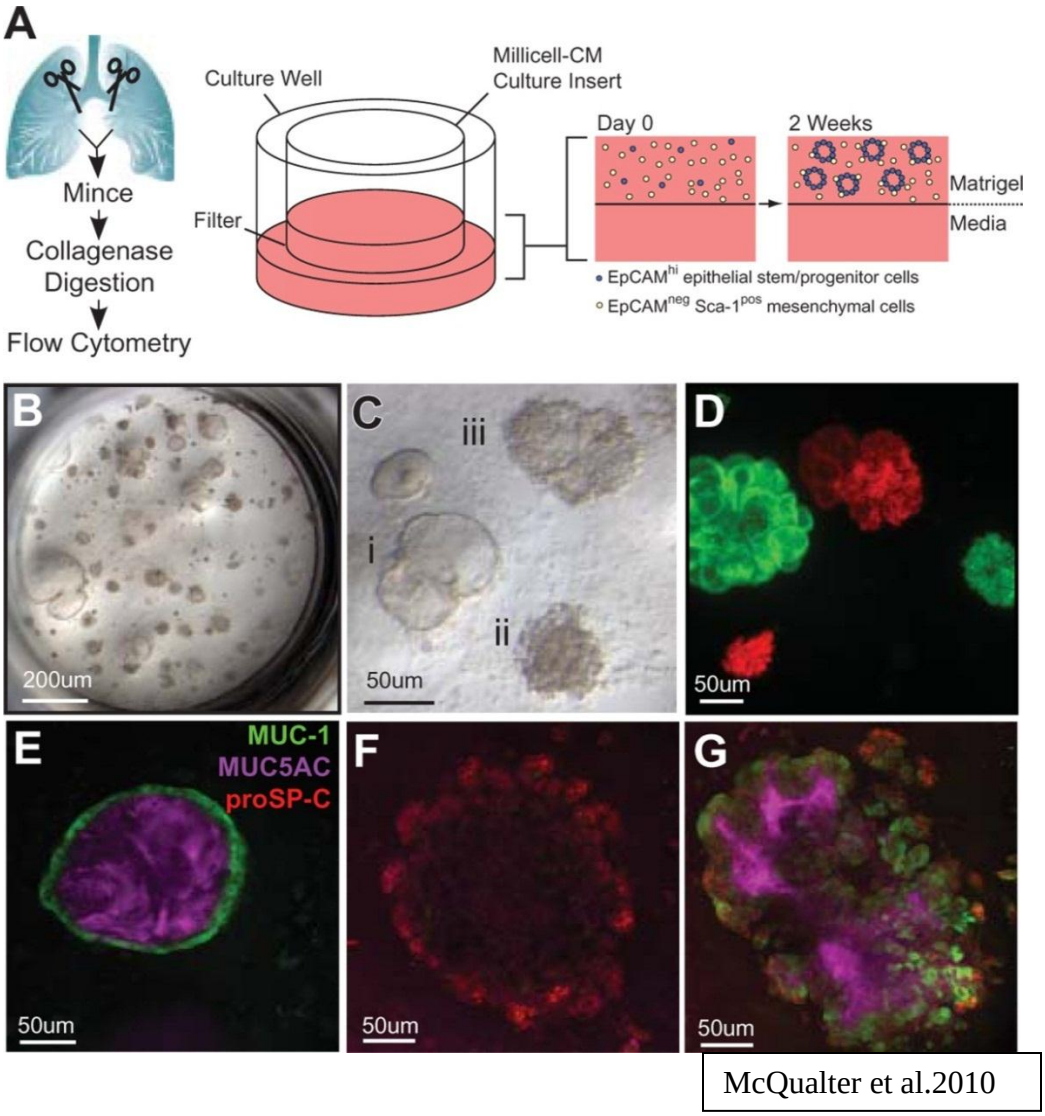
The Clara cell population is divided into two different populations based on naphthalene injury models in lung. These cells, which have a cuboidal shape, play a role in homeostasis of lung tissue, immunologic reaction, and epithelial repair. These non-ciliated cells secrete (CCSP), which is a significant protein for anti-inflammatory and immune reaction, and regulations. In the lung it is a marker for defining Clara cells

(Wang, Rosenberger, Bao, Stark, & Harrod, 2003). One group is the Clara variant cells (Clara^{V} cells) (Rawlins et al., 2008). The Clara^{V}cells are located in NEB and (BADJ). They are resistant to naphthalene injury and are undifferentiated. According to Reynolds et al. bronchiolar epithelium is derived from Clara cells (Susan D. Reynolds & Malkinson, 2010). It is undeniable that Clara cells repair proximal and respiratory structures (Stripp & Reynolds, 2008). However, the property of mucus secretion is not included in stem cells characteristic. Since stem cells have to be undifferentiated, scientist cannot conclude that Clara cells are lung stem cells (Kajstura et al., 2011).

There is also debate about basal cells clonogenicity and their potential to generate various cells rather than just lung epithelium cells. Therefore, scientists have looked for another cell population which could be lung stem cells with all self-renewal, clonogenicity, and multipotency *in-vitro* and *in-vivo* conditions. Based on Anversa et al's previous study on cardiac stem cells and the expression of C-Kit positive cells on cardiac stem cells (CSCs) (Barile, Messina, Giacomello, & Marbán, 2007), they hypothesized this marker can be an efficient marker for isolating endogenous lung stem cells in both adult and human embryos. They found a low number of human lung stem cells in comparison to bone marrow stem cells. Their result from adult human lung tissue showed the existence of one human lung stem cells (hLSCs) per 2400 cells. They found one cell per 6000 cells in the bronchioles and one hLSC per 30000 of alveolar cells. Indeed, 79% of hLSC were located in bronchioles and 21% in alveoli. As a result, each 10 cm^3 of human lung tissue contains 7700 human lung stem cells (Kajstura et al., 2011).

Moreover, their results from research on human embryos showed the existence of, on average,"1 hLSC per 4100 cells in whole tissue" (Kajstura et al., 2011). On the other hand McQualter and et al. isolated a population of epithelial/progenitor stem cells from the lungs of transgenic mice that express either RFP or eGFP. These progenitor cells were CD45 ^{Neg}, CD31^{Neg}, epithelial cell adhesion molecule EpCAM $^{+}$, CD104^{+} (beta 4 integrin), CD49^{+} and heat stable antigen (CD24) positive (McQualter et al., 2010), and were shown that can differentiate into various lineages of lung cells.

These cells were co-cultured with mesenchymal stem cells (MSCs) that obtained from wild type mice. They co-cultured progenitor cells and MSCs in a three-dimensional Matrigel-based condition. As illustrated in the Figure 3A, they analyzed their colony forming potential. These RFP and eGFP express cells were also EpCAM+, CD104, CD24 positive and were isolated from transgenic mice. These lung stem cells could produce three different types of colonies. According to Figure 3 B and C the morphology of these colonies are different. They observed all colonies where either red or green. Therefore, they interpreted that colonies arise from progenitor cells. In fact, none of the existing colonies were derived from Sca-1 MSCs. Also, they observed mesenchymal induction by two growth factors FGF10 and HGF in the culture. Thus, they recommended addition of these two growth factors instead of co-culturing with them. They did not provide any information regarding the relevance of this population and Clara cells *in-vivo*.



McQualter et al.2010

Figure 3. A) Shows the harvesting EpCAM^{hi} + cell from lung and an organotypic lung epithelial colony-forming assay. B) Colonies of co-cultured Sca-1pos MSCs and EpCAMhi epithelial stem/progenitors. C) Three different colonies: i) airway-like, ii) alveolar-like, and iii) mixed colonies. D) Fluorescent image of EpCAMhi cells derived from RFP and eGFP mice co-cultured with Sca-1pos EpCAMneg cells from C57Bl/6 mice showing monochromatic colony-formation (= clonal proliferation). All colonies were fluorescent. Thus, epithelial colonies were derived from EpCAM^{hi} epithelial stem/progenitor cells and not from Sca-1pos MSCs. The MUC-1 is illustrated in green, proSP-C in red, and MUC5AC in purple. E) The airway-like colony stained for MUC-1

and MUC5A. F) Illustration of the red color alveolar-like colony. P) The mixed lung epithelial colonies visualized in purple.

All in all, with a brief review of the available research results on lung stem cells, we can conclude that stem cells do exist in the lung tissue but most studies are carried out *in-vitro*. Therefore, more research is required regarding lung stem cells and their application in cell therapies.

Research Methods for Lung Stem Cells

In-vivo and In-vitro Study Models:

Tissue Source

Most research has been done on murine lungs. Due to the limited sources of human lung tissue as well as ethical issues, animal models are generally well developed regarding lung stem cells. Nevertheless, Anversa et al. (2011) recently published new evidence about human lung stem cells. In addition, other models such as large animals (pig and sheep) have their own complications. Therefore, due to the cost and preparation complications of lung organs in large animals, most studies were done with the use of the murine lungs.

It is common to study regenerate capacity of endogenous and exogenous stem cells through inducing injury to the lung tissue. The most common tools for preparing injured lung are naphthalene or SO2. Anversa et al. (2011) also used a cryoinjury model that involved inserting a cold needle into the lung tissue. After preparing injured models in mouse lungs they tried an injection of stem cells via trachea or intravascular. These research methods, as I mentioned previously, have the problem of homing the stem cells in the exact place of injury where you see the regeneration of lung. Therefore, scientists have attempted to prepare a different method for applying stem cells such as designing

engineered lung organ or a scaffold for culturing lung tissue. For this purpose they have used synthetic and organic materials. They hypothesized that culturing lung stem cells in a condition with properties of natural lung architecture can induce proper differentiation of lung cells.

Manufactured Scaffold

Synthetic Materials

Cortiela et al. (2006) used polyglycolic acid (PGA) (Song & Ott, 2011) and Pluronic F-127 (PF-127) for designing a scaffold. The PGA is made from fiber with porosity up to 0.81 and area/volume ratios as high as 0.05 micron (Mikos et al., 1993). Their *in-vitro* results were promising and they have differentiated cells from lung progenitors. However, *in-vivo* the immune reaction to PGA caused disruption of lung tissue development (Cortiella et al., 2006). Also, PGA fibers in an *in-vivo* experiment incite the immune reaction and hydrolysis of these fibers.

In another study researchers designed a scaffold by Nano fibers: poly-L-lactic-acid (PLLA) and porous sponge of poly-lactic-co-glycolic acid (PLGA) (Mondrinos et al., 2006).These fibers were smaller than 1mm. They tried a culture suspension of fetal pulmonary cells in Matrigel hydrogel in this three dimensional scaffold. Although, Mondrinos et al. (2006) could achieve generation of ATII cells and detect an expression of SP-C gene, the epithelial cell differentiation wasn't maintainable (Cortiella et al., 2006). The bio-compatibility and lack of access to air are challenges that decrease efficiency of synthetic-material based scaffolds.

The previous attempts to culture lung progenitor cells and maintain differentiated ATII or 2-Dimensional scaffolds failed. Therefore, these culture conditions were not efficient in terms of lacking factors which are essential for differentiation of cells and branching out of bronchioles. Modifying culture condition revealed the requirement of Collagen or Matrigel and growth factors. In fact, the essential property of Matrigel is its content which is basal membrane proteins. The proteins in extracellular matrix includes:

Laminin, CollagenIV, and glycosaminoglycans. During development, lung epithelium differentiates on the basal lamina. Matrigel is made from mouse tumor cells and consists of Laminin. Therefore, Matrigel plays a similar role as natural ECM.

According to Mondrinos et al AII2 cells grew in 2-D on Matrigel and differentiated to ATI cells. In contrast the same cell population cultured on plastic surfaces didn't differentiate. (Blau, Guzowski, Siddiqi, Scarpelli, & Bienkowski, 1988; Cecilia Crisanti, Koutzaki, Mondrinos, Lelkes, & Finck, 2008).

As a result, scientists have designed three dimensional matrixes by natural materials. These matrixes not only have three dimensional structure of organs which can induce a generation of specific cell lineages, but are also made from ECM proteins which can recapitulate development of lung.

Natural Materials

Natural material such as collagen (Sugihara, Toda, Miyabara, Fujiyama, & Yonemitsu, 1993), Glycosaminoglycans (P. Chen, Marsilio, Goldstein, Yannas, & Spector, 2005) and compressed skin porcine (Gelfoam) (Andrade, Wong, Waddell, Keshavjee, & Liu, 2007) have been used to design efficient scaffolds. In the study by Chen et al., after seeding cells in Collagen and Glycosaminoglycan (collagen type I-chondroitin 6-sulfate); alveolar-like structures were generated. However, the size of pores in the scaffold wasn't proper and merely differentiation of alveolar cells was observed. Most of these scaffolds lack elasticity and the complexity of normal lung organs. Furthermore, degradation rate was one of the complications of these material that should be considered (Lavik & Langer, 2004). These studies revealed the disadvantage of manufactured matrixes from natural materials. In addition, these results suggested the requirements of other factors such as growth factors that have an effect on differentiation of epithelial cells.

In this regard, particularly FGF2, plays a role in morphogenesis of branches (Mondrinos et al., 2006). Moreover, recently McQualter et al. suggested "mesenchymal

induction" of two growth factors FGF10 and HGF which can be alternatives of mesenchymal cells.

Natural Scaffolds:

The role of ECM is fundamental in cell cross talk and communication. Most cells have integrin receptors for ECM. These receptors contribute to cellular differentiation and function and can direct cell fate from proliferation to formation of a structure (Mondrinos et al., 2006).For example fibronectin is important in branching bronchioles during lung development. One way to access ECM is to decellularize the tissue. Thus, researchers tried to prepare acellular organs to have natural matrixes that include ECM and proper to culture progenitor cells.

The achievements in preparation of acellular organs opened a new horizon towards organ transplantation and cell therapy. Through these techniques, an organ can be depleted from whole cellular and DNA content and only extracellular proteins will remain in the organ's tissue. Therefore, the whole organ can be used as a scaffold which embraces biocompatible natural ECM protein. This scaffold has the three dimensional structure and complexity of a natural organ. Thus, it can encompass seeded cells and provide satisfactory condition for normal differentiation of infused progenitor cells. For instance, one of the first acellular organs used for therapeutic application was skin. *Alloderm1* is an FDA-approved acellular dermal matrix. *CryoValve1 SG* Pulmonary Valve, a decellularized human valve, is currently implanted in right ventricle of a heart with congenital defect. These acellular matrixes showed the possibility of engrafting decellularized organs and successful cell growth, as well as a resistance to infection (Song & Ott, 2011).

In conclusion, preparation of an acellular lung organ via decellularizing techniques and using a natural scaffold can revolutionize the future of lung disease therapies. In other words, a decellularized lung not only has complexity and a 3-D structure but also provides proper ventilation mechanics in a decellularized lung. Thus, cells can be

cultured in a condition similar to *in-vivo* condition. Indeed, this scaffold with collagen and other ECM proteins collaborate to form air-blood barrier in the lung. Also, the problem of immune reaction is addressed. As a result, in this research we tried to prepare a decellularized murine lung based on a technique developed in the Mortari laboratory (Price et al., 2010). Progenitor/stem cells were isolated according to McQualter et al.'s technique (McQualter et al., 2010). In this acellular matrix, elastin and laminin are decreased by about 50% but collagen amount is almost normal (about 80%). Also, results of Pulmonary Function Test showed lower compliance and increased elasticity in the decellularized lung. Indeed, decellularized lungs lack surfactant proteins that decrease its compliance. These decellularized matrixes were placed in bioreactors and maintained their structure after ventilation. This natural scaffold can be a good option for studying differentiation of lung progenitor cells in terms of having enough complexity and three dimensional structure, ability to ventilate and to provide good conditions to generate air and blood barrier (vascular matrix network) (Price et al., 2010). This scaffold contains natural material which can be used for culturing autologous or exogenous stem/progenitor cells. Overall, the problem of limited donated organs and immune reactions could be addressed.

Mortari et al have prepared whole mouse lung as acellular matrixes based on an older protocol to decellularize lung fragments which kept the pattern of ECM (Lwebuga-Mukasa, Ingbar, & Madri, 1986). Therefore, seeding a decellularized lung was done by infusion of stem/progenitor lung cells in a bioreactor. For purpose of recapitulating normal lung condition and cell differentiation in bioreactor the air via ventilator was provided. Also, FGF10 and HGF were added to media to deliver mesenchymal induction.

Material and methods

Mice

C57BL/6 (B6) male or BALB/c female mice were purchased from Jackson Laboratories (Bar Harbor, ME). B10.BR (BR) male mice were bred in The University of Minnesota Research Animal Resources facility from breeders originally purchased from

Jackson Labs. Mice were used at 2-3 months of age. The use of mice was approved by the Institutional Animal Care and Use Committee. To distinguish the injected sorted lung cells from any remaining cells in the decellularized lung, the cells were mismatched. For instance, based on the availability of mouse types in our lab, if the decellularized lungs were prepared from B6 mice, lungs from BR mice were taken to prepare the cell suspension and to sort the cells to inject into decellularized B6 mouse matrix.

Preparing Decellularized Adult Mouse Lung

The mouse was euthanized with a lethal injection of Nembutal and the thoracic cavity opened to expose the lungs (Figure 4). The salivary glands were removed to expose the trachea. After that, the heart was injected with 3 cc of distilled, deionized (DI) water with a 21 gauge needle through the right ventricle. This injection washes the blood from heart and respiratory system. According to the diameter of the trachea, which is 2.5 mm, a 19 gauge needle was used to infuse lungs with 3-cc DI water through the trachea. As Figure 4 shows after infusing lung and tying the suture quickly; the needle was removed. This tie will prevent DI solution from leaking out from lungs. In addition, the thymus was removed and the whole respiratory system, while still attached to the heart, was also removed and placed in the DI solution inside of one well of a sterile six well plate for one hour at room temperature.

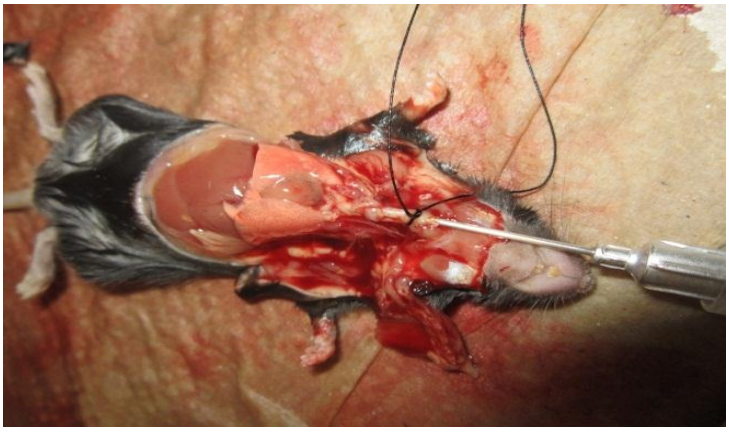


Figure 4. Lung infusion. This figure shows a mouse with opened thoracic cavity and 19 gauge needle injecting DI water through the trachea.

All subsequent wash steps were done in the hood to prepare a sterile matrix. After one hour, the first wash was done with Phosphate-buffered saline (PBS) 1%. Lungs and heart were rinsed three times with 3 cc of PBS solution through the trachea with 19 gauge needle and three times 3-cc rinses through the right ventricle via 21 gauge syringe. After each injection, the needle was removed and the solution was allowed to drain out of the lung.

The second incubation was with 3 cc of Triton detergent solution. Triton X-100, sterile filtered 0.1% in PBS injected via the trachea and into the right ventricle and incubated for 24 h at 4°c.The next day, the lungs were rinsed three times with PBS using the same technique. The second buffer was a 2% sodium deoxycholate solution made in PBS. This buffer was infused with 3-cc through the trachea and 3 cc into the right ventricle and incubated for 24 hours at 4°C. On the third day, the lungs were removed from the sodium deoxycholate solution and rinsed with fresh PBS in a new six well plate. The lungs were injected with 3 cc of 1M NaCl through the trachea and 3 cc into the right ventricle and incubated for 1 h at room temperature. As the last step, rinses of PBS were repeated and 3 cc of 30 mg/mL porcine pancreatic DNase in 1.3mM $MgSO_4$ and 2mM $CaCl_2$ through the trachea and into the right ventricle were injected. The incubation again was 1 hour and once again washed with PBS buffer as described above. Figure 5 shows the acellularized lung after three days of the decellularization process.

This matrix can be used right after the last rinse with PBS or can be stored in PBS 1x

in 4C for at least 4 weeks.

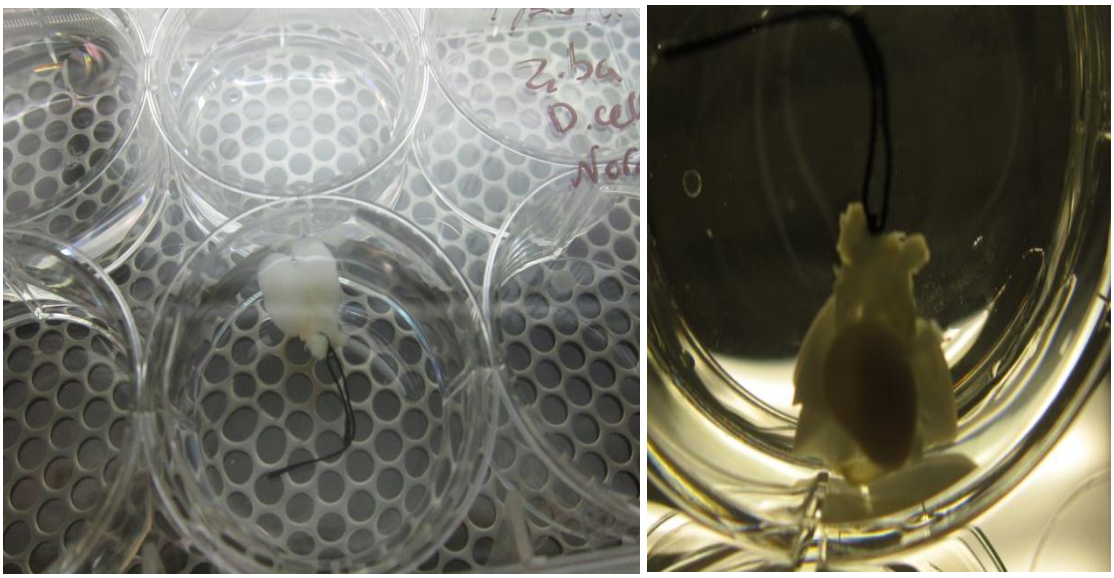


Figure 5. Decellularized lung. Images of a decellularized lung after the three days decellularizition procedure.

Preparing Bioreactor

Each bioreactor was comprised of a T-25 plastic flask attached to a cannula with a 0.2 µm syringe filter and connected to a ventilator. The bioreactors were prepared inside an aseptic laminar flow hood to keep them sterile. To avoid contamination, the silk sutures were kept overnight at 100% ethanol and the cannulae were incubated one hour in 50% bleach. After washing the cannula with PBS, a hole was opened through the filtercap of the culture flask with a 19 gauge needle and the cannulae was placed in the hole and fixed in place by superglue.

The decellularized lungs usually can be stored for 4-5weeks in 1×PBS. It was attached to the cannulae by sliding the trachea over the cannula and tying in place with sterile suture. The 35 ml culture flask was filled with enough media to cover the lungs. The lungs (attached to the cap) were suspended in the flask and hooked up to ventilator set to a volume of 300uL at a rate of 180 breaths per minute (for the mouse). The Figure 6 shows three attached bioreactors to the ventilator in the incubator. As is shown, the

ventilator hoses at were first attached to a blue filtercap then connected to cannulae in the cap of culture flask.

Infusing of cells was done through the cannula and trachea. Before infusing the lung with cells, culture medium was injected to wash out remaining PBS. Injecting cells with1ml syringe through the trachea was done and lungs were inflated. The bioreactor was placed into incubator and attached to the ventilator (Dual Mode Ventilator, VFA-23-BV, Kent Scientific) at 37°C and 5% CO2 for two weeks. Every three days the media was changed and 1ml media infused to the lung for feeding cells that were seeded into the decellularized lung. Moreover, two growth factors were added to induce mesenchymal induction and better cell growth (mHGF at 30 ng/mL and FGF10 at 50 ng /mL).

In each experiment the decellularized lung, without cell infusion, was used as a negative control. Also, a cell population, which was negative for EpCAM and CD-24, was isolated from lung cells suspension through sort purification and was injected to a third decellularized matrix in the third bioreactor.

Figure 6. Bioreactors were attached to ventilators.

Preparation of EpCAM Positive Lung Stem Cells

To prepare a single-cell suspension of lung cells, collagenase was used to degrade connective tissue collagen. Collagen provides structural and organizational framework for tissues (Perumal, Antipova, & Orgel, 2008).

Collagenase type I from *Clostridium histolyticum* at 1mg/ml in sterile PBS was preheated to 37◦C and 3 mL was injected into the exsanguinated lung through the trachea via a 19-gauge needle. The needle was inserted into the trachea and secured with silk suture. Then the needle was removed and a suture was used to prevent leaking of collagenase solution from trachea. In some experiments, 10% Fetal Bovine Serum (FBS) was added to collagenase to increase the viability of cells. The FBS helps cells to survive and stabilize cells during digestion. Moreover, another factor that can adversely affect collagenase activity is excess calcium ions in the tissue. Therefore, to have better tissue harvest results, it is important to pay attention to the amount of calcium in the tissue.

The lung was immersed in a 15ml tube with 3 ml collagenase and incubated for 40 minutes in a 37° C water bath. Lung tissue was then ground through a 40 µm cell strainer into a petri dish with 2ml of media (Epi-CFU expansion medium). The Figure7 shows digested lung tissue which was ground through a 40 µm blue cell strainer. The cell suspension was moved to a sterile 50 ml centrifuge tube and centrifuged 10 minutes at 1200 g.

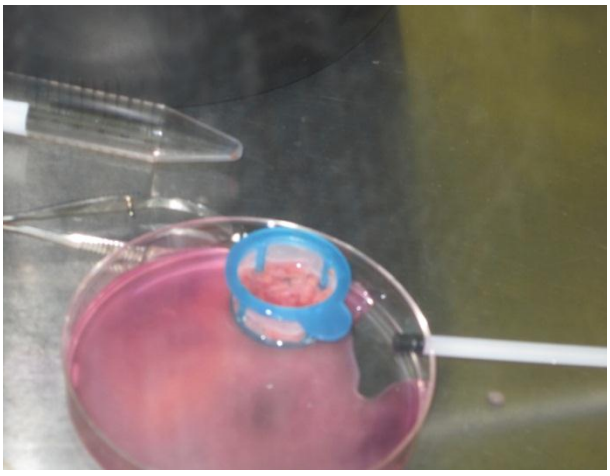


Figure 7. Tissue preparation.

Ground lung tissue cells pass through a strainer. The blue 40 µm strainer contains pink

chunks of digested lung and is placed in the petri dish with media.

Red blood cells were lysed with ACK Red Cell Lysis buffer (2 ml/ 9 lungs). After the lysis step, cells were washed with PBS + 0.1% BSA 5 min and centrifugation at 1200g 4◦C.

Hematopoietic and endothelial cells were removed by magnetic bead depletion. Cells were incubated with biotinylated antibodies against mouse CD31, CD45 and Ter119 for 20 minutes, vortexed and incubated, at 4°C or on ice. Streptavidin-coated-magnetic beads (Promega) were added to the labeled cells and incubated for 10 minute at room temperature. Hematopoietic and endothelial cells were removed by placing the tube in a magnetic stand for 15 minute. After that, the supernatant containing non-hematopoietic/endothelial cells was collected in a clean 50 ml tube and centrifuged for 5 minutes in 4°c, 1200g. Then the cell pellet was re-suspended in the PBS+0.1% Bovine serum albumin (BSA). Cells were counted by hemocytometer and trypan blue stain and were used to distinguish live cells from dead cells.

Cell concentration was adjusted to 6 million/ml of PBS+BSA 0.1% and cells were labeled for sorting.

Antibody Labeling of Cells for Flow Cytometry and Sort

For isolation of EpCAM+, CD-24 ^{Low},CD-104+, CD45^{Neg} and CD-31^{Neg},Sca-1^{Neg} cells, the cells were labeled with the following fluorochrome labeled antibodies: Anti-Mouse CD326 (EpCAM) PE-Cy7, (eBioscience catalog number: 25-5791)

Anti-Mouse Ly-6A/E (Sca-1) FITC, (eBioscience catalog number: 11-5981 with concentration: 0.5 mg/ml) Biotin Rat Anti-Mouse CD31, (eBioscience, catalog number: 13-0311-85)

Biotin Rat Anti-Mouse CD45, (BD Pharmingen catalog number: 553078, 0.5 mg/ml). Streptavidin eFluor® 450 (Pacific Blue® replacement), (eBioscience catalog number: 48-4317, 0.2 mg/ml), as secondary for CD-45 and CD-31 cells.

The PE-CD104, (US Biological, catalog number: C24469).

Rat mAb ,CD24(PE-Cy5), (Abcam, catalog number:ac25281) .TO-PRO-3 (Invitrogen, catalog number: 642/661) to stain nucleic acid and distinguish live cells from dead cells. Cells were labeled in the following combinations:

1. 1million Unstained cells.
2. 1million stained for CD-31 and CD-45 with primary antibodies Biotin Rat Anti-Mouse CD31 ,and Biotin Rat AntiMouse CD45. The secondary antibody which binds to both CD31 and CD45 was: Streptavidin eFluor® 450.
3. 1 million cells for CD-104 stained with anti CD-104 conjugated with PE.
4. 1 million cells for CD-24 stained with Rat mAb CD24 conjugated with PE-Cy5.
5. 1 million cell for EpCAM which binds to PE-Cy7.
6. 1 million for Sca-1 stained with Anti-Mouse Sca-1 conjugated with FITC.
7. 1 million for TOPRO-3 for staining nucleic acids and nuclei.
8. The rest of cells in one tube as a sample/test tube with all antibodies. Cell number ranged from 1.6 million to 23 million.

Therefore, in each sort experiment seven tubes were prepared as compensation tubes. In this study, I tried to have at least one million cells in each compensation and unstained tube. These compensation tubes were used for comparison between intensity and positivity of cells for each marker.

Incubations with primary antibodies were for 30 minute at 4°C and light protected. The Streptavidin eFluor® 450 was added to CD45 and CD31 stained cells and to sample tubes for 15 minutes. Cells were washed to remove excess antibodies and TOPRO-3 was added in the last step of staining. The antibody concentration based on cell number changes due to refine protocol proved to have better sort results. In each experiment where cell numbers were not similar, different antibody concentration were applied. Cells were kept on ice or in cold room until sorting.

Cell Culture Media

After sorting the cells and isolating EpCAM+, CD24 ^{LOW}, CD102^{High} the cell suspension was centrifuged for 5 minute, 4°C, at 1200 g . The cells were then washed with modified media. The media which used was Epi-CFU expansion medium that

Dr.McQualter used for culturing EpCAM positive cells. Also, FGF10 50 ng/ml and mHGF 30 ng/ml were added. This media contains: α-MEM (gibco) containing 500ml with l-Glutamine, 10% (v/v) fetal bovine serum 50 ml (gibco, cat.no: 160000-044),1× penicillin/streptomycin (gibco, cat no.:15070-063) 5ml, 1×insulin/transferrin/selenium (Roche; add from 100× stock, cat no.11074547001) 250 µl, 2 mML-glutamine 5ml and heparin [1/1000 dilution of 0.2% heparin sodium salt (Sigma, cat. no. H4784-250ml). The media was stored for up to 4 weeks at 4◦C.

PCR

The Polymerase chain reaction was done to analyze the existence of Genomic DNA in the decellularized lung. After two weeks of incubation a decellularized lung and a decellularized lung that was infused with sorted cells, both were homogenized separately in TRIZOL reagent (Ambion). Then, DNA was extracted and amplified by PCR for 30 cycles with Taq DNA polymerase (Qiagen) for each of decellularized lungs. The βactin primers were ordered from the Microchemical Facility of the University of Minnesota. The primers sequences were: Fwd 5'GTG GGC CGC TCT AGG CAC CA 3' and Rev 5'CTC TTT GAT GTC ACG CAC GAT TTC3'. Then products of PCR were run out on Agarose (Sigma) and were stained with ethidium bromide (Invitrogen), the gel was visualized with a transilluminator and Alpha Gel Imager (Innotech) with TrackIt 1kb Plus DNA ladder (Invitrogen).

RT-PCR

The decellularized lung after infusion with $EpCAM^{High}$+, $CD24^{+}$ along with two weeks of incubation was analyzed by RT-PCR. Homogenized lung tissue and RNA extracted by TRIZOL, the cDNA were produced from mRNA. In addition, RNA concentration was measured by making a dilution from a sample with RNA free water and then read by a spectrophotometer. After, checking the existence of mRNA in the sample, the total product of RNA extraction from each sample was reverse transcribed into cDNA with Super Script VILO Kit (Invitrogen).Then samples were prepared for reaction in 7500 Real time PCR system from Biosystem and Master Mix for RT-PCR.

Forward and reverse primers, which were ordered from the University of Minnesota. One ml of cDNA was combined to water to a volume of 20 μL. Reactions were run in Halfskirt 96-well Reaction Plates (DOT scientist) in the 7500 Real Time PCR system machine from AB Biosystem for 40 cycles of amplification.

The Ct of samples was compared with endogenous control which was GAPDH. The probe for expected genes was TaqMan®. Through PCR, the probe is cleaved by DNA polymerase and the reporter dye releases from the probe. Genes of interest were normalized to GAPDH to provide a relative mRNA quantity.

By RT-PCR assay we looked for expression of the following genes: GAPDH, SP-C, CCSP, AQP5, CK5, Sca-1.Primers were purchased from Applied Bio-system.

Histologic Assessment

After removal from the bioreactor, lungs were inflated by a mixture of 0.5mL Optimal Cutting Temperature compound (OCT; Miles): phosphate-buffered saline (PBS) (3:1ratio) via the trachea. Then, an aluminum foil cup was prepared and filled with OCT. The lung tissue was placed into the cup, frozen in liquid nitrogen, and stored at 80°C. Frozen blocks were cut on a Leica Cryostat microtome machine into 0.6μm sections and fixed by Acetone (Sigma). Sections were stained by Hematoxylin and Eosin (H&E) and mounted with Permount.

Immunofluorescence

In order to investigate grafted sorted cells in the decellularized lung, sections were prepared from normal lung as positive control and decellularized matrixes with sorted cells. Then, sections with Acetone (Sigma) were fixated and were stained with conjugated antibodies with flourochromes.

Four different markers were investigated by immunofluorescent antibodies for EpCAM, CD-104, C-kit and CD-133. For EpCAM, a primary antibody was used: Anti-mouse CD326 (EpCAM) from eBioscience and secondary anti-rat conjugated with Cy5 from Jackson Immunoreserach, Biotin anti-mouse CD-133 from Biolegend and a

secondary antibody is Cy3 conjugated Streptavidin Jackson Immuno-research, Integrinβ4 (H-101) for CD104 from Rabbit, Santa cruz Biotechnology and secondary is anti-rabbit Cy5 conjugated from Jackson Immuno-research and Alexa Fluor 488anti-mouse – CD117(C-kit) from Biolegend. After staining sections, images were taken by confocal microscopy. We looked for existence of positive cells for stem cells markers. Flow View software (version 5) was used for taking photos.

Results

Decellularized Lung and Genomic DNA PCR

The lung decellulaization was done based on Dr. Mortari's technique. After infusing the lungs through the trachea with Triton-100x, the lung tissue was depleted of cells and with Dnase, the remnant of nuclei was digested completely. The efficiency of decelluarized matrix was examined by placing it into the bioreactor and connected to ventilator's tubes. The lungs showed proper function and ventilation in the rate of 180 breaths per minute. Then PCR results for genomic DNA were analyzed to assess proper depletion of total cell content and genominc DNA in a decellularized lung. The primer of βActin which is a house-keeping gene was used to detect genomic DNA. We examined a band in the 565 BP for existence of any remnant of genomic DNA. As is shown in Figure.8, no βactin DNA was found in decellularized lung samples. In contrast, the normal lung as a positive control does show a band with 565 base pair size.

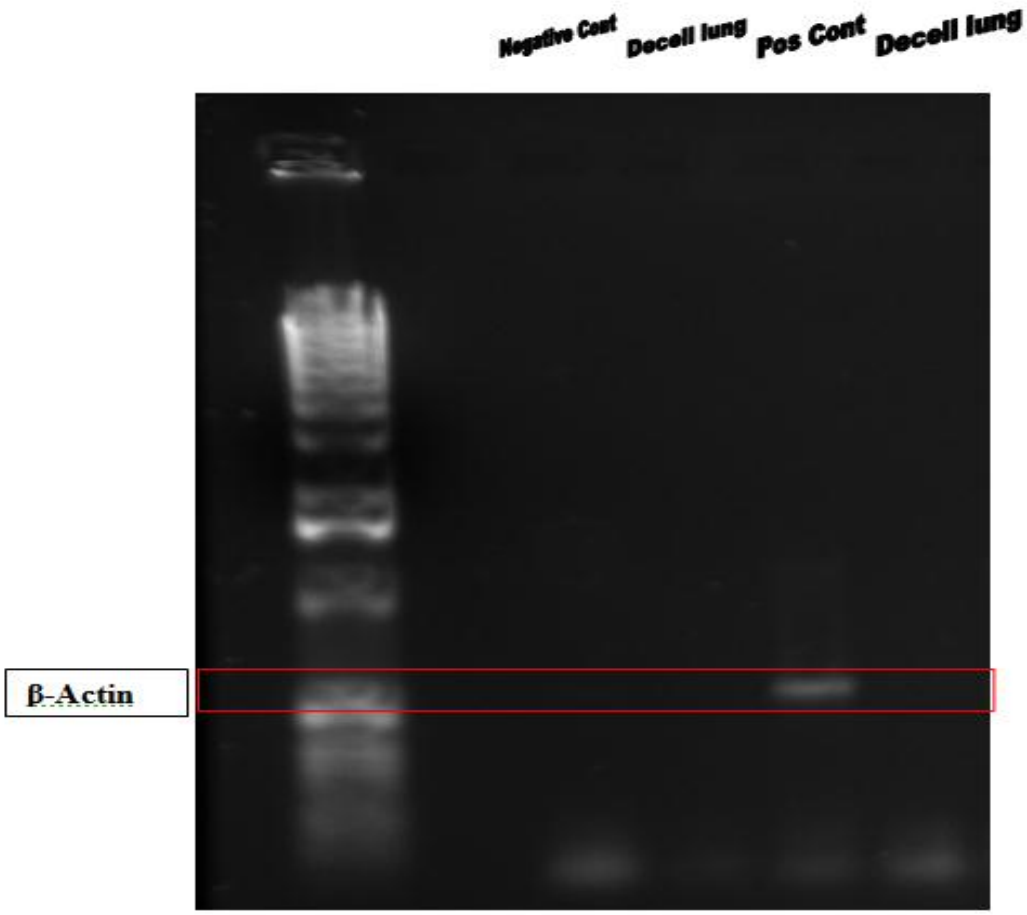


Figure 8.PCR results. PCR for genomic DNA (βActin) showing negative results in 2 decellularized lung samples.

Therefore, we interpreted that the decellularized matrix is deprived of any DNA and cell content.

Histologic assessment of Decellularized matrix

After taking cryosection and mounting slides the images were taken. The pictures in Figure 9, which were taken from a normal lung and a decellularized lung, show the efficient removal of cells from the matrix. In the normal lung in Figure 9A, the alveoli are attached to each other on the basement membrane. The nuclei are in dark pink. The

cytoplasm of cells is in light pink and some ciliated cells can be seen inside of the bronchiole.

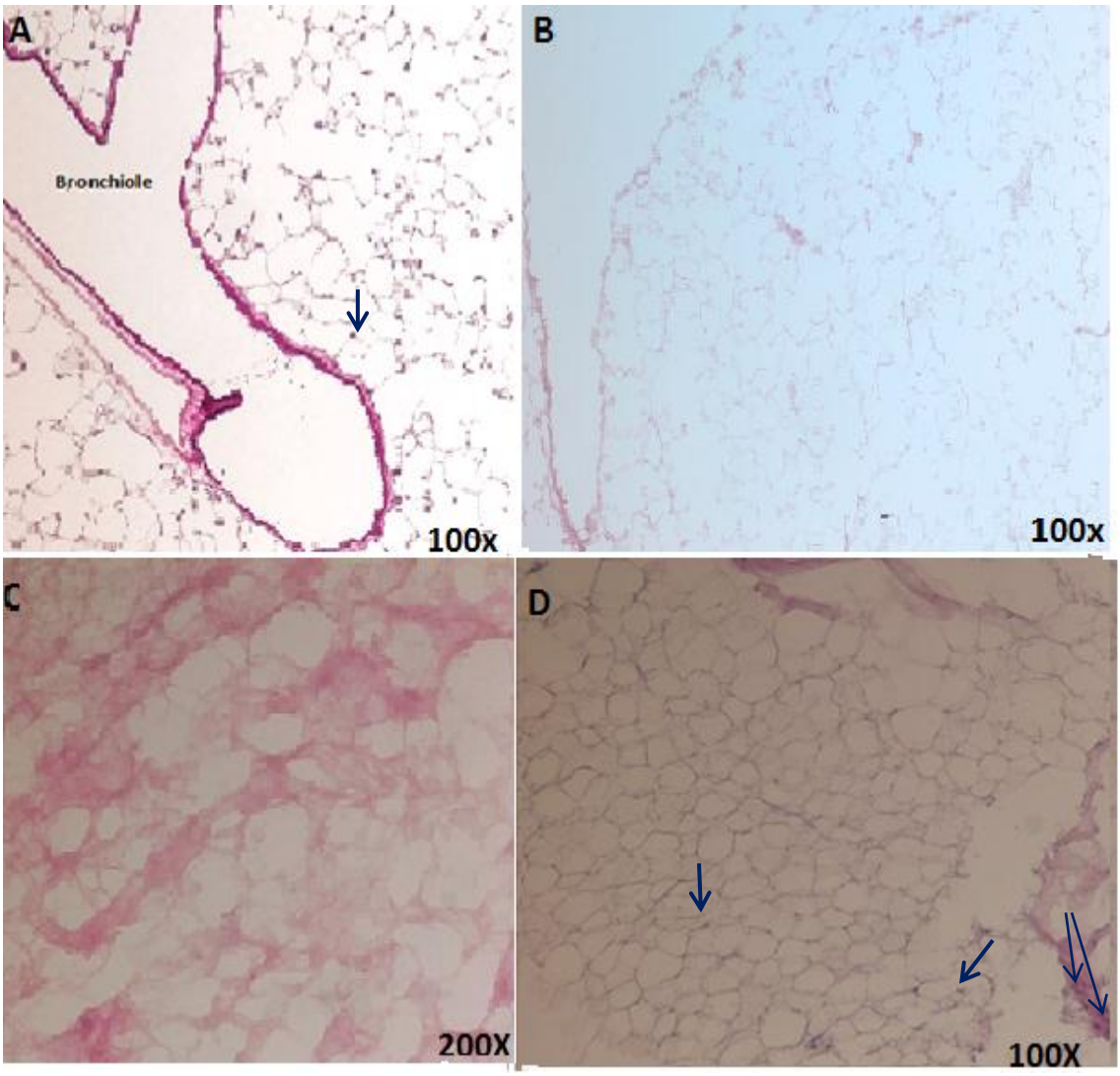


Figure 9. A) H&E section from normal lung. Section prepared from 2 month old B6 male mouse. It was infused with OCT+PBS (100x objective). Also, a nucleus is indicated with arrow. B) H&E section from decellularized lung with infusion by OCT+PBS in PBS. C) A section from two month old BALB/c male mouse after two weeks incubation with media and ventilation in bio-reactor. The nuclei of Alveolar type II cells and other lung tissue cells were depleted. The extracellular matrix proteins were pink in color and

no nuclei can be seen in blue or dark pink. D) Shows a decellularized lung after seeding with sorted cells and being incubated for one week. Blue arrows point to the nucleus of engrafted cells. The slide stained with H&E show the existence of a few cells that can be clearly seen. The nucleus is dark blue and the ECM is pink.

The results demonstrated that the pulmonary matrix was intact and depleted from all cell content. The three dimensional structure of lung tissue and the alveoli, bronchioles and the basement membrane of these structures all remained intact. In addition, Figure 9. shows sorted cells with nuclei in dark blue color. We can conclude in a decellualrized whole mouse lung progenitor cells could engraft.

Isolation EpCAM+^{high}, CD24^{lo}, CD104+ with Sorting Technique and Reseeding Matrices

After enzymatic digestion of lung tissue and depletion of hematopoietic cells, the cell suspension was stained and sorted. Approximately, up to 20 million cells were obtained after digestion of 9 lung mice and depletion of hematopoetic cells, but before staining. The average number of live cells was 1,433,867 in the sample tube. Therefore, 0.5% of total live cells isolated from one mouse were EpCAM+. Also, a bone marrow cell cocktail was used as a negative control for EpCAM positive cells and the efficiency of depletion of CD45 and CD31 positive cells. As a control for accuracy of cell staining, a bone marrow cell suspension was prepared.

Figure10 shows the results of sort of the sample and negative control. Cells, after collection, were infused in to decellularized lung that had been prepared in the bio-reactor. Also, EpCAM CD104, CD24 negative cells were isolated as a negative population that was infused with another decellularized lung in the bio-reactor. In this

research, we also looked for cells which induce “mesenchymal induction” to stem cells and help differentiation of the epithelium. Sca-1 positive, EpCAM negative cells were found. However, in this experiment for the purpose of “mesenchymal induction” the growth factors were added to the media instead of $Epcam^{Neg}$,
$Sca\text{-}1^{+}$ cells. These growth factors can be good alternatives for the generation of epithelial colonies (McQualter et al., 2010).

In addition, a normal decellularized lung was used as a negative control. This acellular lung, without any cell added, was placed into the bio-reactor and incubated for two weeks.

The bone marrow cocktail that was prepared, and used, was highly positive for hematopoietic and endothelial cells. Thus, it was stained with TOPRO-3 and CD45 and CD-31 antibodies.

As is shown in Figure 10f, no EpCAM positive cells were available in the bone marrow cocktail and staining technique were properly done and hematopoietic and endothelial cells labeled correctly.

The following dot plots show how, at first, the labeled cells in the sample tube were gated based on unstained control and then live cells were collected. As Figure10 h) illustrates the live TOPRO-3 negative cells were isolated and then from them EpCAM positive cells were separated. After that CD104 positive and CD24 positive cells were gated. Although, Mcqualter et al. separated CD24 low cells, in most of our experiments all CD24+ were isolated. The number of $CD24^{low}$ positive cells was low in comparison with Mcqualter et al.’s result. Therefore, all CD24 positive cells were collected. Also, the EPCAM negative, Sca-1 positive cells were gated but not collected.

In the Figure 10, dot plots d, e and f show bone marrow control. In Figure 10, live cells are Topro negative cells and these cells were isolated. Then live cells were analyzed for CD31 and CD45. As is shown in Figure10 h), 54.9 percent of cells were TOPRO negative. Then, these live cells were separated. In the second step, EpCAM positive and negative cells were gated. According to Figure 10 i) 14.8 percent of Topro-negative cells were positive for EPCAM and negative for CD31 and CD45. Also, 74 percent of cells were positive for CD31, CD45 but were negative for EpCAM. This population was

analyzed for CD104 and CD24. Figure10 j) and 10 k) demonstrate these cells. These EpCAM+, CD104+, CD24+ cells are our population of interest.

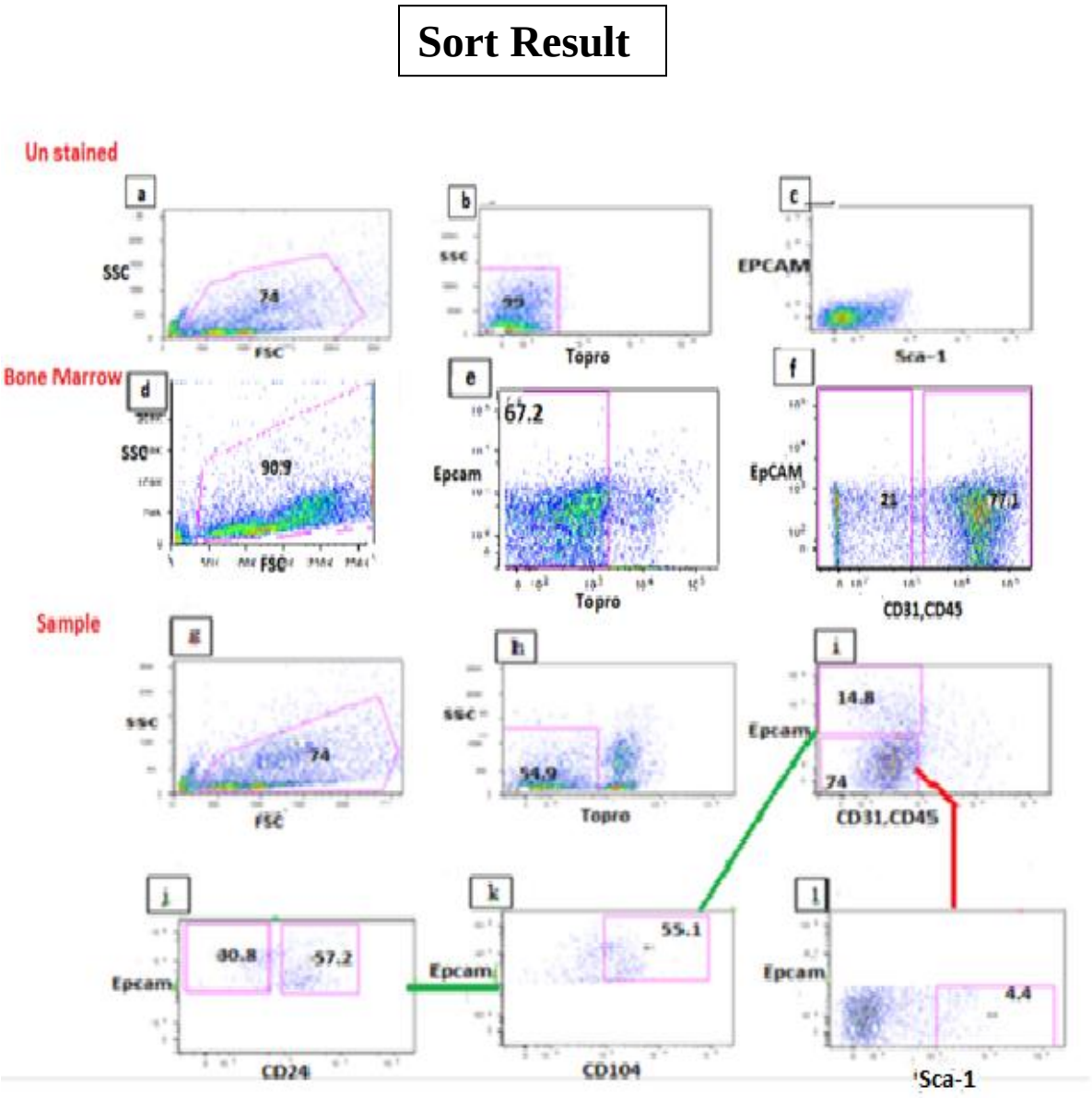


Figure10. Representative example of sorting of EpCAM+,CD104+, CD24+ lung stem cells.

According to dot plot from the EpCAM positive cells, CD104 positive and CD24 low cells were selected.
The dot plot l shows cells that are Sca-1 positive EpCAM negative (mesenchymal cells).

On average, 50863 EpCAM positive cells were injected via trachea to a lung. Table I illustrates the number of attempts made to isolate enough cells and cell culturing. According to Table 2, the average percentage of positive cells for EpCAM, CD24 and CD104 is 0.5%, with one experiment at 14.9%, because we used all CD-24 positive EpCAM positive cells. Table 2 illustrates the result of each experiment and changes that were done to refine the protocol, in order to have a higher yield of cells from the tissue.

Many attempts were made to solve the viability problem. However, the total number of cells before sort, was lower than Mcqualter's cell number. Since the purpose of this study was the investigation of LSC differentiation in the acellular lung, this research failed due to the low number of LSCs.

Table 1: Lung Cell Staining and Sorting Results

Experiment	Total cell before sort $\times 10^6$	Live cells in sort tube $\times 10^6$	%EPCAM+ (P5) %p	%T	EPCAM+CD10 %P	%T	EPCAM+ CD104+ CD24+ (LSC) %P	%T	Cells injected	Bioreactor results
1	5.1	5.2	5.8	0.6	NA*	NA	NA	NA	30599 All EPCAM+	3rd day Bioreactor failure
2	2.8	9.5	14.8	4.7	61.2	2.9	41.4	1.2	33747 (P6)	14 days No cells in slide Neg RT-PCR
3	7.4	2.59	2.2	0.8	53.2	0.41	72.3	0.3	22176 (P8)	14 days No cell
4	1.6	6.3	64.9	38.2	80	20.5	58	14.9	239057 (P8)	14 days No cell seen
5	3.5	2.3	7.7	0.52	50	0.27	74.8	0.2	6907 (P8)	Contamination
6	6.3	3.7	33.8	2.0	22.7	0.45	88.4	0.4	25151 (P8)	14 days No cell survival RT-PCR negative
7	7.0	4.0	0.8	0.05	9.1	NA	NA	NA	3232 All EPCAM+	7 days No cell
8	23.9	2.39	2	0.2	NA	NA	NA	NA	47801 All EPCAM+	7days Cell detected RT-PCR Neg

*NA= Not available, not enough cell to continue sort. P= parent population T= total population P8/P6= Population of cells

Table 2: Troubleshooting

Experiment	# Mice	# Live Cells in sample tube $\times 10^6$	Findings	Change made to protocol
1	10	5.2	Inefficient depletion of hematopoietic cells. Poor CD104, CD24 staining. Purity EpCAM+ cells were low. Tracheal failure in bioreactor. Changed antibody for CD104 and CD24.	Pay more attention to maintain good trachea. Order new antibody for CD104 andCD24 to improve staining and changed fluorochrome combination.
2	9	9.5	Staining of cells successful but inefficient depletion of hematopoietic cells.	Increased the amount of beads to have better depletion.
3	9	2.59	EPCAM+ cell purity was low. Cells didn't survive in the bioreactor.	Refining the homogenizing process by decreasing collagenase concentration and manual homogenization used.
4	9	6.3	Contamination with hematopoietic cells	Depleted with beads two times.
5	9	2.3	Having continuous contamination with hematopoietic cells. Aggregation of hematopoietic cells. Low cell yield.	Kept cells on ice. Decreased the amount of BSA in the FACS buffer to decrease cell aggregation. Addition of FBS to fresh collagenase (5%).
6	9	3.7	The cell viability improved.	Added more serum to collagenase (10%). Collagenase prepared one day before sort.
7	10	4.2	Low efficiency of isolation process. No cell survival in bioreactor.	Rinsed matrix with media before cell infusion. Add more FGF10 and HGF to culture media.
8	9	2.39	Just EpCAM+ cells isolated since CD104, 24 were too low. EpCAM+ cells infused and cells did survive in matrix.	Try pre culture cells after sort with MSCs in the Matrigel based culture condition.

RT-PCR

All samples of EpCAM+ infused decellularized matrixes contained too low a concentration of cDNA. Therefore, RT-PCR results were negative. In addition, cell growth was not seen. In the last experiment (experiment 8), cells were detected, but they were dispersed and too few. The RT-PCR for positive control worked but the concentration of cDNA in sample from decellularized matrix with EpCAM+ cells was too low. The Ct of positive control sample for endogenous control (GAPDH) was 19.8 and Ct of the sample from decellularized matrix with EpCAM+ cells was 32.6. In other words, if the amount of cDNA for GAPDH could be detected earlier, it would be interpreted as an existence of similar amount of cDNA for a specific gene. In fact, it is an indication that it is an expression of the specific gene. In this study, we couldn't detect an expression of genes of interest in experimental samples and only positive controls worked. In contrast, extraction of genomic DNA from sample (experiment 8) was positive. The Figures 11 and 12 show Rn vs Cycle graphs for GAPDH and SP-C which was prepared by 7500 real time PCR software. As shown in Figure 11, one sample from the decellularized matrix without infused sorted cells had Ct 23.7. It means that the matrix contained DNA and had DNA contamination. However, one sample from another decellularized matrix was negative in terms of GAPDH and SP-C, mRNA extracted and cDNA existence.

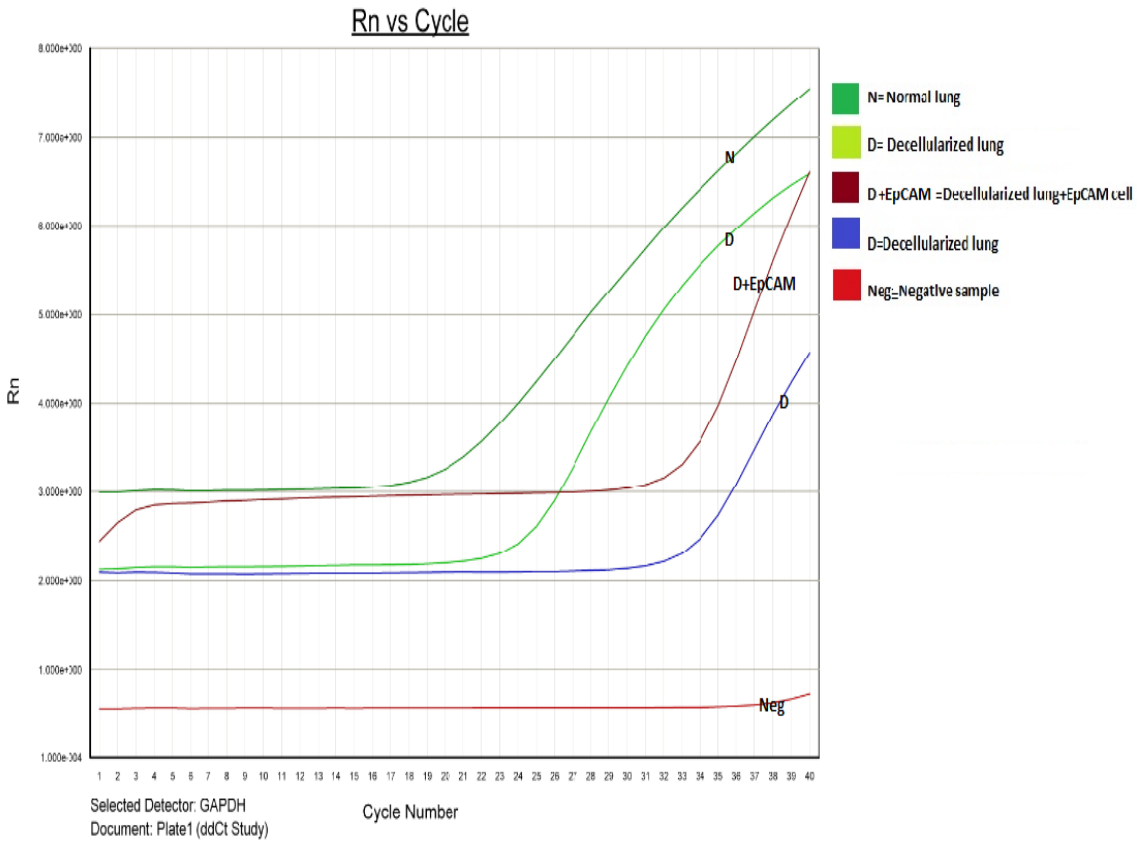


Figure11. RT-PCR results from four samples and one negative control. The sample from the normal lung is the positive control. The dark green line increased sooner than other lines which showed the existence of higher amounts of cDNA. Since there was a higher amount of cDNA, fewer cycles were required to detect cDNA.

In the Figure 12 the SP-C gene cDNA was detected. This protein, which is a marker of ATII cells, was not expressed in the matrix with EpCAM+ cell infusion. The sample from the normal lung (positive control) Ct for SP-C was 17.3. This experiment included two positive controls as normal lung tissue.

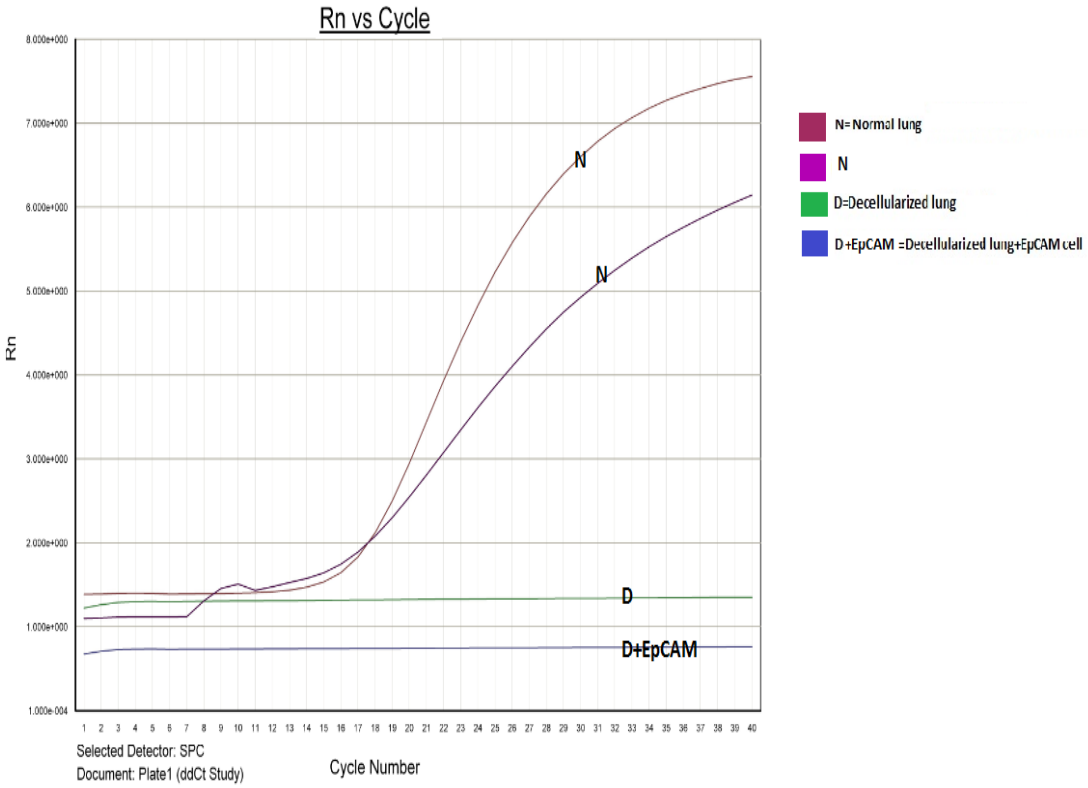


Figure12. The maroon and purple color lines show SP-C gene expression in 2 normal lung tissue samples (Ct at cycles 16.1 and 17.3). However, extracted mRNA from experiment 8 (decellularized matrix with EpCAM+ cells), was negative (blue) as well as the decellularized matrix without infused cells (green).

Immunofluorescence and Confocal Microscope Image

The results from Immunofluorescence (IF) showed the existence of CD-133 positive cells around the bronchiole in the normal lung control sample (Figure13.B). Images from normal lung tissue stained with antibodies for EpCAM and CD-133, indicated the location of these cells were around bronchiole sites. Also, in the decellularized matrix with infused cells, after two weeks nuclei were detected but no cells with stem cell markers were detected in the section. Since the number of lung stem cells

was very low, their location required more sections for study. However, for CD-133, in the positive control cells, positivity was clearly indicated.

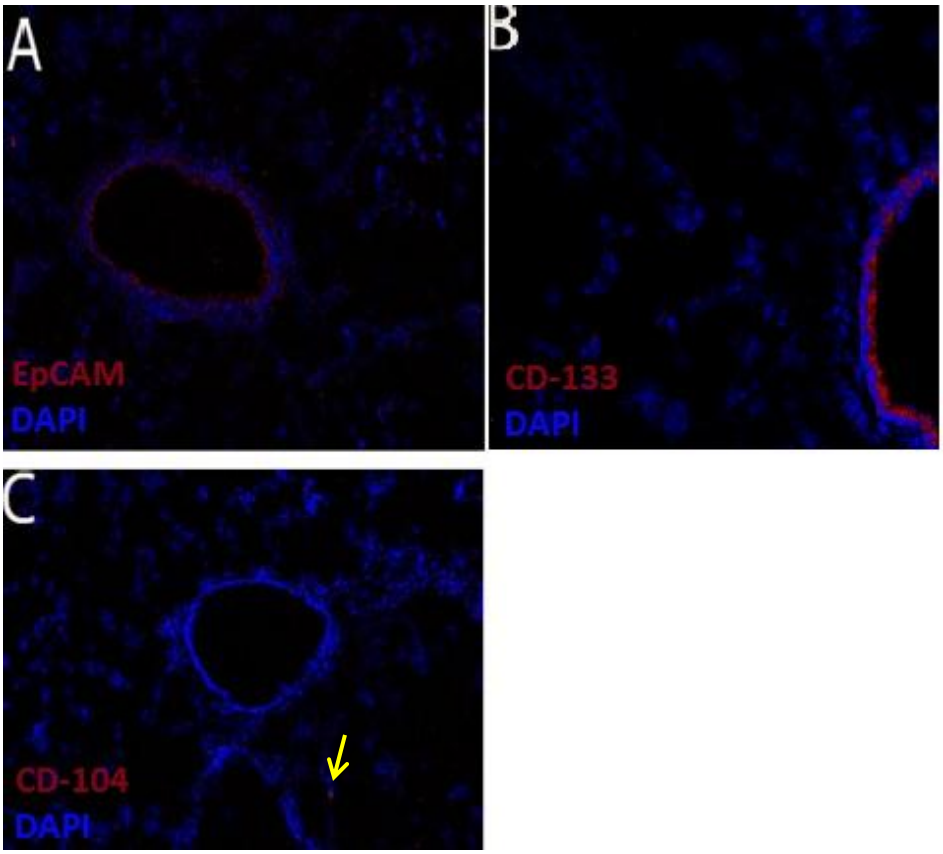


Figure 13. Immunofluorescence analysis of normal lung tissue. These images were taken from positive control slides from a normal lung. As is shown here, nucleated cells are in Blue (DAPI). A) Shows EPCAM positive cells in red color which surrounds around bronchiole epithelium cells. B) Shows cells positive for DAPI in blue and cells positive for CD-133 in red. C) Cells are positive for DAPI but didn't express CD-104 in the epithelium of the normal lung. Just one cell was positive for CD-104 (Yellow arrow)

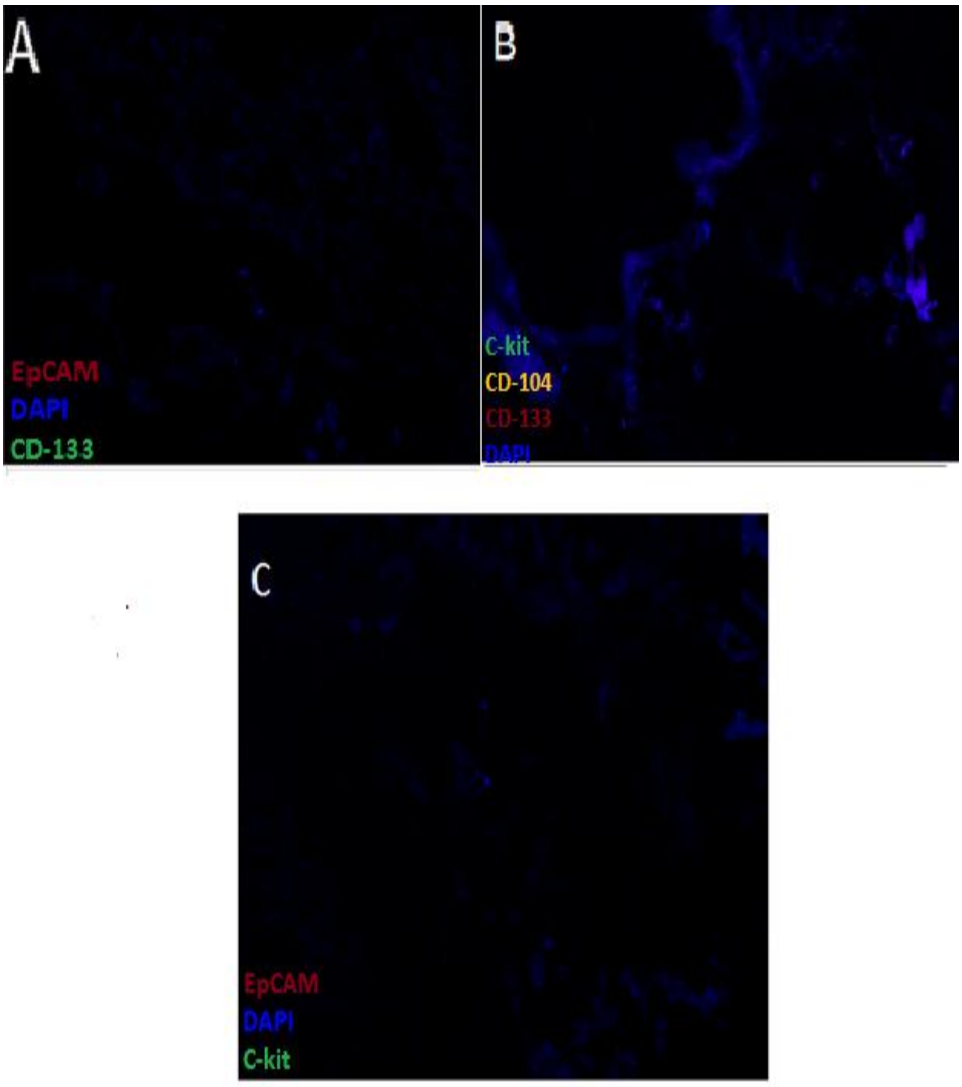


Figure 14 Immunofluorescence analysis of infused decellularized lung tissue with LSC. Images were taken from a decellularized lung with infused EpCAM positive LSCs (experiment number 8 from table one). This slide was stained with four different antibodies for C-kit, CD-133, CD-104 and EpCAM. A) Shows a decellularized lung that was infused with EpCAM positive cells. This figure illustrates the ECM matrix in the black background which is also illustrated in the blue color. For better visualization of nuclei, the intensity of DAPI in the confocal microscope was increased. Therefore, the presence ECM is seen in blue and black and it is negative for any nuclei. ECM reveal the

existence of only one cell in shining blue color with DAPI but negative for EpCAM. B) Shows the slide stained for C-kit cells. As is shown, the slides were negative for c-kit positive cells. C) Decellularized infused lung stained for EpCAM, CD-133, C-kit, and CD104. As is shown in Fig14C, few cells survived and results were negative for EpCAM, CD-133, and C-kit.

Discussion

This study attempted to examine the existence and differentiation potential of an endogenous lung stem cell population (EpCAM+, CD104+, CD24+ cells) through an *in-vitro* experiment. For the first time, this specific cell population was cultured in a natural scaffold. This scaffold was a decellularized mouse lung with the complexity of lung organs. According to this study data, we provided a proper acellular matrix. However, in some cases the bioreactor set up failed or became contaminated. The evidence indicated LSC numbers were low. We observed that these cells didn't survive in the bioreactor and that we couldn't analyze the expression of proteins such as SP-C, AQ5, and CCSP. In addition, since the number of cells was low in most cases, all CD24 positive cells were isolated instead of only a small number of CD24 low cells. In contrast, in the McQualter protocol for isolation of lung stem cells (LSC), just $CD24^{low}$ were separated. Furthermore, in this research with IF analysis, we tried to identify the location of stem cells in the lung. We recognized the location of cells with EPCAM and CD-133. According to previous studies, CD-133 positive cells proposed to be similar to stem cells. This marker is a transmembrane glycoprotein and is also found on endothelial progenitor cells and cancer stem cells (Corbeil et al., 2000). Therefore, we have used antibodies for these markers. The EpCAM marker is an epithelial cell adhesion molecule. These antigens are expressed on normal epithelial cells as well as carcinoma cells. They play a role in cell signals and cell polarity and is associated with WNT pathway and the Cadherin-Catenin pathway. Cadherins are calcium-dependent adhesions. Thus, EpCAM is responsible for intracellular signaling and cell polarity. On the other hand, Integrin β-4

(CD-104) also regulates interactions of adhesion molecules on cells and extracellular matrix or the interaction between adjacent cells. These transmembrane proteins, same as other integrins, are involved in cell migration during development, differentiation and wound healing. Integrins are part of focal signaling. Through focal adhesion signals, the cytoskeleton of a cell connects to the extracellular matrix (Alberts, 2008). Cells that we localized were negative for CD-104 and we didn't stain them for CD-24. Therefore, we cannot interpret that these cells are the same population that McQualter et al suggested as epithelial cell adhesion molecule (EpCAM) which were positive, the CD104 (beta 4 integrin), or the low-level CD24 (Heat Stable Antigen) cells. The CD-24 is a glycoprotein and cell adhesion molecule which plays a role in the process of cell to cell adhesion. However, if we assume that EpCAM and CD-133 are proper markers for lung stem cells, it is possible that some of these identified cells are stem cells. Moreover, in normal lung sample we didn't localize any C-Kit positive cells, which recently Beltrami et al. (2003) suggested are lung stem cells markers. C-kit is a cell surface marker and it is a protein-tyrosine kinase (Edling & Hallberg, 2007).

These results provide evidence of the efficiency of the decellularized lung tissue. Moreover, we tried to identify LSC location in the normal lung. However, viability of cells and the small numbers of cells, after isolation, was a major problem. In the first place, these cells, after sorting, may need to recover from stress. To improve their recovery rates, we suggest co-culturing with feeder cells in a Matrigel-based culture before infusing them to the natural scaffold. Thus, we can expect a better result since cells become enriched and more easily prepared, to engraft into a complex scaffold. In addition, co-culturing them with mesenchymal cells is another factor to increase their survival ability.

The sorted cells were cultured with FGF10 and HGF but not with feeder cells. Therefore, the reason for their death may be a lack of feeder cells. Also, co-culturing them in the acellular matrix with matrigel is another factor which may improve their survival. It is true that the decellularized matrix has many ECM proteins but culturing cell suspension with the matrigel may increase the ability to engraft and differentiate in the decellularized lung.

On the other hand, in order to have proper lung development, various factors should be considered. In other words, factors such as molecular signaling associated with fibroblast growth factor (FGF), retinoic acid, transforming growth factor β (TGFβ), sonic hedgehog (SHH), WNT signaling, and bone morphogenic protein (BMP). Totally, eight mechanisms are required for branching out, morphogenesis and to maintain regeneration of lung epithelium (Cardoso & Lü, 2006).

The cell to cell communication between the mesenchyme and the lung epithelium plays a fundamental role in the development of the lungs. According to studies, FGF10, expressed in the mesenchyme, plays a role in the development of many branched organs including lungs, thyroid, pituitary, and salivary glands (Bellusci, Grindley, Emoto, Itoh, & Hogan, 1997). Also, studies demonstrate the retinoic acid network association with FGF10. Indeed, the most crucial disruption of lung morphogenesis was observed in FGF10 and FGFR2 null mice embryos (Klar et al., 2011; Min et al., 1998). The retinoic acid regulates FGF10 through WNT (via DKK1) or TGFβ-1 (F. Chen et al., 2010). Moreover, previous studies found existence of a feedback loop between SHH, BMP4, FGF10 and a decrease of FGF10-FGFR2. Furthermore, based on McQualter et al.'s studies, hepatocyte growth factor (HGF) can be also an alternative for mesenchymal support.

As a result, presence of mesenchymal cells and growth factors such as HGF and FGF10 are critical in order to improve progenitor cell survival and engraftment in decellularized lung.

In this study, the frequency of EpCAM/CD104/CD24 LSCs in a mouse lung was found to be close to 0.5% in most experiments. In a comparison with other organs, previous studies showed frequency of stem cells in human bone marrow as 1 per 10000 to 20000 cells (0.01% -0.005 %), and in the heart one stem cell in 30000 cells (Bearzi et al., 2007; Craig, Kay, Cutler, & Lansdorp, 1993) which is (0.0033 %.). Therefore, the frequency of LSC in this study was higher at 0.5%.

In conclusion, our results support the existence of the EpCAM/CD104/CD24 population of lung progenitor cells. However, only a small number of cells could be

harvested for culture in decellularized whole lung matrix.To sum up, regeneration of a decellularized lung still requires a lot more work. If the vessels can be recellularized first with endothelial cells, this should help with recellularization and viability of the lung epithelium.

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