Abstract

DINH, PHUONG-UYEN CAO. Therapeutic Potential of Lung Spheroid Cell-Secreted Factors in Rodent Models of Pulmonary Fibrosis. (Under the direction of Dr. Ke Cheng and Dr. Kenneth Adler.)

Idiopathic pulmonary fibrosis (IPF) is a chronic respiratory disease of unknown cause characterized by persistent dry coughing, shortness of breath, clubbing of the fingers, diffused fibrosis, fibroblastic foci and alveolar honeycombing. As fibrotic tissues thicken, the lung becomes stiff and loses function. Currently, IPF has no known cause and few treatment options. Current therapies are primarily palliative and can only delay the disease progression, but do not stop nor reverse the damage that has already occurred. The field of regenerative medicine holds the potential to renew and revitalize damaged cells, tissues and organs. The discovery of resident pulmonary stem and progenitor cells provides a source for pulmonary regeneration. The difficulty of isolating and expanding these cells is overcome by the discovery of lung spheroid cells that have been shown to exert therapeutic benefits even when derived from diseased lung tissue.

It has previously been shown that stem cell secreted factors, or conditioned media, provide similar therapeutic effects as the stem cells themselves. Therefore, stem cell-derived conditioned media is a promising pharmaceutical therapeutic option. Here we present a series of studies utilizing lung spheroid cell-derived conditioned media (LSC-CM) and LSC-exosomes (LSC-EXO) to treat bleomycin- and silica-induced pulmonary fibrosis. Treatment was given for seven consecutive days by inhalation using a nebulizer for clinical relevance. Blood and tissue were collected for protein, immunological and histological examination. Results revealed that LSC-CM and LSC-EXO treatments could attenuate and resolve
bleomycin- and silica-induced fibrosis by reestablishing normal alveolar structure and decreasing collagen accumulation and myofibroblast proliferation. In addition, LSC-CM and LSC-EXOs exhibited superior therapeutic benefits than their counterparts derived from bone marrow mesenchymal stem cells. LSC-CM and LSC-EXOs provide promising therapeutic options for pulmonary fibrosis.
Therapeutic Potential of Lung Spheroid Cell-Secreted Factors in Rodent Models of Pulmonary Fibrosis.

by
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Biography

Phuong-Uyen was born on August 26, 1990, in Ho Chi Minh City, Vietnam to Hieu Dinh and Kim Cao. Her family immigrated to Raleigh, NC in 1994, where she was brought up. She graduated from the University of North Carolina at Greensboro in 2011, where she earned a Bachelor’s of Science in Biology with minors in Chemistry and Studio Arts. Her undergraduate research focus was in evolutionary genetics under the direction of Dr. Malcolm Schug. She started her graduate program in Comparative Biomedical Sciences in the Fall of 2014. She began her doctoral studies under the direction of Dr. Ke Cheng the following summer, where her research focuses on developing therapies for pulmonary diseases.
Acknowledgments

The research presented in this dissertation is the product of a multitude of collaborations and hard work. I am grateful to many people for their support, advice, and contribution to the science presented here. The mentorship and guidance of these colleagues has been essential to my graduate career as well as personal growth.

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I thank my advisors and committee members Drs. Kenneth Adler, Michael Goshe, and Lauren Schnabel for their interest in and feedback on the research. Their advice and suggestions have made this work immensely better.

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Table of Contents

List of Tables ........................................................................................................................................... ix

List of Figures .......................................................................................................................................... x

List of Abbreviations ............................................................................................................................... xii

Chapter 1 .................................................................................................................................................. 1

Introduction ............................................................................................................................................. 1

1.1 Idiopathic Pulmonary Fibrosis: History of a mysterious lung disease ......................... 1

1.2 Modeling the Disease: Bringing bench to bedside ................................................................. 3

1.3 Regenerative Medicine: Approaches, limitations, and future directions ................. 5

1.3.1 Cell-Based Therapies ................................................................................................................. 6

1.3.2 Cell-Free Therapies .................................................................................................................... 7

1.3.3 Exosomes .................................................................................................................................... 8

1.4 Reference ......................................................................................................................................... 11

Chapter 2 ................................................................................................................................................ 17

Derivation of Therapeutic Lung Spheroid Cells from Minimally Invasive Transbronchial Pulmonary Biopsies ........................................................................................................................................ 17

2.1 Introduction ..................................................................................................................................... 18

2.2 Results ........................................................................................................................................... 20

2.2.1 Lung spheroid cells can be expanded from minimally invasive transbronchial biopsies............................................................................................................................................. 20

2.2.2 Lung spheroids shows intricate heterogeneous cell populations............................. 22
2.2.3 Lung spheroid cells exhibit a complex heterogeneous cell phenotype ...........22
2.2.4 Lung spheroid cells promote endothelial cell tube formation in-vitro ..........24
2.2.5 Biodistribution of intravenously-injected lung spheroid cells ..................26

2.3 Discussion .................................................................................................28

2.4 Conclusion .................................................................................................31

2.5 Materials and Methods ................................................................................32
2.5.1 Cell Culture .............................................................................................32
2.5.2 Cell Population Doubling ........................................................................33
2.5.3 Flow Cytometry ........................................................................................33
2.5.4 Immunocytochemistry .............................................................................34
2.5.5 Generation of Heat-Map Images ...............................................................34
2.5.6 Tube Formation Study ..............................................................................35
2.5.7 Biodistribution of LSCs After Intravenous Infusion ..................................35
2.5.8 Statistical Analysis ..................................................................................36

2.6 References ..................................................................................................37

Chapter 3 ..........................................................................................................41

Inhalation of Lung Spheroid Cell-Secreted Factors and Exosomes Promotes Therapeutic Lung Repair in Rodent Models of Pulmonary Fibrosis .................................................................41

3.1 Introduction ................................................................................................42

3.2 Results ........................................................................................................44
3.2.1 Stem cell-conditioned media attenuates and reverses bleomycin-induced fibrosis and fibroblast apoptosis .................................................................44
3.2.2 Stem cell-conditioned media inhalation treatment increases MMP2 expression and promotes vascular and alveolar repair .................................................................46
3.2.3 Therapeutic effects of stem cell-conditioned media therapy in silica-induced pulmonary fibrosis ........................................................................................................48
3.2.4 Protein composition of lung spheroid cell-conditioned media ..................................50
3.2.5 Stem cell exosomes can partially reproduce the therapeutic effects of stem cell-conditioned media ........................................................................................................52
3.2.6 Effects of LSC-CM and LSC-EXO therapy on MMP2 and MCP-1 expression ........54
3.2.7 Effects of stem cell-conditioned media and exosome therapies on lung function following bleomycin-induced fibrosis .................................................................54
3.2.8 Toxicity of conditioned media and exosome therapies .............................................56
3.2.9 The miR-99 family of microRNAs highly upregulated in LSC-EXOs ....................56

3.3 Discussion ..............................................................................................................57

3.4 Materials and Methods ..........................................................................................62

3.4.1 Cell Culture ........................................................................................................62
3.4.2 Conditioned Media Collection and Preparation .....................................................62
3.4.3 Exosome Isolation and Characterization ...............................................................63
3.4.4 Animal Procedures ..............................................................................................63
3.4.5 Pulmonary Function Test (PFT) in Rats .................................................................64
3.4.6 Histology ..............................................................................................................64
3.4.7 Proteomic Analysis ...............................................................................................65
3.4.8 SDS-PAGE and Western Blot ..............................................................................67
3.4.9 Small RNA Library Construction and Sequencing ..............................................68
3.4.10 Mapping and Differential Expression Analysis of miRNAs ........................................ 68
3.4.11 Statistical Analysis .................................................................................................... 69
3.5 References .................................................................................................................. 70

Chapter 4 .......................................................................................................................... 77

Conclusions and Future Outlook ..................................................................................... 77

4.1 Conclusions ................................................................................................................ 77
4.2 Future Outlook ............................................................................................................ 78
4.3 References ................................................................................................................ 80

Appendices ...................................................................................................................... 81

Appendix A ....................................................................................................................... 82
Appendix B ....................................................................................................................... 85
List of Tables

Chapter 2

Table 1 Donor Table ..................................................................................................................20

Chapter 3

Supplemental Table 1 Cell Line Donor Information.................................................................85
Supplemental Table 2 Extracellular protein identified in all three donor CM .........................86
Supplemental Table 3 Donor 1 top 50 most abundant protein................................................91
Supplemental Table 4 Donor 2 top 50 most abundant protein................................................93
Supplemental Table 5 Donor 3 top 50 most abundant protein................................................95
Supplemental Table 6 Top 25 miRNA profile in LSC-EXOs.....................................................97
Supplemental Table 7 Top 25 miRNA profile in MSC-EXOs....................................................99
Supplemental Table 8 miRNA Differentially Expression Table..............................................100
List of Figures

Chapter 2

Figure 1  Growth potential of lung spheroid cells derived from whole lung and biopsy specimens ..........................................................21

Figure 2  Phenotype analysis of lung spheroids .................................................................23

Figure 3  Phenotype analysis of lung spheroid cells ..........................................................25

Figure 4  Lung spheroid cells promote endothelial cell tube formation in-vitro .............26

Figure 5  Biodistribution of LSCs in-vivo after intravenous injection .............................27

Chapter 3

Figure 1  Stem cell conditioned media reverses alveolar epithelial cell damage caused by chronic Bleomycin injury ............................................................45

Figure 2  LSC-CM inhalation treatment promotes alveolar repair .................................47

Figure 3  Lung repair and fibrosis in mice after silica-injury ........................................49

Figure 4  Proteomic analysis of LSC-CM .................................................................51

Figure 5  Therapeutic potential of exosome inhalation treatment in rats post-Bleo Injury .................................................................53

Figure 6  Exosome treatment improves pulmonary function post-Bleo and exosome miRNA profiling .......................................................................55

Chapter 2 Supplemental Figures

Supplemental Figure 1  Negative controls of all immunostaining for phenotype analysis of lung spheroids .....................................................82
Supplemental Figure 2  Negative controls of all immunostaining for biodistribution of LSCs in-vivo after intravenous injection ........................................83

Supplemental Figure 3  Double stained LSCs shows mixed phenotype of mesenchymal and epithelial markers ..........................................................83

Supplemental Figure 4  Representative in-vivo imaging of control athymic nude mice showing auto-fluorescence ............................................84

Chapter 3 Supplemental Figures

Supplemental Figure 1  CD1 mice body weight measures throughout the study ..........113

Supplemental Figure 2  Methylene blue nebulization experiment in CD1 mice ..........113

Supplemental Figure 3  Proteomic analysis of lysed LSCs ........................................114

Supplemental Figure 4  LSC-Exo Study Tunel analysis of apoptotic cells ...............115

Supplemental Figure 5  Blood biochemistry analysis of liver enzymes and kidney metabolite ...........................................................................116

Supplemental Figure 6  Representative H&E staining of heart, kidney, liver and spleen in CM and Exo treated animals ..................................116

Supplemental Figure 7  RNA sequencing analysis of LSC-EXO and MSC-EXO samples ...........................................................................117
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSMA</td>
<td>alpha smooth muscle actin</td>
</tr>
<tr>
<td>ANGPTL2</td>
<td>angiopoietin-like protein 2</td>
</tr>
<tr>
<td>AT1</td>
<td>alveolar type 1 epithelial cells</td>
</tr>
<tr>
<td>AT2</td>
<td>alveolar type 2 epithelial cells</td>
</tr>
<tr>
<td>AQP5</td>
<td>aquaporin 5</td>
</tr>
<tr>
<td>BASC</td>
<td>Bronchioalveolar Stem Cells</td>
</tr>
<tr>
<td>Bleo</td>
<td>bleomycin</td>
</tr>
<tr>
<td>BP</td>
<td>Bipotential Progenitor</td>
</tr>
<tr>
<td>C1r</td>
<td>complement component 1r</td>
</tr>
<tr>
<td>CM</td>
<td>conditioned media</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>Crs</td>
<td>compliance</td>
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<td>DDK3</td>
<td>dickkopf-related protein 3</td>
</tr>
<tr>
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<td>deoxyribonucleic acid</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>EDC</td>
<td>Explant Derived Cells</td>
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<td>FDA</td>
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<td>forced expiratory volume</td>
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<td>FSTL1</td>
<td>follistatin like 1</td>
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<tr>
<td>FVC</td>
<td>forced vital capacity</td>
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<td>growth arrest-specific 6</td>
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<tr>
<td>IC</td>
<td>inspiratory capacity</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>insulin-like growth factor binding protein 2</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove's Modified Dulbecco's Medium</td>
</tr>
<tr>
<td>IPF</td>
<td>idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>i.t.</td>
<td>intra-tracheal</td>
</tr>
<tr>
<td>LC/MS/MS</td>
<td>liquid chromatography-tandem mass spectrometry</td>
</tr>
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<td>LSC</td>
<td>lung spheroid cells</td>
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<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>miR</td>
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<td>matrix metalloproteinase</td>
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<td>resistance</td>
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<td>serpine family E member 1</td>
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<td>SMAD3</td>
<td>mothers against decapentaplegic homolog 3</td>
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<td>secreted proteins acidic and rich in cysteine</td>
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<td>Transbronchial</td>
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<td>Description</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WISP1</td>
<td>Wnt1-inducible signaling pathway protein 1</td>
</tr>
<tr>
<td>WL</td>
<td>Whole Lung</td>
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Chapter 1

Introduction

This chapter aims to provide a brief background introduction into Idiopathic Pulmonary Fibrosis (IPF), the different experimental models, cell-based and cell-free therapies, and current research approaches to studying pulmonary fibrosis.

1.1 Idiopathic Pulmonary Fibrosis: History of a mysterious lung disease

In 1933 two Johns Hopkins' doctors, Louis Hamman and Arnold Rich, were the to describe what is now known as idiopathic pulmonary fibrosis (IPF).¹ Over the next ten years, they reported four unusual cases of patients initially reporting with persistent coughing, shortness of breath (dyspnea), and swelling of the extremities followed by a rapid decline in health who ultimately died with signs of extreme cyanosis, dyspnea, and cardiac failure.² Hamman and Rich termed this new set of symptoms acute diffuse interstitial fibrosis of the lung, later referred to as the Hamman-Rich Syndrome.³,⁴ Due to Hamman and Rich's identification of the disease and detailed description of the clinical and pathological characteristics of the disease, it aided other clinicians to identify future cases of IPF. As IPF slowly became a recognized disorder, the typical symptoms and hallmark characteristics began to emerge. Today, IPF is
known as a progressive and chronic form of interstitial lung disease (ILD) of unknown cause characterized by persistent dry coughing, progressive dyspnea, clubbing of the fingers, dense diffuse fibrosis, fibroblastic foci and alveolar honeycombing. As scar tissue (fibrosis) forms in the lung, the tissue becomes stiff and thickens preventing the lungs to properly perform its necessary function of gas exchange and providing the body's cells with the oxygen they need to survive.

Since the disease's discovery nearly a century ago, research has primarily been focused on refining the clinical and pathological signs of IPF and to better understanding its etiology and risk factors, with very little progress concerning treatment. To date, IPF is still an incurable lung disease with poor diagnosis and prognosis. The median survival of IPF patients once diagnosed is two to five years, with a five-year survival rate estimated at a mere 20%. Even though IPF is regarded as a rare disease, with incident rate rapidly rising worldwide, it has quickly become the most common form of idiopathic interstitial pneumonia (IIP) and the leading type ILD.

Idiopathic pulmonary fibrosis causes some of the same symptoms as other more common lung diseases such as chronic obstructive pulmonary disease (COPD). Patients who suffer from IPF are also typically middle-aged to older adults, who exhibit typical signs of dry cough, shortness of breath and fatigue that is often assumed to be related to their age or physical fitness. Physicians unfamiliar with the disease will often misdiagnose IPF, and it typically requires a specialized pulmonologist for accurate diagnoses. Due to the difficulty of correctly identifying IPF cases, it can take up to one to two years and two to three different doctors to properly diagnose an IPF patient, subsequently delaying proper disease management, allowing the fibrosis to worsen. Even after the first hurdle of accurately
diagnosing the disease, no cure currently exists for IPF. Current treatment for IPF is merely palliative and can help relieve and manage the symptoms.

One of the most significant breakthroughs for IPF treatment came in 2014 when the Food and Drug Administration (FDA) approved two drugs, pirfenidone and nintedanib, as the first pharmaceutical option for IPF. Pirfenidone is a synthetic pyridone compound found to exert anti-fibrotic and anti-inflammatory properties by inhibiting transforming growth factor-beta (TGF-β), a key player in IPF pathogenesis. Nintedanib, which was initially developed as an anti-cancer drug, works by inhibiting multiple tyrosine kinase pathways by targeting vascular endothelial growth factor receptors (VEGFRs), fibroblast growth factor receptors (FGFRs), and platelet-derived growth factor receptors (PDGFRs). Pirfenidone and nintedanib are both pills administered orally and have been shown to delay the progression of scar tissue formation, but they are ineffective in reversing established fibrosis. Both treatments have also been found to be more effective as early intervention treatments; therefore, early diagnosis is critical. A lung transplant remains the only effective treatment option for progressive late stage IPF. However, due to the limited supply of human organs, a majority of IPF patients will never benefit from organ transplantation.

1.2 Modeling the Disease: Bringing bench to bedside

As we better understand the disease, its mechanisms, and the risk factors associated with it, researchers are continuously developing new strategies for not only treatment but also better diagnosis and prevention of IPF. As new therapies continue to develop, regenerative medicine approaches have emerged as a thriving field that holds the potential to heal and replace the damaged cells, tissues, and organs. In-vitro studies are important in studying diseases, but to
more accurately predict the clinical outcome of treatment therapies, in-vivo work is essential. Therefore, utilizing animal models of lung fibrosis is imperative.

Pulmonary fibrosis is a complicated process comprised of cytokine modulations, recruitment of inflammatory cells, fibroblast migration and proliferation, and excessive extracellular matrix accumulation. Over the years, various different animal models have been developed and are continuously improving to better mimic the various pathological features of the disease. Bleomycin is the most commonly used model of experimentally induced pulmonary fibrosis. It is an extremely effective anti-cancer drug discovered in 1962 for the treatment of lymphoma, testicular cancer, ovarian cancer and cervical cancer. However, with increased use of bleomycin, a severe side effect of the drug became apparent. Bleomycin therapy causes diffuse pulmonary fibrosis and impaired lung functions in patients, very similar to IPF. This has led to the wide use of bleomycin in experimental animal models of pulmonary fibrosis. Many clinical trials including those that led to the development of pirfenidone and nintedanib utilized the bleomycin model of lung injury.

Aside from bleomycin, there are many other models used to replicate IPF including, asbestos, silica, fluorescein isothiocyanate (FITC), age-dependent fibrosis, TGF-β overexpression, cytokine overexpression, radiation, and acid/hyperoxia. Out of the different models mentioned, both asbestosis and silica-induced fibrosis are clinically relevant models that occur in humans due to occupational and environmental exposure. Both models cause fibroblastic foci and fibrotic nodules that resembles IPF's histopathology. In both models, the asbestos fibers and silica particles can be easily visualized to locate areas where fibrosis is likely to develop. One main difference in asbestosis and silica-induced fibrosis from
IPF is that they cause fewer fibroblastic foci and the distribution of the fibrosis tends to be uneven, showing a bias to particular lung lobes.

The in-vivo studies presented in chapter three will primarily utilize the bleomycin model of induced-pulmonary fibrosis. However, since no single animal model can completely recapitulate the full pathological features of human IPF, silica-induced fibrosis will also be used in the experimental studies to better validate any therapeutic findings found in the bleomycin model. By utilizing two different models of induced pulmonary fibrosis, it also shows that any treatments effects observed in the studies are likely not model-specific.

1.3 Regenerative Medicine: Approaches, limitations, and future directions

The practice of regenerative medicine started long before the term was first coined in 1992. The idea of cultured organs was first realized by Alexis Carrell in 1937's publication; “The culture of whole organs” describing the techniques of maintaining the thyroid gland ex-vivo in a Lindbergh pump for up to 21 days. Later, in 1954, the first organ transplant was performed between identical twins to avoid rejection and adverse immune responses. The field progressed further in 1956 with the first cell transplantation using bone marrow cells, again in twins. Since the discovery of stem cells in human cord blood in 1978, the field has rapidly grown to generate the first engineered skin tissue in 1981 followed by the first cloned animal, a sheep named Dolly, in 1996. The field of regenerative medicine advances forward unlocking new possibilities of not only replacing and regenerating damaged tissues and organs but also rejuvenating the bodies innate ability to repair itself.
1.3.1 Cell-Based Therapies

Over the years, advances in medicine have helped humans live longer than ever, resulting in an increased demand of healthy tissues and organs, but also a shortage of available donor tissue and organs for transplantation. To alleviate this burden along with the severe immune complications associated with organ transplantation, the field of regenerative medicine utilizes cell biology and engineering techniques to bypass these obstacles. Currently, cell-based therapies primarily focus on stem and progenitor cells along with biological or engineered scaffolds to repair damaged tissues or to grow an entirely new organ. Stem cell therapies today utilize cells from a wide array of sources, from mesenchymal stem cells (MSCs) to embryonic stem cells (ESCs), reprogramed induced pluripotent stem cells (iPSCs), and adult stem cells (ASCs). However, due to ethical and political reasons, ESCs are not widely used. The ideal cell type to mitigate rejection and ethical issues are ASCs, but these cells often occur in very low numbers and in some cases are very difficult to isolate and expand in-vitro. Therefore, the majority of stem cell research has focused on iPSCs and MSCs for their ease of isolation, expansion, and low risk of immunogenicity when allogeneic cells are used.

In preclinical studies of stem cell treatment of IPF, the focus has been on the use of a variety of different MSCs derived from tissues including adipose, bone marrow, the placenta, and cord blood. Many of these studies use the well-established bleomycin model and have shown promising results reporting that MSC therapy can prevent lung epithelial damage caused by bleomycin insult in a cytoprotective manner. However, the majority of the studies started treatment intervention too early for the treatment to be considered therapeutic or clinically relevant, since the majority of IPF patients are not diagnosed in the early stage of the disease.
However, the preclinical data of stem cell therapy in animal models are encouraging and have produced a number of clinical trials to test the safety and efficacy in humans.\textsuperscript{49–51} Unfortunately, to date stem cell-based trials for the treatment of IPF have yielded less than favorable results. Many have been proven to be safe but not effective in reversing fibrosis or improving lung functions. The failure of these clinical trials could be due to many reasons, including patient enrollment criteria as well as the possibility that MSCs are simply not the best cell type for the treatment of IPF.

1.3.2 Cell-Free Therapies

In the last two decades, the surge in stem cell research both experimentally in animal models and clinically have shown beneficial effects in various diseases. However, numerous studies have revealed that stem cells have a very low engraftment rate in-vivo.\textsuperscript{48,52} Therefore, rather than engrafting and differentiating into the cell types needed they are exerting their therapeutic effect through a different route. Accumulating evidence suggests that the beneficial effects of stem cells are due to their paracrine activities.\textsuperscript{53–56} They have been shown to secrete trophic factors, including a variety of growth factors, cytokines, chemokines, and small molecules, to create an ideal microenvironment for tissue repair and regeneration. By collecting the factors secreted by stem cells into the media, known as conditioned media (CM) or secretome, similar therapeutic effects have been observed without the use of the stem cells itself.

This has caused a shift in the field of stem cell research to exploring a cell-free alternative.\textsuperscript{57–61} The use of stem cell-derived conditioned media in place of the stem cells themselves would circumvent many of the challenges of stem cell use including maintaining cell stability and stemness state with prolonged in-vitro cell culture, tumorigenicity risk, and immunogenicity risk. Stem cell CM has the therapeutic benefits of the cell itself, but, because
it does not contain any cells or live products, the need for donor-recipient human leukocyte antigen (HLA) matching is not required and the risk of inducing an immune response and tumor formations such as teratomas, which is common with cell injections, can be avoided.

Although the concept of paracrine mediated stem cell regeneration is generally accepted, the exact mechanism and key factors involved are still unclear. Stem cell CM is complex and contains numerous proteins, lipids, nucleic acids, and small molecules such as extracellular vesicles (EVs). Proteomic studies have identified various cytokines, growth factors, and angiogenic factors in stem cell conditioned media. However, over the last several years many have attributed stem cells' therapeutic abilities to a subset of EVs known as exosomes.62-65

1.3.3 Exosomes
Exosomes are small (50-100nm) membrane vesicles found in most cell types that form inside the cells and are secreted from compartments known as multivesicular bodies (MVBs).66-68 These small vesicles was first discovered in 1983 and were originally considered cellular trash and were thought to merely help traffic unwanted cellular components to lysosomes to get degraded.69 It wasn't until 1996 when Graca Raposo's report of immune cell secreted exosomes and their ability to carry membrane-bound molecules that the field was revitalized. The discovery of B-lymphocyte secreted antigen presenting particles capable of inducing antigen-specific immune responses suggests that exosomes are not just cellular garbage, but could be an entirely new mechanism for intercellular communication.

A decade later, when Jan Lotvall and Mariusz Ratajczak discovered the presence of RNAs, particularly mRNAs and miRNAs, within exosomes that the field saw another surge of interest.70-72 The discovery of genetic material within exosomes that could be translated into
functional proteins in recipient cells helped solidify the notion that exosomes are involved in intercellular signaling. This helped prompt investigation of the clinical applications of exosomes as possible diagnostic tools and therapeutic vectors. Exosomes isolated from stem cell conditioned media have reported therapeutic benefits in many disease models. However to date, very few have explored the possibility of exosomes for treatment of lung disease, and those that have utilize MSC-derived exosomes. Interestingly, proteomic and genomic analysis of exosomes revealed that exosomes from different cell types contain different proteins, lipids, and RNA. Thus suggests that the cells are actively selecting the material that goes into the exosomes. The mechanism behind this selection process remains unclear, but it suggests that exosomes secreted from different cell types could yield varying effects in different disease models. This notion prompted us to assess the efficacy of lung-derived exosomes versus widely used MSC exosomes for the treatment of IPF in chapter 3.

Exosome is a new and rapidly growing field of biomedical research that could make a significant impact on human health, but the clinical use of exosomes still faces many challenges. There are various methods for isolating exosomes using ultracentrifugation, size exclusion, and glucose gradients, but the ability to distinguish exosomes from other secreted vesicles of similar size, including ectosomes and microvesicles, remains a challenge. Also, exosome studies typically use exosomes isolated from cell culture CM, but the length of time the media is allowed to condition and the cell culture conditions vary greatly affecting the number of exosomes secreted as well as the contents inside the exosomes. It is known that exosomes are complex particles containing numerous proteins, lipids, and RNAs, but many studies tend to single out an individual miRNA. This approach to assessing exosome's
therapeutic effect through a specific miRNA is not clinically relevant and could not possibly capture the full biological effect of the exosomes.
1.4 Reference


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Chapter 2

Derivation of Therapeutic Lung Spheroid Cells from Minimally Invasive Transbronchial Pulmonary Biopsies

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This chapter aims to characterize and compare Lung Spheroid Cells (LSCs) derived from different tissue sources highlighting the great therapeutic potential of LSCs generated from transbronchial pulmonary biopsies of diseased patients.

2.1 Introduction

The lung is a highly complex organ; it is responsible for respiration but also acts as a barrier to exterior pathogens and pollutants. It's composed of over forty different cell types that make up the three major pulmonary regions: tracheobronchial, intralobar airway, and alveolar. The adult lung is a highly quiescent organ; however, after injury or other conditions the lung has a remarkable regenerative ability; therefore, the lung is considered an organ with “facultative” stem/progenitor cell populations [1-2]. Thanks to lineage tracing, three main stem/progenitor cell populations in the lung which coordinate the maintenance and regeneration in the three main regions of the lungs have been established [3].

In the proximal trachea, basal cells maintain and give rise to club cells and ciliated cells [4-7]. The club cells found throughout the airway are able to self-renew as well as give rise to ciliated cells. Together the basal and club cells are responsible for maintaining the bronchiolar epithelium [8-9]. The alveolar epithelium is primarily maintained by alveolar type 2 (AT2) cells, which also have the ability to self-renew and give rise to alveolar type 1 (AT1) cells [10-14]. Under certain conditions club and AT1 cells can de-differentiate back into basal and AT2 cells, respectively [8,13]. This plasticity makes the lung a good source of therapeutic cells to treat lung disease, but isolation and study of lung stems cells has been extremely difficult, due in large part to the organ’s heterogeneity and complexity.
Cell-based therapy for lung disease has been primarily focused on the use of non-resident stem cells, particularly mesenchymal stromal cells (MSCs), due to their immunoprivileged properties [15-20]. However, MSCs have a very low rate of engraftment into the lungs, as well as a low rate of differentiating into lung cells [21-23], due at least in part to the fact that these cells are extrinsic to the lung. The use of resident lung stem/progenitor cells for cell-based therapy would have great advantage due to the cells’ inherent ability to engraft and survive in a familiar environment, and a method(s) to utilize these cells for this purpose would be invaluable. The multicellular spheroid method has been used before to generate cardiac stem cells with therapeutic potential [24-25]. We and others have previously demonstrated that regenerative lung spheroid cells (LSCs) could be derived from healthy lung donor tissues, and that these cells have disease-mitigating properties in a mouse model of bleomycin-induced pulmonary fibrosis [26-27]. However, obtaining lung tissues from patients is not a trivial task. Surgical lung biopsies can provide a large amount of lung tissue, but such procedures are associated with high mortality (3-28%) [28]. In contrast, the transbronchial biopsy procedure is much safer (0.20% mortality) [29], but the amount of tissue recovered from each transbronchial biopsy is very limited, and it is unknown whether lung spheroid cells can be derived form this procedure. Therefore, in this study, we sought to develop a rapid and efficient method to derive therapeutic lung spheroid cells from minimally invasive lung biopsies. We compared LSCs derived from transbronchial biopsies and whole lung tissues for their growth potentials and phenotypes.
2.2 Results

2.2.1 Lung spheroid cells can be expanded from minimally invasive transbronchial biopsies

Whole lung (WL) tissue samples and transbronchial (TB) biopsy samples were obtained from the Cystic Fibrosis Center and Pulmonary Diseases Research and Treatment Center at the University of North Carolina at Chapel Hill. Donor comorbidity information is presented in Table 1. Samples were taken from the distal lung region of either whole donor lungs or transbronchial biopsies. Tissue samples were processed using the three-dimensional suspension culture method (Fig. 1a). Both sample types showed an outgrowth of phase-bright and stromal-like cells approximately seven days post-plating (Fig. 1b-i). Those outgrowth cells (or explant-derived cells, EDCs) were then seeded onto ultra-low attachment flasks; under these culture conditions the outgrowth cells spontaneously form a three-dimensional cell suspension termed a “lung spheroid” (Fig. 1b-ii). When plated onto fibronectin-coated surfaces, these lung spheroids generated 10-20 million cells that we termed “lung spheroid cells” (Fig. 1b-iii-iv).

Compared to WL tissue samples, TB samples required more time for the outgrowth of cells from the explant to reach the desired confluency to process into spheres. However, once the cells reached the sphere phase, the LSCs produced from both sample types showed similar

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Figure 1 Growth potential of lung spheroid cells derived from whole lung and biopsy specimens. **a:** Schematic showing the protocol to derive lung spheroid cells (LSCs). **b i-iv:** Bright field image of each stage of WL-LSC generation. **b(i):** Explant tissue in the middle surrounded by outgrowth of cells. **b(ii):** Lung spheroids formed from explant-derived cells (EDCs). **b(iii):** Plated spheres onto fibronectin coated surface allowing lung spheroid cells to grow out from the spheroids. **b(iv):** Expansion of LSCs in adherent culture. **c i-iv:** Bright field image of each stage of TB-LSC generation. **d:** Cumulative population doubling for TB-LSCs and WL-LSCs from three different donors. Scale Bar= 10µm.

growth rates, with 5-10 population doublings every seven to fifteen days (Fig. 1 c-d). This growth rate was maintained for up to 7 passages, as long as 30 days with appropriate freeze-thawing from cryopreservation and long-term cell culture. From one TB lung biopsy, we were able to derive >50 million LSCs cells at Passage 2. This sustainable growth from TB samples
indicates that therapeutic lung cells may be derived and expanded from a small sized tissue biopsy sample.

2.2.2 Lung spheroids shows intricate heterogeneous cell populations

Immunocytochemistry displays positive CD90, CD105, CCSP, ProSPC, AQP5, p63 and krt5 cell markers and negative CD31, CD34 and CD45 markers in both whole lung and transbronchial spheroids (Fig. 2 a-b). This is supported by western blot of the same markers (Fig. 2c). Double staining revealed double positive populations of ProSPC and AQP5, ProSPC and CCSP, and CD105 and CD90 (Fig. 2d-e). Heat maps were generated from each positive marker to examine the spherical distribution of the markers showing the supporting cell types such as CD90, CD105, and krt5 primarily at the sphere edge and CCSP, ProSPC, AQP5 and p63 throughout the sphere (Fig. 2f).

2.2.3 Lung spheroid cells exhibit a complex heterogeneous cell phenotype

Flow cytometry revealed several positive populations of lung progenitor cells (e.g. Club Cells [CCSP] and AT2 cells [Pro-SPC]) along with stromal-like supporting cells (e.g. CD90, CD105) (Fig. 3a). WL-LSCs flow analysis showed the cells were 73.1±5.4% CCSP⁺, 92.3±4.1% AQP5⁺, 72.8±5.3% Pro-SPC⁺, 78.8±1.8% CD90⁺, 63.9±6.1% CD105⁺, and 23.5±6.9% Epcam⁺ (Fig. 3b). There was no significant change in the supporting cell populations from EDCs to the final LSCs, but there was a clear increase in the progenitor cell populations from the EDC stage to LSCs, suggesting three-dimensional culture of lung spheroids may have increased the “stemness” of the cells. There was approximately a 37.2% increase in the proportion of AT2 cells and 36.1% increase in the proportion of club cells from the EDC to LSC stage. There also was a slight but non-significant decrease in the proportion of AT1 cells.
Figure 2 Phenotype analysis of lung spheroids. a-b: Representative immunohistochemistry staining of WL-Spheroids (a) and TB-Spheroids (b) for expression of CD90 in red, CD105 in green, CCSP in red, ProSPC in green, AQP5 in red, p63 in green, krt5 in green, CD31 in green, CD34 in green, CD45 and Dapi in blue. c: Western blot of WL and TB-Spheroids. d-e: Double staining of WL-Spheroids (d) and TB-Spheroids (e) for co-expression of ProSPC/AQP5, ProSPC/CCSP and CD90/CD105. f: Expression intensity heat maps and corresponding histograms visualizing the distribution of the markers within the spheroids. For all spheroids imaged n≥3. Scale Bar= 100µm.
TB-LSCs are also positive for the same markers as WL-LSCs, however, there is a difference in the percentage of positive cell populations (Fig. 3a; c). The levels of CD90+ cells decreased by 23.8%, but CD105+ cells increased by 22.3%. Lung secretory (CCSP) and alveolar (Pro-SPC and AQP5) markers decreased 22.7%, 38.8%, and 25.7% respectively. Overall, the phenotype of TB-derived LSCs was distinguishable from WL-derived LSCs. Double stained flow cytometry revealed a double positive population of lung secretory (CCSP) and alveolar (Pro-SPC and AQP5) markers at approximately 39% and 45% respectively (Fig. 3d). Further analysis of double labeled cells for mesenchymal (CD105) and epithelial (ProSPC) showed a double positive population of approximately 38.29% (Supplemental Fig. 3).

LSC phenotype was further supported by immunocytochemistry which showed the cells are positive for CCSP+, AQP5+, ProSPC+, CD90+, CD105+, and Epcam+ (data not shown). Taken together, these findings show that the LSCs contain a wide mixture of cells including club cells, AT1 cell, AT2 cells, CD90+ and CD105+ stromal-origin cells. It has also been previously reported that both cardiac and lung spheroids exhibit progenitor cells in the core of the spheres, surrounded by supporting cells such as CD90+ and CD105+ cells. This arrangement of cells resembles a stem cell niche environment, necessary to regulate stem cell behavior [30]. When LSCs were cultured with bronchia/trachea epithelial cell growth medium (Sigma-Aldrich) there was a three-fold increase in Epcam expression (Fig. 3e-f) suggesting differentiation of progenitor cells into mature lung cells.

2.2.4 Lung spheroid cells promote endothelial cell tube formation in-vitro

A HUVEC tube formation assay was performed to determine the angiogenic potential of the two LSC types compared to human adipose-derived MSCs (AD-MSCs) (Fig. 4a). Human
Figure 3 Phenotype analysis of lung spheroid cells. a: Representative flow cytometry histograms of WL-EDCs, WL-LSCs, and TB-LSCs for expression of CD90, CD105, AQP5, Pro-SPC and CCSP. Black lines in the histograms are the cell of interest as marked on the left hand labels. Red lines in the histograms are the unstained or isotype controls. b-c: Cumulative data for the expression CD90, CD105, AQP5, Pro-SPC, CCSP and EpCAM showing the expression change between (b) WL-EDCs to WL-LSCs (n= 5-12) and (c) WL-LSCs and TB-LSCs (n=7-12). d: Double stained flow cytometry plot of CCSP versus ProSPC and CCSP versus AQP5. e: Representative flow cytometry histograms of WL-LSCs in either IMDM with 20% FBS or epithelial cell growth medium for expression of CD90, CD105, A15, Pro-SPC, and CCSP. Black lines in the histograms are the cell of interest as marked on the left hand labels. Red lines in the histograms are the unstained or isotype controls. f: Cumulative data for the expression CD90, CD105, A15, Pro-SPC, CCSP, and EpCAM (n=3-4). Data represented as mean ± SD. * p<0.05; **p<0.01
endothelial cells were seeded on Matrigel (BD Biosciences) and cultured with control medium (IMDM) LSC conditioned medium, or human AD-MSC-conditioned medium. The tube formation of human endothelial cells on Matrigel suggested a pro-angiogenic role of LSC-conditioned medium (Fig. 4b).

2.2.5 Biodistribution of intravenously-injected lung spheroid cells

Before beginning in vivo studies of LSCs, a major question to address related to where the cells seed after systemic delivery. To determine where the cells will target and how long they can be retained once administered in-vivo, we fluorescently labeled LSCs with DiD and administered 5 x 10^6 cells via the tail vain of athymic nude mice. The mice were imaged at 24 hrs., 7 days, 12 days, 18 days and finally 21 days’ post injection (Fig. 5a). After the final day 21, an image was acquired, and the mice were then sacrificed and organs harvested. At day 1 post injection, most of the cells were found in the lungs and liver, (Fig. 5b-c), and this persisted up to the endpoint at day 21. With such a large dose of infusion (5 million cells in a mouse can be extrapolated to nearly 5 billion cells for a human), no tumor formation was detected in any of the mice that received LSCs. Hematoxylin & Eosin staining further confirmed the absence of tumors in all harvested organs (Fig. 5d). Immunohistochemistry
Figure 5 Biodistribution of LSCs in-vivo after intravenous injection. 

a: Representative in-vivo imaging of mice intravenously injected with LSCs at day 1, 7, 12, 18 and 21 along with final organ harvest of heart, lungs, liver, spleen and kidneys. 

b-c: Cumulative data for radiant efficiencies in the lungs and liver. 

d-e: Representative hematoxylin and eosin staining (d) and immunohistochemistry (e) of heart, lung, liver, spleen and kidneys. Data represented as mean ± SD. 

f-g: Immunostaining of lung and liver tissue for lung specific markers AQP5, ProSPC, CCSP and Epcam. 

d: Scale Bar= 100µm. 
e-g: Scale Bar= 25µm.
confirmed the presences of engrafted LSCs labeled with DiD in the lungs with DiD labeled cell debris and/or secreted factors in the liver and spleen (Fig. 5e). Lung and liver tissues were further examined for LSC phenotypes, showing positive lung alveolar (AQP5 and ProSPC), secretory (CCSP) and epithelial (Epcam) markers in the lung tissue (Fig. 5f). However, these same markers were absent in liver tissues (Fig. 5g). It is worth noting that despite the fact that two different cell lines were used for the intravenous injection of cells, no differences were found. Thus, using different cell lines did not alter overall cell fate or the rate at which the signal strength subsided with time.

2.3 Discussion

Lung disease remains one of the top causes of morbidity and mortality worldwide [31]. Chronic and degenerative diseases of the airway and alveolar tissues, such as emphysema and idiopathic pulmonary fibrosis (IPF), are particularly devastating and to date have no cure [32-33]. Despite advances in supportive care and symptomatic treatments, allogeneic lung transplantation is the only effective treatment for these disorders, but the procedures are highly complicated and highly invasive. Complicating this fact is the lifelong immunosuppression required to prevent rejection. Further, lung transplantation has a high five-year mortality rate at approximately 50% [34]. Therefore, new treatments approaches are desperately needed.

Stem cell-based therapy appears to be a potential major advance in treating these lung diseases. Major advances in the field of lung regenerative medicine have been made in the past few decades as various stem and progenitor cell populations have been identified and characterized, such as bronchioalveolar stem cells (BASC) [35], alveolar bipotential progenitor (BP) cells [3], and other putative distal lung stem and progenitor cell populations. This provides cells for potential new approaches for treating different lung diseases. Although these
stem and progenitor cell populations have been defined, it remains difficult to isolate pure populations of these cells due to a lack of specific surface markers, since many markers are shared between these cell populations. Therefore, mesenchymal stromal cells remain the preferred choice for cell-based therapy in several lung diseases due to their ease of isolation and production, despite the identification of resident lung stem and progenitor cells.

It is important to keep in mind that stem cells do not act alone, but rather interact with surrounding cells (i.e. niche) to perform their necessary functions in both homeostasis and in response to injury. Multicellular spheroids have been used for neural and cardiac stem cells [30, 36-37] with great success. We have recently shown that lung spheroid cells have regenerative abilities in treating early stage pulmonary fibrosis in a murine model [26]. In the present study, we present two distinct sources for LSC generations using a simple and robust method. Whether generated from whole lung donors or a small transbronchial biopsy, we are able to derive therapeutic cells from both tissue sources. However, it should be noted that whole lung tissue sources were from deceased donors with no history of lung disease (i.e. healthy lungs) while transbronchial tissue sources were from donors suffering from advanced lung disease (Table 1). This fundamental difference in the tissue source could account for the significant phenotypic difference in the proportion of mesenchymal (CD90 and CD105) and alveolar (AQP5 and ProSPC) markers (Fig. 3a; c); however, despite tissue source, both WL and TB-LSCs express the same overall phenotypes and growth potential (Fig. 1-3). The cell yield and growth potential were comparable for both types of LSCs and are suitable for clinical applications (Fig. 1). The ease of our method is due in large part to the lack of segregation of different cell types, precluding the need for cell sorting. In addition, our method of lung spheroid generation is without bias for any particular cell type (Fig. 2; 3a-c). Therefore, LSCs
express a heterogeneous phenotype of both mesenchymal and epithelial markers. We believe the stem and progenitor cells are important for regeneration, and their effect is maximized when non-stem epithelial and mesenchymal cells provide the necessary niche environment for proper stem cell function (Fig. 2). This is especially true for the stromal-like supporting cells, as it's been shown that fibroblasts and growth factors, such as FGF 9 and 10, can regulate stem cell homeostasis and activation through Wnt signaling [38-41].

The lung is characterized as having “facultative” progenitor cell populations where differentiated cells such as club cells may be induced to re-enter the cell cycle and proliferate in response to stimuli [42]. This type of response is similar to the liver which has the ability to regenerate in response to injury, but otherwise is quiescent. This is different from organs with high cellular turnover and require a dedicated stem cell population, such as the intestine or skin and hair; or organs such as the brain which has limited ability to regenerate even after injury. As shown via flow cytometry, the markers for lung secretory (CCSP) and alveolar (Pro-SPC) cells, widely accepted as “facultative” lung progenitor cells, showed a significant increase from explants (EDC) to LSCs (Fig. 3b). This suggests that progenitor cell populations are enriched through the 3-dimensional spheroid culture, perhaps by recapitulating the natural niche environment of the cells. The high percentage of both club and AT2 cells suggest that there is a subpopulation that coexpress both markers, which is a hallmark of BASC. There also appears to be an overlap in AT1 and AT2 cell expression, which could be due to the presence of BP cells or intermediate cells in AT2 to AT1 differentiation. It's also has been suggested that AT1 have phenotypic plasticity and may not be terminal cells as commonly believed [14]. AT1 cells cultured on fibronectin surfaces in 20% FBS without additional growth factors, similar to the culture condition used in this study, have been shown to proliferate and express
phenotypic markers of other cell types such as AT2 cells (Fig. 3). AT1 cells cultured in keratinocyte growth factor have also been shown to lose expression of AT1 markers while reacquiring AT2 markers.

As an important in vitro potency indicator of cell therapy, we performed an endothelial tube formation assay to demonstrate the therapeutic potential of WL-LSCs and TB-LSCs in comparison to MSCs (Fig. 4). We showed that LSCs outperformed AD-MSCs in their angiogenic ability, as shown through endothelial cell tube formation. In vivo, we were able to show, using fluorescently labeled LSCs, that the majority of the cells could be seen in the lungs, where they persisted for up to 21 days (Fig. 5a-g). Even though the liver has high fluorescence, immunostaining shows the absence of cells engrafted in the liver as compared to cell clusters found in the lung (Fig. 5e). This suggests that the high fluorescence in the liver may be due to the natural auto-fluorescent nature of the liver shown in Supplemental Figure 4 and/or a combination of a leak in the fluorescence tracer and cell debris from dead cells that were labeled with the fluorescence tracer that has migrated and subsequently taken up by the liver. Further immunostaining for lung specific markers showed that if any LSCs were to engraft in the liver, they do not retain lung phenotype (Fig. 5f-g). There was also a large amount of off-target cell migration to the liver, but otherwise no complications or tumor formation were observed in any animal subjects throughout the study.

2.4 Conclusion

The results show that lung cells have great plasticity, due in large part to cell culture conditions and crosstalk between cells and between cells and their environment. Thus, lung stem and progenitor cell “organoid/spheres” with their niche cells can be considered as superior to pure stem cell populations because they provide the proper cellular support and allow cell signaling.
There remains much to be elucidated about the dynamic feedback between stem cells and their niche and how feasible and effective these cells are at treating severely damaged lung epithelium. Lung spheroids and lung spheroid cells provide a new avenue to explore those questions.

Thus, to the best of our knowledge, we are the first to derive lung spheroid cells containing potential therapeutic lung cells from minimally invasive transbronchial biopsy specimens. Through our simple and highly reproducible three-dimensional culture method, therapeutic lung cells can be generated from small biopsy sized tissues in high efficiency and in clinically relevant numbers. Future studies will focus on the therapeutic potential of transbronchial biopsy-derived lung spheroid cells in animal models of lung diseases.

2.5 Materials and Methods

2.5.1 Cell Culture

Human LSCs were generated from whole lung (WL) and transbronchial (TB) samples and expanded as described [30]. Briefly, tissue samples were washed with phosphate buffered saline (PBS) (Life Technologies), followed by enzymatic digestion at 37°C in 5mg/mL collagenase IV solution (Sigma-Aldrich) for 5 minutes. Iscove’s Modified Dulbecco’s Media (IMDM; Life Technologies) containing 20% fetal bovine serum (FBS; Corning) was then added to the sample to inactivate the collagenase. The tissue samples were further minced into smaller tissue explants (~0.5 x 0.5 mm). Approximately 15-50 pieces of tissue explants were then placed onto a fibronectin-coated plate with approximately 1.5 cm between each explant, and covered with 2 mL of IMDM with 20% FBS overnight. The cultures were maintained in IMDM with 20% FBS and media change was performed every other day. In about seven days, cells started to grow out from the tissue explants. Once these outgrowth cells were about 70-
80% confluent, usually around day 17-25, they were harvested after 5-10 minutes of incubation with TryPLE Select™ (Life Technologies). The cells were then seeded into an Ultra-Low attachment flask (Corning) at a density of 100,000 cells/cm² and cultured in IMDM with 10% FBS. Phase-bright lung spheroids started to form in 5-7 days. Lung spheroids were then collected from the low-attachment flasks and re-plated onto fibronectin-coated surfaces to produce adherent LSCs. LSCs were cultured in IMDM with 20% FBS media and passaged every 5-7 days. We used passage 2-5 LSCs for all in-vitro and in-vivo testing. Bronchia/trachea epithelial cell growth medium (Sigma-Aldrich; 511-500) was used for testing effects of media on cell markers.

2.5.2 Cell Population Doubling

We started with a known amount of cells plated to a flask. On the next passage, cells are counted and the amount was compared to the original cell count plated. Using these numbers and the known amount of time in between the cell counts, the rate of population doubling can be calculated. This process was repeated for each passage of cells. The following equation was used: 

$$\text{log}\left(\frac{\text{cell count at end passage}}{\text{cell count at plating}}\right)/\text{log}(2)$$

2.5.3 Flow Cytometry

Cells were washed with MACS flow buffer (MACS, 130-091-222) and permeabilized with BD Cytofix/Cytoperm (BD, 554714) prior to incubation with antibodies. Cells were labeled for antibodies against CD90 (Abcam, ab3105; Abcam, ab124527; Abcam, ab23894; BD, 555595), CD105 (Abcam, ab107595; Abcam, ab2529; Abcam, ab11414; R&D Systems, Fab10971p), Pro-SPC (Biosis, bs 10067R; Abcam, ab40879), CCSP (Abcam, ab171957), Epcam (Abcam, ab71916, Abcam, ab168828; Life Technologies, a15755), and Aqp5 (Abcam, ab78486;
Abcam, ab85905) and detected by Alexa Fluor 488 (Abcam, ab150117, ab150077) or fluorescein isothiocyanate (FitC) (Abcam, ab6840) secondary antibodies. Both unstained and isotype controls (Abcam, ab18419; BD, 559320; Abcam, ab125938) were utilized as controls. Human adipose-derived mesenchymal stem cells (AD-MSCs) were obtained from Lonza. Flow Cytometry was performed on the CytoFlex (Beckman Coulter, Indianapolis, IN, http://www.beckman.com) and analyzed using FCS Express (De Novo Software, Glendale, CA, https://www.denovosoftware.com) or CytExpert ((Beckman Coulter, Indianapolis, IN, http://www.beckman.com).

2.5.4 Immunocytochemistry

Cells were plated onto fibronectin-coated 4-well chamber slides (Millipore; PEZGS0416). Once the desired confluency was achieved the slides were fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences; 15710) followed by permeabilization and blocking with Dako Protein blocking solution (DAKO; X0909) containing 0.1% saponin (Sigma-Aldrich; 47036) prior to immunocytochemistry. Cells were stained for antibodies against CD90, CD105, Pro-SPC, CCSP, Epcam, and Aqp5 and detected by Alexa Fluor 488 or FitC secondary antibodies. Slides were imaged on a fluorescent microscope (Olympus; Olympus IX81, Center Valley PA, http://www.olympusamerica.com).

2.5.5 Generation of Heat-Map Images

Single channel immunostained images of the spheroids were imported into ImageJ (https://imagej.nih.gov/ij/). The Rainbow RGB lookup table was applied to each image to visualize cell marker density distribution throughout the spheroids. A plot profile histogram is then generated using the rainbow RGB image.
2.5.6 Tube Formation Study

Human umbilical vein endothelial cells (HUVECs; American Type Culture Collection) were seeded onto Matrigel in a 96-well plate at a density of \(2 \times 10^4\) cells per well with 100 µl of plain IMDM, LSC conditioned media, or adipose derived-MSC conditioned media. After 5 hours, the wells were imaged with the Nikon TE-200 (Nikon, Tokyo, Japan, http://www.nikon.com). The average tube length was measured with NIH ImageJ software.

2.5.7 Biodistribution of LSCs After Intravenous Infusion

All studies were in compliance with the requirements from the Institutional Animal Care and Use Committee at North Carolina State University. Athymic nude mice (n=10; Crl:NU(NCr)-Foxn1\(^{nu}\); Charles River Laboratories) were intravenously injected with \(5 \times 10^6\) LSCs from passage 1-3 suspended in 300 µL of a 1:10 heparin/PBS solution. Two different LSC lines were used and mice were randomly assigned to the two different lines. Before injection, all cells were labeled with the lipophilic tracer DiD (Invitrogen) as per their protocol for subsequent live imaging using the Xenogen Live Imager. After taking baseline images, mice were injected with the DiD-labeled LSCs. Subsequent images were taken at 1, 4, 7, 11, and 20-day time points, after which the mice were euthanized. The hearts, lungs, livers, spleens, and kidneys were removed and imaged separately to allow examination without interference from other tissues.

For each nude mouse, an image showing luminescence was analyzed using the region of interest (ROI) tools from the tools palette window of the Living Image 4.2 software package. First, the image was adjusted so that each subject’s radiant efficiency reading was set to a manually fixed scale (a minimum and maximum range). This is important for visual
comparisons of the different time points but not necessary for using ROI tools. The color table was set to Rainbow 2 and reversed for visual clarity of the signal. A circular ROI was chosen and the diameter was fixed to encompass the entire signal range. The same diameter was used for every nude mouse image. One ROI was placed around the lungs and another around the liver of each subject. As the signal began to fade from the organs, a heuristic technique was used to separate the liver from the lungs. The liver signal remained quite discernable so it was used as a starting point. The upper extremity of the liver signal was used as the outer-most edge of the lower-most region of the lung signal. The two ROI perimeters were set so that they never touched. The measurement type was set to radiant efficiency and was then measured. The average radiant efficiency output was saved and exported to Excel where it could be compared across the time points.

2.5.8 Statistical Analysis

All results are expressed as mean ± standard deviation (SD) and Gaussian distribution of data is tested using Kolmogorov-Smirnov test and/or D’Agostino and Pearson omnibus normality test. Comparison between two groups was conducted by two-tailed Student's $t$-test. One-way ANOVA was used for comparison among three or more groups with Bonferroni post hoc correction. Differences were considered statistically significant at P-values <0.05.
2.6 References


Chapter 3

Inhalation of Lung Spheroid Cell-Secreted Factors and Exosomes Promotes Therapeutic Lung Repair in Rodent Models of Pulmonary Fibrosis

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This chapter aims to assess the therapeutic effect(s) of lung spheroid cells (LSCs) derived conditioned media and exosomes in different models of lung injury highlighting potential key factors within the LSC secretome including MMPs, TIMPs, miR99 family and let-7 family of miRNAs.

3.1 Introduction

Our entire body and all its organs are covered with a protective layer of epithelial cells. The epithelial cells in our lungs not only facilitate oxygen exchange but also defend against continuous exposure to inhaled irritants and toxins. Fortunately, our lungs have the ability to undergo facultative regeneration because of the resident stem and progenitor cell populations that are essential for maintaining tissue homeostasis and repair in response to acute and/or chronic lung injuries.

However, respiratory-related morbidity and mortality are on the rise. While some chronic lung injuries have been linked to environmental factors or lifestyle choices such as smoking, the cause of Idiopathic Pulmonary Fibrosis (IPF) remains unknown (as the name implies). IPF is actively being studied by many groups as it is a progressive and fatal form of interstitial lung disease, characterized by fibroblastic foci, alveolar honeycombing and persistent and unremitting fibrosis that ultimately leads to destruction of lung architecture and fulminant respiratory failure. However, the exact pathological mechanism(s) and cause of IPF remain unknown, resulting in poor prognosis. There remain few treatment options for IPF.
To date, only two Food and Drug Administration (FDA) approved therapies exist, pirfenidone and nintedanib, yet these treatments are only palliative and merely delay disease progression.\textsuperscript{7–11} They do not halt or reverse the fibrosis that is already established.

As new treatment strategies continue to evolve, cell-based therapies have emerged as a promising option with multiple clinical trials utilizing stem cells.\textsuperscript{12–14} Although stem cells have beneficial effects, their clinical applications face many challenges including extensive labor, high cost, and safety concerns. Cell-based therapy carries a certain risk of tumorigenicity and immunogenicity, along with cell stability concerns, as stem and progenitor cells all have an inherent risk of transformation during prolonged \textit{in-vitro} cell cultures. Also, cell therapy products need to be carefully preserved and processed before clinical applications. Cumulating evidence indicates that the regenerative ability of adult stem cells is primarily due to their paracrine activity.\textsuperscript{15–20} Therefore, utilization of the stem cell secretome or stem cell conditioned media (CM) in place of the actual cells would mitigate not only these limitations but retain the regenerative benefits of cell-based therapies.

Our previous studies have shown that therapeutic adult lung cells termed LSCs, containing a heterogeneous population of cells expressing lung epithelial (Epcam, AQP5, ProSPC and CCSP) and mesenchymal (CD90 and CD105) markers.\textsuperscript{20–22} The purpose of the present study is to examine the therapeutic effects of lung spheroid cell (LSCs) derived conditioned media in pulmonary fibrosis. It has been reported that the mesenchymal stem cell (MSCs) secretome or conditioned media indeed reproduces the therapeutic effects seen in MSC cell therapy in some diseases, such as osteoarthritis, bronchopulmonary dysplasia, and multiple sclerosis.\textsuperscript{17,19,23–26} Therefore, our study will include MSC-derived CM as a comparator treatment to test for non-inferiority. Our over-arching hypothesis is that lung spheroid cell-
conditioned media (LSC-CM) promotes lung repair in pulmonary fibrosis, in a fashion superior to its MSC counterpart.

3.2 Results

3.2.1 Stem cell-conditioned media attenuates and reverses bleomycin-induced fibrosis and fibroblast apoptosis

To test the impact of LSC-CM on lung repair and fibrosis, we used a single high dose intratracheal (i.t.) bleomycin (Bleo)-injection model in immunocompetent CD1 mice. Body weight was monitored throughout the study as a sign of disease burden (Supplementary Fig. 1). Our goal was to treat fibrosis and not inflammation; therefore, we allowed the initial inflammation phase to pass and the fibrosis to set in before starting treatment. Our previous experience with the Bleo model showed that inflammation peaks around day seven post instillation and transitions to fibrosis around day nine; therefore, we waited until day ten after Bleo insult to begin intervention (Fig. 1a). At day ten, the mice were given inhalation treatment using a nebulizer for seven consecutive days with a dose of 10 mg of CM protein per kg of body weight or an equal volume of PBS. To verify that nebulized substances were able to reach the distal lung we tested mice with nebulized methylene blue dye. We immediately harvested lungs from one group of animals after one nebulization treatment for histological examination and verified that the dye indeed reached the distal lung (Supplementary Fig. 2).

At initial macroscopic examination of therapeutic effects, all groups that received Bleo showed hemorrhagic necrosis (Fig. 1b), which was decreased with either LSC- or MSC-CM treatment. Since Bleo induces DNA damage as its molecular mechanism, we examined effects of CM treatment on apoptosis (Fig. 1 c-d). LSC-CM treatment led to a reduction in apoptosis in the lung as compared to the PBS treated group. Both LSC-CM and MSC-CM treatments
Figure 1 Stem cell conditioned media reverses alveolar epithelial cell damage caused by chronic Bleomycin injury. (a) The schematic of conditioned media procedure and the experimental study schematic; n=6-7 per group. (b) Macroscopic view of the explanted lungs at study endpoint. (c-d) Representative Tunel staining of apoptotic cells for each treatment group (c) and quantification of percent of Tunel positive cells; Scale bar= 100µm *P≤0.05; each dot represents data from one animal; n=6 (d). (e) Representative H&E staining. Top: Scale bar= 500µm Bottom: Scale bar= 200µm (f) Quantification of fibrosis by Ashcroft score; *P≤0.05; ***P≤0.001; each dot represents data from one animal; n=7. (g) Representative Gomori's trichrome staining; muscle fibers (red), collagen (blue), nuclei (black-purple) and erythrocytes (red). Scale bar= 1000µm (h) Representative picrosirius red staining; Collagen types I and III (red). Scale bar= 50µm (i) Quantification of pulmonary hydroxyproline levels; *P≤0.05; ***P≤0.001; each dot represents data from one animal; n=6.
showed an apparent reduction in fibrosis by preserving alveolar epithelial structures (Fig. 1 e-f) and reduced collagen deposition (Fig. 1 g-i). Histological examination of fibrosis via hematoxylin and eosin (H&E) staining and corresponding Ashcroft score revealed that only LSC-CM was able to reduce the fibrotic area and reverse the alveolar epithelial damage back to healthy levels as observed in sham controls.

**3.2.2 Stem cell-conditioned media inhalation treatment increases MMP2 expression and promotes vascular and alveolar repair**

The alveoli are terminal structures of the distal airway. The alveolar epithelium is comprised of alveolar type 1 epithelial cells (AT1) which are the specialized cell type in the lungs that mediate gas exchange, and alveolar type 2 epithelial cells (AT2) which produces and releases pulmonary surfactants, antioxidants, cytokines/chemokines, and other molecules important for the lung’s defense, response to insult, and maintaining pulmonary homeostasis. Therefore, we examined the changes in AT1 and AT2 distribution in response to CM treatment post-Bleo injury to assess epithelial damage and rescue (Fig. 2a). Immunostaining of AT1 marker aquaporin 5 (AQP5) showed that LSC-CM treatment was able to reverse the epithelial damage caused by Bleo (Fig. 2a, d). Additionally, LSC-CM treatment significantly increased the proliferation of surfactant protein C (ProSPC) positive AT2 cells (Fig. 2a, c), which synthesize and secrete pulmonary surfactant as well as maintaining alveolar structure and integrity by proliferating and differentiating into AT1 cells. This response still occurred with MSC-CM treatment, but at a lower level and not significantly different from the PBS treated group. Only LSC-CM treatment was able to increase the expression of von Willebrand factor (vWF) positive vasculatures in the PF lungs (Fig. 2a-b).
Figure 2 LSC-CM inhalation treatment promotes alveolar repair. (a) Representative immunostaining of lung sections for von Willebrand Factor (vWF), pro-surfactant protein C (Pro-SPC) and aquaporin 5 (AQP5). Scale bar= 100µm (b-d) Quantification of percent pixel intensity of vWF+ (b), ProSPC+ (c), and AQP5+ (d). *P≤0.05; **P≤0.01; each dot represents data from one animal; n=4. (e-f) Western blot analysis of alpha smooth muscle actin (αSMA) (e), matrix metalloproteinase 2 (MMP2) (f), and GAPDH loading control with corresponding quantification of protein levels as fold of sham control; *P≤0.05; each dot represents data from one animal; n=3. (g) Representative cytokine array with quantification of relative intensity; * P≤0.05 compared to sham; # P≤0.05 compared to PBS.

In parallel, we examined the fibrotic response by measuring protein levels of alpha-smooth muscle actin (αSMA) (Fig. 2e), which is a marker of fibroblast to myofibroblast transition in IPF. Both CM treated groups showed a drastic decrease in αSMA when compared to PBS treated controls. Interestingly, the protein expression of matrix metalloproteinase 2 (MMP2) were increased in both CM treated groups (Fig. 2f). We employed a cytokine array...
to access systemic cytokine expression. Only LSC-CM was able to significantly decrease IL-4 expression compared to the PBS controls (Fig. 2g).

3.2.3 Therapeutic effects of stem cell-conditioned media therapy in silica-induced pulmonary fibrosis

To test whether the regenerative effects of LSC-CM could be applied to other models of lung injury, we used the well-established silica model of induced pulmonary fibrosis. Unlike Bleo, a biochemical agent that causes direct cellular injury, instillation of fine silica particles into the lungs causes fibrotic nodules to develop around the particles. Again, because our goal was to treat the fibrosis and not the inflammation that precedes it in animal models, we waited until the inflammation phase had passed and fibrosis had set in before treating the mice. For the silica model we waited until day 28 post-silica instillation to start treatment and like the previous Bleo study, we gave inhalation treatment of CM or saline for seven consecutive days via a nebulizer (Fig. 3a).

The physical presence of the particles embedded in the lungs aided in localization and visualization of the fibrotic response. There was no significant difference in any of the treated groups regarding effects on apoptotic cells (Fig. 3b). However, LSC-CM was able to significantly decrease the severity of the fibrosis as compared to the PBS treated group (Fig. 3c-d). The fibrotic tissue around the silica-induced nodules were less intense and widespread but persisted in both LSC- and MSC-CM treated groups (Fig. 3c, e, f). CM treatment also reduced collagen deposition and prevented alveolar epithelial damage as compared to the PBS treatment, although collagen deposition was still significantly higher than that of the healthy sham controls (Fig. 3f-g).
Figure 3 Lung repair and fibrosis in mice after silica-injury

(a) The experimental study schematic of the silica-induced fibrosis study in A/J mice; n=10 per group. (b) Representative Tunel staining of apoptotic cells for each treatment group and quantification of percent of Tunel positive cells; Scale bar= 50µm; each dot represents data from one animal; n=5 (c) Representative H&E staining. Scale bar= 100µm (d) Quantification of fibrosis by Ashcroft score; *P≤0.05; ***P≤0.001; ****P≤0.0001; each dot represents data from one animal; n=4. (e) Gomori's trichrome staining (bottom); muscle fibers (red), collagen (blue), nuclei (black-purple) and erythrocytes (red). Scale bar= 100µm (f) Representative picrosirius red staining; Collagen types I and III (red). Scale bar= 100µm (g) Quantification of pulmonary hydroxyproline levels; *P≤0.05; each dot represents data from one animal; n=4. (h) Representative immunostaining of lung sections for aquaporin 5 (AQP5), pro-surfactant protein C (Pro-SPC) and von Willebrand Factor (vWF) Scale bar= 50µm. (i-k) Quantification of percent pixel intensity of AQP5+ (i), ProSPC+ (j), and vWF+ (k). *P≤0.05; each dot represents data from one animal; n=4
Examination of the AQP5+ AT1 cells and ProSPC+ AT2 cells revealed a significant decline in both alveolar markers in all silica-injured lungs (Fig. 3 j-k). However, LSC-CM was able to reduce the alveolar epithelial damage as compared to the PBS group by promoting ProSPC+ AT2 cell expression and maintaining the AQP5+ AT1 cell population (Fig. 3i-j). As expected, the fibrotic nodules were absent of AQP5 expressing AT1 cells, but, interestingly, still contained limited expression of ProSPC- and vWF-positive cells (Fig. 3k).

3.2.4 Protein composition of lung spheroid cell-conditioned media

To better understand the molecular processes that mediate the observed regenerative abilities of LSC-CM, we sought to define its proteomic composition. We used pooled CM from three different donor LSC lines (Supplementary Table 1) and employed liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis to examine their proteome and compare them to well-reported stem cell secretomes. Interestingly, the three LSC lines had remarkably similar proteomes, considering that the cell lines were derived from individuals of different sex, race and age (Fig. 4a). Of the shared proteins, 29.6% were annotated as extracellular proteins with known membrane receptors for secretion and 47.5% were annotated as cytoplasmic proteins with no known secretion pathways (Fig. 4b).

The high percentage of cytoplasmic proteins prompted us to verify whether the proteins identified in the CM were truly secreted proteins from the cells or were released from dead cells. Thus, we compared the identified cytoplasmic proteins from the CM with those proteins identified from lysed cells (Supplementary Fig. 3a-c). As expected, when LSCs were lysed, the majority of the proteome consisted of annotated cytoplasmic proteins (65%) and only 5% of annotated extracellular proteins. When comparing the cytoplasmic proteins detected in the LSC-CM with the lysed cells, we found that only 56 out of 222
Figure 4 Proteomic analysis of LSC-CM (a) Venn diagram of proteins identified in three LSC-CM. (b) Pie chart of protein subcellular location of all common proteins in all three LSC-CM (c) Relative abundance of 20 of the 103 extracellular proteins identified in all three LSC-CM. (d) Gene ontology pie chart of biological process associated with the shared extracellular proteins.

(~25%) proteins classified as cytoplasmic proteins in the CM could be from potentially lysed or dead cells. These findings suggest that the other ~75% of the proteins annotated as cytoplasmic proteins identified in LSC-CM are proteins that are released from the cells via a mechanism or pathway that is currently unknown.

The majority of the 103 common extracellular proteins identified in all three donors were growth factors and extracellular matrix-related proteins (Supplementary Table 2). Most notably from this list were proteins involved in Wnt signaling (dickkopf WNT signaling pathway inhibitor 3 [DKK3]), the complement system (complement C1r subcomponent [C1r] and decorin), angiogenesis (angiopoietin-like 2 [ANGPTL2] and extracellular matrix protein 1[ECM1]), lung development (follistatin-like 1 [FSTL1] and nidogen 2), cell proliferation (growth arrest-specific 6 [GAS6] and insulin like growth factor binding protein 2 [IGFBP2]) and extracellular matrix (ECM) remodeling (MMP 1, 2, 3, tissue inhibitor of metalloproteinase
TIMP] 1, 2, procollagen C-endopeptidase enhancer [PCOLCE], serpin family E member 1 [SERPINE1] and secreted protein acidic and rich in cysteine [SPARC]) (Fig. 4c). Gene ontology (GO) analysis of biological processes associated with the LSC secretome identified genes important in developmental process, response to stimulus, and cellular component organization or biogenesis (Fig. 4d).

3.2.5 Stem cell exosomes can partially reproduce the therapeutic effects of stem cell-conditioned media

We and others have demonstrated that the therapeutic components in stem cells CM is comprised of not only soluble proteins but also extracellular vesicles (EVs), particularly exosomes (EXOs). We characterized the LSC-EXOs and MSC-EXOs by size, morphology and common exosomal markers (CD63 and CD81) (Fig. 5 a-c). To test the functional effects of EXOs, we employed a Bleo-induced PF model in SD rats similar to the mouse model previously presented (Fig. 5d).

All CM and EXO treated groups showed therapeutic effects in terms of maintaining normal lung architecture (Fig. 5e) and having decreased fibrosis (Fig. 5f), lung apoptosis (Supplementary Fig. 5), and collagen deposition (Fig. 5g-h). Notably, only LSC-CM and LSC-EXO significantly decreased collagen deposition as compared to the PBS treated group. Additionally, only LSC-CM significantly decreased the fibrosis (Ashcroft score) back to similar levels as the healthy sham control. Protein levels of αSMA showed a non-significant trend of decline in treatment groups (Fig. 5i). LSC-CM and LSC-EXO treatment attenuated alveolar epithelial and vascular injury and reduced fibrotic response, as shown by increased AQP5^+ and vWF^+ cells and decreased αSMA^+ cells (Fig. 5j-l).
Figure 5 Therapeutic potential of exosome inhalation treatment in rats post-Bleo injury

(a) Size analysis of fresh, frozen and lyophilized exosome particles by NanoSight. (b) Representative transmission electron micrograph (TEM) of LSC-EXO. Left scale bar = 0.2µm. Right scale bar = 50nm. (c) Western blot analysis of CD63, CD81 and GAPDH protein in LSC- and MSC-EXO. (d) The experimental study schematic of the exosome study in SD rats; n= 12 per group. (e) Representative H&E staining Top: Scale bar= 100µm Bottom: Scale bar= 50µm (bottom); muscle fibers (red), collagen (blue), nuclei (black-purple) and erythrocytes (red). (f) Quantification of fibrosis by Ashcroft score; Scale bar= µm ***P≤0.001; ****P≤0.0001; each dot represents data from one animal; n=12. (g) Quantification of pulmonary hydroxyproline levels; *P≤0.05; each dot represents data from one animal; n=4. (h) Representative picrosirius red staining; Collagen types I and III (red); Scale bar= 50µm. (i) Western blot analysis of matrix metalloproteinase 2 (MMP2), alpha smooth muscle actin (αSMA), SMAD3 and GAPDH loading control. (j) Representative immunostaining of lung sections for aquaporin 5 (AQP5), alpha- smooth muscle actin (αSMA), and von Willebrand Factor (vWF). Scale bar= 75µm. (k) Quantification of pixel intensity of AQP5 and vWF. *P≤0.05; **P≤0.01; each dot represents data from one animal; n=4. (l) Quantification of pixel intensity of αSMA. *P≤0.05; each dot represents data from one animal; n=4.
3.2.6 Effects of LSC-CM and LSC-EXO therapy on MMP2 and MCP-1 expression

To examine potential molecular mechanisms involved in the response to CM- and EXO-initiated responses, we examined systemic cytokine expression and local tissue protein levels of MMP2 and SMAD3. Unlike what we observed in the murine Bleo model, here we found no significant change in MMP2 protein levels in lung tissue of treated animals (Fig. 5i). However, protein levels of SMAD3 in lung tissue of treated animals were significantly upregulated in the PBS group, which was alleviated by LSC-CM and LSC-EXO treatment. Because the immunomodulating effects of cytokines are systemic rather than local, we examined cytokine levels in blood serum of treated animals (Fig. 6a). Monocyte chemoattractant protein-1 (MCP-1/CLL2) was found to be significantly upregulated by Bleo injury, which was rescued by stem cell-CM and -EXO treatment.

3.2.7 Effects of stem cell-conditioned media and exosome therapies on lung function following bleomycin-induced fibrosis

Next, we sought to predict the clinical impact of LSC-CM and LSC-EXO treatment on impaired lung architecture and fibrosis. We, therefore, performed multiple pulmonary function tests in parallel with the inhalation treatment to monitor the decline in lung function following Bleo injury and any effects of the various treatments. Baseline reads were recorded immediately before Bleo instillation, midpoint reads were taken at day nine post-Bleo (the day before treatment started) and endpoint measurements were taken at day 17 post-Bleo (one day after the last inhalation treatment) (Fig. 6b). As expected, inspiratory capacity (IC), resistance (Rrs), compliance (Crs), hysteresis area, and forced expiratory volume (FEV) to forced vital capacity (FVC) ratio all showed a decline at midpoint measurements as compared to baseline (Fig. 6 c-e, g, and h). Consistently, respiratory elastance (Ers), which is the inverse of
Figure 6 Exosome treatment improves pulmonary function post-Bleo and exosome miRNA profiling (a) Representative rat cytokine array detecting 19 rat proteins from blood serum. *P ≤ 0.05 from PBS group; # P ≤ 0.05 from Sham group (b) The schematic of pulmonary function measurements. (c-g) Quantification of lung function measurements; *P ≤ 0.05; **P ≤ 0.01 (c) pulmonary inspiratory capacity; (d) pulmonary resistance; (e) pulmonary compliance; (f) hysteresis area; (g) forced expiratory volume (FEV) to forced vital capacity (FVC) ratio. (i) Principal component analysis chart of LSC- and MSC-exosome microRNA content. (j) Volcano plot of differentially expressed miRNA of LSC- and MSC-exosomes miRNA content. (k) Distribution of the top 10 miRNAs in LSC-exosomes (top) and MSC-exosomes (bottom). (l) LSC secreted exosomes and soluble factors modulate alveolar repair and fibrosis.
compliance, consistently increased at midpoint analysis (Fig. 6f). Altogether, pulmonary function of all Bleo animals was consistently impaired as expected with pulmonary injury resulting in increased tissue stiffness and elastic recoil (elastance).

At endpoint analysis, pulmonary function was only partially rescued by CM and EXO treatment. Inspiratory capacity and respiratory compliance were both significantly improved after LSC-CM, LSC-EXO or MSC-EXO treatment. Respiratory resistance had a significant recovery only with LSC-EXO treatment. Elastance, hysteresis area and FEV/FVC ratio all had no significant changes after treatment.

3.2.8 Toxicity of conditioned media and exosome therapies

In addition to the effectiveness of CM and EXO treatment, we also assessed the safety of the treatments. Liver and kidney function were found to be within an acceptable range in all treatment groups (Supplementary Fig. 5). Histological analysis of the heart, kidney, liver, and spleen tissues of treated animals in both the silica and Bleo studies did not reveal any apparent damage nor toxicity (Supplementary Fig. 6).

3.2.9 The miR-99 family of microRNAs highly upregulated in LSC-EXOs

Exosomes contain a variety of different RNAs, proteins, and lipids, but miRNAs have garnered significant interest since their discovery within EXO\textsuperscript{27–29}. It is believed that EXOs utilize miRNAs as a mechanism of genetic exchange between cells. We then performed a global small RNA deep sequencing of LSC- and MSC-EXO, and analyzed the differences in their miRNA populations (Supplementary Fig. 7). Together, over 600 unique miRNAs were detected in these LSC-EXO and MSC-EXO samples, indicating that exosomal-derived miRNAs could have numerous regulatory roles in these samples. After removing less abundant miRNAs, 142 remained for downstream analysis (Fig. 6 i-j, Supplementary Fig. 7b, and Supplementary
Tables 6-7). In total, 42 miRNAs were found to be differentially expressed between these two EXO types (minimum fold change of two and adjusted p-value < 0.05). Among the top upregulated miRNAs in LSC-EXO were hsa-miR-99a-5p (log2 fold change 1.25, adjusted p-value 0.0005) and hsa-miR-100-5p (log2 fold change 1.27, adjusted p-value 0.0005) (Fig. 6j, Supplementary Table 8). They were also the two most abundant miRNAs in LSC-EXO and are members of the miR-99 family (Fig. 6k). Notably, antifibrotic miR-30a-3p was also significantly upregulated in LSC-EXO compared to MSC-EXO (log2 fold change 2.28, adjusted p-value 0.00002). hsa-let-7a-5p (log2 fold change 1.09, adjusted p-value 0.001) and has-let-7f-5p (log2 fold change 1.28, adjusted p-value 0.008) were the most upregulated in the MSC-EXO sample and are part of the highly conserved let-7 family.

3.3 Discussion

Various animal models have been developed to mimic the pathological hallmarks of human IPF30–32. Due to the unknown cause of the disease, a true IPF animal model does not exist. The Bleo model of induced pulmonary fibrosis is widely used for the study of IPF and is arguably the most clinically relevant33–36. To support any therapeutic effects found in the Bleo model, we also tested using another model, silica-induced pulmonary fibrosis.

Both the Bleo and silica mouse models presented significant alveolar epithelial damage and ECM deposition (Fig. 1e-i, 3c-g, and 5e-h). In the rat model, we also showed a significant decline in pulmonary function following Bleo injury (Fig. 6c-i). We and others have demonstrated that the cell-free CM derived from stem cells can achieve similar or superior protective and regenerative abilities as compared to the cells themselves itself19,23,37–40. Our observations showed that LSC-CM was capable of reversing fibrosis caused by Bleo, and also that caused by silica particles (Fig. 1-3). However, the regenerative effects of LSC-CM were
more robust in the Bleo model than in the silica model. LSC-CM inhalation treatment in the Bleo-instilled mice was able to rescue the damaged lungs back to similar levels as the healthy sham control in terms of decreased apoptotic cells, fibrotic score, hydroxyproline content, and significant recovery of AT1 and AT2 cell populations (Fig. 1c-i and 2a-d).

The effects of CM treatment in the silica model, while reversing fibrosis compared to the non-treated controls (PBS treated), did not reverse the damage back to levels as seen in healthy sham controls. This could be due to the difference in the animal models. Silica, unlike Bleo induced injury, is caused by the physical presence of the particles depositing into the lung, causing formation of fibrotic nodules. Histological examination showed a decrease in fibrotic tissues surrounding the nodules in CM treated groups as compared to the PBS control, but CM therapy was not enough to resolve the nodules themselves (Fig. 3c, e, and f). It is possible that seven days of treatment was not sufficient to reverse the damage caused by the silica particles. It would be beneficial to see if extended CM treatment would be more effective. For certain types of lung injury where particulate matter is retained in the lungs causing dysfunction, such as silicosis it may require longer continuous CM treatment to not only resolve the fibrosis, but also clear the particles.

The field of regenerative medicine utilizes many different types of stem cells for research and clinical applications, but mesenchymal stem cells remain the most widely used, partly due to their immunomodulating abilities and ease of isolation. Therefore, we wanted to compare the regenerative benefits of LSC-CM against MSC-CM. Our observations in both Bleo and silica models demonstrated that while MSC-CM inhalation therapy had regenerative activities in treating pulmonary fibrosis, LSC-CM was superior to MSC-CM in all measures (Fig. 1-3).
We aimed to elucidate the molecular mechanism(s) underlying CM-mediated lung repair through proteomic analyses. Such analyses revealed downregulation of αSMA (a myofibroblast marker) and pro-inflammatory/pro-fibrotic cytokines, possibly through the upregulation of MMP2 activity (Fig. 2e-g). Studies have shown a diverse role of MMP activities in PF, suggesting that in the early stages of Bleo-induced PF MMPs play a role in disease pathogenesis\(^ {43-45}\). However, MMP activity in the late stage may play a role in the repair process. Mass spectrometry analysis of the LSC secretome revealed proteins related to the complement system (C1r and decorin) as well as Wnt signaling pathway (DKK3) (Fig. 4 and Supplementary Table 2). Additionally, an abundance of ECM remodeling, pro-angiogenic, and cell proliferation proteins was also identified, suggesting that LSC-CM contains proteins able to not only break down and reverse the fibrosis already in place, but also promote epithelial and vascular repair.

Conditioned media does not merely contain soluble proteins, but also insoluble substances, such as exosomes. Exosomes are an emerging field of biomedical research that has seen an explosion of discoveries and interest in the last two decades. In fact, the therapeutic effects of stem cells have been attributed to the miRNAs found in the secreted exosomes\(^ {29,46-52}\). Therefore, we sought to determine if the regenerative benefits observed in response to LSC-CM can be accounted for by EXOs. Our findings indicated that LSC-EXOs were indeed capable of reproducing part of the regenerative abilities of the CM from which they were isolated (Fig. 5-6). We also demonstrated that LSC-EXOs outperformed MSC-EXOs in reversing pulmonary fibrosis and restoring healthy lung function. As expected all CM and EXO treated groups showed significant improvement in terms of decreased apoptotic cells, reduced fibrosis severity, and improved lung function and restoration of the alveolar
epithelium. However, both LSC-CM and LSC-EXOs exhibited superior therapeutic effects as compared to both MSC-CM and -EXOs. LSC-CM was the only treatment capable of reversing the alveolar damage, reducing the fibrotic score and hydroxyproline levels to that of the sham control. Interestingly, in regards to pulmonary function measures, only LSC-EXO showed a significant recovery of total respiratory resistance.

RNA sequencing analysis of LSC-EXOs and MSC-EXOs showed the enrichment of miRNAs in the let-7 and miR99 families, both present in the top ten miRNAs identified in both LSC-EXOs and MSC-EXOs (Fig. 6k). The let-7 family of miRNAs was the first miRNA discovered and is highly conserved in plants and animals\(^53^{–57}\). Let-7 miRNA has been found to play an essential role in biological development, stem cell differentiation, and tumorigenesis. Similarly, the miR99 family (miR99a, miR99b, and miR100) of miRNAs are also highly conserved miRNAs found to be highly expressed in stem cells and have been found to be downregulated in lung injury and cancer\(^58^{–61}\). Different subtypes of let-7 miRNAs are found in specific tissues, cells, and cancer types. Interestingly, let-7a, c, g, and miR100 are downregulated in lung cancer, and all four types are found to be in the top 10 miRNA types detected in both LSC-EXOs and MSC-EXOs. miR99a, miR100, and miR10a have also been found to be modulated in response to DNA damage and in lungs exposed to cigarette smoke\(^62\). The miR99 family of microRNAs, along with miR-151a, miR-10a and miR-30a, is significantly upregulated in LSC-EXOs compared to the other miRNAs identified. Likewise, let-7a and -f are significantly upregulated in MSC-EXOs. Notably, miR-30a expression has been reported to be downregulated in IPF patients\(^63^{–66}\). Studies have shown antifibrotic properties of miR-30a by reversing transforming growth factor-beta (TGF-β) induced Wnt1-inducible signaling pathway protein 1 (WISP1) and inhibiting mitochondria fission preventing
apoptosis. WISP1 is a known profibrotic mediator in IPF patients shown to enhance ECM deposition and promoting fibrotic progression\textsuperscript{67}. Even though miR-30a and the let-7 and miR99 family of miRNAs are identified in both LSC- and MSC-EXOs their differential expression may explain the difference in treatment effects observed.

To assess any systemic immunogenicity or off-target effects of inhaled CM and EXO therapies, we examined systemic cytokine expression, blood biochemistry analysis and histology of all major organs (Fig. 2g, 6a, and Supplementary Fig. 6-7). Normal tissue histology in all organs, along with normal liver and kidney functions, suggests that CM and EXO treatment does not elicit any local or systemic immune reaction and does not cause any aberrant cellular or tissue alterations. Interestingly, our analysis of systemic cytokine expression showed a downregulation of monocyte chemoattractant protein 1 (MCP-1) in blood serum of treated animals (Fig. 6a). Profibrotic cytokine MCP-1 has been reported to play a key role in lung inflammation and increase in MCP-1 levels has been linked to poor prognosis for IPF patients\textsuperscript{68–73}.

We acknowledge several limitations to this work. First, we used only one dose of CM and EXO, determined arbitrarily and guided by previous experience, respectively. It would be sensible for future studies to include a dose-response study to determine the least effective dosage of both CM and EXO. Secondly, our treatment in all three studies was delivered via inhalation using a nebulizer. An investigation into different administration routes to determine the optimal delivery method and frequency for CM and EXOs can help maximize their benefits. Lastly, we presented here the proteomic and genomic composition of LSC-CM and –EXO, respectively, but it would be clinically and pharmaceutically beneficial to determine which proteins, small molecules or RNAs are capable of achieving the therapeutic effects.
In summary, we report here novel acellular therapeutic agents, namely LSC-CM and LSC-EXO, that are shown to be safe and effective in the treatment of bleomycin- and silica-induced pulmonary fibrosis in rodents. The evidence presented here suggests that the mechanism of CM-mediated regeneration may relate to the EXOs, MMP2 activity, and a host of proteins found in the CM. Conditioned media and EXOs are considerably less immunogenic then their parent cells and administration of these factors can overcome the limitations of stem cells while still maintaining similar effects. The identification of miR-30a and the miR-99 and let-7 family of miRNAs in CM warrants future investigations, since miR-30a is known to be downregulated in IPF patients, and the miR-99 and let-7 families of miRNAs are also known to be downregulated in various cancers including lung cancer. Idiopathic pulmonary fibrosis is a fatal respiratory disorder with increasing rates of morbidity and mortality; with no effective therapies currently available, LSC-CM and LSC-EXO provide promising candidates for the development of IPF therapies.

3.4 Materials and Methods

3.4.1 Cell Culture

Human LSCs were generated from whole lung samples and expanded as described\textsuperscript{20–22}. Human LSCs were generated from healthy whole lung donors acquired from the Cystic Fibrosis and Pulmonary Diseases Research and Treatment Center at the University of North Carolina at Chapel Hill and expanded as described in Fig. 1a. We used passage 2-5 LSCs for all \textit{in-vitro} and \textit{in-vivo} testing. Cells were also analyzed by flow cytometry for the appropriate markers before use (CD90\textsuperscript{+}, CD105\textsuperscript{+}, CCSP\textsuperscript{+}, AQP5\textsuperscript{+}, ProSPC\textsuperscript{+}, Epcam\textsuperscript{+}, CD31\textsuperscript{−}, CD34\textsuperscript{−} and CD45\textsuperscript{−}).

3.4.2 Conditioned Media Collection and Preparation

Human LSCs and MSCs were cultured to approximately 75% confluence before the serum –
containing media was removed and replaced with serum-free media (IMDM). The following day the cells were washed six times for 30 min each with fresh IMDM to deplete the albumin on the cells before the media was allowed to condition, as albumin can interfere with some experiments (especially LC/MS/MS analysis). The media (IMDM) was allowed to condition for three days before it was harvested and filtered through a 0.22 µm filter to remove any cells and cell debris. The filtered CM was stored at -80°C for at least 24 hrs or until solid. The frozen CM vial(s) were lyophilized using a LABCONCO FreeZone 2.5 Liter Freeze Dry System overnight or until samples were dehydrated. Once samples were lyophilized, they were stored at -20°C until ready for use.

3.4.3 Exosome Isolation and Characterization

Exosomes were collected and purified from human LSC-CM using an ultrafiltration method\textsuperscript{74,75}. LSC-CM was first filtered through a 0.22 µm filter to remove any cells or cell debris. The filtered media was then placed in a 100 kDa MWCO ultrafiltration filter (Millipore) and centrifuged at 5,000 RCF for 10-15 min depending on volume. Any media contents or small proteins were removed by the filtered centrifugation, and the remaining exosomes were suspended in PBS then filtered and washed. Before use, all exosome samples were analyzed for proper size by nanoparticle tracking analysis (NTA; NanoSight, Malvern) and morphology by transmission electron microscopy (TEM). Additionally, successful exosome isolation was confirmed by immunoblotting for known exosome markers (CD63 and CD81).

3.4.4 Animal Procedures

Six to eight week old male CD1 mice [Crl: CD1(ICR)] and CD (SD) IGS rats [Crl: CD(SD)] were obtained from Charles River Laboratory (Massachusetts, USA) and A/J mice were
obtained from Jackson Laboratory (Maine, USA). Pulmonary fibrosis was induced by a single intratracheal injection of 3 U/kg bleomycin sulfate (EMD; 203401) solution in CD1 mice and CD (SD) rats and a single oropharyngeal aspiration of a 100 mg/kg silica (MIN-U-SIL-5) suspension in A/J mice. Nebulizer treatment started 10 days post bleomycin insult and 28 days post silica insult. Conditioned media, exosome or saline inhalation treatment was given for approximately 30 min/day for seven consecutive days using a nebulizer (Pari Trek S Portable Compressor Nebulizer Aerosol System; 047F45-LCS). Animals were euthanized, and blood and tissues were collected for RNA, protein, and histological examination. All studies complied with the requirements of the Institutional Animal Care and Use Committee at North Carolina State University.

3.4.5 Pulmonary Function Test (PFT) in Rats

Pulmonary function measurements were performed on the FlexiVent (SCIREQ Inc., Montreal, Canada). Prior to measurements, animals were anesthetized with an intraperitoneal injection of ketamine and xylazine solution (2:1 ratio). The animals were intubated with a 14-gauge cannula.

3.4.6 Histology

Immunostaining was performed on tissue slides fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences; 15710) followed by permeabilization and blocking with Dako Protein blocking solution (DAKO; X0909) containing 0.1% saponin (Sigma-Aldrich; 47036) prior to antibody staining. Cells were stained with antibodies against AQP5 (ab78486, Abcam), ProSPC (ab90716, Abcam), vWF (F3520, Sigma-Aldrich), αSMA (ab5694, Abcam). Tunel staining was performed on cryosectioned tissues using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer’s instructions.
Gomori’s Trichrome and Hematoxylin and eosin were performed on paraffin embedded tissue sections. Hematoxylin and eosin stained sections were used for Ashcroft scoring. Ashcroft score was performed by averaging the score from one blinded and one non-blinded scorer.

3.4.7 Proteomic Analysis

To concentrate the samples, 15 ml of the conditioned media was lyophilized. The samples dried to completion in 24 h and were re-suspended in 1 ml of 100 mM ammonium bicarbonate (Sigma Aldrich) buffer at pH 8.6. For protein precipitation, 10 ml of cold acetone (Optima grade, ThermoFisher Scientific) was added to the samples, incubated at -20°C overnight, and then centrifuged for 30 min at 10,000 rpm to separate the precipitated proteins from the supernatant. Total protein concentration was determined using the Bradford Assay (Pierce, ThermoFisher Scientific). All samples were loaded in triplicate (10 µl) onto a microtiter plate, measured at an absorbance of 595 nm using a Tecan Genios microplate reader, and protein concentrations estimated using reference absorbance of BSA standard protein. Precipitated proteins were digested using the Filter Aided Sample Preparation (FASP) method as described by Wisniewski et al.\textsuperscript{76} In a 30 kDa molecular weight cutoff Vivacon filter (Sartorius, ThermoFisher Scientific), approximately 100 µg of protein from each sample were added, reduced with 5 mM dithiothreitol (ThermoFisher Scientific) at 56°C for 30 min and then alkylated with 10 mM iodoacetamide (Sigma-Aldrich) for 20 min in the dark at RT. Digestion on-filter was carried out using sequencing grade trypsin (Promega) at a 1:100 trypsin-to-protein ratio overnight at 37°C. Peptides were removed from the filter with 100 mM ammonium bicarbonate buffer, pH 8.6, and the solvent was evaporated using vacuum centrifugation before storage at -20°C for further processing.

LC/MS/MS analysis for all samples was performed on an Easy nano ultra-high pressure
liquid chromatograph coupled to an LTQ Orbitrap Elite mass spectrometer (ThermoFisher Scientific). Samples were injected onto a PepMap C18, 5 µm trapping column (ThermoFisher Scientific) then separated by in-line gradient onto a New Objective Self Pack PicoFrit column (packed in house with 3.0 µm Reprosil C18 stationary phase (Dr. Maisch GmbH). The linear gradient for separation was 5-40% mobile phase B over 90 min at 300 nl/min flow rate, where mobile phase A was 2% acetonitrile/0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The Orbitrap Elite operated in a data-dependent mode, where the five most intense precursors were selected for subsequent fragmentation. Resolution for the precursor scan ($m/z$ 400–2000) was set to 60,000 at $m/z$ 400 with a target value of $1 \times 10^6$ ions. The MS/MS scans were also acquired in the orbitrap with a normalized collision energy setting of 27 for HCD. For internal mass calibration, the ion of polycyclodimethylsiloxane with $m/z$ 445.120025 was used as the lock mass. Monoisotopic precursor selection was enabled, and precursors with unknown charge or a charge state of +1 were excluded.

Raw data files were processed using Proteome Discoverer (1.4, ThermoFisher Scientific). Peak lists were searched against a forward and reverse Homo sapiens UniProt database using Mascot (1.4.1.14 Matrix Science, www.matrixscience.com). The parameters used for identification of tryptic peptides were: 10 ppm precursor ion mass tolerance, 0.01 Da fragment mass tolerance; up to two missed cleavage sites; carbamidomethylation of Cys was set as a fixed modification; oxidation of Met was set as a variable modification. Scaffold (4.8.4 Proteome software, www.proteomesoftware.com) was used to filter the data, quantify peptides/proteins, and perform statistical analysis. A minimum of 2 peptides per protein at a peptide and protein threshold of 95% were required for high confidence identification. Ingenuity Proteomic Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity)
was used for classification of subcellular localization of the common proteins. The listed common secreted proteins were classified using Panther (Protein Analysis Through Evolutionary Relationships, http://pantherdb.org) and DAVID (The Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov) to explore molecular function, cellular components and pathways.

3.4.8 SDS-PAGE and Western Blot

Samples were reduced by β-mercaptoethanol and denatured at 90°C for 5 min. Proteins were separated by gel electrophoresis carried out in triplicate on a 4-15% Tris-Glycine stain-free gel (Bio-Rad) along with a molecular weight standard (Bio-Rad, Precision Plus Protein Unstained Standards MW Ladder 161-0363). The gel was run at a stack voltage at 100V for approximately 5 min followed by a constant 200V. The gel was activated and visualized by UV light in a Bio-Rad Imager.

The Bio-Rad Mini-PROTEAN Tetra Cell system was used for a wet transfer. The SDS-PAGE gel was assembled into the apparatus with a PVDF membrane stacked between filter papers. Following the transfer, the membrane was washed three times in PBS-T for 5 min each then blocked using 5% milk in PBS-T for one hour. A 4°C overnight incubation of primary antibody was performed for MMP2 (ab86607, Abcam), αSMA (ab5694, Abcam), SMAD3 (MA5-14939, ThermoFisher Scientific), CD63 (nb100-77913, Novus Biologicals), CD81 (MA5-13548, ThermoFisher Scientific), and GAPDH (MA5-15738-HRP, ThermoFisher Scientific) and then followed by a 1-hr incubation with the corresponding HRP conjugated secondary antibodies.
3.4.9  Small RNA Library Construction and Sequencing

Exosomal RNA was isolated using a commercially available total exosome RNA isolation kit (Qiagen’s exoRNeasy Serum Plasma Kit). Once exosomal RNA was isolated, it was further analyzed by sequencing. RNA quantity, 260/280 ratios, and 260/230 ratios were assessed by Nanodrop (ThermoFisher). RNA integrity was verified on a Bioanalyzer 2100 using an Agilent RNA 6000 Nano Assay. Small RNA libraries were prepared according to the manufacturer's protocol using a NEBNext® Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs). Libraries were size selected using a Pippen Prep (Sage Science), and quality-checked by Bioanalyzer using an Agilent High Sensitivity DNA Kit. Libraries were quantified by a Quant-iT™ dsDNA High Sensitivity Assay Kit (ThermoFisher) and sequenced on an Illumina NextSeq500 using a mid-output V2 kit.

3.4.10  Mapping and Differential Expression Analysis of miRNAs

Raw reads were demultiplexed and quality trimmed using the standard Illumina bcl2fastq conversion software. Using mirDeep2, reads had adapters removed and were clustered using default parameters. Reads were then aligned to human rRNAs using Bowtie and the remaining unaligned reads were mapped to miRBase 22 also using Bowtie. For downstream analysis, identified miRNAs were filtered by requiring that a miRNA have at least ten read counts in at least two of the five samples. Differential expression analysis between MSC samples and LSC samples was performed using the edgeR and limma R packages and upper quantile normalized read counts. miRNAs were considered differentially expressed if the absolute log2 fold change was greater than 1 and if the adjusted p-value (FDR) was less than 0.05.
3.4.11 Statistical Analysis

All results are expressed as the mean ± standard deviation (SD). Two-tailed Student's t-test was used for comparison between two groups. Non-parametric one-way ANOVA (Kruskal-Wallis test) was used for comparison of three or more groups with additional Bonferroni post hoc correction. Differences were considered statistically significant at P-values ≤0.05. * P-values ≤0.05; ** P-values ≤0.01; *** P-values ≤0.001; **** P-values ≤0.0001.
3.5 References


17. Linero, I. Paracrine Effect of Mesenchymal Stem Cells Derived from Human Adipose Tissue in Bone Regeneration. 9, (2014).


68. Rose, C. E. J., Sung, S.-S. J. & Fu, S. M. Significant involvement of CCL2 (MCP-1) in


This chapter aims to provide a brief summary of the field of idiopathic pulmonary fibrosis research, how the work presented in this dissertation contributed to the field and future directions.

4.1 Conclusions

There is still much to learn in the field of idiopathic pulmonary fibrosis (IPF), but in recent years many advances have been made in regards to the diagnostics and treatment of IPF.\textsuperscript{1–6} The first major obstacle for IPF patients is to be accurately diagnosed. Early diagnosis remains a challenge, but as we better understand the disease, more accurate guidelines are being established to better identify IPF cases. Recently in 2014, the approval of pirfenidone and nintedanib, two pharmaceutical drugs for the management of IPF, has aided in delaying disease progression. However, significant challenges remain in terms of resolving the fibrosis, improving patient prognosis and overall quality of life.

The respiratory system is vital for life providing the body with oxygen and mediating gas exchange. It is also one of the body's essential barriers to inhaled substances. With the rise in chronic respiratory diseases, such as IPF and the lack of effective therapies, it is vital to develop novel strategies for pulmonary regeneration. This dissertation has presented several
potential therapeutic options for pulmonary fibrosis utilizing patient-derived lung spheroid cells (LSCs) [Chapter 2], LSC-conditioned media (CM), and LSC-exosomes (EXOs) [Chapter 3]. The first study presented in Chapter 2 demonstrated that therapeutic cells could be generated from not only healthy lung tissues but also from diseased lung tissues via a minimally invasive transbronchial biopsy procedure. Next, in Chapter 3, a set of three studies demonstrated the regenerative effects of LSC secreted factors (CM and EXOs) in both bleomycin and silica models of experimental pulmonary fibrosis. The proteome of LSC-CM along with the genome of LSC-EXOs was also described. MMP2, miR-30a, miR-99a, miR-100 and let-7 family of miRNA were identifying as potential key factors within the LSC secretome that may be responsible for the therapeutic effects observed.

### 4.2 Future Outlook

While IPF is still an incurable disease with few treatment options, the work presented in this dissertation describes novel treatment strategies that have been shown to be safe and effective in both mice and rats as well as in two different experimental model of pulmonary fibrosis. The clinical applications of stem cell-CM and -EXO inhalation therapy could make an enormous impact on respiratory disease. However, many challenges in CM and EXO research remains, including the characterization and standardization of CM and EXO. A standard protocol for the isolation and characterization of stem cell-CM and -EXO currently does not exist. Also, because CM and EXOs are actively secreted and isolated from cells, there is an inherent variation in the contents of the CM and EXO from batch to batch. This batch variation must be addressed before the clinical applications of CM and EXO therapies can be realized.
One potential solution to batch variation is to develop synthetic CM and EXOs, but the main challenge with this approach is that the key factors in CM and EXOs remain unknown and are actively being investigated. Presented in Chapter 3 are the proteomic analysis of LSC-CM and the miRNA profiling of LSC-EXOs and MSC-EXOs, identifying a few potential key factors. However, further studies are required to determine the exact proteins, miRNA, and other molecules responsible for the observed effects. Different cell types secrete different factors; therefore, further studies to elucidate the key differences between LSCs and MSCs factors and their functional and biological responses in-vivo is required. Many studies utilizing CM and EXOs have taken a candidate approach and singled out a specific protein or miRNA, but it is unlikely that any single factor can capture the full effect of CM and EXO as a whole. The animal models presented in Chapter 3 showed that LSC-CM, LSC-EXO, MSC-CM and MSC-EXO are all safe and did not elicit any adverse effects in-vivo and did not result in any abnormal cell growth such as tumors. However, extensive immunogenicity and toxicity tests must be done before such treatments can reach clinics. The task of elucidating stem cell-CM and EXO is difficult, but if successful CM and EXO therapies could close the gap in chronic respiratory disease.
4.3 References


Appendices
**Supplemental Figure 1** Negative controls of all immunostaining for phenotype analysis of lung spheroids.
**Supplemental Figure 2** Negative controls of all immunostaining for biodistribution of LSCs in-vivo after intravenous injection.

**Supplemental Figure 3** Double stained LSCs shows mixed phenotype of mesenchymal and epithelial markers.
Supplemental Figure 4 Representative in-vivo imaging of control athymic nude mice showing auto-fluorescence.
Appendix B

Supplemental Data for Chapter 3

**Supplemental Table 1**: Cell Line Donor Information

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</table>
Supplemental Table 2: Extracellular protein identified in all three donor CM.

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<th>Accession No.</th>
<th>Symbol</th>
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<th>Donor 2</th>
<th>Donor 3</th>
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<tr>
<td>A8KAJ3_HUMAN</td>
<td>EFEMP1</td>
<td>EGF containing fibulin like extracellular matrix protein 1</td>
<td>55 kDa</td>
<td>8.93E+08</td>
<td>2.90E+08</td>
<td>4.09E+08</td>
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<td>B2RCM5_HUMAN</td>
<td>EFEMP2</td>
<td>EGF containing fibulin like extracellular matrix protein 2</td>
<td>49 kDa</td>
<td>2.79E+07</td>
<td>9.00E+06</td>
<td>0.00E+00</td>
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<tr>
<td>EMIL1_HUMAN</td>
<td>EMILIN1</td>
<td>elastin microfibril interfacier 1</td>
<td>107 kDa</td>
<td>2.81E+07</td>
<td>2.52E+07</td>
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<td>B4DUV1_HUMAN</td>
<td>FBLN1</td>
<td>fibulin 1</td>
<td>70 kDa</td>
<td>4.21E+08</td>
<td>3.31E+07</td>
<td>3.68E+07</td>
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<td>FBLN1</td>
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<td>9.54E+08</td>
<td>3.25E+08</td>
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<td>5.52E+07</td>
<td>2.04E+07</td>
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<td>FBN1_HUMAN</td>
<td>FBN1</td>
<td>fibrillin 1</td>
<td>312 kDa</td>
<td>1.99E+08</td>
<td>7.01E+07</td>
<td>4.38E+08</td>
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<td>FINC_HUMAN</td>
<td>FN1</td>
<td>fibronectin 1</td>
<td>263 kDa</td>
<td>8.14E+10</td>
<td>1.29E+11</td>
<td>8.12E+10</td>
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<td>H0Y4K8_HUMAN</td>
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<td>fibronectin 1</td>
<td>27 kDa</td>
<td>5.45E+09</td>
<td>1.35E+10</td>
<td>7.78E+09</td>
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<tr>
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<td>angiopoein</td>
<td>fibronectin 1/ thrombospondin 1 variant</td>
<td>235 kDa</td>
<td>4.05E+10</td>
<td>8.64E+10</td>
<td>4.71E+10</td>
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<td>Q6MZM7_HUMAN</td>
<td>angio</td>
<td>fibronectin 1/ uncharacterized protein</td>
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<td>6.22E+07</td>
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<td>FSTL1</td>
<td>follistatin like 1</td>
<td>35 kDa</td>
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<td>1.52E+09</td>
<td>5.06E+08</td>
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<td>B4DZY7_HUMAN</td>
<td>GAS6</td>
<td>growth arrest specific 6</td>
<td>60 kDa</td>
<td>8.28E+07</td>
<td>3.80E+07</td>
<td>1.88E+07</td>
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<tr>
<td>GPX3_HUMAN</td>
<td>GPX3</td>
<td>glutathione peroxidase 3</td>
<td>26 kDa</td>
<td>8.81E+07</td>
<td>1.00E+07</td>
<td>2.44E+08</td>
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<td>B4DJJ2_HUMAN</td>
<td>GRN</td>
<td>granulin</td>
<td>57 kDa</td>
<td>5.20E+07</td>
<td>0.00E+00</td>
<td>1.70E+07</td>
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<td>B7Z4U6_HUMAN</td>
<td>GSN</td>
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<td>76 kDa</td>
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<td>HBA1/HB A2</td>
<td>hemoglobin subunit alpha 2</td>
<td>11 kDa</td>
<td>2.28E+08</td>
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<td>G3V1N2_HUMAN</td>
<td>HBA1/HB A2</td>
<td>hemoglobin subunit alpha 2</td>
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<td>9.47E+07</td>
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<td>Accession No.</td>
<td>Symbol</td>
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<td>Molecular Weight</td>
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<td>Donor 2</td>
<td>Donor 3</td>
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<td>HMCN1_HUMAN</td>
<td>HMCN1</td>
<td>hemicentin 1</td>
<td>613 kDa</td>
<td>1.21E+06</td>
<td>8.50E+06</td>
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<td>HSPG2</td>
<td>heparan sulfate proteoglycan 2</td>
<td>469 kDa</td>
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<td>4.61E+08</td>
<td>1.94E+08</td>
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<td>HTRA1</td>
<td>HtrA serine peptidase 1</td>
<td>48 kDa</td>
<td>4.67E+07</td>
<td>3.25E+07</td>
<td>0.00E+0</td>
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<tr>
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<td>IGFBP3</td>
<td>insulin like growth factor binding protein 3</td>
<td>29 kDa</td>
<td>9.64E+08</td>
<td>2.41E+09</td>
<td>5.47E+08</td>
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<td>insulin like growth factor binding protein 4</td>
<td>28 kDa</td>
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<td>1.81E+09</td>
<td>5.15E+08</td>
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<td>IBP5_HUMAN</td>
<td>IGFBP5</td>
<td>insulin like growth factor binding protein 5</td>
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<td>0.00E+00</td>
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<td>F8VYK9_HUMAN</td>
<td>IGFBP6</td>
<td>insulin like growth factor binding protein 6</td>
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<td>laminin subunit alpha 2</td>
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<td>H0U149_HUMAN</td>
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<td>laminin subunit alpha 4</td>
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<td>4.24E+09</td>
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<td>LAMB1</td>
<td>laminin subunit beta 1</td>
<td>200 kDa</td>
<td>8.90E+08</td>
<td>1.93E+09</td>
<td>2.83E+08</td>
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<td>LAMB2_HUMAN</td>
<td>LAMB2</td>
<td>laminin subunit beta 2</td>
<td>196 kDa</td>
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<td>6.70E+08</td>
<td>8.84E+07</td>
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<td>2.05E+09</td>
<td>4.28E+09</td>
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<td>LEG1_HUMAN</td>
<td>LGALS1</td>
<td>galectin 1</td>
<td>15 kDa</td>
<td>4.81E+08</td>
<td>9.96E+07</td>
<td>1.82E+09</td>
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<td>LGALS3</td>
<td>lectin, galactoside binding soluble 3</td>
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<td>3.20E+08</td>
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<td>LOX</td>
<td>lysyl oxidase</td>
<td>47 kDa</td>
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<td>0.00E+00</td>
<td>1.63E+07</td>
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<tr>
<td>LOXL2_HUMAN</td>
<td>LOXL2</td>
<td>lysyl oxidase like 2</td>
<td>87 kDa</td>
<td>1.25E+07</td>
<td>3.42E+07</td>
<td>2.95E+07</td>
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<td>LTBP1</td>
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<td>190 kDa</td>
<td>4.31E+08</td>
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<tr>
<td>LUM_HUMAN</td>
<td>LUM</td>
<td>lumican</td>
<td>38 kDa</td>
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<td>MASP1</td>
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<td>0.00E+00</td>
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<td></td>
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<tr>
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<td>42 kDa</td>
<td>3.63E+08</td>
<td>4.08E+08</td>
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<td>macrophage migration inhibitory factor</td>
<td>12 kDa</td>
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<td>6.30E+07</td>
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<td>(glycosylation-inhibiting factor)</td>
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<td>46 kDa</td>
<td>3.11E+08</td>
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<td>MMP3</td>
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<td>54 kDa</td>
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<td>NID1_HUMAN</td>
<td>NID1</td>
<td>nidogen 1</td>
<td>136 kDa</td>
<td>1.47E+08</td>
<td>2.15E+08</td>
<td>1.87E+08</td>
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<td>NID2</td>
<td>nidogen 2</td>
<td>151 kDa</td>
<td>1.25E+09</td>
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<td>olfactomedin like 3</td>
<td>39 kDa</td>
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<td>1.33E+07</td>
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<td>PAPPA</td>
<td>pappalysin 1</td>
<td>74 kDa</td>
<td>3.32E+07</td>
<td>1.66E+07</td>
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<td>PCOLCE</td>
<td>procollagen C-endopeptidase enhancer</td>
<td>48 kDa</td>
<td>1.02E+09</td>
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<td>3.77E+08</td>
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<td>PLTP</td>
<td>phospholipid transfer protein</td>
<td>57 kDa</td>
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<td>3.21E+08</td>
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<td>periostin</td>
<td>87 kDa</td>
<td>2.09E+08</td>
<td>7.07E+07</td>
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<td>B4DEK5_HUMAN</td>
<td>PSAP</td>
<td>prosaposin</td>
<td>51 kDa</td>
<td>5.08E+08</td>
<td>9.37E+07</td>
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<td>7.73E+07</td>
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<td>7.25E+07</td>
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<td>Accession No.</td>
<td>Symbol</td>
<td>Protein Name</td>
<td>Molecular Weight</td>
<td>Donor 1</td>
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<td>B7ZAB0_HUMAN</td>
<td>SERPINE1</td>
<td>serpin family E member 1</td>
<td>157 kDa</td>
<td>1.45E+09</td>
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<td>SERPINF1</td>
<td>serpin family F member 1</td>
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<td>9.59E+07</td>
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<td>7.78E+07</td>
<td>5.59E+07</td>
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<td>SPARC</td>
<td>secreted protein acidic and cysteine rich</td>
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<td>1.47E+09</td>
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<td>SPON2</td>
<td>spondin 2</td>
<td>36 kDa</td>
<td>8.88E+08</td>
<td>3.46E+08</td>
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<td>STC2</td>
<td>stanniocalcin 2</td>
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<td>THBS2</td>
<td>thrombospondin 2</td>
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<tr>
<td>B3KQF4_HUMAN</td>
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<td>TIMP metallopeptidase inhibitor 1</td>
<td>23 kDa</td>
<td>2.59E+09</td>
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<td>2.89E+09</td>
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<td>TIMP2</td>
<td>TIMP metallopeptidase inhibitor 2</td>
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<td>136 kDa</td>
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</table>
Supplemental Table 3: Donor 1 top 50 most abundant protein.

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<tr>
<th>Identified Proteins (316)</th>
<th>Accession Number</th>
<th>Molecular Weight</th>
<th>Donor 1</th>
</tr>
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<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens</td>
<td>G3P_HUMAN</td>
<td>36 kDa</td>
<td>2.04E+10</td>
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<tr>
<td>Actin, cytoplasmic 1 OS=Homo sapiens</td>
<td>ACTB_HUMAN</td>
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<td>Keratin 1 OS=Homo sapiens</td>
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<td>Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens</td>
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<td>Filamin A OS=Homo sapians</td>
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<td>278 kDa</td>
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<td>14-3-3 protein zeta/delta OS=Homo sapiens</td>
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<td>Heat shock protein beta-1 OS=Homo sapiens</td>
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<td>Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1 SV=1</td>
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<tr>
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<td>Nucleoside diphosphate kinase OS=Homo sapiens GN=NME1-NME2 PE=2 SV=1</td>
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<tr>
<td>Actin, alpha skeletal muscle OS=Homo sapiens GN=ACTA1 PE=1 SV=1</td>
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<tr>
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<tr>
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<td>5.09E+09</td>
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<tr>
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<td>Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2</td>
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<td>Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2</td>
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<td>PGK1_HUMAN</td>
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<td>65 kDa</td>
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<td>Heat shock protein beta-1 OS=Homo sapiens GN=HSPB1 PE=1 SV=2</td>
<td>HSPB1_HUMAN</td>
<td>23 kDa</td>
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<td>14-3-3 protein zeta/delta OS=Homo sapiens</td>
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<td>28 kDa</td>
<td>2.48E+09</td>
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<td>Annexin A5 OS=Homo sapiens GN=ANXA5 PE=1 SV=2</td>
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<td>36 kDa</td>
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<td>Protein DJ-1 OS=Homo sapiens GN=PARK7 PE=1 SV=2</td>
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<td>2.22E+09</td>
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<td>Thioredoxin OS=Homo sapiens GN=TXN PE=1 SV=3</td>
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<td>12 kDa</td>
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<tr>
<td>Histidine triad nucleotide-binding protein 1 OS=Homo sapiens GN=HINT1 PE=1 SV=2</td>
<td>HINT1_HUMAN</td>
<td>14 kDa</td>
<td>1.97E+09</td>
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<td>28 kDa</td>
<td>1.96E+09</td>
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<td>Transgelin-2 OS=Homo sapiens GN=TAGLN2 PE=1 SV=3</td>
<td>TAGL2_HUMAN</td>
<td>22 kDa</td>
<td>1.76E+09</td>
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<tr>
<td>Profilin-1 OS=Homo sapiens GN=PFN1 PE=1 SV=2</td>
<td>PROF1_HUMAN</td>
<td>15 kDa</td>
<td>1.66E+09</td>
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<td>Ubiquitin carboxyl-terminal hydrolase isozyme L1 OS=Homo sapiens GN=UCHL1 PE=1 SV=2</td>
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<td>103 kDa</td>
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</tr>
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<td>Vinculin OS=Homo sapiens GN=VCL PE=1 SV=4</td>
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<tr>
<td>Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4</td>
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<td>83 kDa</td>
<td>1.31E+09</td>
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<td>Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3</td>
<td>K1C9_HUMAN</td>
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<tr>
<td>Tubulin-specific chaperone A OS=Homo sapiens GN=TBCA PE=4 SV=1</td>
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<td>1.29E+09</td>
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<tr>
<td>Endoplasmmin OS=Homo sapiens GN=HSP90B1 PE=1 SV=1</td>
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<td>Alpha-actinin-4 OS=Homo sapiens GN=ACTN4 PE=1 SV=2</td>
<td>ACTN4_HUMAN</td>
<td>105 kDa</td>
<td>1.23E+09</td>
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### Supplemental Table 5: Donor 3 top 50 most abundant protein.

<table>
<thead>
<tr>
<th>Identified Proteins (612)</th>
<th>Accession Number</th>
<th>Molecular Weight</th>
<th>Donor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1</td>
<td>ACTB_HUMAN</td>
<td>42 kDa</td>
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<tr>
<td>Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3</td>
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<tr>
<td>Triosephosphate isomerase OS=Homo sapiens GN=TP11 PE=1 SV=3</td>
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<tr>
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<td>Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2</td>
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<td>Identified Proteins (612)</td>
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<td>Rho GDP-dissociation inhibitor 1 OS=Homo sapiens GN=ARHGDI1A PE=1 SV=3</td>
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<tr>
<td>Endoplasmin OS=Homo sapiens GN=HSP90B1 PE=1 SV=1</td>
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</tr>
<tr>
<td>Tropomyosin alpha-4 chain (Fragment) OS=Homo sapiens GN=TMPO4 PE=4 SV=1</td>
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<td>Galectin-3 OS=Homo sapiens GN=LGALS3 PE=1 SV=5</td>
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<td>26 kDa</td>
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<tr>
<td>Pyruvate kinase (Fragment) OS=Homo sapiens GN=PKM PE=3 SV=1</td>
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Supplemental Table 6: Top 25 miRNA profile in LSC-EXOs

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<tr>
<th>miRNAs LSC1</th>
<th>Raw Counts LSC1</th>
<th>Percent LSC1</th>
<th>miRNAs LSC2</th>
<th>Raw Counts LSC2</th>
<th>Percent LSC2</th>
<th>miRNAs LSC3</th>
<th>Raw Counts LSC3</th>
<th>Percent LSC3</th>
</tr>
</thead>
<tbody>
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<td>hsa-miR-100-5p</td>
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**Notes:**
- Log2 Fold Change: Log2 fold change values for each miRNA.
- p. value: Raw p-values for miRNA expression levels.
- adj.p. value: Adjusted p-values for miRNA expression levels.
- LS C1 raw: Log2 transformed raw counts.
- LSC1 UQ norm: Log2 transformed raw counts normalized.
- LS C2 raw: Log2 transformed raw counts.
- LSC2 UQ norm: Log2 transformed raw counts normalized.
- LS C3 raw: Log2 transformed raw counts.
- LSC3 UQ norm: Log2 transformed raw counts normalized.
- MS C1 raw: Log2 transformed raw counts.
- MSC1 UQ norm: Log2 transformed raw counts normalized.
- MS C2 raw: Log2 transformed raw counts.
- MSC2 UQ norm: Log2 transformed raw counts normalized.
- UQavg: Average UQ value.
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**Supplemental Figure 1:** CD1 mice body weight measures throughout the study.

**Supplemental Figure 2:** Methylene blue nebulization experiment in CD1 mice. Left: explanted mice lung and heart. Right: Unstained cryosectioned lung tissue. Scale bar= 50 µm.
Supplemental Figure 3: Proteomic analysis of lysed LSCs. (a) SDS-PAGE gel of LSC lysate. 001 = donor 1, 002 = donor 2, 003 = donor 3. (b) Quantification of subcellular localization of lysed LSC proteins. (c) Comparison of cytoplasmic proteins identified in LSC-CM versus LSC lysate.
Supplemental Figure 4: LSC-Exo Study Tunel analysis of apoptotic cells. (a) Representative Tunel staining of apoptotic cells for each treatment group (b) and quantification of percent of Tunel positive cells; Scale bar= 100µm; each dot represents data from one animal; n=6
Supplemental Figure 5: Blood biochemistry analysis of (a-c) liver enzymes alanine transaminase (ALT) (a), aspartate transaminase (AST) (b), ratio of ALT: AST (c) and (d-f) kidney metabolite blood urea nitrogen (BUN) (d), creatinine (e) and the ratio of BUN: creatinine (f); each data point represents data from one animal; n=11-12; **P≤0.01.

Supplemental Figure 6: Representative H&E staining of heart, kidney, liver and spleen in CM and Exo treated animals in both the A/J mice silica study and SD rats bleomycin study showing no tumors.
**Supplemental Figure 7**: RNA sequencing analysis of LSC-EXO and MSC-EXO samples. (a) Global RNA profile of LSC-EXOs from the three donor cell lines and MSC-EXOs from two different cell lines. (b) Top 10 miRNAs in each of the EXO samples.